State of Hawaii
Department of Agriculture
Agricultural Loan Division

March 23, 2021

Department of Agriculture
Honolulu, Hawaii

Subject: **Loan Presentation**

APPLICANT(S):

Bradley F. Smith
P.O. Box 152
Kilauea, HI 96754

Amy Arnett-Smith
P.O. Box 152
Kilauea, HI 96754

CLASSIFICATION & ELIGIBILITY:

Sole Proprietorship
The applicants meet the definition of a "Qualified Farmer," as stated in Chapter 155-1 and General Eligibility Requirements stated in 155-10 of the Hawaii Revised Statutes. Bradley F. Smith (hereinafter "Brad"), and Amy Arnett-Smith (hereinafter "Amy") have been farming full time since 2006 doing business as Viva Rain farms. Brad has been a Hawaii resident for the past 20 years and Amy for the past 34 years.

COMMODITY:

Avocado, Rambutan, Longan, Lettuce, Tomatoes, Cucumbers, Mangosteen and Onions

CREDIT HISTORY:

SEE EXHIBIT A (CONFIDENTIAL)

OTHER STATE AGRICULTURAL LOANS:

Loan No.	Approval Date	Loan Amount	Balance	Status
DA- 6284	02/23/2010	$ 200,000	$142,677	Current

In 2010, the State Agricultural Loan Division (SALD) provided a loan to purchase the 5 acre fee simple agricultural property in Moloa'a on Kauai. The loan is being paid as agreed.

LOAN REQUEST & PURPOSE:

Amount	Class
$50,000	D- Emergency Operating Loan
$50,000	Total Request

The requested loan proceeds will be used to cover farm related expenses due to the loss of sales from the COVID-19 pandemic.

TERMS:

Class D

Loan Amount:	$50,000
Term:	7 years
Interest rate:	**3.00%** per annum, fixed
Repayment:	Monthly principal and interest payments of $660.67 until maturity.

SECURITY:

The requested loan will be secured by the following:

- A second position UCC-1 blanket filing to include all crops, receivables, inventory, furniture, fixtures, equipment, supplies, etc. The SALD already maintains the first position priority lien with the existing class A loan. The primary security for the current SALD class A loan is real property valued at $982,900. The real estate collateral more than adequately covers the existing loan of $142,677.

Brad and Amy also own a Kubota Tractor valued at $38,000 but is lease financed through Lien Solutions, with a specific UCC filing; thus, the tractor was excluded in the farm equipment calculations

Description	***No.***	***Year***	***Condition***	***Value***
Tow Sprayer	1	2018	Good	3,000
Hustler Mower	1	2014	Good	1,800
Yurt	1	2012	Good	50,000
Green house	1	2016	Good	6,000
Green House (10 x 20)	9	2018	Good	9,000
Tractor Mount Sprayer	1	2018	Good	5,000
Tractor Mowing Deck	1	2015	Fair	2,000
Walk behind tiller	1	2014	Good	600
Fork Tractor Implement	1	2019	Good	800
Bucket Tractor Implement	1	2019	Good	1,200
Seed Nursery Houses	3	2014	Fair	1,000
Aluminum Trailer	1	2013	Fair	1,200
Farm Quad	1	2018	Good	600
Tractor Tiller	1	2019	Good	1,000
Tractor Ripper	1	2018	Good	1,200
Total:				**84,400**

The farm equipment and machinery are valued at $84,400. Farm equipment includes a sprayer, mower, yurt, ten greenhouses, tiller, farm quad, and assorted tools. The values were provided by the borrowers and verified via internet searches, and reflect fair market value.

GUARANTORS: None

FINANCIAL CONDITION: SEE EXHIBIT A (CONFIDENTIAL)

REPAYMENT ABILITY: SEE EXHIBIT A (CONFIDENTIAL)

INSURANCE: Farm liability insurance with the SALD listed as certificate holder.

BACKGROUND/ MANAGEMENT ABILITY:

Brad and Amy have been farming on 22 acres in Kilauea and Moloa'a, Kauai, for over fifteen years. Brad and Amy's relationship with the State Department of Agriculture started in 2010 when Brad and Amy purchased the 5- acre farm in Moloa'a. The farm includes fruit trees and other exotics such as Longan, Rambutan, Mangosteen, Moyas, Chiku, Starfruit, Jackfruit, Lanzone & Avocado.

Amy has been farming with her husband Brad since 2006 and manages the business side of the farm. Brad and Amy emphasize the importance of managing their harvest and overall volume to keep labor minimal. Additionally, they use part-time laborers and the student work exchange program to assist the farm during harvesting periods.

The farm operates as a sole proprietorship dba "Viva Rain Farms" named after Amy's daughter Vivian Rain who began the family farm journey with Brad and Amy and later passed away. Brad and Amy now farm with their son and continue to pride themselves on farming family style.

The Moloa'a farm includes 5 acres of avocado, longan, rambutan, mango, pineapple, onions and ten greenhouses for tomatoes and lettuce, and various other vegetables. The Kilauea farm consists of 17 acres of fruit orchards, longan, rambutan, avocado, star fruit, and mangosteen trees. Brad and Amy have been farming on the Kilauea property for the past fifteen years through an agreement to farm from the land

owners in exchange for maintenance of the property. Farm visits over the years confirmed the operation to be well organized, productive and managed. The crops appear to be healthy, productive, and thriving.

COMMENTS:

This proposed loan will provide the necessary funds to continue farm operations due to sales loss from the ongoing COVID-19 pandemic. Brad and Amy suffered a reduction in income due to the loss of tourism resulting from Statewide shutdowns and travel restrictions. During the pandemic, Brad and Amy lost their Chinatown markets and lost two of the five farmers markets where they actively sold their produce. With the COVID pandemic prolonging with no immediate rapid turnaround of the economy in sight for Hawaii. Brad and Amy had no other choice but to seek financial assistance with Department of Agriculture in efforts to sustain the farm operations through the remainder of this pandemic.

With the loss arising from the COVID-19 pandemic, Brad and Amy have added another source of revenue with a "online farmers market" where local customers can purchase their products on a Monday or Thursday, Tuesday & Friday pick up. Additionally, they pivoted to focus more on the local markets and the local buyers' specific demands. This adjustment has resulted in a move from garden vegetables to mangosteen, avocadoes, longan, and rambutan that targets the local sales.

This loan will benefit Brad and Amy by providing much-needed relief for the daily operational expenses and allowing them to continue farming and sustain local agriculture, further supporting the Department of Agriculture's goal. The State will also greatly benefit by keeping a business operating in an economically depressed area, keeping people employed, and preserving food self-sufficiency.

TURNDOWNS: Turndowns for emergency loans of $100,000 and under have been waived by the Board of Agriculture.

RECOMMENDATIONS: This loan is recommended for approval based on the applicants proven farming experience, the need for emergency assistance due to the COVID 19 pandemic, historical performance and excellent repayment history with SALD.

Date

03-05-21

Recommended by:

Christine Asing
Business Loan Officer I

Date

3/5/21

Reviewed by:

Dean M. Matsukawa
Agricultural Loan Administrator

Date

3/15/2021

Approved for submission:

Phyllis Shimabukuro-Geiser
Chairperson, Board of Agriculture

STATE OF HAWAII
DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESOURCE MANAGEMENT DIVISION
HONOLULU, HAWAII

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

Subject:	REQUEST FOR CONSENT TO ASSIGNMENT OF GENERAL LEASE NO. S-6014, DANA DAODY KIAT-A NAN, LESSEE/ASSIGNOR, TO KLK FARM, LLC, ASSIGNEE; TMK: 1ST DIV/5-6-006:042, LOT NO. 14, KAHUKU AGRICULTURAL PARK, KOOLAULOA, KAHUKU, ISLAND OF OAHU
Authority:	Sections 166-7 and 166-9, Hawaii Revised Statutes (HRS), and Section 4-153-33(a)(6)(A), Hawaii Administrative Rules (HAR)
Lessor/Assignor:	Dana Daody Kiat-A-Nan
Assignee:	KLK Farm, LLC
Land Area:	6.196 gross acres
Tax Map Key:	1st Div/5-6-006:042 (see Exhibit "A")
Land Status:	Encumbered by Governor's Executive Order No. 3867 to the Department of Agriculture for agricultural park purposes
Rental:	$2,580.00 per year until rental re-opening – April 1, 2024
Additional Rent:	The amount by which 3% of the gross proceeds from the sale of commodities produced on the demised premises that exceeds the base rental
Character of Use:	Diversified Agriculture purposes
Lease Term:	45 years, April 1, 1999 to March 31, 2044
Consideration:	None

B1

B2

REMARKS:

In 1999, General Lease No. S-6014 was awarded to Jimmy S. Inthasone by the Board of Agriculture, and in 2004 the subject lease was assigned to Dana D. Sourinthone and McArt Sourinthone (husband). By mesne assignment, in 2017 the subject lease was assigned to Dana Daody Kiat-A Nan. Ms. Kiat-A Nan has developed her lot into a successful farm that produces banana, mango, lychee, and dragon fruit, which are sold at farmers markets.

For estate purposes, Ms. Kiat-A Nan requests an assignment of General Lease No. S-6014 to KLK Farm, LLC. Ms. Kiat-A Nan is named member-manager of the Hawaii Limited Liability Complany. Ms. Kiat-A Nan lives and works fulltime at the farm premises which is her principal residence. Assignment and transfer of a lease may be made if the lease contains the principal residence of the Lessee pursuant to Section 4-153-33(a)(6)(A) HAR and Paragraph 17(b)(1) of General Lease No. S-6014.

There is a no consideration for the assignment.

RECOMMENDATION:

That the Board of Agriculture consent to the assignment of General Lease No. S-6014 from Dana Daody Kiat-A Nan, Assignor, to KLK Farm, LLC, Assignee, subject to the approval as to form of the assignment and consent documents by the Department of the Attorney General.

Respectfully submitted,

FOR

BRIAN KAU, P.E.
Administrator and Chief Engineer
Agricultural Resource Management Division

Attachment – Exhibit "A"

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

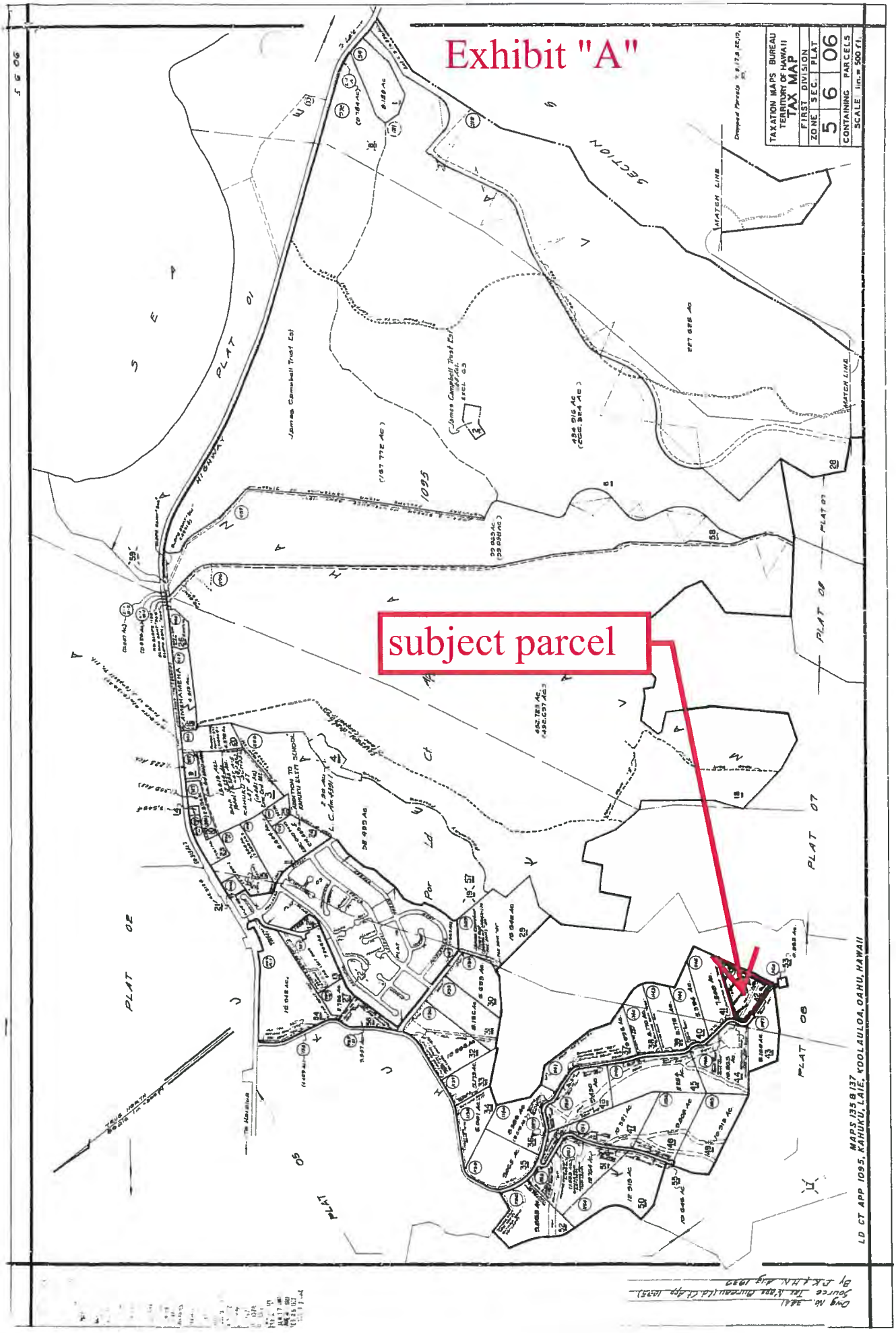


Exhibit "A"
subject parcel
TAXATION MAPS BUREAU
TERRITORY OF HAWAII
TAX MAP
ZONE 5
SEC. 6
PLAT 06
CONTAINING PARCELS
SCALE 1in.= 500 ft.
SECTION 6
PLAT 01
PLAT 02
PLAT 05
PLAT 07
PLAT 08
MATCH LINE
MAPS 135 & 137
LD CT APP 1095, KAHUKU, LAIE, KOOLAULOA, OAHU, HAWAII

B3

STATE OF HAWAII
DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESOURCE MANAGEMENT DIVISION
HONOLULU, HAWAII

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

Subject:	REQUEST FOR CONSENT TO ASSIGNMENT OF GENERAL LEASE NO. S-4753; PHILIP J. ITO AND CAROLE K. ITO, LESSEE/ASSIGNOR; KEITH K. KUROIWA, ASSIGNEE; TMK:3RDDIV/2-2-056:032, LOT NO. 06, PANAEWA AGRICULTURAL PARK, WAIAKEA, SOUTH HILO, ISLAND OF HAWAII
Authority:	Sections 166-7 and 166-9, Hawaii Revised Statutes ("HRS"), and Section 4-153-33(a)(6)(B), Hawaii Administrative Rules ("HAR")
Lessee/Assignor:	Philip J. Ito and Carole K. Ito
Assignee:	Keith K. Kuroiwa
Land Area:	10.212 gross acres
Tax Map Key:	3rd Div/2-2-056:032 (Exhibit "A")
Land Status:	Encumbered by Governor's Executive Order No. 3378 to the Department of Agriculture for agricultural park land purposes.
Lease Term:	January 1, 1982 through December 31, 2036 (55 years)
Annual Base Rent:	$2,715.00 Per Year until January 1, 2022 (rent reopening)
Character of Use:	Orchard purposes
Consideration:	$60,000.00

B4

BACKGROUND:

Philip J. Ito and Carole K. Ito are the original Lessees of General Lease No. S-4753. On January 1, 1982, the Board of Land and Natural Resources awarded General Lease No. S-4753, Lot No. 06, located in the Panaewa Agricultural Park, to Mr. and Mrs. Ito. The products grown were pineapple, banana, rambutan and lychee.

Mr. and Mrs. Ito are tenants of record in good standing and request to assign General Lease No. S-4753 to Keith K. Kuroiwa, due to physical disability, a permitted basis for an assignment under the lease and pursuant to Section 4-153-33(a)(6)(B).

Keith K. Kuroiwa will continue to focus on the current seven (7) acres of planted lychee. In addition to lychee, avocados of various varieties will be planted along with citrus including lemon, lime-orange, and tangerine. One acre of land will be comprised exclusively of fast-growing vegetable crops so revenue can be produced sooner. A COOLBOT will be brought in to provide a cold storage as most of the products will be shipped to Oahu, while some will be sold at the local farmer's market. Future endeavors include the planting of a variety of exotic fruit trees to include durian, eggfruit, jackfruit and mango.

Mr. Kuroiwa qualifies as a bona fide farmer with more than two years of full-time farming experience and meets the eligibility residency requirements of three years, commensurate with Sections 4-153-1 and 13.

There is a consideration of $60,000.00 for the assignment of lease. Staff does not recommend an adjustment of the annual rental rate as the consideration amount appears to be consistent with fair market values.

RECOMMENDATION:

That the Board of Agriculture consent to the assignment of General Lease No. S-4753 from Philip J. Ito and Carole K. Ito, Lessee/Assignor, to Keith K. Kuroiwa, Assignee, subject to the approval as to form of the assignment and consent documents by the Department of the Attorney General, and such other terms and conditions as may be prescribed by the Chairperson to best serve the interests of the State.

Respectfully submitted,

FOR

BRIAN KAU, P.E.
Administrator and Chief Engineer
Agricultural Resource Management Division

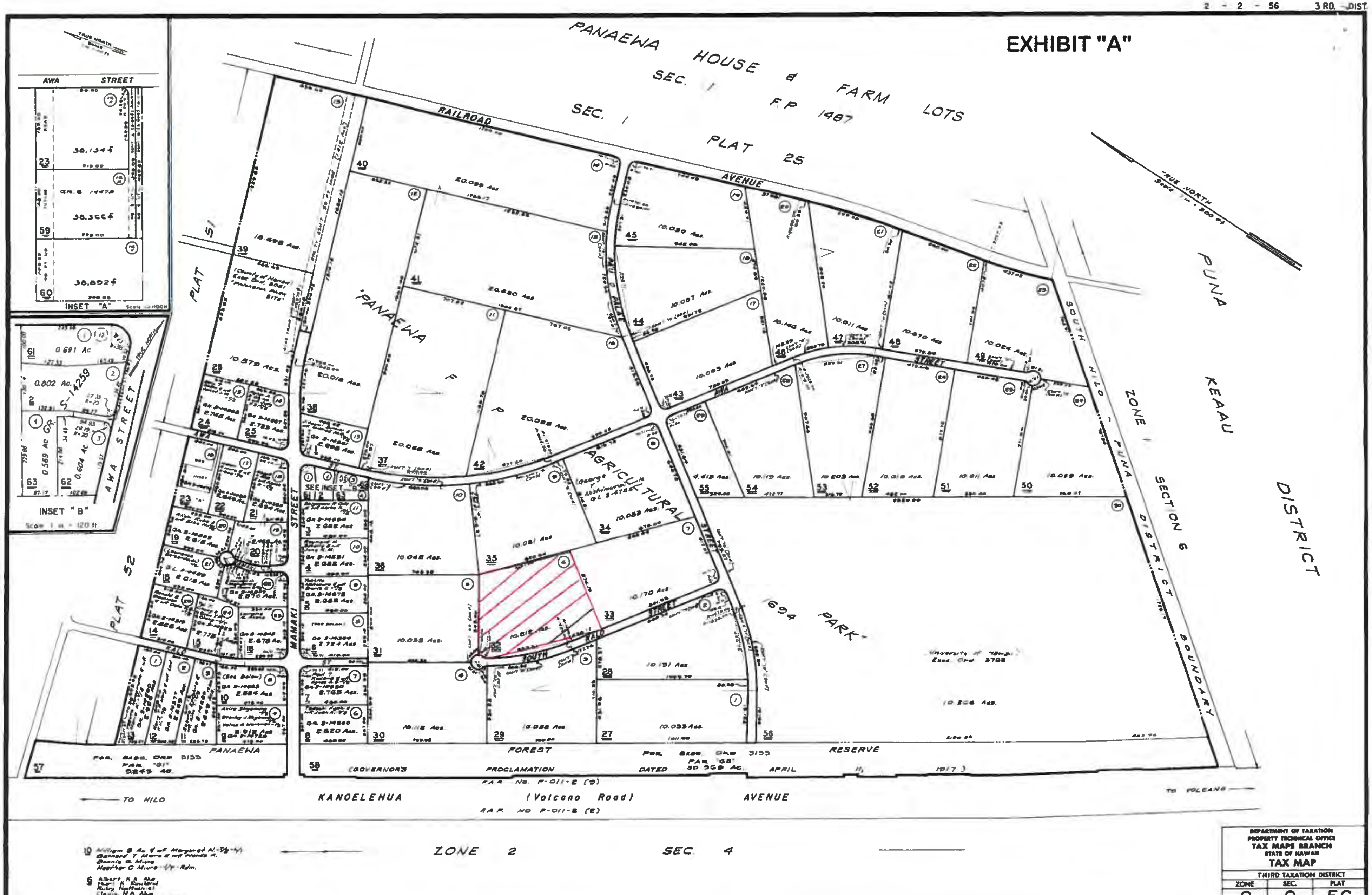
Attachment – Exhibit "A"

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

2 - 2 - 56 3 RD. DIST.

EXHIBIT "A"



REVISED October 6, 1980 Revised J.Y.-G.S.-M.H.
DATE April 9, 1964 BY J.Y.-S.Y.
SOURCE T.M.B.
DWG NO 5508

"PANAEWA AGRICULTURAL PARK"
"PANAEWA HOUSE LOTS - 2 ND. SERIES" Por. WAIAKEA, SOUTH HILO, HAWAII (formerly por. 2-2-48)

FOR PROPERTY ASSESSMENT PURPOSES
SUBJECT TO CHANGE

DEPARTMENT OF TAXATION
PROPERTY TECHNICAL OFFICE
TAX MAPS BRANCH
STATE OF HAWAII
TAX MAP

THIRD TAXATION DISTRICT		
ZONE	SEC.	PLAT
2	2	56

SCALE: 1 IN. = 300 FT.

B6

STATE OF HAWAII
DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESOURCE MANAGEMENT DIVISION
HONOLULU, HAWAII

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

Subject:	REQUEST FOR APPROVAL TO SUBLEASE BETWEEN THE HAMAKUA AGRICULTURAL COOPERATIVE, LESSEE/SUBLESSOR AND JOSHUA YANG, SUBLESSEE; GENERAL LEASE NO. S-7011, TMK: 3RD DIV/4-3-005:018(por), LOT W10, HAMAKUA POHAKUHAKU AND KEMAU 1ST, HAMAKUA, ISLAND OF HAWAII
Authority:	Section 166-6, Hawaii Revised Statutes, (HRS), and Section 4-153-33(a)(7), Hawaii Administrative Rules (HAR)
Lessee/Sublessor:	Hamakua Agricultural Cooperative
Sublessee:	Joshua Yang
Land Area:	4.050 acres
Tax Map Key:	3rd Div/4-3-005:018(por) (see Exhibit "A")
Land Status:	Hamakua Agricultural Park lands were acquired in fee by the Department of Agriculture under foreclosure and Bankruptcy Settlement Agreement with Hamakua Sugar Company, Inc.
Lease Term:	June 30, 1998 to June 29, 2033
Sublease Term:	March 1, 2021 to June 29, 2033
Sublease Base Annual Rental:	$203.77/year – Lot W10 until June 29, 2028 (Reopening Date)
Character of Use:	General Agriculture and pasture purposes in accordance with a Plan of Utilization and Development approved by the Department.

B7

B8

Joshua Yang is the sole proprietor of K Farm and has been in operation since 2001. His farm is situated in Paauilo on the Hamakua Coast where he grows lemon grass, cacao, turmeric, kava, beans, peas, tomatoes, carrots, fennel, basil, cilantro, calamansi, rollinia, starfruit, and more. Initially, he sold his produce directly to restaurants throughout the Big Island. In 2013, Mr. Yang began selling Kava, his personal line of Chocolate, and Turmeric on the internet. He also started a chocolate making company called Paauilo Chocolate. In 2019, he began selling lemongrass to Ola Brew in Kona. His current farm is 100% in production. With the additional 4.050 acres leased from the Hamakua Agricultural Cooperative, Joshua Yang plans to focus on the expansion of his kava, lemongrass, and turmeric crops as well as galangal, pineapple and bananas.

Mr. Joshua Yang qualifies as a bona fide farmer with more than two years of full-time farming experience and meets the residency requirements of three years commensurate with Sections 4-153-1 and 13, HAR.

RECOMMENDATIONS:

That the Board of Agriculture approve the request to sublease Lot W10 in the Hamakua Agricultural Park under General Lease No. S-7011 to Joshua Yang until the expiration date of June 29, 2033 and further subject to the approval as to form of the consent document by the Department of the Attorney General, and such other terms and conditions as may be prescribed by the Chairperson to best serve the interest of the State.

Respectfully submitted,

FOR

BRIAN KAU, P.E.
Administrator and Chief Engineer
Agricultural Resource Management Division

Attachment – Exhibit "A"

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

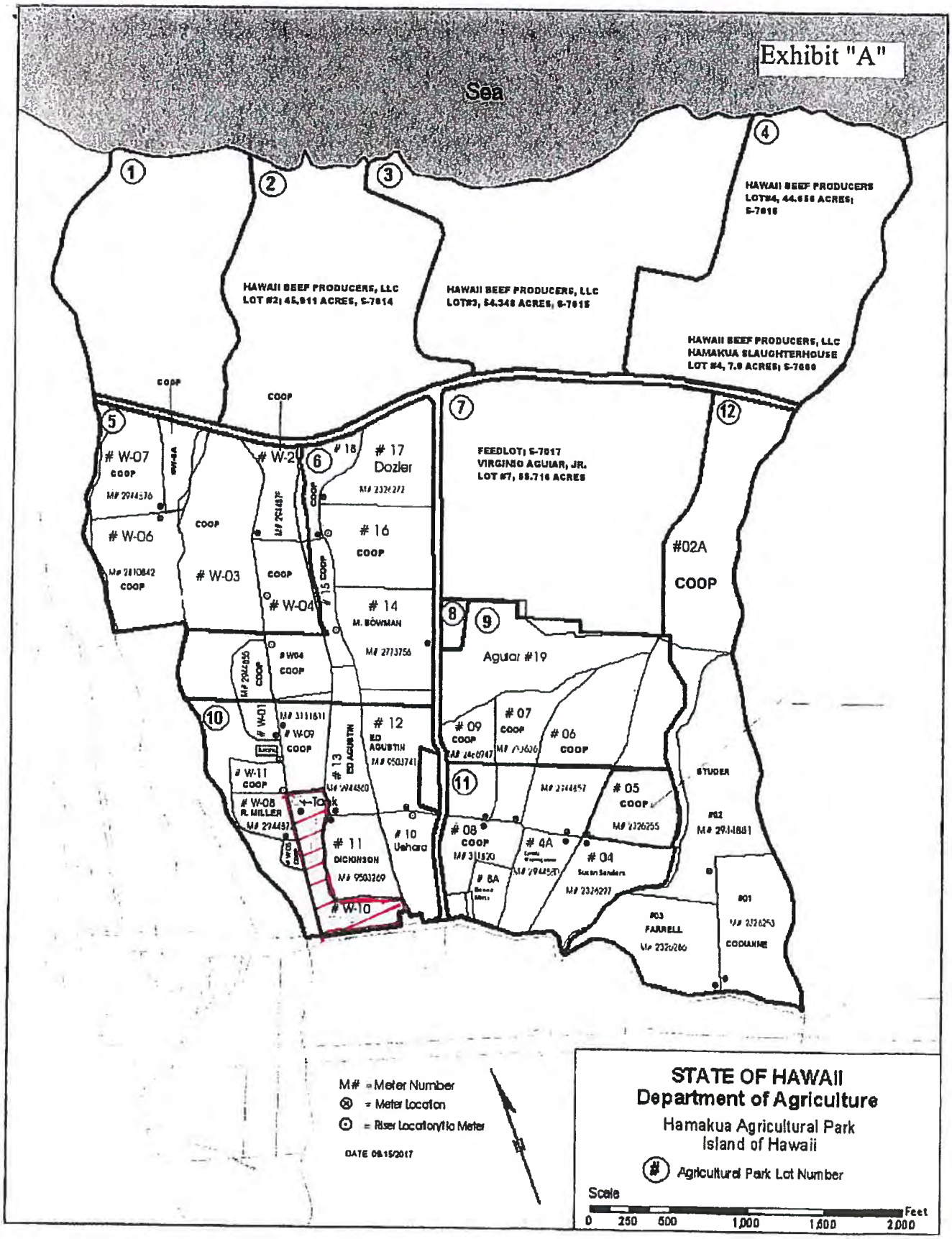


Exhibit "A"
Sea
HAWAII BEEF PRODUCERS, LLC
LOT #2; 45.911 ACRES, S-7614
HAWAII BEEF PRODUCERS, LLC
LOT#3, 54.348 ACRES, S-7615
HAWAII BEEF PRODUCERS
LOT#4, 44.656 ACRES;
S-7616
HAWAII BEEF PRODUCERS, LLC
HAMAKUA SLAUGHTERHOUSE
LOT #4, 7.0 ACRES; S-7660
FEEDLOT; S-7617
VIRGINIO AGUIAR, JR.
LOT #7, 58.716 ACRES
W-07
W-06
W-03
W-04
W-2
17
Dozler
16
14
M. BOWMAN
12
ED AGUSTIN
13
ED AGUSTIN
11
DICKINSON
W-10
W-11
W-08
R. MILLER
W-09
Tank
10
Uehara
Aguiar #19
09
07
06
08
4A
04
Susan Sanders
8A
05
#02A
COOP
STUDER
#02
#01
#03
FARRELL
COOP
M# = Meter Number
= Meter Location
= Riser Location/to Meter
DATE 08/15/2017
STATE OF HAWAII
Department of Agriculture
Hamakua Agricultural Park
Island of Hawaii
Agricultural Park Lot Number
Scale
0 250 500 1,000 1,500 2,000
Feet

B9

STATE OF HAWAII
DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESOURCE MANAGEMENT DIVISION
HONOLULU, HAWAII

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

Subject:	REQUEST FOR CONSENT TO ASSIGNMENT OF GENERAL LEASE NO. S-5643; THOMAS PAUL CALLAGHAN, LESSEE/ASSIGNOR, TO KEIKI O KA 'AINA FARMS, INC., ASSIGNEE; TMK: 1ST DIV/4-1-018:051, KOOLAUPOKO, WAIMANALO, ISLAND OF OAHU
Authority:	Section 166E-6, Hawaii Revised Statutes (HRS), and Sections 4-158-2(a)(9) and 4-158-19(a)(4)(A) Hawaii Administrative Rules (HAR)
Lessee/Assignor:	Thomas Paul Callaghan
Assignee:	Keiki O Ka 'Aina Farms, Inc.
Land Area:	Approximately 2.2 acres
Tax Map Key:	1st Div/4-1-018:051 (see Exhibit "A")
Land Status:	Encumbered by Governor's Executive Order No. 4239 to the Department of Agriculture for Non-Agricultural Park Lands purposes
Rental:	$6,270.00 per year until the October 1, 2022 rental reopening
Character of Use:	Diversified Agriculture
Lease Term:	35 years, October 1, 2002 to September 30, 2037
Consideration:	$395,000.00

B10

REMARKS:

General Lease No. S-5643 was awarded to Patrick Vahey in 2002 by the Board of Land and Natural Resources. In 2008, General Lease No. S-5643 was set aside and transferred to the Department of Agriculture by Governor's Executive Order No. 4239.

In 2014 when the Board of Agriculture approved the assignment of lease to Thomas Callahan, the premises was in extremely poor condition as Patrick Vahey had been unable to farm due to his physical quadriplegic state and deteriorating health. Mr. Callaghan invested a tremendous amount of time, effort and finances clearing the overgrown land, hauling debris, planting over 100 lemon trees and fully renovating the existing combined dwelling/processing/storage facility. Mr. Callaghan is a retired military combat veteran who suffered traumatic brain injury during his active duty tour overseas and suffers from post-traumatic stress (PTS). With the onset of COVID-19, he experienced increased mental anxiety and stress for which he is receiving professional treatment. Due to his mental disability he is requesting to assign the lease to Keiki O Ka 'Aina Farms, Inc. (KOKA) pursuant to Section 4-158-19(a)(4)(A), HAR.

KOKA Board of Directors consists of a team of experienced Bona fide farmers including Dane Kanaloa Bishop, Robert Bence, Jacob Holcomb and Momi Akana. They plan to maximize agricultural food production on the premises by adding lettuce, taro (kalo), ti plants, breadfruit (ulu), sweet potato ('uala), turmeric (olena), ginger root, various herbs, etc., in addition to the existing citrus trees. These crops were chosen as they are staples in the traditional Hawaiian diet. The primary target market for distribution of these products is the general public through food distribution centers in Kalihi and Ma'ili.

Pursuant to Section 4-158-1 and 27, HAR, KOKA qualifies as an agricultural corporation with at least 75 percent of its corporate members who qualify as Bona fide farmers and meet residency eligibility requirements.

There is a consideration of $395,000.00 for the assignment of lease. In accordance with Exhibit "C" ASSIGNMENT OF LEASE EVALUATION POLICY of General Lease No. S-5643, any net proceeds are subject to a Premium Percentage charge benefiting the Lessor. Calculations in accordance with this provision net $19,633.00 to the Lessor (see Exhibit "B").

RECOMMENDATION:

That the Board of Agriculture consent to the assignment of General Lease No. S-5643 from Thomas Callaghan, Lessee/Assignor, to Keiki O Ka 'Aina Farms, Inc., Assignee, pursuant to Section 4-158-2(a)(9), HAR, and approve the consideration amount of $395,000.00 for the Assignment of General Lease No. S-5643 to be paid by the Assignee. All documents shall be subject to review and approval as to form by the Department of the Attorney General, and such other terms and conditions as may be prescribed by the Chairperson to best serve the interests of the State.

Respectfully Submitted,

FOR

BRIAN KAU, P.E.
Administrator and Chief Engineer
Agricultural Resource Management Division

Attachments – Exhibits "A" & "B"

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

B12

B13

Exhibit "A"

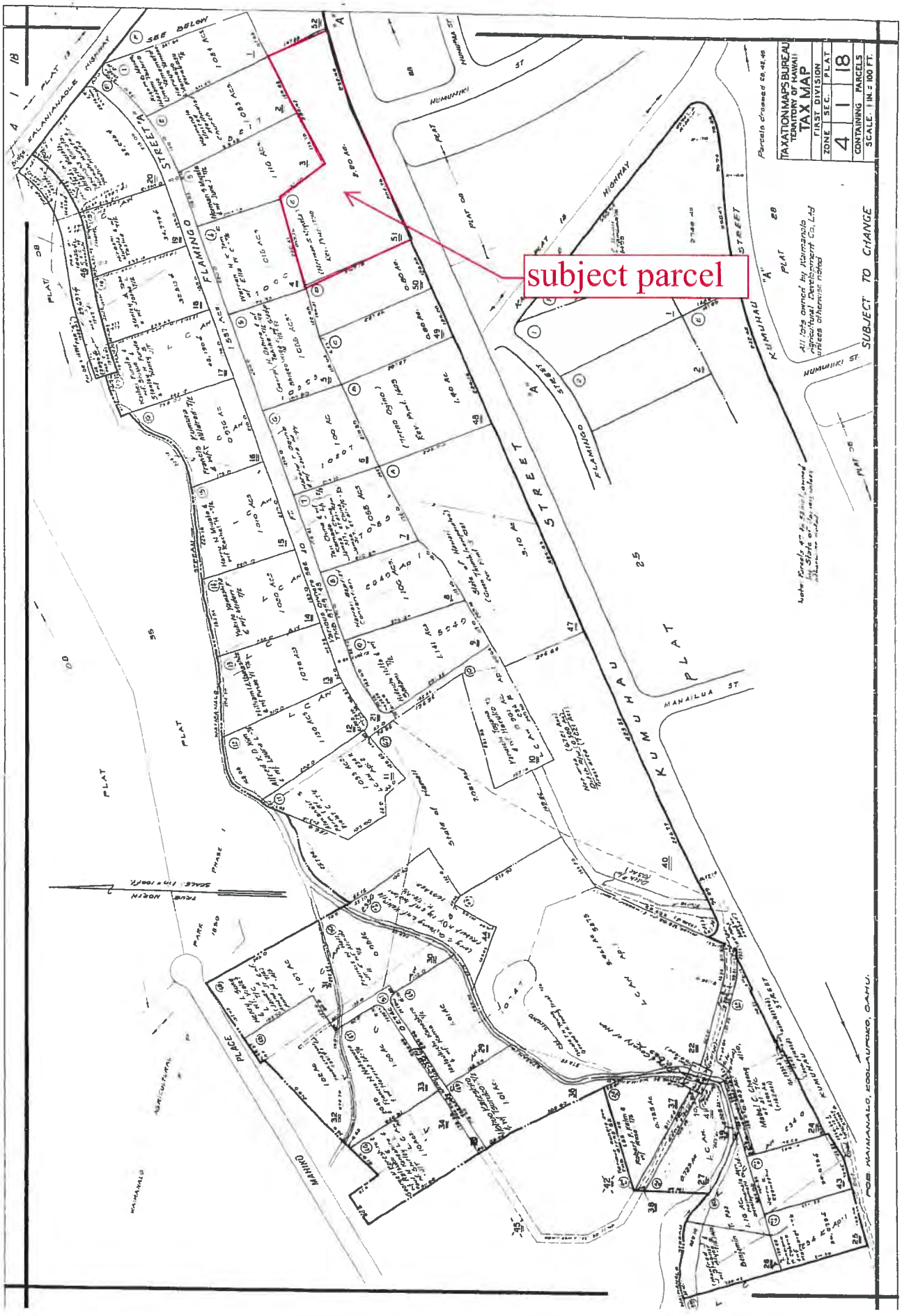


EXHIBIT "B"

ASSIGNMENT OF LEASE CALCULATIONS FOR
GENERAL LEASE NO. S-5643

Adjusted Depreciation Cost of Improvements or Renovations

Actual Cost:	$257,935.00
CCI (most recent):	11627
CCI (base):	9870
Expired Term:	221
Whole Term:	420

1. Adjusted Cost of Improvments or Renovations:

Actual Cost x CCI (most recent)/CCI (Base)

CCI (recent)	11627
CCI (base)	9870
CCIR/CCIB	1.18

Actual Cost x CCI(R)/CCI(B) =

$257,935.00	1.18	$303,851.09

2. Depreciation:

Adjust. Cost Impr./Whole Term x Expired Term =

$303,851.09	420	221	$159,884.00

3. Adjusted Depreciated Cost of Improvements:

Adjust cost - Depreciated cost =

$ 303,851.09	$ 159,884.00	$ 143,967.09

1. TOTAL NET CONSIDERATION		$ 222,500.00
2. Adj Cost of Imp/Renov	$ 303,851.09	
	$ 159,884.00	$ 143,967.09
3. Adj. cost of Trade Fixtures	$ -	
	$ -	
4. Excess		$ 78,532.91
5. Premium	Percentage: 25%	$ 19,633.00

Total Consideration:	$ 395,000.00
Less Lease Purchase:	$ 110,000.00
Less Inventory:	$ 62,500.00
Net Consideration:	$ 222,500.00

B14

STATE OF HAWAII
DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESOURCE MANAGEMENT DIVISION
HONOLULU, HAWAII

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

Subject:	REQUEST FOR (1) CONSENT TO ASSIGNMENT OF GENERAL LEASE NO. S-5380; THOMAS AKI, LESSEE/ASSIGNOR, TO HALA TOA MUI FARMS LLC, ASSIGNEE; AND (2) CONVERSION OF GENERAL LEASE NO. S-5380; TMK: 1ST DIV/4-1-009:266, KOOLAUPOKO, WAIMANALO, ISLAND OF OAHU
Authority:	Sections 166E-6, Hawaii Revised Statutes (HRS) and Sections 4-158-2(a)(9), 4-158-8, and 4-158-19(a)(4)(B) Hawaii Administrative Rules (HAR)
Lessee/Assignor:	Thomas Aki
Assignee:	Hala Toa Mui Farms LLC
Land Area:	31.170 gross acres
Tax Map Key:	1st Div/4-1-009:266 (see Exhibit "A")
Land Status:	Encumbered by Governor's Executive Order No. 4535 to the Department of Agriculture for Non-Agricultural Park Lands purposes
Rental:	$2,120.00 per year
Character of Use:	Pasture purposes
Lease Term:	35 years, January 1, 1995 to December 31, 2030
Consideration:	$1,000,000.00

B15

REMARKS:

General Lease No. S-5380 was awarded to Robert and Theresia Lee in 1995 for pasture purposes by the Board of Land and Natural Resources (BLNR). In 2017, General Lease No. S-5380 was set aside and transferred to the Department of Agriculture by Governor's Executive Order No. 4535.

In 2015 the BLNR approved the assignment of lease to Thomas Aki and Elizabeth Lee, and they invested a tremendous amount of time, effort and finances clearing the land, hauling debris, and constructing a new horse stable. Mr. Aki and Ms. Lee parted ways in 2019, and General Lease No. S-5380 was assigned solely to Thomas Aki. Subsequently, Mr. Aki suffered economic hardship operating and managing the business alone. Mr. Aki requests to assign the lease to Hala Toa Mui Farms LLC pursuant to Section 4-158-19(a)(4)(B), HAR.

Hala Toa Mui Farms LLC, owned and operated by Tevita Tongotea, plans to cultivate sweet potato, yam, taro, banana, avocado, and coconut. These crops will be for distribution to Polynesian markets and direct to consumers. Mr. Tongotea was born, raised, and worked on his family's farmland in Tonga. From 2000 to present he leases a 5-acre parcel in Punaluu growing yam, avocado, banana, sweet potato, taro and spinach. Mr. Tongotea would like to expand his business with the assignment of this lease as this Waimanalo parcel is larger and closer to his home in Kailua.

Pursuant to Section 4-158-1 and 27, HAR, Hala Toa Mui Farms LLC qualifies as an agricultural company with at least 75 percent of its members qualifying as Bona Fide farmers and meeting residency eligibility requirements.

There is a consideration of $1,000,000.00 for the assignment of the leasehold position and improvements on the premises. In accordance with Exhibit "C" ASSIGNMENT OF LEASE EVALUATION POLICY of General Lease No. S-5380, any net proceeds are subject to a Premium Percentage charge benefiting the Lessor. Calculations in accordance with this provision net $75,267.00 to the Lessor (see Exhibit "B").

Mr. Tongotea also requests a conversion of General Lease No. S-5380 in accordance with Section 4-158-8, HAR, to a new 35-year lease subject to Board approval of the assignment of lease. An appraisal has been ordered pursuant to Section 4-58-21, HAR to determine the fair market rental of the subject parcel for diversified agriculture purposes. The new appraised annual rental for this lease will be applied to the converted new lease as of the commencement date. Additionally, commensurate with the administrative rules, the lessee will pay a premium equal to 25% of the rental for the new lease for a period not to exceed seven (7) years from the commencement of the converted lease term.

RECOMMENDATION:

That the Board of Agriculture (1) consent to the assignment of General Lease No. S-5380 from Thomas Aki, Lessee/Assignor, to Hala Toa Mui Farms LLC, Assignee, pursuant to Section 4-158-2(a)(9), HAR; and (2) approve the conversion of General Lease No. S-5380 to a new 35-year lease term pursuant to Section 4-158-8, HAR, subject to approval of the assignment of lesae. All documents shall be subject to review and approval as to form by the Department of the Attorney General, and such other terms and conditions as may be prescribed by the Chairperson to best serve the interests of the State.

Respectfully Submitted,

BK FOR

BRIAN KAU, P.E.
Administrator and Chief Engineer
Agricultural Resource Management Division

Attachments – Exhibits "A" & "B"

APPROVED FOR SUBMISSION:

Phyllis Shimabukuro Geiser

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

B17

B18

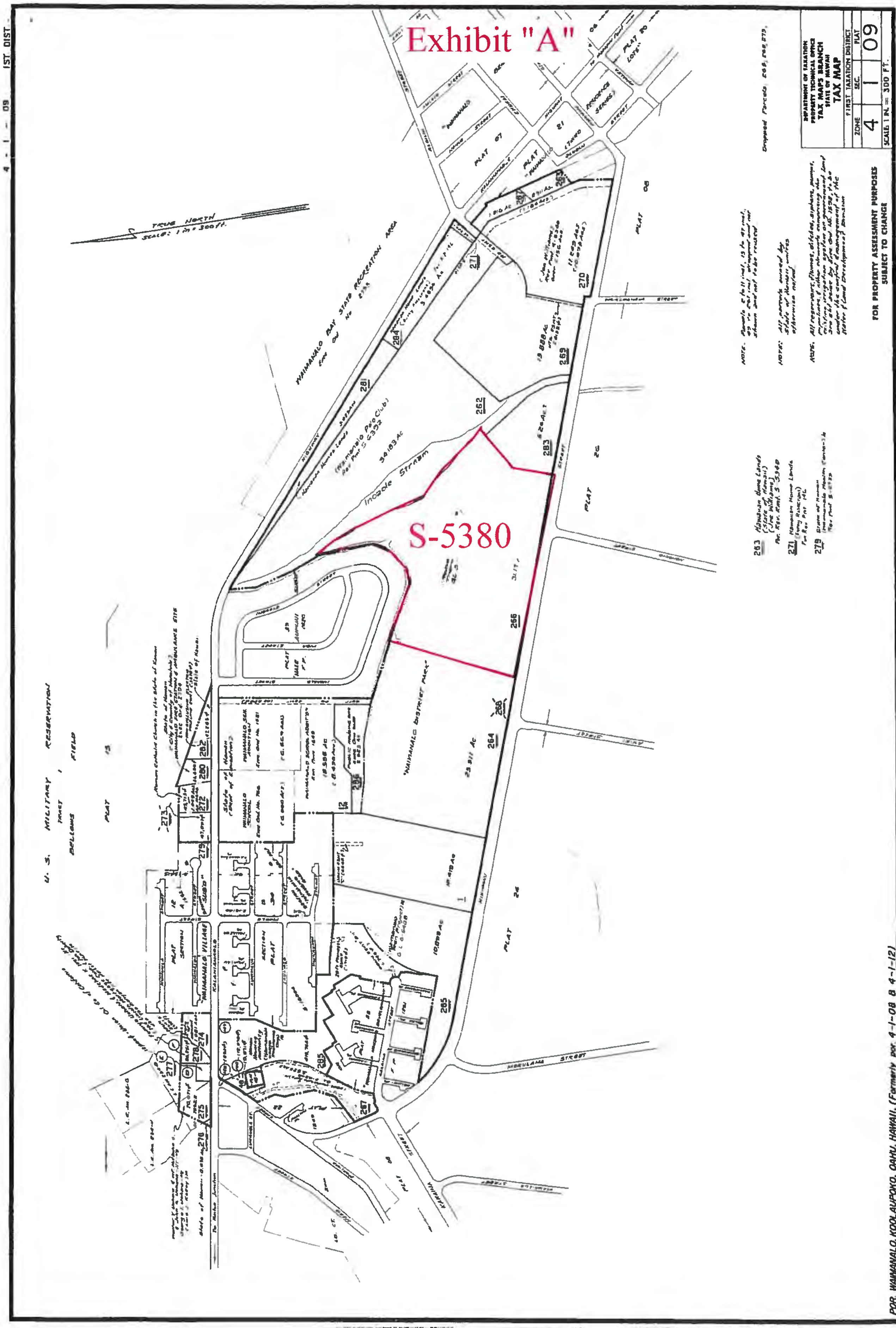


EXHIBIT "B"

ASSIGNMENT OF LEASE CALCULATIONS FOR
GENERAL LEASE NO. S-5380

Adjusted Depreciation Cost of Improvements or Renovations

Actual Cost:	$663,396.00
CCI (most recent):	11627
CCI (base):	11213
Expired Term:	314
Whole Term:	420

1. Adjusted Cost of Improvments or Renovations:

Actual Cost x CCI (most recent)/CCI (Base)

CCI (recent)	11627
CCI (base)	11213
CCIR/CCIB	1.04

Actual Cost x CCI(R)/CCI(B) =

$663,396.00	1.04	$687,889.53

2. Depreciation:

Adjust. Cost Impr./Whole Term x Expired Term =

$687,889.53	420	314	$514,279.00

3. Adjusted Depreciated Cost of Improvements:

Adjust cost - Depreciated cost =

$ 687,889.53	$ 514,279.00	$ 173,610.53

1. TOTAL NET CONSIDERATION		$ 926,280.00
2. Adj Cost of Imp/Renov	$ 687,889.53	
	$ 514,279.00	$ 173,610.53
3. Adj. cost of Trade Fixtures	$ -	
	$ -	
4. Excess		$ 752,669.47
5. Premium	Percentage: 10%	$ 75,267.00

Total Consideration:	$ 1,000,000.00
Less Inventory:	$ 73,720.00
Net Consideration:	$ 926,280.00

B19

STATE OF HAWAII
DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESOURCE MANAGEMENT DIVISION
HONOLULU, HAWAII 96814

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

Subject: REQUEST FOR APPROVAL TO WITHDRAW SIX ENCUMBERED LAND PARCELS FROM GOVERNOR'S EXECUTIVE ORDER NO. 4535 AND RE-SET ASIDE TO THE DEPARTMENT OF LAND AND NATURAL RESOURCES PURSUANT TO ACT 90, SLH 2003, CODIFIED AS CHAPTER 166E, HAWAII REVISED STATUTES, TMK NOS. 1ST DIV/4-1-008:046, 1ST DIV/4-1-013:017, 1ST DIV/4-1-027:005, 1ST DIV/5-8-001:038, ISLAND OF OAHU

Authority: Section 166E-3, Hawaii Revised Statutes (HRS)

Land Area: 44.866 gross acres, more or less

Tax Map Keys: (1) 4-1-008:046, (1) 4-1-013:017, (1) 4-1-027:005, (1) 5-8-001:038

Land Status: Encumbered by Governor's Executive Order No. 4535

BACKGROUND:

Act 90, Session Laws of Hawaii (SLH 2003), established the Non-Agricultural Park Lands Program to which certain public lands classified for agricultural use by DLNR should be transferred to Department of Agriculture (DOA) in a manner consistent with article XI, section 10 of the State Constitution. Therefore, Hawaii Revised Statutes, Chapter 166E entitled Non-Agricultural Park Lands was established. Under section HRS 166-E transfer and management of Non-Agricultural Park Lands and related facilities to the DOA, "Upon mutual agreement and approval of the Board (of Agriculture) and the Board of Land and Natural Resources, the DOA may accept the transfer of and manage certain qualifying non-agricultural park lands..." Further, the program shall include the following conditions pertaining to encumbered Non-Agricultural Park Lands:

(1) The lessee or permittee shall perform in full compliance with the existing lease or permit;
(2) The lessee or permittee shall not be in arrears in the payment of taxes, rents, or other obligations owed to the State or any county;
(3) The lessee's or permittee's agricultural operations shall be economically viable...

B20

Governor's Executive Order No. 4535 dated August 29, 2017 transferred a total of 25 leases and revocable permits without the approval of the Board of Agriculture. DOA declined to formally accept certain lease/revocable permits for transfer until additional due diligence was completed. When DOA staff reviewed the lease files and performed site visits to each of the corresponding premises, it was determined that certain of the leases and revocable permits were not in compliance with lease provisions or not suitable for farming activities, and therefore, are unacceptable for transfer. DOA is requesting that the following leases and revocable permit be reset aside to DLNR.

Doc No.	TMK	State Land Use	Leased Area (Acres)
gl5491	(1) 4-1-008:046	Agriculture	5.055
gl5309	(1) 4-1-013:017	Agriculture	4.134
gl5313	(1) 4-1-027:005	Agriculture	1.146
rp7825	(1) 5-8-001:038	Agriculture	2.164

RECOMMENDATION:

That the Board of Agriculture approve staff's request to have the lands identified and listed above and delineated on the attached maps as Exhibits "A" be withdrawn from the respective Governor's Executive Order No. 4535 and re-set aside to DLNR.

Respectfully submitted,

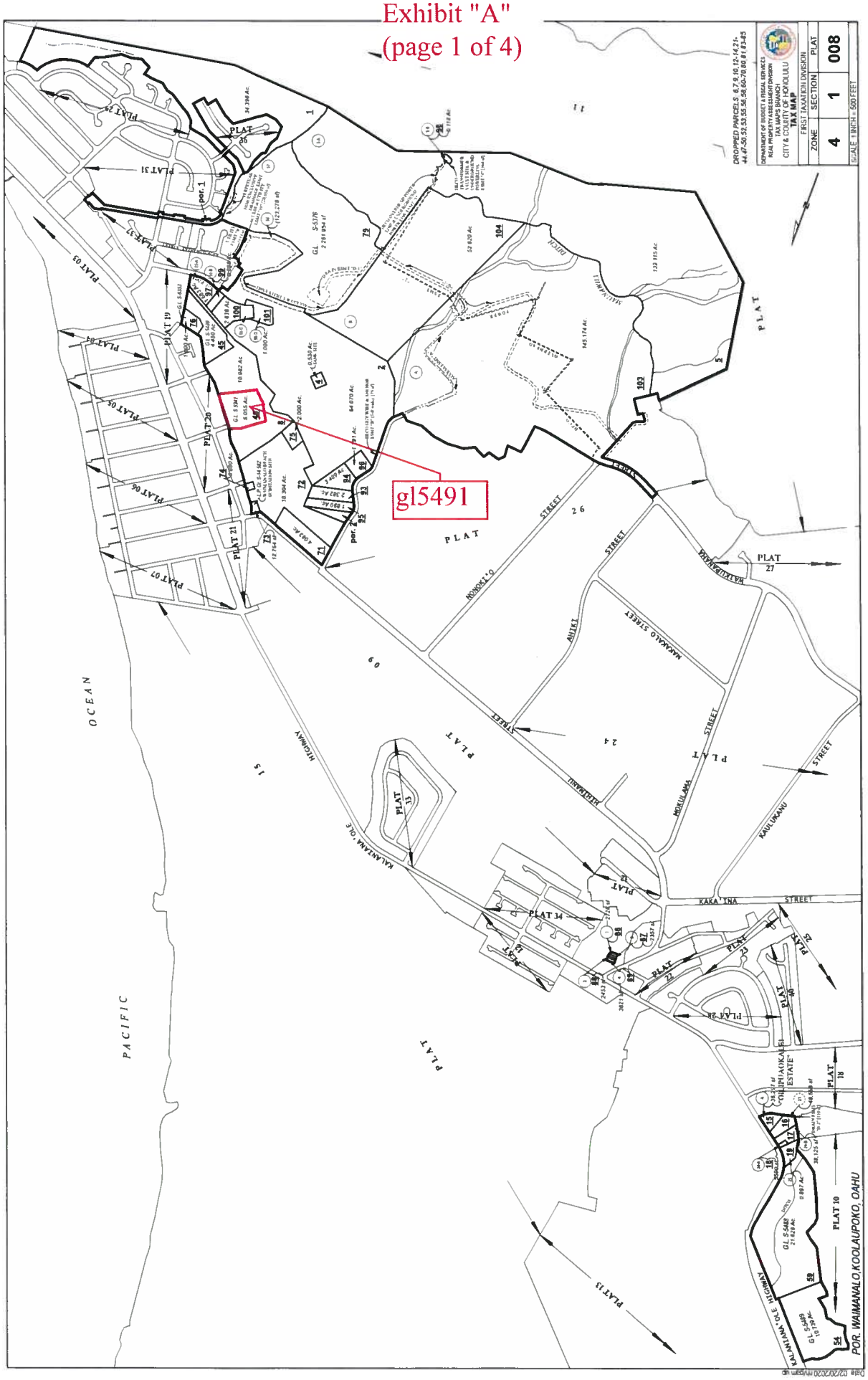
FOR BRIAN KAU, P.E.
Administrator and Chief Engineer
Agricultural Resource Management Division

Attachment – Exhibit "A"

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

Exhibit "A"
(page 1 of 4)



B22

Exhibit "A"
(page 2 of 4)

B23

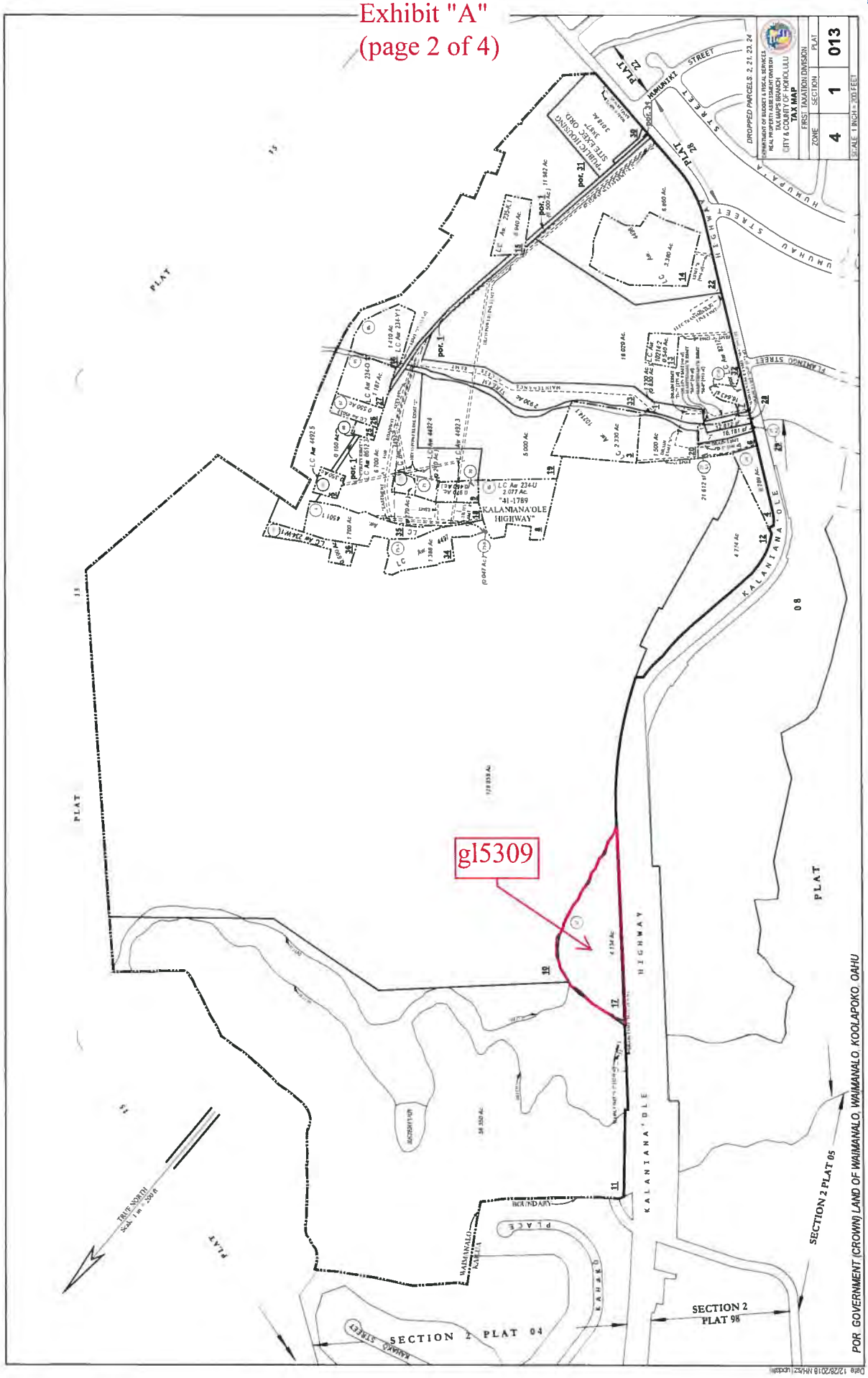
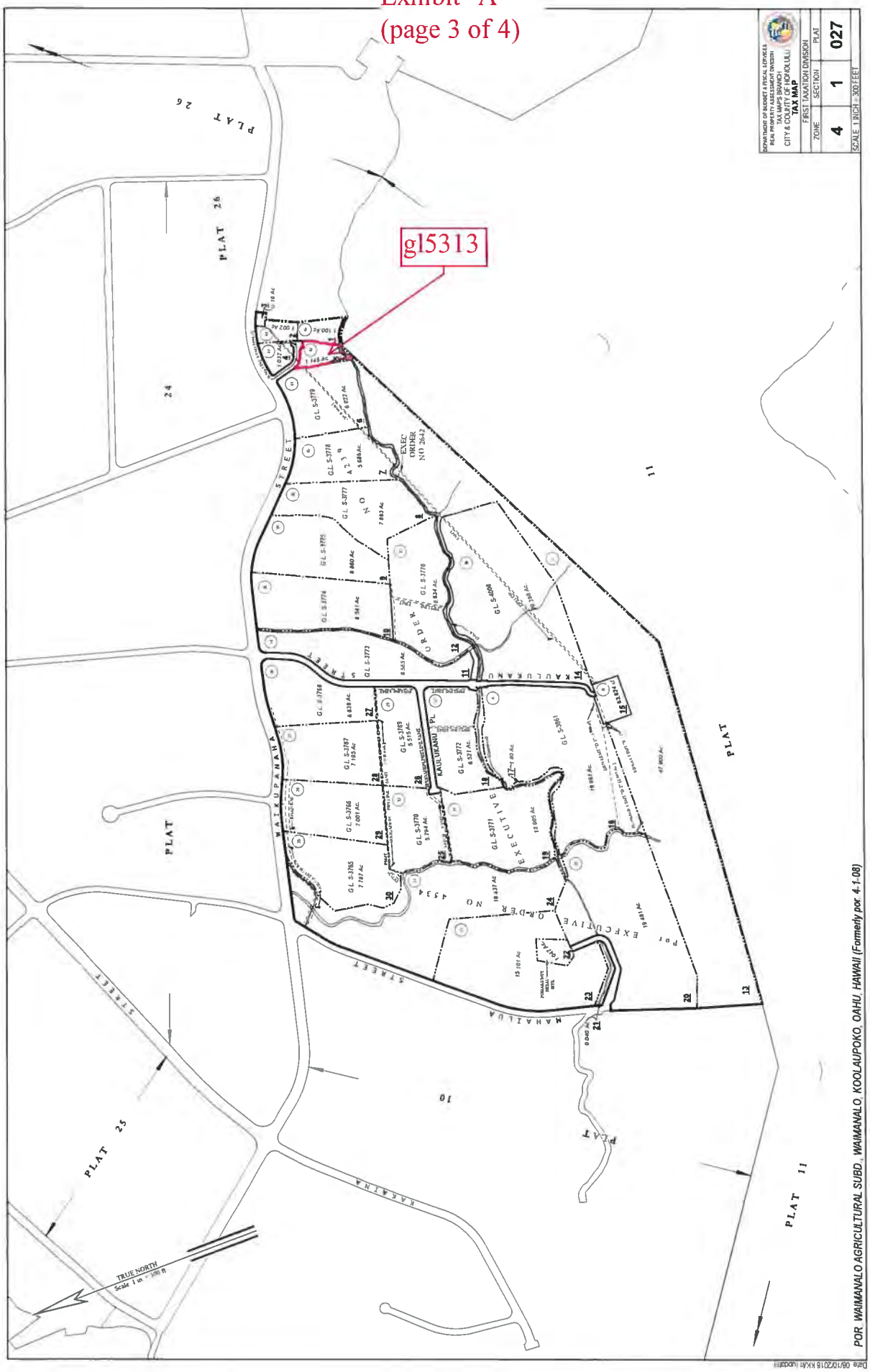
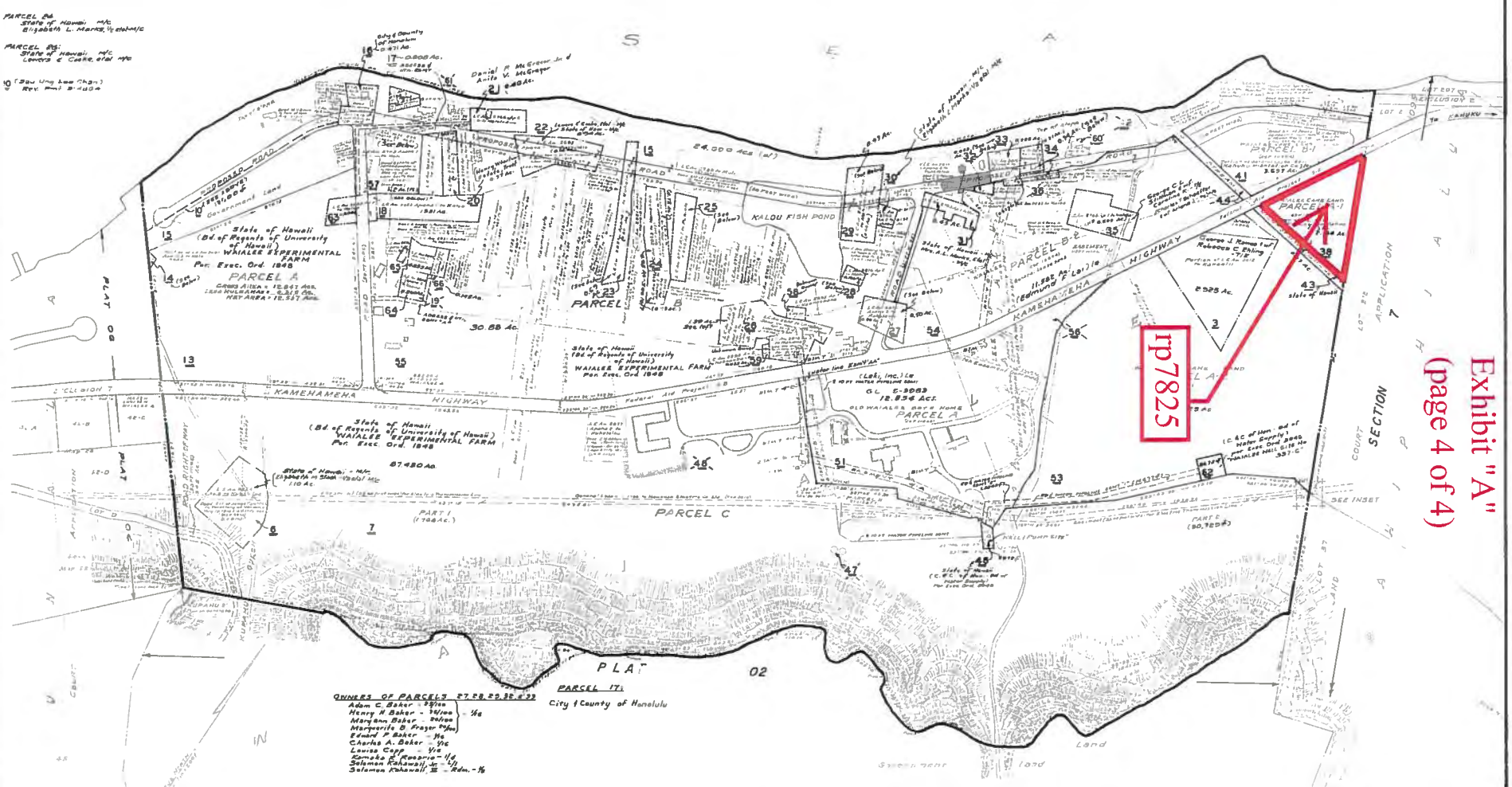


Exhibit "A"
(page 3 of 4)



B24

5-8-01 FIRST DIV.



DWG NO. 1779 (Revised)
SOURCE: M.T.S. PLAT 2147-B
BY: MM/J.M. March 19, 1982

Por. WAIALEE, KOOLAULOA, OAHU

DEPARTMENT OF TAXATION
TAXATION MAPS BUREAU
STATE OF HAWAII
TAX MAP

FIRST ZONE	SEC.	DIVISION PLAT
5	8	01

CONTAINING PARCELS
SCALE: 1 IN. = 200 FT.

B25

State of Hawaii
Department of Agriculture
Plant Industry Division
Plant Quarantine Branch
Honolulu, Hawaii

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

SUBJECT: Request to Designate the Islands of Oahu and Lanai as Expanded Coffee Leaf Rust Infested Areas, Pursuant to Plant Quarantine Interim Rule 20-1, Subject to Quarantine Measures to Prevent the movement of Coffee Leaf Rust, *Hemileia vastatrix*, on Coffee Plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. Including Hybrids and Varietals) and Plant Parts Such as Roasted and Unroasted Beans, Fruits, Leaves, Stems, Twigs, Cuttings, Wood, Logs, and Mulch or Greenwaste, Used Coffee Related Packing Materials Such as Coffee Bags, and Any Equipment Used to Harvest, Transport or Process Coffee Plants or Plant Parts, All of Which are Hosts or Harbor the Fungus, Except by Permit.

I. Introduction

The Hawaii Department of Agriculture (HDOA) Plant Quarantine Branch (PQB) is proposing to expand the coffee leaf rust (CLR), *Hemileia vastatrix,* quarantine areas that are designated in Plant Quarantine Interim Rule 20-1 (See Appendix A – Maui and Hawaii Island) to now include the islands of Oahu and Lanai, to restrict the movement of all *Coffea spp.* plants and plant parts, including green waste, and used coffee packing, harvesting, processing and transporting equipment, to prevent the spread of CLR.

PATHOGEN: CLR, *H. vastatrix*, is a devastating coffee pathogen and was first discovered in Sri Lanka in 1869 and has subsequently spread to all major coffee producing areas worldwide. CLR can cause severe defoliation of coffee plants resulting in premature defoliation, greatly reducing photosynthetic capacity. Depending on CLR prevalence in a given year, both vegetative and berry growth are greatly reduced. There are multiple long-term impacts of CLR, including dieback, and results in an impact to the following years' crops, with estimated losses ranging from 30 percent to 80 percent.

SYMPTOMS: Initial symptoms of CLR are yellow-orange circular spots on the upper sides of the leaves, followed by yellowish-orange powdery rust on the underside of

C116

leaves. The fungus spreads rapidly via wind and water and eventually leads to premature leaf drop, slow development of fruits, and potentially the death of the plant.

II. Background

On October 21, 2020, leaf samples displaying CLR symptoms from managed coffee plants in the Haiku area of Maui were turned in to Maui HDOA staff. Based on physical characteristics and host expression, HDOA staff tentatively identified the pathogen as CLR. Subsequent surveys on Maui conducted after the initial detection found plants with similar symptoms at five additional locations, including wild coffee.

On October 23, 2020, HDOA sent a memo to members of the coffee industry throughout the state to alert them to the situation. Samples were submitted to the University of Hawaii College of Tropical Agriculture and Human Resources (UH-CTAHR) and the U.S. Department of Agriculture (USDA) National Identification Services (NIS) for identification confirmation. On October 26, 2020, a statewide press release of the CLR detection was issued and UH-CTAHR also tentatively identified the pathogen as CLR. USDA NIS confirmed the identification as CLR, *H. vastatrix* on October 29, 2020.

On October 26, 2020, HDOA staff found a coffee plant suspected to be infected with CLR on the east side of Hawaii Island. That sample was submitted to USDA Agricultural Research Services (ARS). On October 29, 2020, USDA ARS tentatively identified the sample as CLR. This sample was also submitted to USDA NIS for formal confirmation. HDOA notified coffee industry members on October 29, 2020 and a statewide press release was issued on October 30,2020.

On October 31, 2020, UH-CTAHR staff received a report of CLR on the west side of Hawaii Island. That sample was submitted to USDA ARS and tentatively identified as CLR as well. This sample was submitted for formal confirmation by USDA NIS.

On November 10, 2020, HDOA was notified that USDA NIS determined the East Hawaii Island samples were negative for CLR; however, they confirmed the West Hawaii samples as positive.

On November 20, 2020, Plant Quarantine Interim Rule 20-1 went into effect.

In early January 2021, HDOA received notification of possible CLR on the island of Lanai. On January 15, 2021, HDOA preliminarily identified a sample as CLR. Subsequently, six samples were submitted to the USDA NIS for formal confirmation.

On January 22, 2021, HDOA received confirmation that the samples from Lanai were positive for CLR.

On January 25, 2021, the Hawaii Agriculture Research Center located in Maunawili, Oahu submitted samples of possible CLR collected on its property. HDOA submitted samples to USDA NIS for formal confirmation on January 27, 2021. On February 5, 2021, USDA NIS confirmed the presence of CLR.

On February 2, 2021, samples were collected from Lyon Arboretum, located in Manoa, Oahu, to be examined for possible CLR. Samples were submitted to USDA NIS for confirmation. On February 9, 2021, the samples were confirmed as positive for CLR.

Currently, HDOA is continuing its efforts to survey Maui, Hawaii Island, Oahu, and Kauai to determine the full extent of the infestation within the State. Partners on both Lanai and Molokai have been informed and asked to look for the disease. To date, no positive samples have been found on Molokai or Kauai.

III. Procedural Background

Pursuant to 150A-9.5, Hawaii Revised Statutes, the HDOA established Plant Quarantine Interim Rule 20-1 on November 20, 2020. This interim rule shall not be effective for more than one year.

Section 4-72-4.5, Hawaii Administrative Rules (HAR), provides that the designated infested area may be expanded by: receiving advice from qualified persons with relevant expertise; identifying the revised geographical extent of the infestation; Board action at a meeting on the island where the new infestation has occurred, after written notice to industry groups likely to be affected; and after the Board's action, certain public notification and industry notification requirements, either prior to the effective date of the Board's action or within 12 days of the Board's action, as applicable.

Due to the Governor's emergency proclamations relating to the COVID-19 Pandemic, all public meetings may be conducted virtually. Affected industry members were notified verbally that the Board would be considering the proposed quarantine expansion at the Hawaii Coffee Association CLR meeting on Thursday, March 11, 2021. Written notice about the proposed quarantine expansion was provided to industry associations on Monday, March 15, 2021. A departmental press release was issued on Tuesday, March 16, 2021.

C118

IV. Authority

Chapter 4-72, HAR, the HDOA's Plant and Non-Domestic Animal Quarantine, Plant Intrastate Rules, regulates the intrastate movement of plants, generally. Section 4-72-3, HAR, requires inspection of propagative plants and plant parts prior to being transported between islands of the State. Section 4-72-4, HAR, prohibits interisland movement of commodities infested with a pest unless treated with a pesticide that exterminates the pest. An interim rule provides the means for quarantine and safeguard measures to restrict or prohibit the movement of pests and their plant or commodity hosts to prevent the spread and establishment of pests that are detrimental to agriculture, horticultural industries, and forest lands on uninfested islands and in uninfested localities of the State.

V. Proposed Interim Rule Expansion

The current interim rule establishes a quarantine to restrict the movement of coffee plants (*Coffea arabica*, *C. canephora* and other *Coffea spp.*, including hybrids and varietals), plant parts such as roasted and unroasted beans, fruits, leaves, stems, twigs, cuttings, wood, logs, and mulch or greenwaste, used coffee-related packing materials such as coffee bags and any equipment used to harvest, transport or process coffee plants or plant parts from the island of Maui and Hawaii Island to other non-infested islands in the State to prevent the further spread and establishment of CLR, *H. vastatrix,* found on coffee plants.

Based on the most recent findings on Oahu and Lanai, the proposed expansion of the quarantine shall include the entire islands of Oahu and Lanai, subject to the same mitigation measures as already stated in Plant Quarantine Interim Rule 20-1. See Appendix B for Plant Quarantine Interim Rule 20-1, which includes Oahu and Lanai.

Impact of Quarantine Expansion: CLR will severely impact commercial coffee production on Oahu. Currently, there is no large-scale commercial coffee production on Lanai, but the presence of CLR there would almost eliminate the likelihood of future commercial production. Productivity will continue to decline if effective measures are not taken immediately to control the spread of this disease. Economic burdens will be placed on the coffee growing and roasting industries. Feral coffee plants on forest and private lands will impact the ability to contain the spread of CLR within infested islands.

Boundaries of Proposed Expanded Quarantine Zones: HDOA proposes to expand the quarantine to include the entire islands of Oahu and Lanai. Additional CLR infected areas on Oahu and Lanai, as well as the expanding infestations on Maui and Hawaii

Island, increase the likelihood that other infested areas may continue to be found as more extensive sampling occurs. It is suspected that the area infested with CLR is larger than what is currently known. To protect Kauai and Molokai, expanding the restrictions of the inter-island movement of potentially infected material to include Oahu and Lanai is critical. Past quarantines utilized established, defined quarantine zones within a specific island (ex: Banana Bunchy Top and Coffee Berry Borer on Hawaii Island); however, the established quarantine zones were quickly breached, and island-wide spread quickly occurred. Focusing limited resources at the ports of entry allows for much greater control of the spread of host materials.

Quarantine Exceptions: The expansion of the current interim rule will not change quarantine restrictions and still allows the movement of green coffee beans, roasted coffee beans, and coffee plants and plant parts for specific purposes under permits issued by the PQB.

VI. Advisory Subcommittee Review

This request was submitted to the Advisory Subcommittee on Plants and the Advisory Committee on Fungi for their review and recommendation. Their recommendations and comments are below:

1. I recommend approval ____/ ____ disapproval that the unrestricted movement of coffee plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. including hybrids and varietals) and plant parts such as roasted and unroasted beans, fruits, leaves, stems, twigs, cuttings, wood, logs, and mulch or greenwaste, used coffee related packing materials such as coffee bags, and any equipment used to harvest, transport or process coffee plants or plant parts, all of which are hosts or harbor the fungus, Coffee Leaf Rust, *Hemileia vastatrix*, from the Islands of Oahu and Lanai constitutes justifying an expansion of the infested areas indicated in Plant Quarantine Interim Rule 20-1.

ADVISORY COMMITTEE ON PLANTS:

Dr. Susan Schenck: Recommends Approval.

Comments: none.

Dr. J. B. Friday: Recommends Approval.

C120

Comments: "The spread of coffee leaf rust from Maui to Oahu and Lanai is regrettable but not unforeseen. Efforts to slow the spread to Kauai and Molokai, both of which islands have commercial coffee production, are useful in that they will give growers on those islands time to prepare before the rust spreads to their islands."

External Expert, University of Hawaii, College of Tropical Agriculture and Human Resources:

Andrea Kawabata: Recommends Approval.

Comments: none.

ADVISORY COMMITTEE ON FUNGI:

Dr. Susan Schenck: Recommends Approval.

Comments: none.

Mr. George Wong: Recommends Approval.

Comments: "Actually, coffee leaves are the part of the plant that is infected, but it can't hurt to include other items associated with the coffee plant since the uredospores can readily fall onto anything that is near the plant."

Dr. David Clements: Recommends Approval.

Comments: none.

Dr. A. Chris Whelen: Recommends Approval.

Comments: none.

Dr. Raquel Wong: Recommends Approval.

Comments: none.

Dr. Stephen Ferreira: Recommends Disapproval.

Comments: Please refer to comments below.

2. I recommend approval ____/ ____ disapproval to adopt an expansion of the infested areas listed in Plant Quarantine Interim Rule 20-1 to restrict the movement of coffee plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. including hybrids and varietals) and plant parts such as roasted and unroasted beans, fruits, leaves, stems, twigs, cuttings, wood, logs, and mulch or greenwaste, used coffee related packing materials such as coffee bags, and any equipment used to harvest, transport or process coffee plants or plant parts, all of which are hosts or harbor the fungus, Coffee Leaf Rust, *Hemileia vastatrix*, to include the entire islands of Oahu and Lanai.

ADVISORY COMMITTEE ON PLANTS:

Dr. Susan Schenck: Recommends Approval.

Comments: none.

Dr. J.B. Friday: Recommends Approval.

Comments: "Coffee leaf rust is easily spread not only on coffee plants but on machinery and equipment used in coffee plantations. Good inter-island biosecurity can help slow the spread of this disease between islands."

External Expert, University of Hawaii, College of Tropical Agriculture and Human Resources:

Andrea Kawabata: Recommends Approval.

Comments: none.

ADVISORY COMMITTEE ON FUNGI:

Dr. Susan Schenck: Recommends Approval.

Comments: none.

Mr. George Wong: Recommends Approval.

CVR

Comment: "I recommend that Molokai and Kauai also be included even though Coffee Rust has not been identified from those islands. All it takes is a couple of leaves from an infected plant, which could have thousands of uredospores on them and can be readily dispersed by wind to expand its distribution in Hawaii."

Dr. David Clements: Recommends Approval.

Comments: none.

Dr. A. Chris Whelen: Recommends Approval.

Comments: none.

Dr. Raquel Wong: Recommends Approval.

Comments: none.

Dr. Stephen Ferreira: Recommends Disapproval.

Comments: "Absent an effort to eradicate the presence of Coffee Rust on coffee already established on Maui, I can **not** support any attempt to restrict movement of coffee rust between the islands. The reason for this viewpoint is that coffee rust is very efficiently dispersed and spread via wind movement. The impact controlled spread by human intervention makes no sense since it spreads effectively via wind. It's been long before it was discovered and has already likely been distributed (though not yet discovered) to the other islands. Restricting movement via plant materials or containers and bags will not likely impact distribution as the fungus is so readily distributed naturally via wind.

If the department actively implementing an eradication effort (which I would not recommend) I could somewhat support the change in rules for restricting movement. But with an active attempt to eradicate, human interventions for restricting movement make no sense from a scientific perspective."

STAFF RECOMMENDATION: Based upon the recommendation and comments of the Advisory Subcommittee on Plants, Advisory Subcommittee on Fungi, and University of Hawaii outside expertise, the PQB recommends approval of the expansion of the Plant Quarantine Interim Rule 20-1 to include Oahu and Lanai as expanded quarantine areas.

Coffee Rust Interim Rule Expansion - Oahu & Lanai
Board of Agriculture
March 23, 2021

Respectfully Submitted,

JONATHAN K. HO
Acting Manager, Plant Quarantine Branch

CONCURRED:

BECKY N. AZAMA
Acting Administrator, Plant Industry Division

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

C124

APPENDIX A C128

DAVID Y. IGE
Governor

JOSH GREEN
Lt. Governor

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

MORRIS M. ATTA
Deputy to the Chairperson

State of Hawaii
DEPARTMENT OF AGRICULTURE
1428 South King Street
Honolulu, Hawaii 96814-2512
Phone: (808) 973-9600 FAX: (808) 973-9613

HAWAII DEPARTMENT OF AGRICULTURE

PLANT QUARANTINE INTERIM RULE 20-1

Prohibits the Intrastate Movement of Coffee Plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. Including Hybrids and Varietals) and Plant Parts Such as Green Beans, Fruits, Leaves, Stems, Twigs, Cuttings, Wood, Logs, and Mulch or Greenwaste, Used Coffee-Related Packing Materials Such as Coffee Bags, and Any Previously-Used Equipment Used to Harvest, Transport, or Process Coffee Plants or Plant Parts, All of Which Are Potential Carriers of the Fungus, Coffee Leaf Rust, *Hemileia vastatrix*, From the Island of Maui, Hawaii Island, or Any Other Island Confirmed with Coffee Leaf Rust
Except by Permit Issued by the Hawaii Department of Agriculture

Under authorization granted in Section 150-9.5, Hawaii Revised Statutes (HRS), the Hawaii Department of Agriculture (Department) hereby establishes this interim rule to impose a quarantine on the movement of coffee plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. including hybrids and varietals), plant parts such as green beans, fruits, leaves, stems, twigs, cuttings, wood, logs, and mulch or greenwaste, used coffee-related packing materials such as coffee bags, and any previously-used equipment used to harvest, transport, or process coffee plants or plant parts from the Island of Maui and Hawaii Island. These quarantine areas are established to prevent the spread of the Coffee Leaf Rust, *Hemileia vastatrix,* a fungal pathogen, from areas infested by *H. vastatrix* to uninfested areas within the State.

Movement or transportation of coffee plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. including hybrids and varietals), plant parts such as green beans, fruits, leaves, stems, twigs, cuttings, wood, logs, and mulch or greenwaste, used coffee-related packing materials such as coffee bags, and any previously-used equipment used to harvest, transport, or process coffee plants or plant parts from the Island of Maui and Hawaii Island is prohibited except by permit issued by the Department: 1) for green coffee beans for roasting, or other non-propagative plant parts, that are shipped to an approved facility located in an uninfested area that is located at least two miles from a commercial coffee growing area, or are subjected to an approved treatment; 2) for plants from Department certified nurseries, green coffee beans for roasting, or other non-propagative plant parts, that are transshipped through an uninfested area of the State with a final destination outside the State; 3) for previously-used equipment that is designed to harvest, process or transport coffee plants or plant parts; 4) for plants and plant parts for scientific studies or other diagnostic uses at approved facilities, provided the contents and packing materials used for shipping are subjected to a treatment approved by the PQB chief after the contents are unloaded; 5) coffee plants, beans for roasting, other non-propagative plant parts, used coffee bags, and previously-used coffee harvesting, processing or transporting equipment, that are shipped between infested areas; and 6) for limited quantities of coffee plants for propagation from an infested area to a non-infested area, subject to a one-year quarantine in a state-run facility, provided that the Board of Agriculture may reduce the quarantine period. All movement is subject to inspection and approved mitigation and decontamination

measures. This interim rule does not affect the movement of roasted coffee beans, or restrict the export of coffee plants and plant parts, green coffee beans, used coffee bags, or other CLR carriers, provided they are shipped directly from an infested area to a destination outside of the State.

Any person who violates this rule shall be guilty of a misdemeanor and fined not less than $100. The provisions of HRS section 706-640 notwithstanding, the maximum fine shall be $10,000. For a second offense committed within five years of a prior conviction, the person or organization shall be fined not less than $500 and not more than $25,000.

This interim rule shall become effective on Friday, November 20th, 2020 and is valid for no longer than one year from its inception.

Phyllis Shimabukuro-Geiser

PHYLLIS SHIMABUKURO-GEISER
~~Chairperson, Board of Agriculture~~

APPENDIX B C120

DAVID Y. IGE
Governor

JOSH GREEN
Lt. Governor

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

MORRIS M. ATTA
Deputy to the Chairperson

State of Hawaii
DEPARTMENT OF AGRICULTURE
1428 South King Street
Honolulu, Hawaii 96814-2512
Phone: (808) 973-9600 FAX: (808) 973-9613

HAWAII DEPARTMENT OF AGRICULTURE

PLANT QUARANTINE INTERIM RULE 20-1
(To Include Oahu and Lanai as expanded Quarantine Areas)

Prohibits the Intrastate Movement of Coffee Plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. Including Hybrids and Varietals) and Plant Parts Such as Green Beans, Fruits, Leaves, Stems, Twigs, Cuttings, Wood, Logs, and Mulch or Greenwaste, Used Coffee-Related Packing Materials Such as Coffee Bags, and Any Previously-Used Equipment Used to Harvest, Transport, or Process Coffee Plants or Plant Parts, All of Which Are Potential Carriers of the Fungus, Coffee Leaf Rust, *Hemileia vastatrix*, From the Islands of Maui, Hawaii Island, Oahu, and Lanai
Except by Permit Issued by the Hawaii Department of Agriculture

Under authorization granted in Section 150A-9.5, Hawaii Revised Statutes (HRS), the Hawaii Department of Agriculture (Department) hereby establishes this interim rule to impose a quarantine on the movement of coffee plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. including hybrids and varietals), plant parts such as green beans, fruits, leaves, stems, twigs, cuttings, wood, logs, and mulch or greenwaste, used coffee-related packing materials such as coffee bags, and any previously-used equipment used to harvest, transport, or process coffee plants or plant parts from the Islands of Maui, Hawaii Island, Oahu and Lanai. These quarantine areas are established to prevent the spread of the Coffee Leaf Rust, *Hemileia vastatrix,* a fungal pathogen, from areas infested by *H. vastatrix* to uninfested areas within the State.

Movement or transportation of coffee plants (*Coffea arabica*, *C. canephora* and other *Coffea* spp. including hybrids and varietals), plant parts such as green beans, fruits, leaves, stems, twigs, cuttings, wood, logs, and mulch or greenwaste, used coffee-related packing materials such as coffee bags, and any previously-used equipment used to harvest, transport, or process coffee plants or plant parts from the Island of Maui and Hawaii Island is prohibited except by permit issued by the Department: 1) for green coffee beans for roasting, or other non-propagative plant parts, that are shipped to an approved facility located in an uninfested area that is located at least two miles from a commercial coffee growing area, or are subjected to an approved treatment; 2) for plants from Department certified nurseries, green coffee beans for roasting, or other non-propagative plant parts, that are transshipped thorough an uninfested area of the State with a final destination outside the State; 3) for previously-used equipment that is designed to harvest, process or transport coffee plants or plant parts; 4) for plants and plant parts for scientific studies or other diagnostic uses at approved facilities, provided the contents and packing materials used for shipping are subjected to a treatment approved by the PQB chief after the contents are unloaded; 5) coffee plants, beans for roasting, other non-propagative plant parts, used coffee bags, and previously-used coffee harvesting, processing or transporting equipment, that are shipped between infested areas; and 6) for limited quantities of coffee plants for propagation from an infested area to a non-infested area, subject to a one-year quarantine in a state-run facility, provided that the Board of Agriculture may reduce the quarantine period. All movement is subject to inspection and approved mitigation and decontamination

measures. This interim rule does not affect the movement of roasted coffee beans, or restrict the export of coffee plants and plant parts, green coffee beans, used coffee bags, or other CLR carriers, provided they are shipped directly from an infested area to a destination outside of the State.

Any person who violates this rule shall be guilty of a misdemeanor and fined not less than $100. The provisions of HRS section 706-640 notwithstanding, the maximum fine shall be $10,000. For a second offense committed within five years of a prior conviction, the person or organization shall be fined not less than $500 and not more than $25,000.

This interim rule shall become effective on Friday, November 20th, 2020 and is valid for no longer than one year from its inception.

Effective (Insert Date Here), pursuant to section 4-72-4.5, Hawaii Administrative Rules, the quarantine areas are expanded to include the Islands of Oahu and Lanai.

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

State of Hawaii
Department of Agriculture
Plant Industry Division
Plant Quarantine Branch
Honolulu, Hawaii

March 23, 2021

Board of Agriculture
Honolulu, Hawaii

Subject: Request to Review the Petition from Tracie Matsumoto, U.S. Department of Agriculture, Agricultural Research Service, Daniel K. Inouye Pacific Basin Agricultural Research Station, to Shorten or Exempt the Duration of Quarantine for Tissue Cultured, Coffee Leaf Rust Resistant Coffee Plants, *Coffea* spp. subject to alternative propagation or import procedures

I. Summary Description of the Request

PQB NOTES: *The Plant Quarantine Branch (PQB) submittal for requests for petition review, as revised, distinguishes information provided by the applicant from procedural information and advisory comment and evaluation presented by PQB. With the exception of PQB notes, hereafter "PQB NOTES," the text shown below in Section II from page 2 through page 8 of the submittal was taken directly from Dr. Matsumoto's petition and subsequent written communications provided by the applicant, Dr. Tracie Matsumoto. For instance, the statements on pages 7 and 8, regarding effects on the environment are the applicant's statements in response to standard PQB questions and are not PQB's statements. This approach for PQB submittals aims for greater applicant participation in presenting import requests in order to move these requests to the Board of Agriculture (Board) more quickly, while distinguishing applicant provided information from PQB information. The portions of the submittal prepared by PQB, including procedural background, Summary of Proposed Shortening and Exemption of Quarantine, and Advisory Subcommittee Review, are identified as Sections II, III and V of the submittal, which start at pages 2, 3, and 8, respectively.*

We have a request to review the following request for exemption for 1-year quarantine for coffee permit 21-08-H-P1886:

COMMODITY: 1107 tissue cultured coffee plants, one shipment, *Coffea* species

C107

SHIPPER: David Lawson, Agristarts Inc.,1728 Kelly Park Road, Apopka, FL 32712 through cooperative project with Solene Pruvot-Woehl at World Coffee Research.

IMPORTER: Tracie Matsumoto, Supervisory Research Horticulturist, U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) Daniel K. Inouye Pacific Basin Agricultural Research Center (DKI PBARC), 65 Nowelo Street, Hilo, HI 96720

CATEGORY: Coffee plants are currently restricted for importation and require a 1-year quarantine prior to release to the importer. The importer may petition the Board of Agriculture to shorten or exempt the quarantine subject to alternative importation or propagation procedures to mitigate risk or pest entry and/or spread.

II. Procedural Background

Dr. Matsumoto's petition the Board for shortening and exempting the duration of quarantine is brought under Section 4-70-6, Hawaii Administrative Rules (HAR), which states: "Unless otherwise specified for specific plants in subsequent subchapters, the duration of quarantine shall be one year". However, Section 4-70-6 also states that the Board may exempt or shorten the period of quarantine under certain conditions of importation or propagation procedure.

To be considered by the Board, a petition must contain certain substantive items, specifically: (1) the alternative propagation or import procedures that would justify the shortening or exemption of the one-year quarantine; (2) a statement of the petitioner's interest in the subject matter; and (3) a statement of the reasons in support of the proposed shortening or exemption of quarantine. Dr. Matsumoto's petition appears to conform to these prerequisites for consideration and the information she provided is contained in Section IV.

PQB NOTES: *Advisory Subcommittee review is not a prerequisite for a petition to be submitted to the Board. However, due to the critical nature of the situation regarding coffee leaf rust's potential affect to the industry, the PQB had Dr. Matsumoto's petition reviewed by the Advisory Subcommittee on Plants and Advisory Committee on Fungi so that the Board would have a more complete picture of the petitioner's proposal.*

III. Summary of Proposed Shortening and Exemption of Quarantine

Dr. Matsumoto is requesting that the Board reduce the one-year quarantine by six (6) months for all plants imported under permit 21-08-H-P1886, subject to the proposed procedures listed in Section IV below; thus allowing the plants to be released immediately from quarantine.

IV. Information Provided by the Applicant in Support of the Application

PROJECT: The mission of the USDA ARS DKI PBARC Tropical Plant Genetic Resources and Disease Research Unit (TPGRDRU) is to support our stakeholders by safeguarding tropical fruits and nuts for future generations by efficiently and strategically collecting, conserving, regenerating, evaluating and distributing tropical genetic resources and information and by conducting research to enhance agricultural sustainability through improved plant growth and development, value added products and disease resistance and develop improved environmentally sound and economically beneficial practices for sustainable, diversified crop production. The imported coffee plants contain varieties that are resistant to Coffee Leaf Rust, the most devastating coffee disease that was recently found in Hawaii.

OBJECTIVE: Coffee has been a significant focus of the USDA ARS DKI PBARC TPGRDRU research and was recently added to our list of conserved crops for our germplasm repository in Hilo. Coffee Leaf Rust (CLR), *Hemileia vastatrix*, is the most devastating disease for coffee production. In anticipation that CLR would be found in Hawaii one day, this project aimed to find new CLR resistant cultivars suitable for the Hawaii Specialty Coffee Market. USDA ARS and Hawaii Agriculture Research Center (HARC) are participants in the World Coffee Research International Multi-Location Variety Trial. This trial aims to determine how 30 different coffee varieties perform in different coffee growing regions globally; many of these lines are resistant to CLR. We received the tissue cultured plants under permit 21-08-H-P1886 and started the 1-year quarantine on September 4, 2020. Once quarantine was complete, the goal was to do the field test of this material in Kalaheo, HI, with Kauai Coffee. However, with the recent discovery of CLR on Maui on October 20, 2020, and subsequent discovery in Kona, Lanai and Oahu evaluation of this material is critical for the Hawaii coffee industry and the plant material will be grown on in additional areas in Hawaii. To propagate these lines for evaluation, I would like to request an exemption for the 1-year quarantine to be reduced to 6 months.

C103

Although official quarantine will end, plants will be kept in tissue culture until enough material can be propagated for the trials. Plants will be acclimatized in the greenhouse for 6 months before being transferred into the fields that are infested with CLR. For all practical purposes, plants will be in quarantine like conditions for over 1 year. If the location does not have CLR, plants can be either sent in culture from PBARC and transferred to the greenhouse at the grower location or grown in the greenhouse and treated with fungicide at PBARC prior to shipment.

PROCEDURE: Plants were received and began quarantine on September 4, 2020.These plants had been in tissue culture in Florida for 7 years prior to shipment. Clones of these plants have been sent to 30 countries with no notation of diseases (see Agristart email in Appendix 4 – Letters). Since arriving and placed in quarantine at PBARC, plants are visually monitored for the presence of contaminants (bacterial and fungal diseases) in the growing media and plant and plants monitored for abnormal growth. All contaminated cultures are disposed by autoclaving and records are kept on temperature readings during sterilization cycle. Most bacterial and fungal contaminants will grow on the culture media that the plants are grown. However, as an added precaution, when propagation begins and before plants are released to the environment, we will remove a section of the plant and growing media will be subjected to disease indexing using protocols described (Thomas, 2004). Most fungal diseases are readily apparent when the plant is first placed into culture, however there is a possibility of latent bacterial contamination. Both fungal and bacterial growth will be evident in the media described by Thomas (2004). The bacteria *Xylella* require special media to grow, thus plants will be screened for *Xylella fastidiosa* as described (Minisavage et al, 1994; Harper et al., 2010). (Appendix 6 – Protocols)

DISCUSSION:

1. Person Responsible:

Tracie Matsumoto, Supervisory Research Horticulturist, USDA ARS DKI PBARC TPGRDRU, 64 Nowelo Street, Hilo, HI 96720. Cell: (808) 313-0712 FAX (808) 959-5470 tracie.matsumoto@usda.gov
Tracie Matsumoto has a PhD in Horticulture from Purdue University and a certificate in Plant Tissue Culture from the University of California, Riverside under Dr. Toshio Murashige has over 30 years working with plant tissue culture in both a commercial and research settings. She currently serves as the

Collateral Duty Biosafety Officer for the USDA ARS DKI PBARC. (Appendix 1 CV).

2. Safeguard Facility and Practices:

USDA ARS DKI PBARC, 64 Nowelo Street, Hilo, HI 96720. The USDA ARS DKI PBARC is not located near bodies of water and is on a sewer system. The perimeter of the property is enclosed by a fence and the building is locked to authorized personnel only. The property is monitored by security cameras. The facility is built to Biological Safety level 2 standards which includes but not limited to sealed windows and rodent control. There are functional autoclaves in both the laboratory building and greenhouse/headhouse facility and devitalization can be monitored by temperature recordings from the autoclave as well as biological indicators. Transfers are conducted in lockable rooms under certified Biological Safety Cabinets and the tissue culture room where the plants are stored is double locked with limited access. Personnel have years of experience working with tissue cultured plants and are trained and sign SOPs working with regulated material. Greenhouse is also locked with limited access to personnel and monitored by security cameras. Please see attached for map of facilities (Appendix 2-3).

Plants were received and began quarantine on September 4, 2020 in tissue culture. Roughly ten plants were placed in the same tissue culture vessel but was transferred to single tubes upon arrival. Tissue culture is a method to grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. This technique is used to clonally propagate plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens. These plants have been in culture in Florida for 7 years prior to shipment. Clones of these plants have been sent to 30 countries with no notation of diseases (e-mail from Agristart in Appendix 4 - letter).

While under quarantine, we monitor plants every 3 to 4 weeks for the presence of contaminants (bacterial and fungal diseases) in the growing media and plants monitored for abnormal growth. Since the growing media contains sugar, if accidentally exposed to non-sterile and filtered air, other contaminants can grow. Like food that is kept too long, will grow mold. All contaminated cultures are disposed by autoclaving and records are kept on temperature readings during sterilization cycle. Most fungal contaminants are readily visible on the plant tissue culture media and can be detected using the same nutrient media used to detect latent bacterial contaminants (Leifert et al, 1991). When propagation begins and before plants are released to the environment, sections of the plants and growing media will be subjected to disease indexing using protocols described (Thomas,

2004). This protocol has been found to be effective in detecting latent bacterial contamination in plants. If latent contaminants are detected, the cultures will be isolated and identified by PCR or through Plant Pathologist Dr. Lisa Keith. Since the bacteria *Xylella fastidiosa* requires special media that might not be detected, these will be specifically tested for as described (Minisavage et al, 1994; Harper et al., 2010). (Appendix 6 – Protocols)

3. Method of Disposition:

All contaminated cultures are disposed by autoclaving and records are kept on temperature readings during sterilization cycle. At the end of the quarantine, the plants will be propagated in tissue culture, acclimatized in the greenhouse for approximately 6 months at PBARC and placed at different grower locations around the state of Hawaii. Field plants times will vary depending on when adequate numbers of plants have been propagated. Plants will be in quarantine like conditions for over 1 year. If the location does not have CLR, plants can be either sent in culture from PBARC and transferred to the greenhouse at the grower location or grown in the greenhouse and treated with fungicide at PBARC prior to shipment. Plants have been and will be continued to be monitored weekly for presence of any diseases.

4. Abstract of Organism:

a. Organism's available levels of classification including scientific name. If common names are known then they should also be submitted.
   - Plantae, Gentianales, Rubiaceae, Coffea common name coffee

b. Organism's life history (e.g., biology, reproductive habits, temperature requirements, natural habitat, growth rate, biotic potential, size at maturity, longevity, etc.) including its dispersal capabilities.
   - The plants were in tissue culture for at least 7 years and clones have been distributed to over 30 countries (Appendix 4 - Letter)

c. What are the habitats (e.g., wet forest, ocean reef, etc.) and niche requirements?
   - Coffee does well in all environments and is grown on all islands in Hawaii.

d. What is the native range of the organism? Is it naturalized in Hawaii?
   - Coffee originated in the plateau in Ethiopia and is grown throughout the world in sub-tropical and tropical regions.

e. How might it become established in Hawaii?

- The plant is already established in Hawaii.

f. Has the species established viable populations beyond its native range?
- Yes, the species established viable populations beyond its native range.

g. What is the host range and what does it feed on? Are hosts and alternate hosts present in Hawaii and how abundant or widely is it distributed?
- Coffee is widely distributed throughout Hawaii in both cultivated and feral populations.

h. Is the species highly domesticated, cultivated or cultured for commercial purposes?
- Yes, coffee is grown for commercial production.

i. In its naturalized range, are there impacts to wild stocks, commercial species, aquaculture, aquarium and/or ornamental species, etc.?
- There is both feral and abandoned coffee throughout the islands.

j. List diseases or other pests of concern.
- For a complete list of infectious Diseases for Coffee please see attached APS chapter (Appendix 5). Tissue culture eliminates the threat of insect pest. Coffee Leaf Rust (*Hemileia vastatrix*) is the most damaging coffee disease and is already present in Hawaii. Coffee Berry Disease (*Colletotrichum kahawae*) is limited to Africa but presents a major economic threat to coffee production. Fungal and bacterial diseases should be detected with indexing methods (see attached protocol) except for Atrophy of Coffee Branches (*Xylella fastidiosa*) which will be specifically tested.

k. Does the organism have potential to be toxic and pathogenic?
- No, introduced coffee will not be toxic and pathogenic beyond existing cultivars in Hawaii.

5. Effects on the Environment:

a. Assessment of the probability of introduction and spread.
- The introduced coffee plants should pose no additional risk than existing coffee varieties. Some of these cultivars are resistant to Coffee Leaf Rust and Coffee Berry Disease which is beneficial to the coffee industry in Hawaii.

b. Assessment of potential economic and environmental consequences. What are the specific potential impacts to humans, animals, plants and natural resources in Hawaii?
   - Should not pose a threat to humans, animals, plants, and natural resources in Hawaii.

c. Assessment of the probability of establishment in Hawaii.
   - If production, bean quality and cupping are favorable, these cultivars should be favorable for the economic growth of Hawaii's coffee industry.

d. What specific safeguards or procedures are used to assure that the organism requested for import presents probably minimal or no significant effects on the environment?
   - Tissue culture eliminated the introduction of insect pests. Since plants are sterilized and grown in a media and environment suitable for fungal and bacterial growth. Most diseases should be readily apparent within a few days or weeks of initiation. Indexing for latent bacterial or fungal diseases decreases the chances of introduction even further (Appendix 4 – letters).

6. References:

Harper, S. J., Ward, L. I., and Clover, G. R. G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. Phytopathology 100:1282-1288.

Leifert, C., J.Y. Ritchie, and W.M. Waites. 1991. Contaminants of plant-tissue and cell cultures. World Journal of Microbiology and Biotechnology. 7: 452-469.

Minisavage, G.V., C.M. Thompson, D.L. Hopkins, R.M.V.B.C. Leite, and R. E. Stall. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology. 84: 456-461.

Thomas, P.2004. A three-step screening procedure for detection of covert and endophytic bacteria in plant tissue cultures. Current Sci. 87:67-72

V. Advisory Subcommittee Review:

This request was submitted to the Advisory Subcommittee on Plants and Advisory Committee on Fungi for their review and recommendations. Their recommendations and comments are as follows:

Are the proposed alternative importation and procedures for propagation or quarantine by Tracie Matsumoto, USDA-ARS, sufficient to justify a six-month reduction in the standard one-year quarantine for Coffee plants.

___ Yes
___ No (If "No", please explain and suggest appropriate procedures if applicable).

ADVISORY SUBCOMMITTEE ON PLANTS:

Dr. Susan Schenck: Yes.

Comments: "In my opinion tissue cultured plants should be treated differently that field grown plants. That would require a rule change. In this instance, it is critical to get fust resistant clones into production ASAP."

Dr. James B. Friday: Yes.

Comments: None.

Dr. Steven L. Montgomery: Yes.

Comments: None.

ADVISORY SUBCOMMITTEE ON FUNGI:

Dr. Raquel Wong: Yes.

Comments: "Proposed alternative importation and procedures is sufficient to justify a 6-month reduction. Based on the cleanliness of the source, housing and monitoring that is ongoing, and adequate destruction of any waste, the submitter has demonstrated item is free from clinical disease and will not increase the risk greater that locally grown plants have in developing disease."

Dr. Edward Desmond: Yes.

Comments: None.

Dr. George Wong: Yes.

Comments: None.

Dr. David Clements: Yes.

Comments: None.

STAFF RECOMMENDATION: Based upon the recommendations and comments of the Advisory Subcommittee on Plants and Advisory Subcommittee on Fungi, the PQB is recommending approval of this request.

Respectfully Submitted,

JONATHAN K. HO
Acting Manager, Plant Quarantine Branch

CONCURRED:

BECKY N. AZAMA
Acting Administrator, Plant Industry Division

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

Curriculum Vitae for Tracie K. Matsumoto

EDUCATION

1999 Ph.D. Horticulture, Purdue University, West Lafayette, IN (advisor Paul M. Hasegawa)
1994 M.S. Horticulture, University of Hawaii, Honolulu, HI (advisor Adelheid R. Kuehnle)
1991 B.S. Agriculture, University of Hawaii, Hilo, HI
1989 Plant Tissue Culture, University of California, Riverside, CA (Toshio Murashige)

EMPLOYMENT

2015 to present	Research Leader, USDA ARS DKI PBARC, Hilo, HI
2002-2015	Research Horticulturist, USDA ARS DKI PBARC, Hilo, HI
2001- 2002	Post-Doctoral Research Associate, Dept. Bio Sci, Purdue University
1999-2001	Post-Doctoral Research Associate, Center for Plant Environmental Stress Physiology Purdue University
1995-1999	Graduate Research Assistant, Purdue University
1987-1989	Laboratory Technician / Production Assistant ManagerTropculture Laboratories Ltd. Hilo, HI
1986-1987	Laboratory Technician, Hiromi's Nursery Hilo, HI
1985-1986	Laboratory Technician, Matsumoto Nursery Inc.

SELECT PUBLICATIONS

Puig, A.S., Marelli, J.P., Matsumoto, T.K., Keith, L.M. and Gutierrez, O.A. 2019. First Report of *Neofusicoccum parvum* Causing Pod Rot on Cacao in Hawaii. Plant Disease. https://doi.org/10.1094/PDIS-10-18-1719-PDN

Wraight, S.P., Wraight-Galaini, S., Castrillo, L.A., Griggs, M., Keith, L.M., **Matsumoto, T.K.** 2018. Collection, isolation, in vitro culture, and laboratory transmission of Hirsutella eleutheratorum (Hypocreales: Ophiocordycipitaceae) from coffee berry borer on Hawaii Island. Journal of Invertebrate Pathology. 157:53-66.

Wraight, S.P., Galaini-Wraight, S., Howes, R.L., Castrillo, L.A., Carruthers, R.I., Smith, R.H., **Matsumoto, T.K.**, Keith, L.M. 2018. Prevalence of naturally-occurring strains of *Beauveria bassiana* in populations of coffee berry borer *Hypothenemus hampei* on Hawaii Island, with observations on coffee plant-H. hampei-B. bassiana interactions. Journal of Invertebrate Pathology. 156:54-72.

Aristizabal, L.F., Shriner, S., Hollingsworth, R., Mascarin, G.M., Chavez, B., **Matsumoto, T**., Arthurs, S.P. 2018. Field sampling strategies for coffee berry borer (Coleoptra: Curculionidae: Scolytinae) infesting berries in coffee farms in Hawaii. International Journal of Tropical Insect Science. 38:418-426.

Matsumoto, T.K. and J. Lopez. 2016. Coffee harvest management by manipulation of coffee flowering with plant growth regulators. Acta Hort. 1130: 219:223.

Souza, F.V., Ergun, K., Vieria De Jesus, L., De Souza, E.H., Amorim, V., Skogerboe, D.M., **Matsumoto, T.K.**, Alves, A.A., Ledo, C., Jenderek, M.M. 2015. Droplet-vitrification and morphohistological studies of cryopreserved shoot tips of cultivated and wild pineapple genotypes. Plant Cell Tissue and Organ Culture. 124: 351-360.

Matsumoto, T.K., L.M. Keith, R.Y.M. Cabos, J.Y. Suzuki, D. Gonsalves and R. Thilmony. 2013. Screening promoters for *Anthurium* transformation using transient expression. Plant Cell Rep. 32:443-451.

Keith, L. M. and **T. K. Matsumoto.** 2013. First report of *Pestalotiopsis* leaf blotch on Mangosteen in Hawaii. Plant Disease. 97:146.

Keith, L. M., **T. K. Matsumoto.**, and G. T. McQuate. 2013. First report of *Dolabra nepheliae* associated with corky bark disease of langsat in Hawaii. Plant Disease.97:990

Keith, L. M., **T.K. Matsumoto,** F.T. and Zee. 2013. First report of *Calonectria* leaf spot on Ohelo in Hawaii. Plant Disease. 97:990.

Melzer, M.J., J.S. Sugano, D. Cabanas, K.K. Dey, B. Kandouh, D. Mauro, I. Rushanaedy, S. Srivastava, S. Watanabe, W.B. Borth, S. Tripathi, **T. Matsumoto**, L. Keith, D. Gonsalves,

J.S. Hu. 2012. First report of Pepper mottle virus infecting tomato in Hawaii. Plant Dis. 96(6):917.

Gonsalves, D., C. Gonsalves, J. Carr, S. Tripathi, **T. Matsumoto**, J. Suzuki, S. Ferreia and K. Pitz. 2012. Assaying for Pollen Drift from Transgenic 'Rainbow' to Nontransgenic 'Kapoho' Papaya under Commercial and Experimental Field Conditions in Hawaii. Tropical Plant Biology. 5: 153-160. DOI: 10.1007/s12042-011-9090-5

Hollingsworth, R., A. Lysy and **T.K. Matsumoto.** 2011. Preliminary study of genetic variation in Hawaiian isolates of *Beauveria bassiana* [Hypocreales, Clavicipitaceae]. Journalof Invertebrate Pathology. 106: 422-425.

Keith, L., **T. Matsumoto**, K. Nishijima, M. Wall and M. Nagao. 2011. Field survey and fungicide screening of fungal pathogens of rambutan (*Nephelium lappaceum*) fruit rot in Hawaii. HortScience. 46: 730-735.

Narasimhan, M.L., H. Lee, B. Damsz, N. K. Singh, J. I. Ibeas, **T. K. Matsumoto**, C.P. Woloshuk, and R.A. Bressan. 2003. Overexpression of a cell wall glycoprotein in *Fusarium oxysporum* increases virulence and resistance to a plant PR-5 protein. Plant J.36: 390-400

Matsumoto, T.K., A.J. Ellsmore, S.G. Cessna, P.S. Low, J.M. Pardo, R.A. Bressan and P.M. Hasegawa. 2002. An osmotically induced cytosolic Ca^{2+} transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of *Saccharomyces cerevisiae. J. Biol. Chem.* 277: 33075-33080.

Watad, A.A., D.J. Yun, **T. Matsumoto**, X. Niu, Y. Wu, A.K. Kononowicz, R.A. Bressan, and P.M. Hasegawa. 1998. Microprojectile bombardent mediated transformation of *Lilium longiflorum*. *Plant Cell Rep.* 17:262-267.

BOOK CHAPTERS AND OTHER RELEVANT PUBLICATIONS

Matsumoto, T. K. and D. Gonsalves. 2012. Biolistic and other non-agrobacterium technologies of plant transformation. *In* Plant biotechnology and agriculture: Prospects for the 21st century, A. Altman and P.M. Haegawa eds., Elsevier Press, Oxford, U.K. pp 117-122.

Matsumoto, T.K., and A.R. Kuehnle. 1997. Micropropagation of Anthurium. *In* Biotechnology in Agriculture and Forestry High-Tech and Micropropagation VI . Bajaj, Y.P.S. (ed.) Springer Verlag, Berlin.40: 14-29.

Tanabe, M. J., English, J., Moriyasu, P., Arakawa, C., and **Matsumoto, T.** Anthurium in vitro cultures. Proc. Third Anthurium Blight Conference. Hawaii Inst. Trop. Agri. and Human Resources 05.07.90. p. 54-55. 1990. (Conference Proceedings)

Tanabe, M. J., Arakawa, C., **Matsumoto**, **T**., Tanaka, R., and English, J. Anthurium in vitro propagation. Proc. Fourth Hawaii Anthurium Industry Conference. Hawaii Inst. Trop. Agri. and Human Resources. 06.18.91. p. 4-6. 1991. (Conference Proceedings)

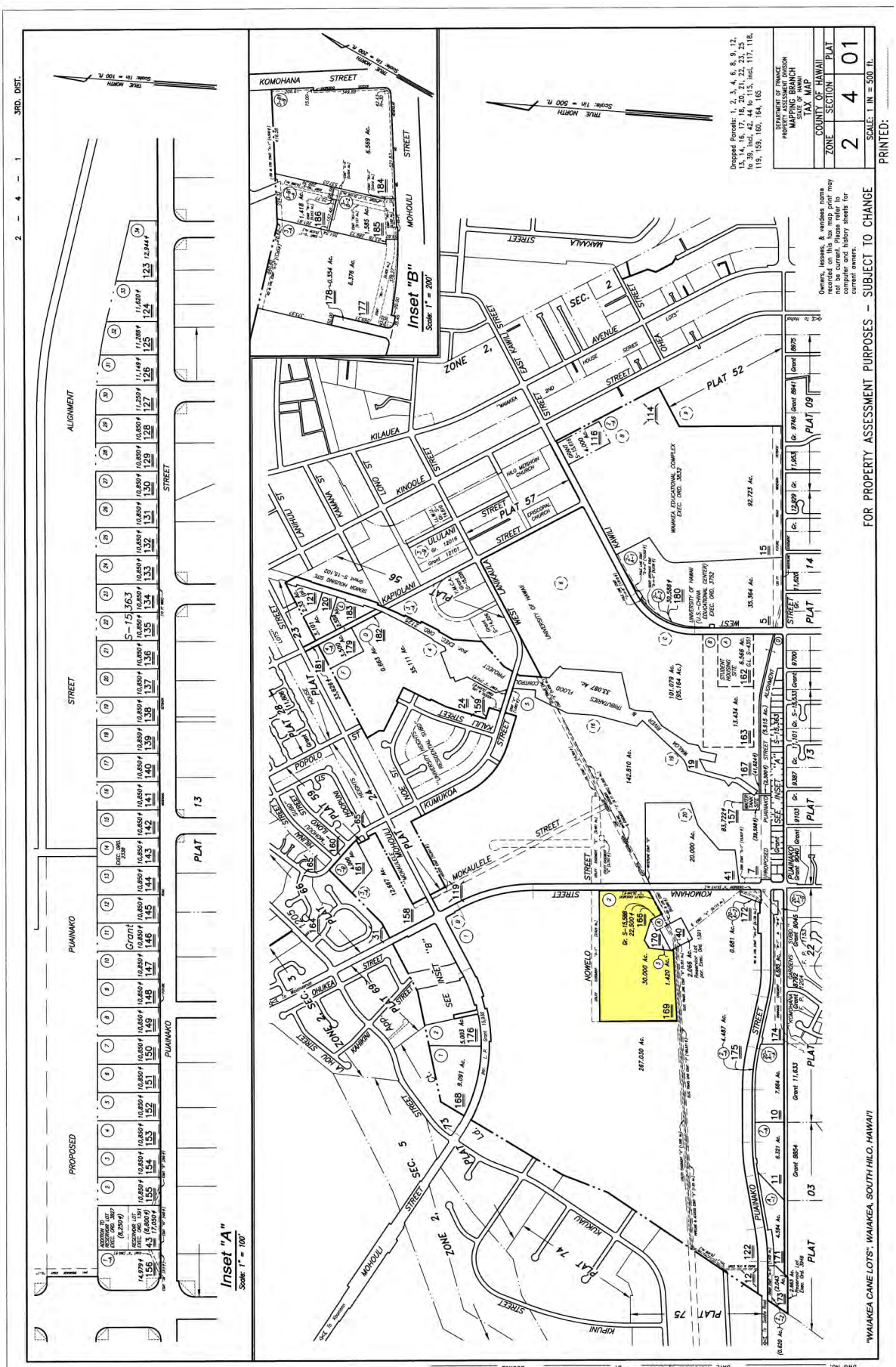
Tanabe, M. J. and **Matsumoto, T**. Anthurium explant surface disinfestation. Proc. Fifth Hawaii Anthurium Industry Conference. Hawaii Inst. Trop. Agri. and Human Resources. 02.02.94. p. 10-11. 1992. (Conference Proceedings)

BIOSAFETY EXPERIENCE

Committees

Co-chair, Biosafety Committee, USDA-ARS Hilo location, 2002-2008.
Co-chair, Safety Committee, USDA-ARS-PBARC-TPGRMU Hilo, location 2002-2008.
Collatoral Duty Biosafety Officer, USDA, ARS Hilo location 2008-present.
Conduct yearly Biosafety training using ARS/CDC training materialParticipate in ARS Biosafety Officer's monthly teleconference
SHEM Committee member, USDA, ARS Hilo location 2008-presentMember of the PBARC BQMS Committee (certified March 2012) PWA IBC Committee (February 2013 - present)
ARS Biosafety, Safety and Health Operation Committee (July 2013 –present)

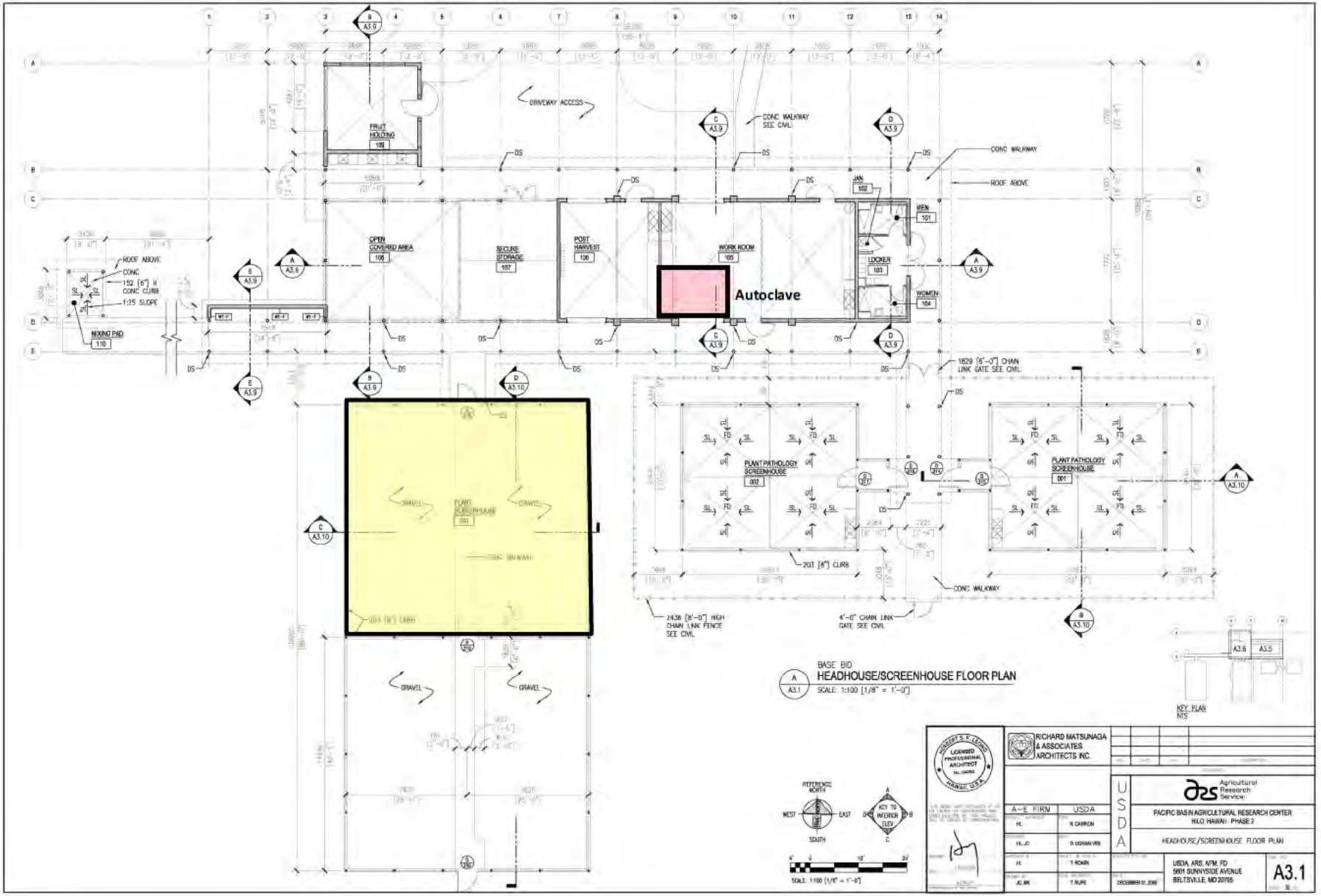
Appendix 1. Demarcation of property (highlighted in yellow) with laboratory and greenhouse for proposed coffee research on Hawaii county Tax Map.



Appendix 2. Blue print of laboratory with rooms for transfer and storage highlighted in yellow. Red indicates autoclave for devitalization.

APPENDIX 3

Appendix 2 (cont) Schematic of greenhouse with proposed area for growing coffee highlighted in yellow. Red indicates autoclave for devitalization



APPENDIX 3

From: David Lawson
To: Siaska Vieira de Castro; Matsumoto, Tracie
Cc: Vern Long
Subject: [External Email]RE: Tissue cultured coffee plants
Date: Thursday, February 25, 2021 9:34:18 AM

Hi Tracie,

Glancing back at all my logs. I can safely say that all the plants you received had been maintained in vitro for nearly 7 years. Everything except for H1, H16(EC16) and Marsellessa were solely sourced from imported seed material. The seed material was surface sterilized for 45 minutes in 20% bleach before the embryos were extracted and germinated in vitro. The H1, H16(EC16) and a portion of the Marsellessa were imported as in vitro plant embryos and callus from embryogenesis which we then germinated and began micropropagation from the plantlets.

We have shipped these plants in vitro to over 30 locations world wide and to my knowledge none have experienced disease issues from the tissue culture material. In most scenarios countries received at least 52 plants per variety. WCR would be best to answer this.

Let me know if you need anything else.

David

From: Siaska Vieira de Castro <siaska@worldcoffeeresearch.org>
Sent: Wednesday, February 24, 2021 2:28 PM
To: Matsumoto, Tracie <tracie.matsumoto@usda.gov>
Cc: David Lawson <David@agristarts.com>; Lee Goode <Lee@agristarts.com>; Nick Ashby <Nick@agristarts.com>; Vern Long <vern@worldcoffeeresearch.org>
Subject: Re: Tissue cultured coffee plants

Hello Tracie,

I hope that David can help you here. David, we appreciate it!

Best,
Siaska

Siaska Castro
Financial Director

United States Department of Agriculture

Research, Education and Economics
Agricultural Research Service

February 9, 2021

Lance S. Sakaino
Hawaii Department of Agriculture
Plant Quarantine Branch
1849 Auiki Street
Honolulu, Hawaii 96819-3100
Phone: (808) 832-0566
Fax: (808) 832-0584
Email: Lance.S.Sakaino@hawaii.gov

Dear Lance,

The Hawaii coffee industry, conservatively valued at $100M per year, is facing their toughest challenge with an invasive new pathogen, *Hemileia vastatrix*, the causal agent of coffee leaf rust (CLR). CLR, the most devastating pathogen of coffee worldwide, was recently discovered on Maui and in Kona on Hawaii Island in late 2020. Since then it has rapidly spread throughout the coffee farms in Kona and has most recently been found on Lanai and Oahu. Farms left un-managed or ill-managed will suffer crop loss and ultimately tree death since cultivars currently grown in Hawaii are highly susceptible to CLR. A critical component of a CLR IPM strategy is the use of resistant coffee varieties and since very few are available in Hawaii, these need to be propagated, multiplied, and provided to the growers as quickly and as safely as possible.

Propagating coffee in tissue culture should pose no additional risk of introducing new plant diseases to Hawaii. Tissue culture plants are grown in a sterile environment with media such as salt, vitamins, and sugars so any fungi or bacteria on this material will become evident in the culture within a few weeks which can be immediately devitalized. Tissue culture media lacks contaminant suppressants, so if a latent contaminant is detected the plants are devitalized by autoclaving. The media and plant material are also routinely tested to detect any latent infection. As an additional precaution, the tissue culture plantlets are screened for pathogens before moving to the greenhouse. For these reasons, I fully support Dr. Tracie Matsumoto's request to allow propagation of coffee while under quarantine and believe it is a critical step necessary for the success of a CLR mitigation and management program.

Thank you very much for your time.
Sincerely,

Lisa Keith

Lisa Keith, Ph.D.
Research Plant Pathologist

Pacific West Area
Pacific Basin Agricultural Research Center
Tropical Plant Genetic Resource and Disease Research Unit
64 Nowelo St., Hilo, HI 96720
Voice: 808-959-4357 Fax: 808-959-5470 E-mail: lisa.keith@usda.gov

College of Tropical Agriculture and Human Resources
Department of Plant and Environmental Protection Sciences

UNIVERSITY of HAWAI'I® MĀNOA

February 9, 2021

Hawaii Department of Agriculture
Plant Quarantine Branch
1849 Auiki St.
Honolulu HI 96819

Dear HDOA-PQ,

My name is Michael Melzer and I am an Associate Researcher in Agricultural Security at the University of Hawaii's College of Tropical Agriculture and Human Resources. I am writing to support the permit application of Dr. Tracie Matsumoto Brower (USDA-ARS) to propagate coffee (*Coffea arabica*) plants in aseptic tissue culture while under quarantine. In my opinion the positive impact of having a large number of plants to evaluate for coffee leaf rust resistance immediately upon the conclusion of quarantine far outweighs any perceived risk to Hawaii's agricultural or natural ecosystems that may be associated with their propagation during the quarantine period. Aseptic culture inherently removes the risk of propagating most pests and pathogens of coffee of concern to Hawaii. Arthropod pests are almost immediately identified, the fungal spores of *Hemileia* spp. would not survive the one year quarantine, and the propagules of other fungal pathogens would be easily spotted as culture contaminants and destroyed. There are no major systemic viral pathogens of coffee, leaving the only potential concern being propagation of *Xylella fastidiosa*. Propagation of the coffee plants, however, will not introduce infection by this fastidious pathogen of coffee.

Dr. Matsumoto Brower's work to identify solutions for Hawaii's coffee industry is critical, but I would not support her permit application if I thought it would threaten this industry in any manner. I see no such threat with her application and fully endorse the issuance of her propagation permit. Please feel free to contact me should you have any questions about this correspondence.

Sincerely,

Michael Melzer
Associate Researcher in Agrosecurity
(808) 956-7887
melzer@hawaii.edu

3050 Maile Way, Gilmore Hall 310, Honolulu, Hawai'i 96822
Telephone: (808) 956-7076, Fax: (808) 956-2428
Email: peps@ctahr.hawaii.edu, Web: www2.ctahr.hawaii.edu

Compendium of Coffee Diseases and Pests
A. L. Gaitán, M. A. Cristancho, B. L. Castro Caicedo, C. A. Rivillas, and G. Cadena Gómez, eds.

ISBN: 978-0-89054-472-3

Part I. Infectious Diseases

Disease Caused by a Virus

Coffee Ringspot

Coffee ringspot virus (CoRSV) was first reported in Brazil, in the state of São Paulo, and since then it has been found on coffee plantations in other states in Brazil (Federal District, Minas Gerais, and Paraná) and in Costa Rica, Central America. There were reports of a ringspot-like disease in the Philippines in the 1950s and symptoms were again observed in 1968 (H. David Thurston, *personal communication*), but confirmation of its relationship with CoRSV is not available. When first described, the disease was considered to be of secondary importance, but in recent years, severe symptoms followed by intense plant defoliation and dieback have elevated the concern about this disease in coffee-growing areas of Minas Gerais and São Paulo States.

Symptoms

Affected coffee plants develop conspicuous, local ringspot symptoms mainly on leaves and berries and less commonly on twigs. On leaves, symptoms start as small, chlorotic spots that gradually increase in size, usually becoming ring shaped and sometimes fusing and covering larger foliar areas (Fig. 1). The initial spots are generally rounded, and as they become larger, a necrotic center may develop. Sometimes lesions follow the midrib or secondary veins, becoming brown as they age. In senescent leaves, the ringspot area may stay green while the rest of the leaf yellows. Green fruits develop whitish, rounded spots on the skin that turn into ringspots as the fruits mature. On mature berries, the ringspots are frequently depressed and discolored, which reduces the quality of the berries. When heavily affected, coffee trees may drop their leaves and fruits.

Causal Agent

Coffee ringspot virus is a short bacilliform particle, 35–40 × 100–110 nm (Fig. 2). Transmission electron microscopy of thin sections of infected leaves reveals that these particles are present only within the lesion, scattered in either the nucleoplasm or the cytoplasm. These particles tend to be associated with the nuclear envelope or endoplasmic reticulum membranes, arranged perpendicularly as though involved in a budding process, which apparently is hardly ever completed (Fig. 3). In rare instances, membrane-bound particles (60–80 × 180–220 nm) have been seen within the cisternae of the endoplasmic reticulum. Another remarkable feature is the presence in many nuclei of an electron lucent inclusion, referred to as a viroplasm, in which the rod shaped particles may appear interspersed (Fig. 4). Similar alterations have also been found in local lesions in mechanically inoculated *Chenopodium amaranticolor* Coste & A. Reyn. and *Chenopodium quinoa* Willd. leaves. In São Paulo, a new morphologic virus type (CoRSV SP) causes smaller lesions with necrotic spots and shows bacilliform viruslike particles in the endoplasmic reticulum. An enzyme linked immunosorbent assay (ELISA) has been developed and is being

Fig. 1. Symptoms of *Coffee ringspot virus* infection on coffee leaves. (Courtesy C. M. Chagas)

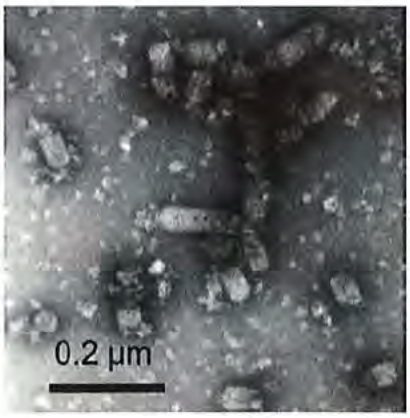


Fig. 2. Virus particles in a purified preparation of *Coffee ringspot virus*. (Courtesy C. M. Chagas)

used for diagnosis. Part of the CoRSV genome has been sequenced and primers have been designed for its detection by reverse transcription polymerase chain reaction (RT PCR).

Disease Cycle and Epidemiology

Coffee ringspot has been demonstrated to be transmissible by the false spider mite *Brevipalpus phoenicis* (Geijskes) (Acari: Tenuipalpidae), which is a polyphagous species complex with hundreds of host plants. Also, a mite species complex has been reported. Under experimental conditions, transmission efficiency was about 24% using female adult mites that had access to infected leaves. Viruslike particles were observed in viruliferous *Brevipalpus* mites. So far, no transovarial passage of CoRSV has been observed. Mechanical inoculation using inoculum from coffee tissues with ringspots caused local lesions on *Beta vulgaris* L., *Chenopodium amaranticolor*, *Chenopodium quinoa*, and *Alternanthera tenella* Colla. *Chenopodium quinoa* and *Chenopodium amaranticolor* plants mechanically inoculated with CoRSV develop local chlorotic lesions, and if kept at 28–30°C, systemic infection may result. A survey of the coffee germplasm bank of the Centro APTA Café, Instituto Agronômico de Campinas, São Paulo, Brazil, revealed that many species and hybrids of the genus *Coffea* (e.g., *C. kapakata* (A. Chev.) Bridson, *C. dewevrei* De Wild. & T. Durand cv. Excelsa, *C. canephora* Pierre ex A. Froehner cv. Robusta, and hybrids *C. arabica* L. × *C. racemosa* Lour., *C. arabica* × *C. dewevrei*, and 'Timor Hybrid' [a natural hybrid between *C. arabica* and *C. canephora*]), as well as noncoffee Rubiaceae species, such as *Psilanthus ebracteolatus* Hiern, are susceptible to CoRSV. No seed transmission of CoRSV has been reported.

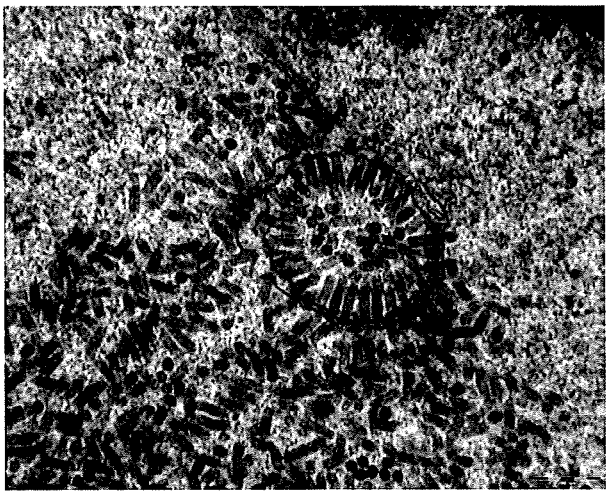


Fig. 3. Bacilliform particles in endoplasmic reticulum arranged in a "spoke wheel" configuration from coffee leaf tissue infected with *Coffee ringspot virus* São Paulo type (CoRSV SP). (Courtesy C. M. Chagas)

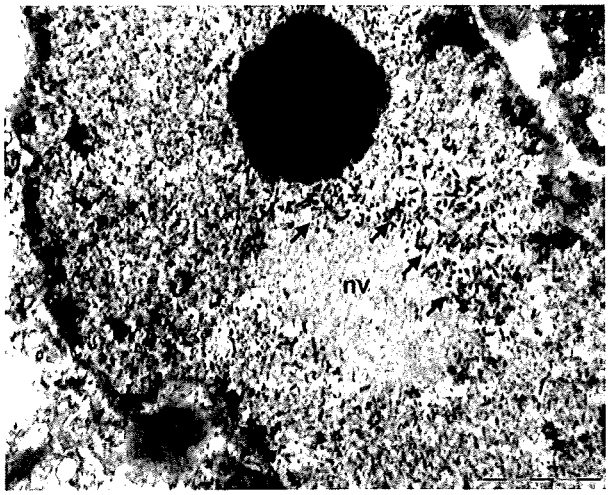


Fig. 4. Nuclear viroplasm (nv) and short bacilliform particles (arrows). (Courtesy C. M. Chagas)

Management

Strategies to manage the disease may be applied at several levels. To succeed with the management strategies, it is necessary to consider the variables that are affecting the virus host–vector relationships. To avoid introducing CoRSV into a new plantation, growers must use only virus free plants from the nursery and take measures to avoid introducing viruliferous mites (e.g., establish windbreaks with plants immune to CoRSV; control weeds that may be alternate hosts to the virus and vector; and control people, tools, boxes, vehicles, etc., coming into coffee orchard sites). These measures are particularly critical now that there is evidence that CoRSV multiplies in the mite vector. Chemical spraying to reduce the vector mite population is also a common practice based upon an empirical sampling process and threshold levels. In the states of São Paulo and Minas Gerais, Brazil, products such as acrinathrin, azocyclotin, bifenthrin, cyhexatin, dicofol, hexythiazox, fenbutatin oxide, propargite, and quinomethionate are being used. Because resistance of the mites to most of these miticides develops after repeated applications, rotation of different active ingredients is important. In infected orchards, besides constant chemical treatment, pruning affected branches is also recommended. Biological control using predaceous mites or entomopathogenic fungi is being studied. There is no information on plant resistance variability to CoRSV in coffee.

Selected References

Bitancourt, A. A. 1938. A mancha anular, uma nova doença do cafeeiro. Biológico 4:404 405.

Boari, A. J., Figueira, A. R., Neder, D. G., Infiesta, L. R., Nogueira, N. L., Rossi, M. L., and Kitajima, E. W. 2003. Efeito da temperatura na infecção sistêmica de *Chenopodium quinoa* pelo Coffee ringspot vírus (CoRSV). (Abstr.) Fitopatol. Bras. 28(Suppl.):S246 S247.

Boari, A. J., Freitas Astua, J., Ferreira, P. T. O., Neder, D. G., Nogueira, N. L., Rossi, M. L., and Kitajima, E. W. 2004. Purification and serology of the coffee ringspot virus. Summa Phytopathol. 30:453 458.

Carvalho, C. M., and Figueira, A. R. 1998. *Alternanthera tenella* as a potential wild host for coffee ringspot virus. (Abstr.) Virus Res. Rev. 3(Suppl. 1):146.

Chagas, C. M., Kitajima, E. W., and Rodrigues, J. C. V. 2003. Coffee ringspot virus vectored by *Brevipalpus phoenicis* (Acari: Tenuipalpidae) in coffee. Exp. Appl. Acarol. 30:203 213.

Chagas, C. M., Kitajima, E. W., and Locali Fabris, E. 2007. Isolado distinto do vírus da mancha anular do cafeeiro (CoRSV). (Abstr.) Fitopatol. Bras. 32(Suppl.):S135.

Childers, C. C., French, J. V., and Rodrigues, J. C. V. 2003. *Brevipalpus californicus*, *B. obovatus*, *B. phoenicis* and *B. lewisi* (Acari: Tenuipalpidae): A review of their biology, feeding injury and economic importance. Exp. Appl. Acarol. 30:5 28.

Childers, C. C., Rodrigues, J. C. V., and Welbourn, W. C. 2003. Host plants of *Brevipalpus californicus*, *B. obovatus* and *B. phoenicis* (Acari: Tenuipalpidae) and their potential involvement in the spread of viral diseases vectored by these mites. Exp. Appl. Acarol. 30:29 105.

Kitajima, E. W., Chagas, C. M., Braghini, M. T., Fazuoli, L. C., and Locali Fabris, E. 2007. Infecção natural de várias espécies e híbridos de *Coffea* e uma outra espécie de Rubiaceae pelo vírus da mancha anular de cafeeiro (CoRSV). (Abstr.) Fitopatol. Bras. 32(Suppl.):S134.

Kitajima, E. W., Boari, A. J., and Chagas, C. M. 2007. Detecção do vírus da mancha anular do cafeeiro nos tecidos do ácaro vetor *Bre-*

vipalpus phoenicis (Acari: Tenuipalpidae). (Abstr.) Fitopatol. Bras. 32(Suppl.):S135.
Locali, E. C., Freitas Astua, J., Antonioni Luizon, R., Boari, A. J., and Machado, M. A. 2005. Diagnose da mancha anular do cafeeiro através do RT-PCR. (Abstr.) Fitopatol. Bras. 30(Suppl.):S185.
Magalhães, B. P., Rodrigues, J. C. V., Boucias, D. G., and Childers, C. C. 2005. Pathogenicity of *Metarhizium anisopliae* var. *acridum* to the false spider mite *Brevipalpus phoenicis* (Acari: Tenuipalpi dae). Fla. Entomol. 88:195 198.
Reyes, T. T. 1959. Ringspot of coffee in the Philippines. FAO Plant Prot. Bull. 8:11-12.
Rodrigues, J. C. V., Childers, C. C., Gallo Meagher, M., Ochoa, R., and Adams, B. J. 2004. Mitochondrial DNA and RAPD polymor phisms in the haploid mite *Brevipalpus phoenicis* (Acari: Tenuipal- pidae). Exp. Appl. Acarol. 34:274 290.

(Prepared by J. C. Rodrigues, E. W. Kitajima, and C. M. Chagas)

Disease Caused by Phytoplasmas

Coffee Crispiness Disease

Coffee crispiness disease is an endemic disease on Colom bian coffee plantations that affects the normal physiological de velopment of the aerial parts of the plant, especially the leaves and floral buds. The presence of unusual curly leaves, together with massive vegetative growth in the branches, gave the local name *crespera* (crispiness) to the disease. Coffee crispiness dis ease was reported for the first time in 1940, affecting restricted areas of the western department of Antioquia, and by 1950, it slowly spread to south-central regions of the country, mainly in the departments of Caldas, Risaralda, and Huila. Throughout the years, its persistence has been confirmed in those areas, especially in places located more than 1,500 m above sea level. All the *Coffea arabica* L. cultivars planted in Colombia, in cluding Typica, Bourbon, Caturra, and the bred variety Co- lombia, are susceptible to the disease. Although considered a mild disease that normally has no effect on yield or quality, severe attacks of coffee crispiness disease can dramatically re- duce productivity, particularly in association with pruning and stumping practices, which are commonly used in Colombia for plantation regrowth.

Symptoms

Coffee crispiness disease symptoms are related to interfer- ence in nutrient balance and alteration of protein and auxin synthesis, which result in leaf proliferation and phyllody, alter- ing also the fruit by increasing the percentages of monosper mic berries. Affected leaves are very narrow and curly, taking a ribbonlike appearance, and sometimes exhibiting chlorosis on the edges. Multiple bud activation generates a broom mor phology at the tip of affected branches (Fig. 5). Inflorescence is scarce or completely absent. When the disease is mild, trees are tolerant and leaf symptoms can disappear during changing environmental conditions, leading to an apparent recovery. It is normal to find either healthy new leaves on branches that are symptomatic or healthy branches on symptomatic trees. Severely diseased plants, however, are dwarfed as a result of shorter internodal distances in the trunk and branches and their yields are severely reduced (Fig. 6).

Grafting tests of diseased scions on healthy rootstock can be used to resolve confusing symptoms, such as toxic effects of herbicides. Glyphosate, for example, can induce alterations in vegetative buds, producing numerous long and narrow leaves. Grafting results in a disease transmission rate of 90% after 44 days. There is accelerated bud formation during the first 15 days, with symptoms appearing at day 30, consisting of necro sis of the first pair of axillary buds produced by the rootstock at the site of grafting, followed by a proliferation of short and narrow leaves from the new buds, and resulting in plant devel opment similar to that observed in diseased field plants. None of these effects is observed when herbicide treated scions are grafted.

Causal Organisms

Throughout the years, coffee crispiness disease has been at- tributed to various causes, from viral problems to micronutrient deficiency (zinc), mineral malnutrition, and physical and chemi cal soil limitations through herbicide toxicity. More recently, comparative symptomatology and direct evidence suggest an

Fig. 5. Phyllody and leaf deformation symptoms of coffee crispi ness disease. (Reproduced from Galvis et al., 2007)

Fig. 6. Stunted plant severely infected with coffee crispiness disease. (Courtesy J. E. Leguizamón, Cenicafé)

association of phytoplasmas with coffee crispiness disease. In petioles of diseased leaves, pleomorphic phytoplasma bodies lacking a cell wall and measuring 300–400 nm in length are located in the vacuole of phloem cells. Coffee crispiness disease phytoplasmas exhibit abundant peripheral cytoplasm, containing ribosomes and a central net of fibrils (probably DNA). Sequencing of nested polymerase chain reaction products amplified with primers FU5/rU3 from affected tissues resulted in a consensus 941-base pair fragment corresponding to the 16S rDNA (GenBank accession number AY525125). The coffee crispiness disease sequence clustered with the 16Sr III group, described as the X-disease group, and was close to the phytoplasma identified as the causal organism for *machorreo* in lulo or naranjilla (*Solanum quitoense* Lam.).

Disease Cycle and Epidemiology

Although an alternate plant host can be involved, a proposed disease cycle starts with the inoculum already present in coffee plants that may be expressing disease symptoms, depending on the severity of the phytoplasma infection. The organism then moves systemically throughout the plant and maintains a reservoir in the roots. After stumping, which removes all the aerial parts of the plant, except for 30 cm of the base of the trunk, new stems normally exhibit increased symptom severity. No seed or mechanical transmission of the disease has been obtained.

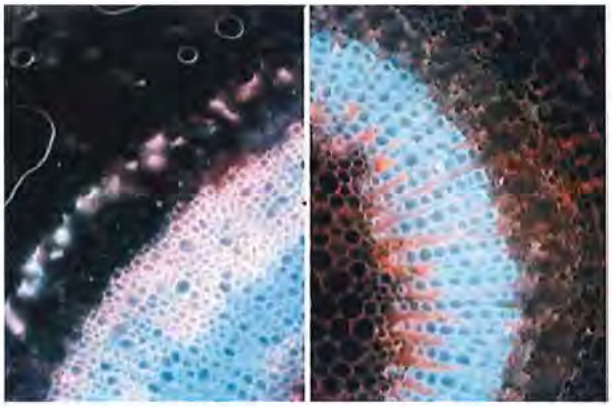


Fig. 7. 4',6-Diamidino-2-phenylindole (DAPI) staining of the phloem in a petiole affected by coffee crispiness disease (left) compared with healthy tissue (right). (Reproduced from Galvis et al., 2007)

However, *Clinonella declivata* (Osborn) and a *Graphocephala* sp., both of the order Hemiptera and family Cicadellidae, have been successfully demonstrated as vectors for coffee crispiness disease. These insects, commonly present on coffee plantations but mostly associated with weeds and other cover crop plants, can then disperse the phytoplasmas along the plots in a process that can take years before it becomes evident to the coffee growers. The sudden outbreaks of the disease in past and recent years could be the result of a combination of phytoplasma presence, excessive weed control with chemical herbicides, and interaction between environmental conditions and vector populations. The elimination of main or alternate plant hosts leaves coffee plants as the only food and protection sources for the insects. Special consideration must be taken for those regions directly influenced by weather changes that originate during El Niño Southern Oscillation (ENSO).

Management

Proper diagnosis is important to rule out cases of herbicide toxicity. Histological studies using Dienes' staining produce a deep blue coloration in the phloem cells of affected petioles, which is absent in healthy tissues. Similarly, fluorescence can be observed using 4',6 diamidino 2 phenylindole (DAPI) stain (Fig. 7). Although it takes longer, transmission by grafting is the most effective way to confirm coffee crispiness disease. Stumping or pruning within a plantation is counterproductive because either can exacerbate the symptoms. Planting new healthy nursery plants and preserving a green coverage on the space between plantation lines with an integrated weed management program are recommended when yield levels are severely affected by the disease. No additional fertilization or soil amendments are necessary besides those indicated under normal conditions.

Selected References

Galvis, C. A. 2006. Identificación de insectos vectores del fitoplasma causante de la crespera del cafeto. (Abstr.) Page 135 in: Resúmenes Congreso de la Sociedad Colombiana de Entomología, 33rd. Sociedad Colombiana de Entomología, Bogotá, Colombia.

Galvis, C. A., Leguizamón, J. E., Gaitán, A. L., Mejía, J. F., Álvarez, E., and Arroyave, J. 2007. Detection and identification of a group 16SrIII-related phytoplasma associated with coffee crispiness disease in Colombia. Plant Dis. 91:248-252.

Mejia F., R. 1950. Sanidad y defensa de las plantaciones de café en el país. Cenicafé 1:22-27.

(Prepared by A. Gaitán)

Diseases Caused by Bacteria

Bacterial Halo Blight

Bacterial halo blight of coffee, caused by *Pseudomonas syringae* pv. *garcae*, inflicts losses in coffee-growing regions of Brazil and Kenya, especially on young plantations. The disease was first reported in Brazil in the 1950s, when the causal organism was described as *P. garcae* Amaral, Teixeira & Pinheiro. After its appearance on coffee plantations in the state of São Paulo, Brazil, the disease diminished in importance. However, since the 1970s, epidemics of the disease have been observed in nurseries and on young plantations in other Brazilian coffee growing regions. *P. syringae* pv. *garcae* also causes a bacterial blight of coffee in Kenya at altitudes above 1,800 m. It is known as Elgon/Solai dieback, named after the places where it has been observed since the 1960s.

Symptoms

In the nursery, initial symptoms on young leaves are dark green, punctate lesions that appear water soaked and translucent when viewed with backlighting. These lesions later become irregular and necrotic and may be surrounded by a yellow halo. When a leaf is viewed with backlighting, a translucent line can sometimes be seen encircling the necrotic area between the necrotic tissue and the yellow halo. A large proportion of the area of affected leaves may exhibit dried, necrotic, or greasy symptoms that are red or dark in color (Fig. 8). Under conditions favorable for the pathogen, the punctiform lesions on young leaf tissues may expand and involve the entire leaf surface in a few days, also reaching the orthotropic branches (those growing along the vertical axis). The leaves and tips of these branches become dehydrated and collapse. Spread of the

disease to the orthotropic branches may cause leaf drop and death of coffee plantlets in nurseries (Fig. 9).

In the field, coffee leaves develop necrotic lesions, with or without a yellow halo. The leaves appear comma shaped because of the lesions located on the leaf edges. These necrotic lesions prevent leaf expansion, while the nearby tissues develop normally (Figs. 10 and 11). Other important symptoms of this disease in the field are the apical necrosis of both the orthotropic and plagiotropic (those growing at horizontal angles) branches (Fig. 12), flower abortion, necrosis of the rosette and young fruits, and necrosis of the epidermis of plagiotropic branches at the junction points of the rosettes, young fruits, branches, or young leaves.

Causal Organism

Pseudomonas syringae van Hall pv. *garcae* (Amaral, Teixeira & Pinheiro) Young, Dye & Wilkie belongs to the class

Fig. 8. Lesions caused by *Pseudomonas syringae* pv. *garcae* on a *Coffea arabica* plantlet in a nursery. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

Fig. 9. Death of a *Coffea arabica* plantlet caused by *Pseudomonas syringae* pv. *garcae* in a nursery. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

Fig. 10. Typical lesions caused by *Pseudomonas syringae* pv. *garcae* on *Coffea arabica* in a field. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

Fig. 11. Typical lesions caused by *Pseudomonas syringae* pv. *garcae* on *Coffea arabica* in a field. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

Fig. 12. Apical necrosis of an orthotropic branch caused by *Pseudomonas syringae* pv. *garcae*. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

Gammaproteobacteria, order *Pseudomonadales*, and family *Pseudomonadaceae*. There are various pathovars of *P. syringae*. *P. syringae* pv. *garcae* is gram negative, oxidase negative, and levan positive, and it liquefies gelatin and produces yellow fluorescent pigments in King's medium B. In artificial inoculations, it is pathogenic to *Phaseolus vulgaris* L. 'Moruna' and causes typical symptoms of bacterial halo blight. Forty-eight pathovars of *P. syringae* and eight related species were studied through the DNA–DNA hybridization method (S1 nuclease method) and ribotyping. Nine genome species were identified, one of which included pathovars that correspond to *P. coronafaciens* (Elliott) F. L. Stevens, *P. syringae* pv. *porri* Samson, Poutier & Rat, *P. syringae* pv. *garcae*, *P. syringae* pv. *striafaciens* (Elliott) Young, Dye & Wilkie, *P. syringae* pv. *atropurpurea* (Reddy & Godkin) Young, Dye & Wilkie, *P. syringae* pv. *oryzae* (ex Kuwata) Young, Bradbury, Davis, Dickey, Ercolani, Hayward & Vidaver, and *P. syringae* pv. *zizaniae* (ex Bowden & Percich) Young, Bradbury, Davis, Dickey, Ercolani, Hayward & Vidaver.

Differences were found in biochemical and pathogenic characteristics among isolates of *P. syringae* pv. *garcae* from Kenya and Brazil, demonstrating that this bacterium has at least two strains, a Kenyan isolate and a Brazilian isolate, and leaving open the possibility that other strains may be found.

Disease Cycle and Epidemiology

P. syringae pv. *garcae* survives as an epiphyte on coffee leaves. During the winter, cold winds and humidity predispose the leaves to infection by the pathogen and favor the development of bacterial halo blight. In nurseries, beyond the mentioned factors that favor its proliferation, the bacterium is rapidly disseminated by mechanical transmission during nursery operations. In the field, the presence of inoculum generated in coffee leaves during the winter, the flushing of new leaves, flowers, and fruits in the spring (September to December in the Southern Hemisphere), and intense rainfall promote rapid development of the disease. *P. syringae* pv. *garcae* has the capability to penetrate these juvenile structures and other soft tissues of the coffee plant through natural openings or through mechanical injuries caused by the abrasive action of windblown soil particles or by friction among plant tissues.

In several municipalities of the state of Parana, Brazil, the disease occurs in 2- to 4-year-old coffee plants during the winter (July 21 to September 22), when temperatures vary between 13 and 26°C and the relative humidity is 58–67%, with a mean monthly precipitation of 133.5 mm, an average of 7.6 days of rain, and a mean wind speed of 4.6 m/s. The disease reaches the highest incidence on coffee plantations during the spring (September 23 to December 21), mainly in October when temperatures are 13–32°C and the average relative humidity is between 57 and 73%, with a mean monthly precipitation of 111.3 mm, an average of 9.1 days of rain, and a mean wind speed of 3.4 m/s. The disease progression slows during the summer months (December to March), when the temperatures increase coincident with reductions of the relative humidity, number of days of rainfall, and wind speed.

Management

The main control measures are preventive and aimed at avoiding or reducing damages caused by the pathogen. In the winter and beginning of spring, nurseries must be protected from the wind and plants must be under strict monitoring and careful manipulation to avoid the development of disease foci and the proliferation of the bacteria. Patches of the disease in nurseries are controlled by eliminating infected plants and chemical spraying the remaining plant material with copper hydroxide-based fungicides. In Kenya, use of captafol has been found to exacerbate the disease. Plantations located at high elevations are more frequently exposed to cold winds and should be protected with temporary or permanent windbreaks. Disinfected instruments can be used to prune affected plant parts when the disease incidence is low or during the initial stages of infection in the field. In cases of an epidemic, the use of mixtures of streptomycin sulfate and copper oxychloride formulations is recommended. Biological control has not yet been investigated.

A vast germplasm of commercial varieties, *Coffea* spp., accessions of *C. arabica* from Ethiopia, and lineages and progenies of *C. arabica* resistant to *Hemileia vastatrix* Berk. & Broome have been tested in Brazil since 1978. Several genotypes showed resistance to isolates of this bacterium and, simultaneously, resistance to races of coffee leaf rust, caused by *H. vastatrix*. This behavior was not observed when materials with different *H. vastatrix*-resistant genotypes were inoculated with an isolate of *P. syringae* pv. *garcae* from Kenya. Because commercial varieties with *H. vastatrix* resistance showed lower susceptibility to Brazilian isolates of the bacterium, screening is underway to obtain progenies with resistance to both diseases.

Selected References

Amaral, J. F. do, Teixeira, C., and Pinheiro, E. D. A. 1956. Bactéria causadora da mancha aureolada do cafeeiro. Arq. Inst. Biol. Sao Paulo 23:151-155.

Cardoso, R. M. L. 1978. As doenças do cafeeiro. Doenças infecciosas. Crestamento bacteriano ou mancha aureolada. Pages 195 197 in: Manual Agropecuário do Paraná. Fundação Instituto Agronômico do Paraná (IAPAR), Londrina, Paraná, Brazil.

Cardoso, R. M. L., and Mohan, S. K. 1979. Ocorrência e intensidade do crestamento bacteriano causado por *Pseudomonas garcae* em relação a alguns fatores climáticos, no estado do Paraná. Pages 65 66 in: Congresso Brasileiro de Pesquisas Cafeeiras, 7th. Resumos. Instituto Brasileiro do Café, Grupo Executivo da Racionalizacão da Cultura Caféeira (GERCA), Rio de Janeiro, Brazil.

Cardoso, R. M. L., and Sera, T. 1984. Obtenção de cultivares de *Coffea arabica* L. com resistência simultânea a *Hemileia vastatrix* Berk. & Br. e *Pseudomonas syringae* pv. *garcae* Amaral et al. no estado do Paraná. Pages 417 419 in: Simposio sobre Ferrugens do Cafeeiro. Centro de Investigação das Ferrugens do Cafeeiro, Instituto de Investigação Científica Tropical (IICT), Lisboa, Portugal.

Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F, and Grimont, P. A. D. 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (*ex* Sutic and Dowson 1959). Int. J. Syst. Bacteriol. 49:469 478.

Kairu, G. M. 1997. Biochemical and pathogenic differences between Kenyan and Brazilian isolates of *Pseudomonas syringae* pv. *garcae*. Plant Pathol. 46:239 246.

Kairu, G. M., Nyangena, C. M. S., Javed, Z. U. R., and Crosse, J. E. 1984. Iatrogenic effects of captafol on bacterial blight of coffee. Plant Pathol. 33:131-132.

Kimura, O., Robbs, C. F., and Ferrari, J. A. R. 1976. Algumas observações relacionadas com as bacterioses do cafeeiro. Page 104 in: Congresso Brasileiro de Pesquisas Cafeeiras, 4th. Resumos. Instituto Brasileiro do Café, Grupo Executivo da Racionalizacão da Cultura Caféeira (GERCA), Rio de Janeiro, Brazil.

Mohan, S. K. 1976. Investigações sobre *Pseudomonas garcae* em cafeeiros. Page 56 in: Congresso Brasileiro de Pesquisas Cafeeiras, 4th. Resumos. Instituto Brasileiro do Café, Grupo Executivo da Racionalizacão da Cultura Caféeira (GERCA), Rio de Janeiro, Brazil.

Mohan, S. K., Cardoso, R. M. L., and Pavan, M. A. 1978. Resistência em germoplasma de *Coffea* ao crestamento bacteriano incitado por *Pseudomonas garcae* Amaral et al. Pesqui. Agropecu. Bras. 13:53-64.

Petek, M. R., Sera, T., Sera, G. H., Fonseca, I. C. B., and Ito, D. S. 2006. Selection of progênies of *Coffea arabica* with simultaneous resistance to bacterial blight and leaf rust. Bragantia 65:65 73.

Waller, J. M., Bigger, M., and Hillocks, R. J. 2007. Bacterial blight. Pages 197 198 in: Coffee Pests, Diseases and Their Management. CAB International, Wallingford, United Kingdom.

(Prepared by R. M. de Lemos Cardoso)

Atrophy of Coffee Branches

Atrophy of coffee branches caused by *Xylella fastidiosa* has been attributed to nutritional deficiency. It became important in Brazil when infected plantations needed to be destroyed in Minas Gerais State and production losses occurred in São Paulo State. In Brazil, the disease was reported in 1995 on coffee plantations in São Paulo State and afterward in other Brazilian coffee growing regions. In Costa Rica, it was reported in 2001. Other names suggested for the disease are coffee stem atrophy and coffee leaf scorch.

Symptoms

The main cause of the disease is a dysfunctional water-conducting system due to the occlusion of the elements of the xylem by "gums", tyloses, or bacterium cells. Other proposed hypotheses are phytotoxins and the imbalance of growth regulators. The external symptoms in *Coffea arabica* L. branches are shortened internodes, leaves in a rosette with reduced leaf area and petioles, apical and marginal leaf scorch, senescence of mature leaves, and small number of leaves at the apex (Fig. 13). Shortened pedicels and internodes cause the clustering of flowers and fruits, reducing the size of these structures. During disease development, branches may become dry and lose their leaves or they may sprout elongated leaves at the nodes.

In severe cases, branches develop at least twice the number of internodes found in healthy branches. When the external symptoms are severe, the internal tissues of the branches have "gum" deposits in the xylem vessels and abnormal divisions in the cells of the mesophyll, xylem, phloem, and cortex of the petiole and stem, with calcium oxalate crystals sometimes present (Fig. 14). In affected leaves, the number of chloroplasts is reduced as the concentration of calcium oxalate crystals increases, turning the leaves light green. The level of xylem vessel blockage by bacterial cell aggregates and of phytotoxin production may be correlated with the level of foliage damage and of hormonal disequilibrium in the plant. Nutritional deficiency, inadequate cultural and sanitary practices, senescence, and water stress are factors that predispose the host to attack by *X. fastidiosa.*

Causal Organism

Xylella fastidiosa Wells, Raju, Hung, Weisburg, Mandelco-Paul & Brenner belongs to the class *Gammaproteobacteria*, order *Xanthomonadales*, family *Xanthomonadaceae*. The bacterium is rod shaped, gram negative, and filamentous and has wrinkled cell walls. It measures 0.25 0.35 × 0.9 3.5 µm and is strictly aerobic, static, and not pigmented. It grows slowly in artificial media, requiring specific culture media, an optimum temperature around 26–28°C, and a pH of 6.5–6.9. To date, three strains of *X. fastidiosa* have been classified: *X. fastidiosa* subsp. *fastidiosa* Schaad, Postnikova, Lacy, Fatmi & Chang; *X. fastidiosa* subsp. *multiplex* Schaad, Postnikova, Lacy, Fatmi & Chang; and *X. fastidiosa* subsp. *pauca* Schaad, Postnikova, Lacy, Fatmi & Chang. There is evidence for the existence of other subspecies. The majority of the pathotypes have not been characterized or compared against each other. Different strains may also cause the same symptoms in common hosts, as is the case of the citrus variegated chlorosis strains, which are capable of causing coffee leaf scorch, and of the citrus variegated chlorosis and coffee leaf scorch strains, both of which may cause Pierce's disease in vineyards.

Disease Cycle and Epidemiology

Twenty eight families of monocotyledonous and dicotyledonous plants (including trees) and perennial annual herbs are known hosts, with or without symptoms, of this bacterium. Asymptomatic hosts may act as alternate inoculum sources. In North, Central, and South America, *X. fastidiosa* causes important diseases in *Citrus* spp., *Prunus persica* (L.) Batsch, *Prunus salicina* Lindl., *Vitis vinifera* L., and *Coffea* spp. Other sources of inoculum are *Bidens pilosa* L., *Brachiaria decumbens* Stapf, *Brachiaria plantaginea* (Link) Hitchc., *Digitaria horizontalis* Willd., *Echinochloa crus galli* (L.) P. Beauv., *Medicago sativa* L., and *Solanum americanum* Mill. The presence of the bacterium on coffee plantations located in regions without citrus and the occurrence of citrus variegated chlorosis in regions with several coffee plantations suggest that coffee may be a primary source of inoculum for *Citrus* spp.

Coffee plants naturally infected by *X. fastidiosa* in different climatic regions of Brazil sustain more severe damage during the dry season because of increased obstruction of the xylem. Symptoms are less evident during the rainy season, although there is an increase of inoculum and potential for vector transmission. In coffee plants, the bacterium is transmitted by leafhopper vectors of the families Cicadellidae (subfamily Cicadellinae) and Cercopidae that feed in branches and leaves, preferably in the xylem tissues. In coffee, the transmission by *Bucephalogonia xanthophis* (Berg), *Dilobopterus costalimai* Young, *Homalodisca ignorata* Melichar, and *Oncometopia facialis* (Signoret) has been confirmed; the same leafhopper species are also vectors of the disease in citrus.

Management

The infection caused by *X. fastidiosa* is limited to the xylem vessels of the hosts, with irregular distribution. To reduce the

Fig. 13. Atrophy of a coffee branch caused by *Xylella fastidiosa*. (Courtesy R. B. Queiroz Voltan, IAC, Brazil)

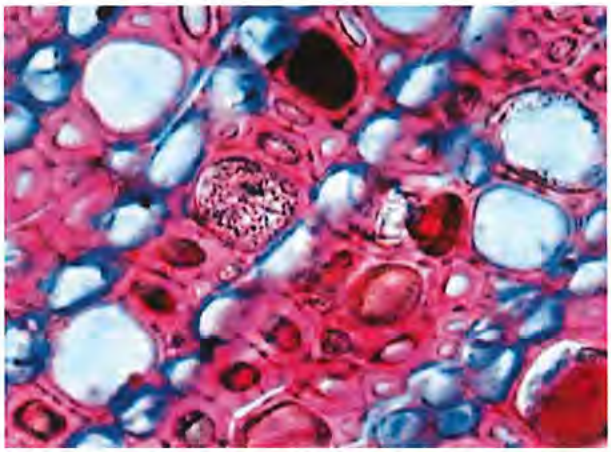


Fig. 14. Xylem cells in the trunk of *Xylella fastidiosa*-infected *Coffea arabica*, some with calcium oxalate and others with "gums". Uninfected cells have a whitish interior. (Courtesy R. B. Queiroz-Voltan, IAC, Brazil)

incidence of the pathogen in the field, management of the coffee crop should begin immediately by planting bacterium-free or resistant seedlings and controlling the sharpshooter leafhoppers that act as vectors. Pruning should be used to eliminate parts of the plant with advanced symptoms caused by *X. fastidiosa*. When disease severity is high, pruning plagiotropic branches in a "skeleton" fashion and stumping orthotropic branches are suggested methods to control *X. fastidiosa*. These types of more drastic pruning eliminate almost the entire upper part of the coffee plant or most of the plagiotropic branches and reduce a large portion of the root system, which is recovered after new shoots sprout. Pruning is also recommended for control of the *Xylella* diseases in citrus and grapevines.

Management with chemical products is not economically feasible. Management of leafhoppers is controversial because of the reservoir of alternate hosts for the vector and the bacterium. Currently, crop management with nutrients and other measures that maintain adequate vigor of the coffee plant increases tolerance to the disease.

Selected References

de Lima, J. E. O., Miranda, V. S., Hartung, J. S., Brlansky, R. H., Coutinho, A., Roberto, S. R., and Carlos, E. F. 1998. Coffee leaf scorch bacterium: Axenic culture, pathogenicity, and comparison with *Xylella fastidiosa* of citrus. Plant Dis. 82:94 97.

Esau, K. 1948. Anatomic effects of the viruses of Pierce's disease and phony peach. Hilgardia 18:423 482.

French, W. J., and Stassi, D. L. 1978. Response of phony infected peach trees to gibberellic acid. HortScience 13:158 159.

Garrity, G. M., and Holt, J. G. 2001. The road map to the *Manual*. Page 119 166 in: Bergey's Manual of Systematic Bacteriology, Vol. 1, 2nd ed. D. R. Boone and R. W. Castenholz, eds. Springer Verlag, New York.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. 1994. Page 100 in: Bergey's Manual of Determinative Bacteriology, 9th ed. Williams & Wilkins, Baltimore, MD.

Li, W. B., Pria, W. D., Jr., Teixeira, D. C., Miranda, V. S., Ayres, A. J., Franco, C. F., Costa, M. G., He, C. X., Costa, P. I., and Hartung, J. S. 2001. Coffee leaf scorch caused by a strain of *Xylella fastidiosa* from citrus. Plant Dis. 85:501 505.

Mircetich, S. M., Lowe, S. K., Moller, W. J., and Nyland, G. 1976. Etiology of almond leaf scorch disease and transmission of the causal agent. Phytopathology 66:17 24.

Paradela Filho, O., Sugimori, M. H., Ribeiro, I. J. A., Machado, M. A., Laranjeira, F. F., Garcia, A., Jr., Beretta, M. J. G., Harakawa, R., Rodriques Neto, J., and Beriam, L. O. S. 1995. Primeira constatação em cafeeiro no Brasil da *Xylella fastidiosa* causadora da clorose variegada dos citros. Laranja 16:135 136.

Queiroz Voltan, R. B., Cabral, L. P., Paradela Filho, O., and Fazuoli, L. C. 2006. Prune efficiency in the control of *Xylella fastidiosa* in coffee trees. Bragantia 65:433 440.

Randall, J. J., Goldberg, N. P., Kemp, J. D., Radionenko, M., French, J. M., Olsen, M. W., and Hanson, S. F. 2009. Genetic analysis of a novel *Xylella fastidiosa* subspecies found in the southwestern United States. Appl. Environ. Microbiol. 75:5631 5638.

Rodríguez, C. M., Obando, J. J., Villalobos, W., Moreira, L., and Rivera, C. 2001. First report of *Xylella fastidiosa* infecting coffee in Costa Rica. Plant Dis. 85:1027.

Wells, J. M., Raju, B. C., Hung, H. Y., Weisburg, W. G., Mandelco Paul, L., and Brenner, D. J. 1987. *Xylella fastidiosa* gen. nov. sp. nov.: Gram negative, xylem limited fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Syst. Bacteriol. 37:136 143.

Yorinori, M. A., Ribas, A. F., Ueno, B., Massola, N. S., Jr., and Leite, R. P., Jr. 2003. Detection of *Xylella fastidiosa* in coffee germplasm. Fitopatol. Bras. 28:427 430.

Zambolim, L., Vale, F. X. R., and Zambolim, E. M. 2005. Doenças do cafeeiro. Pages 165 180 in: Manual de Fitopatologia: Doenças das Plantas Cultivadas, Vol. 2. H. Kimati, L. Amorim, J. A. M. Rezende, A. Bergamin Filho, and L. E. A. Camargo, eds. Agronômica Ceres, São Paulo, Brazil.

(Prepared by R. M. de Lemos Cardoso)

Bacterial Leaf Blight

Bacterial leaf blight was described for the first time in coffee plants in Brazil in 1974, and the causal organism was identified as *Pseudomonas cichorii*. In that year, in Minas Gerais State, this bacterium almost completely destroyed the plantlets in a commercial nursery and caused damage on adult plants. Attacks similar to those attributed to bacterial leaf blight were reported in 1971 in coffee plants of other municipalities in the states of Minas Gerais and Espírito Santo. From 1990 to 2001, bacterial leaf blight was responsible for severe damage to coffee plants in nurseries in the Lavras area of Minas Gerais State.

Symptoms

The bacterium *P. cichorii*, causal organism of bacterial leaf blight, is pathogenic to coffee and rapidly colonizes mature leaves. Dark, water soaked areas develop from penetration points on a leaf (Fig. 15) and can encompass large areas of the leaf surface (Fig. 16). This bacterium readily penetrates wounds in foliar tissues but can also infect the coffee plant through natural openings (Fig. 17). In this case, most of the lesions form on the lateral margins of the leaf or at the apex. In the field or in nursery plantlets under favorable conditions, the foliar lesions cause leaf drop because of the rapid development of infections. Concentric rings can usually be seen around the point of infection when affected leaves are viewed with a backlight (Fig. 18). In nursery plantlets and field plants in Minas Gerais State, there has been frequent association between lesions caused by *P. cichorii* and lesions caused by the fungus *Cercospora coffeicola* Berk. & Cooke. In coffee plants, symptoms caused by *P. cichorii* are on mature leaves, whereas symptoms caused by *P. syringae* van Hall pv. *garcae* (Amaral, Teixeira & Pinheiro) Young, Dye & Wilkie are on young leaf tissues, sprouts, and apical tissues.

Causal Organism

Pseudomonas cichorii (Swingle) Stapp belongs to the class *Gammaproteobacteria*, order *Pseudomonadales*, and family *Pseudomonadaceae*. *P. cichorii* is a rod-shaped bacterium that

Fig. 15. Punctual lesion caused by *Pseudomonas cichorii*, with a water soaked area around necrotic tissue. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

is mobile by a single polar flagellum and produces colonies on seed agar. The colonies are white gray, slightly elevated, and circular with irregular edges. The bacterium is gram negative, oxidase positive, and levan negative; does not liquefy gelatin; produces fluorescent pigments in King's medium B; has alkaline action on bromocresol purple milk medium; induces hypersensitivity in tobacco leaves; and presents an oxidative reaction in glycolysis metabolism. *P. cichorii* is fundamentally distinct from *P. syringae* pv. *garcae* because it is oxidase positive, while *P. syringae* pv. *garcae* is oxidase negative. Although both are fluorescent pseudomonads, *P. cichorii* and *P. syringae* pv. *garcae* are in different groups in the genus *Pseudomonas*. The most practical method to separate the two species is to isolate them in King's medium B and perform the oxidase test.

Disease Cycle and Epidemiology

There are numerous hosts of *P. cichorii*, including *Allium cepa* L., *Arracacia xanthorrhiza* Bancroft, *Beta vulgaris* L., *Brassica oleracea* L. var. *capitata* L., *Capsicum annuum* L., *Cichorium endivia* L., *Curcuma longa* L., *Dendranthema grandiflora* Tzvelev, *Eucalyptus* spp., *Helianthus annuus* L., *Lactuca sativa* L., *Mentha arvensis* L., *Ocimum basilicum* L., *Phaseolus vulgaris* L., *Philodendron* spp., *Pisum sativum* L., *Saintpaulia ionantha* H. Wendl., *Solanum lycopersicon* L., and *Solanum tuberosum* L. Some of the hosts listed are cultivated near coffee plantations and may act as sources of the bacterial inoculum.

P. cichorii is an epiphyte on the surface of coffee leaves and epidemics occur during periods of high temperatures with abundant and frequent rainfall. The outbreaks end when these favorable conditions are followed by a dry period with lower temperatures. This behavior differentiates it from *P. syringae* pv. *garcae*, whose incidence in coffee increases during cooler and more humid months of the year. As an epiphyte on mature leaves, *P. cichorii* penetrates through natural openings, such as stomata and lenticels; through mechanical injuries caused by windblown soil particles; and as a secondary infection of lesions caused by fungi, such as *Cercospora coffeicola* or *Phoma* spp., or by pests, such as *Perileucoptera coffeella* (Guérin Méneville). *P. cichorii* does not easily infect foliar tissues without wounding, but penetration can be facilitated by adding a surfactant to the suspension of bacterial inoculum. Under natural conditions, undetermined mechanisms contribute to adherence of the bacterium to the leaf surface and to penetration through natural openings.

Management

Preventive control of *P. cichorii* is practiced by minimizing favorable conditions for disease development. Nurseries should

Fig. 16. Necrotic lesions caused by *Pseudomonas cichorii* on mature leaves of *Coffea arabica*. The pathogen entered through injuries caused by the abrasive action of windblown soil particles. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

Fig. 17. Necrotic lesions caused by *Pseudomonas cichorii* on mature leaves of *Coffea arabica*. The pathogen entered through natural openings. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

Fig. 18. Punctual lesion caused by *Pseudomonas cichorii*, with concentric rings around the point of infection. (Courtesy R. M. de Lemos Cardoso, IAPAR, Brazil)

be located in areas that are protected from the wind to prevent soil particles from causing wounds that predispose the nursery plants to bacterial infection. Nurseries, although protected, should maintain proper air ventilation to ensure the appropriate temperature and humidity. Foci of disease should be managed according to their size and severity. In nurseries, under low disease incidence or at the initial stage of infection, affected leaves may be removed by sanitary pruning methods, followed by treatment of the plants with copper oxychloride (50% metallic copper). Under higher disease severity, foci should be isolated and treated with a mixture of streptomycin sulfate and copper oxychloride. Plantlets surrounding these patches should be treated only with copper oxychloride.

In the field, coffee plants should be protected from the wind by temporary or permanent windbreaks. Under low disease incidence or at initial infection stages, injured leaves can be selectively removed with sanitized pruning tools. Plants with high disease severity should be sprayed with a mixture of streptomy cin sulfate and copper oxychloride. Cultivars Mundo Novo and Catuai are susceptible to the pathogen. The behavior of newer cultivars used in Brazil is not yet known. In susceptible cultivars, the young leaves are not infected by the pathogen. Resistance of coffee plants to *P. cichorii* is not correlated with the presence of phenolic compounds in extracts of young and mature leaves.

Selected References

Garcia, D., Jr., Pozza, E. A., Souza, P. E., Talamini, V., Castro, H. A., Souza, R. M., Abreu, M. S., and Pfenning, L. H. 2003. Origin of coffee samples, frequency of occurrence of etiological agents and symptoms in twelve years of the plant disease clinic at UFLA. Cienc. Agrotecnol. 27:173 177.

Gilmar, P. H. 2002. Doenças da mandioquinha-salsa e sua situação atual no Brasil. (Present situation of arracacha (*Arracacia xanthorrhiza*) diseases in Brazil.) Hortic. Bras. 20:135 144.

Gonçalves, R. C. 2003. Etiologia da mancha bacteriana com eucalipto no Brasil. Ph.D. thesis. Universidade Federal de Viçosa, Viçosa, Brazil.

Kimati, H., Amorim, L., Bergamin Filho, A., Camargo, L. E. A., and Rezende, J. A. M., eds. 2005. Manual de Fitopatologia: Doenças das Plantas Cultivadas, Vol. 2, 4th ed. Agronômica Ceres, São Paulo, Brazil.

Kimura, O., Robbs, C. F., and Ferrari, J. A. 1976. Algumas observa ções relacionadas com as bacterioses do cafeeiro. Page 104 in: Con gresso Brasileiro de Pesquisas Cafeeiras, 4th. Resumos. Instituto Brasileiro do Café, Grupo Executivo da Racionalizacão da Cultura Caféeira (GERCA), Rio de Janeiro, Brazil.

Maringoni, A. C., Theodoro, G. F., Ming, L. C., Cardoso, J. C., and Kurozawa, C. 2003. First report of *Pseudomonas cichorii* on tur meric (*Curcuma longa*) in Brazil. Plant Pathol. 52:794.

Oliveira, J. R., and Romeiro, R. S. 1990. Reaction of new and old cof fee leaves to *Pseudomonas cichorii* and *Pseudomonas syringae* pv. *garcae*. Fitopatol. Bras. 15:355 356.

Oliveira, J. R., and Romeiro, R. S. 1991. Phenolics compounds, leaf age and coffee resistance to *Pseudomonas cichorii* and *Pseudomonas syringae* pv. *garcae*. Rev. Ceres 38:445 452.

Robbs, C. F., Kimura, O., Ribeiro, R. L., and Oyadomari, L. C. 1974. "Crestamento bacteriano das folhas", nova enfermidade do cafeeiro (*Coffea arábica* L.) incitado por *Pseudomonas cichorii* (Swingle) Stapp. Arq. Univ. Fed. Rural Rio de Janeiro 4:1 5.

Soto, W. R., López, N. M., González, J., Oliva, P., and Dueñas, M. 2003. *Ocimum basilicum* L. nuevo hospedante em Cuba de *Pseudomonas cichorii*. Fitosanidad 7(3):3 5.

(Prepared by R. M. de Lemos Cardoso)

Diseases Caused by Fungi

Damping-Off

Coffee seeds are planted in seeding beds at densities close to 4,000 seeds per square meter, which under proper viability conditions should have a germination percentage of around 90%. Consequently, seedlings in the cotyledon stage are in close contact and susceptible to soilborne diseases such as damping off.

Symptoms

Damping off starts as patches in the germination bed, which are evident 15 days after sowing (Fig. 19). Early infection takes place in the immature radical tissue, resulting in seed rot. Preemergence damping off occurs once the seed has sprouted but before the young stem reaches the surface. After the seedlings break through the soil mass, the point where the stem is in contact with the soil is attacked, resulting in postemergence damping off (Fig. 20). The pathogen advances around the stem, constricting it and moving upward. The cell walls become weakened and the plant topples once the structural support is reduced, usually with its leaves still green. Diseased tissues exhibit a dark brown color and expand rapidly until the cotyledon is completely involved. Hypocotyls can survive late infections with the development of suberized tissue, several cell layers deep, that surrounds the infection point. By the time the first and second pairs of true leaves are produced, the epicotyl is no longer susceptible.

Fig. 19. *Rhizoctonia solani* attack on a *Coffea arabica* seeding bed. (Courtesy G. Hoyos, Cenicafé)

Causal Organism

Damping off is caused by *Rhizoctonia solani* Kühn, whose teleomorph is the basidiomycete *Thanatephorus cucumeris* (A. B. Frank) Donk (order Ceratobasidiales and family Cerato basidiaceae). *R. solani* lacks conidia; therefore, morphological characteristics are limited to vegetative mycelium. Young hyphae have a length to-width ratio of 5:1 and a diameter of 5–8 µm, with branching close to the distal septum and a constriction of the new hyphae at the branching point. The septum is doliporum, and clamp connections and rhizomorphs are absent. Mycelial pigmentation varies in a wide range of browns, and hyphae are multinucleated with four to nine nuclei (Fig. 21), in contrast to other binucleated species of the genus. Intraspecific divisions in *R. solani* are defined by anastomosis groups (AG), or the capacity of hyphae from two separate isolates to fuse in a parasexual process. In Brazil and Colombia, *R. solani* AG-4

Fig. 20. Pre and postemergence damping-off caused by *Rhizoctonia solani* on coffee seedlings. (Courtesy G. Hoyos, Cenicafé)

Fig. 21. *Rhizoctonia solani* multinucleated cell stained with safranin O (×400). (Courtesy A. Gaitán, Cenicafé)

Fig. 22. Epifluorescent microscopy of the infection cushion of *Rhizoctonia solani* on coffee hypocotyls. (Courtesy A. Gaitán, Cenicafé)

has been reported to cause disease in coffee plants. Anastomosis in vitro generates heterokaryotic tufts (white, cottonlike mycelium). The perfect stage, *T. cucumeris*, has been observed in advanced lesions on the lower surface of fallen cotyledons, with abundant basidiospore production. The sporulating area does not wet easily. Under culture conditions, microsclerotia are formed.

Disease Cycle and Epidemiology

Disease inoculum is naturally present in the soil as sclerotia or in plant debris, as well as in organic compost, which is sometimes added as a surface dressing on seedbeds. The effective dose 50 (ED_{50}) varies from 400 to 760 propagules from ground mycelium per 100 g of soil. During the infection process, the fungal hyphae grow on the host surface along the cellular wall borders, and then a multicellular infection cushion is formed to initiate penetration (Fig. 22). Enzymatic cell wall degradation proceeds just below the infection peg, and finally there is intercellular growth once the epidermis is penetrated. The infection process lasts 5 days. Under favorable moisture and temperature conditions, fungal hyphae spread rapidly to reach the coffee seeds or move from seedling to seedling, involving all the surrounding cotyledons as they progress. *R. solani* sclerotia then form on the cortex and remain in the soil. They can survive for up to 225 days on the soil surface or for 375 days when buried.

Management

Genetic resistance against *R. solani* has not been found in cultivars or in coffee germplasm collections. Damping off is a soilborne disease that does not require seed treatment before planting. As a natural soil inhabitant, reduction of soil inoculum is therefore necessary and can be achieved by several means. Large plant debris can be removed by sieving the soil through a fine mesh. Additionally, splashing inoculum from the surrounding soil can be avoided by constructing seedbeds 50 cm above the ground. Washed river sand can be used as a substrate for seed germination instead of soil and can be heat treated with aerated steam. Soil or sand may be solarized or treated with a fungicide or a biological control agent before planting. Copper sulfate and copper oxide are toxic when applied at 25 1,000 ppm to seedbeds, causing root atrophy and signs of toxicity. Products based on thiabendazole can be applied as a liquid suspension (10 ml in 2 L of water per square meter). Pencycuron, tolclofos methyl, and flutolanil are similarly effective for preventive control, as are commercial preparations of *Trichoderma harzianum* Rifai. Because sclerotia have prolonged survival periods, seedbed substrates must be treated or replaced annually or before use. Once the disease is observed, immediate removal of the affected seedlings and their surroundings is necessary, as is replacing the substrate mass with a new one. Chemical disinfectant can be applied locally in the area.

Selected References

Castro, A. M., and Rivillas, C. A. 2005. Biorregulacion de *Rhizoctonia solani* en germinadores de café. Av. Téc. Cenicafé 336:1 8.

Gaitán B., A. L., and Leguizamon C., J. E. 1992. Biología y patogénesis de *Rhizoctonia solani* en café. Fitopatol. Colomb. 16:165-171.

Sussel, A. A. B., Fenille, R. C., Kuramae, E. E., and de Souza, N. L. 2001. Caracterizacao de *Rhizoctonia solani*, agente etiológico do tombamento de mudas de café. Summa Phytopathol. 27:348 352.

Venkatasubbaiah, P., and Safeeulla, K. M. 1983. Studies on the viability of sclerotia of *Rhizoctonia solani* in the soil. J. Coffee Res. 13:30 32.

Venkatasubbaiah, P., and Safeeulla, K. M. 1983. Importance of inoculum density of *Rhizoctonia solani* in collar rot disease of coffee. J. Coffee Res. 13:53 55.

Venkatasubbaiah, P., Prakash, H. S., Shetty, H. S., and Safeeulla, K. M. 1984. Histopathological and histochemical changes due to *Rhizoctonia solani* infection on coffee seedlings. J. Coffee Res. 14:26 33.

Venkatasubbaiah, P., Safeeulla, K. M., and Somashelar, R. K. 1984. Efficacy of *Trichoderma harzianum* as a biocontrol agent for *Rhizoctonia solani*, the incitant of collar rot in coffee seedlings. Biol. Sci. 50:525 529.

(Prepared by A. Gaitán)

Rosellinia Root Rot

Rosellinia root rot affects a large number of plant species in the tropics and subtropics, including cacao, citrus, coffee, avocado, rubber, inga, macadamia, plantain, banana, cassava, and various noncommercial trees. In 1870, Berkeley and Broome detected a *Rosellinia* species in coffee roots in Ceylon, now Sri Lanka. Recently, it has been reported as a very aggressive pathogen in coffee growing areas of Central America, South America, and India, causing dieback and death of trees. The *Rosellinia* spp. on coffee plants are *R. bunodes*, causing black root rot in Central America; *R. pepo*, causing star-shaped rot in South America; and *R. arcuata* Petch, causing black root disease in India.

Fig. 23. General chlorosis and wilting of leaves from root rot caused by *Rosellinia* spp. (Courtesy B. L. Castro Caicedo, Cenicafé)

Fig. 24. Mycelial black dot and short line symptoms of *Rosellinia bunodes* root rot. (Courtesy B. L. Castro Caicedo, Cenicafé)

Symptoms

The external symptoms can be difficult to distinguish from those caused by other vascular pathogens such as *Ceratocystis fimbriata* Ellis & Halst., flooded soils, poorly developed root systems with twisted taproots, or insect attacks on young plants. The coffee plants affected are located in foci and the first symptoms are general chlorosis, followed by wilting of leaves, dieback, and death of trees (Fig. 23). The primary site of infection is at the root collar area, where the bark is superficially cracked and masses of mycelium can be found as black dots or short lines embedded in the internal wood when caused by *R. bunodes* (Fig. 24) or as a white, stellate mycelial growth under the bark of affected roots when caused by *R. pepo* (Fig. 25).

Causal Organisms

Rosellinia root rot is caused by the species *Rosellinia bunodes* (Berk. & Broome) Sacc. and *Rosellinia pepo* Pat. (order Xylariales and family Xylariaceae). The fungi have two forms: the ascosporic (teleomorph) *Rosellinia* spp., and the conidial (anamorph) *Dematophora* sp., which is rarely found in nature. Asci are hyaline, cylindrical, 9–12 × 250–350 µm, long stalked, and unitunicate with eight ascospores. Ascospores are ellipsoidal, without cellular appendages. Several dark reddish to brown synnemata arise from a common enlarged base, are 1.5 mm × 40.0–140.0 µm long, and are composed of septate hyphae with a 3–4 µm diameter. Conidiophores are formed on the top of the synnema, up to 70 µm long, frequently di- or trichotomously branched, and light brown. Conidia are one celled, simple, ellipsoidal or ovoid, hyaline to pale brown, and smooth. The *R. bunodes* conidia are 3.0–3.5 4.0 × 6.0–7.0 µm and the *R. pepo* conidia are 2.0–3.0 × 5.0 9.0 µm.

Under special in vitro culture conditions, such as malt extract or cornmeal agar containing ampicillin, white septate mycelium develops initially. With age, *R. bunodes* becomes black and feltlike and *R. pepo* becomes smoky with radial lines and white, stellate structures on the back of the petri dish. The most

Fig. 25. White, stellate mycelial growth symptoms of *Rosellinia pepo* root rot. (Courtesy B. L. Castro Caicedo, Cenicafé)

important morphological characteristic in the hyphae of both species is the pear-shaped or pyriform swellings immediately above the septum (Fig. 26). Both species have an optimal temperature of 22 27°C for mycelial growth on media from pH 5.5 to 7.0 under lighted conditions and from pH 4.0 to 8.0 in the dark. Ascospores and conidia do not germinate in vitro.

Disease Cycle and Epidemiology

R. bunodes and *R. pepo* are soilborne, facultative saprophytes that become pathogenic on dead stumps or logs on newly cleared land and spread to nearby living trees, thereby affecting a circle of trees around the infection center by root contact. The pathogen starts to colonize with a thin, brilliant white mycelium that superficially invades the larger roots, changing progressively until it turns gray. *R. bunodes* mycelium then forms black masses on the wood and black stripes and dots under the bark, while *R. pepo* produces dense gray, cottony mycelium superficially over the root and fan or star-shaped mycelium under the bark. After the substrate has been consumed, synnemata emerge from the root or on the infested soil. *Rosellinia* spp. can survive saprophytically for several years on rotted roots in the soil. Infested soil and diseased root debris may be transported by farm operations. Cassava debris left on the soil is a rich medium for fungal growth. Optimum natural conditions for development of *Rosellinia* spp. include soils rich in organic matter, temperatures of 20–28°C, soil moisture of 70 80%, and pH of 4.0 7.0. Extremely well drained or flooded soils have strong inhibitory effects on pathogen growth. The disease cycle has been studied by artificially inoculating seedlings and plantlets of *Coffea arabica* L. 'Colombia' with *R. bunodes* grown on sterilized sorghum seeds. Symptoms develop after 9 days on seedlings and 20 days on plantlets, when the pathogen reaches the root collar. The mycelium initially develops on the root surface and then penetrates the cells until reaching conducting vessels and the medulla. Black mycelia colonize the conducting vessels, forming the black stripes and dots in the roots that characterize the disease.

Management

The main control measures are preventing spread of the fungus in the soil and removing possible inoculum sources. All affected trees and their neighbors should be uprooted and destroyed, and care should be taken to prevent movement of the remaining fragments of diseased roots in the soil. Foci of affected trees must be isolated by trenching, and immediate replanting must be avoided. Soil solarization or a drench application of thiophanate methyl helps prevent fungal development during the early stages of infection. Studies on the use of biological antagonists support the effectiveness of *Trichoderma koningii* Oudem. and the bacterium *Burkholderia cepacia* (Palleroni & Holmes) Yabuuchi, Kosako, Oyaizu, Yano, Hotta, Hashimoto, Ezakio & Arakawa. Arbuscular mycorrhizal fungi *Glomus fistulosum* Skou & I. Jakobsen and *Entrophospora colombiana* Spain & N. C. Schenck seem to confer tolerance on coffee plantlets when inoculated on seedlings. Resistance in coffee varieties is unknown.

Fig. 26. *Rosellinia bunodes* basal enlargement at the septum (x400). (Courtesy B. L. Castro Caicedo, Cenicafé)

Selected References

Bermudes, M., and Carranza M., J. 1992. Estado anamórfico de *Rosellinia bunodes* (Berk & Br.) Sacc. y *Rosellinia pepo* (Ascomytina: Xilariaceae). Rev. Biol. Trop. 40:43 46.

Castro C., B. L. 1995. Antagonismo de algunos aislamientos de *Trichoderma koningii*, originarios del suelo colombiano, contra *Rosellinia bunodes*, *Sclerotinia sclerotiorum* y *Pythium ultimum*. Fitopatol. Colomb. 19:7 17.

Castro T., A. M., and Rivillas O., C. A. 2002. *Entrophospora colombiana*, *Glomus manihotis* y *Burkholderia cepacia* en el control de *Rosellinia bunodes* agente causante de la llaga negra del cafeto. Cenicafé 53:193-218.

Castro, B. L., Carreño, A. J., Galeano, N. F., Roux, J., Wingfield, M. J., and Gaitán, A. L. 2013. Identification and genetic diversity of *Rosellinia* spp. associated with root rot of coffee in Colombia. Australas. Plant Pathol. 42:515 523.

Fernández B., O., and López D., S. 1964. Llagas radiculares negras (*Rosellinia bunodes*) y estrellada (*Rosellinia pepo*) del cafeto. I. Patogenicidad c influencia dc la clasc dc inóculo en la infección. Cenicafé 15:126-144.

Gutierrez G., R. A., Castro C., B. L., and Rivillas O., C. A. 2004. Manejo de focos de llagas radicales en cafetales. Av. Téc. Cenicafé 327:1 8.

Ibarra G., N. L., Castro C., B. L., and Ponce, C. A. 1999. Estudio del proceso infectivo de *Rosellinia bunodes* Berk y Sac. en café. Fitopatol. Colomb. 23:59 64.

Kannan, N. 1995. Technical report on diseases affecting coffee in India. Indian Coffee 59:11-17.

Lopez D., S., and Fernández B., O. 1966. Llaga radical negra (*Rosellinia bunodes*) y estrellada (*Rosellinia pepo*) del cafeto. II. Efecto de la humedad y pH del suelo en el desarrollo micelial e infección. Cenicafé 17:61-69.

Petrini, L. E., and Petrini, O. 2005. Morphological studies in *Rosellinia* (Xylareaceae): The first step towards a polyphasic taxonomy. Mycol. Res. 109:569 580.

Saccas, A. M. 1956. Les *Rosellinia* de cefeires en Oubangui-Chari. Agron. Trop. 11:551 595, 687 706.

(Prepared by B. L. Castro Caicedo)

Root Diseases

In addition to Rosellinia root rot, three types of root diseases, namely the brown, red, and Santavery, have been recorded on coffee plantations in India. The first two are generally observed on both arabica and robusta coffee, while Santavery root disease is specific to arabica coffee. These root diseases are sporadic in nature. Control measures are adopted when these diseases are noticed in the fields.

The aerial symptoms for all four types of root diseases are alike in that the affected plants gradually wilt, yellow, and drop their leaves, followed by complete defoliation and death.

Brown Root Disease (Stump Rot)

Brown root disease is commonly known as stump rot and is caused by *Phellinus noxius* (Corner) G. Cunn. (previously *Fomes noxius* Corner). The fungus belongs to the phylum Basidiomycota, order Hymenochaetales, and family Hymenochaetaceae. The common name refers to the everyday association of the pathogen with the rotting stumps of shade trees. Disease spreads to neighboring plants by means of contact with the roots of infected stumps or plants.

The stem of the affected plant becomes spongy and soft near the ground level. The root system develops thick, brown encrustations that adhere to small stones. The fungal mycelium appears either as tiny, brown, wooly hyphae or as brown crusts on the root surface. The name brown root disease comes from the brown fungal encrustation. Affected roots are brittle and readily snap off. The interior of the root has wavy lines that are dark brown or black. These lines consist of tangled fungal hyphae. Detailed studies on brown root disease (*Fomes lamaoensis* (Murrill) Sacc. & Trotter) were done as early as 1930. Disease was found on *Coffea arabica* L., *Coffea robusta* L. Linden, *Thea sinensis* L., cocoa, nutmeg, oil palm, rubber, citrus, *Tephrosia candida* (Roxb.) DC., *Erythrina* sp., *Grevillea* sp., and a number of jungle stumps.

A common control strategy is to remove and destroy the affected plants and isolate the healthy plants by digging trenches 30 cm wide and 60 cm deep to avoid spread of the disease. Agricultural lime should be added to the soil at a rate of 1–2 kg per square meter after uprooting the affected plants and the site should be monitored for 6–12 months to check the soil reaction. Drenching the soil with carbendazim 50% wettable powder (WP) at 0.4% (8 g per liter of water) or carboxin 75% WP at 0.3% (4 g per liter of water) at the rate of 3 L of fungicide solution per plant during the initial stages of wilting, when green leaves are falling, helps in the recovery of the plant. Whenever a shade tree falls, it is advisable to uproot the stump to avoid spread of the disease. Soil application of the biological control agent *Trichoderma* sp. has reduced disease incidence.

Red Root Disease

The causal organism of red root disease, *Schizopora flavipora* (Berk. & M. A. Curtis ex Cooke) Ryvarden (previously *Poria flavipora* Berk. & M. A. Curtis ex Cooke), is in the phylum Basidiomycota, order Hymenochaetales, and family Schizoporaceae. The disease was first reported on coffee in 1975, but since 1923 this fungus has been known to cause red root disease of tea in Sri Lanka, where it is a serious plantation problem. Several shade trees have been identified as being susceptible to *Schizopora* infection in Sri Lanka. Coffee plants become infected from neighboring shade tree stumps infected with *S. flavipora.*

Efforts have been made to spot resistant shade trees to arrest the spread of the disease. Artificial inoculations of *Maesopsis eminii* Engl., commonly referred to as African shade tree, demonstrated resistance to *S. flavipora*, after healthy trees were observed standing in the midst of red root disease affected areas. In southern India, *S. flavipora* is widespread throughout all the tea-growing areas, including Coorg and Chikmagalur Districts of Karnataka. Of plantation shade trees, generally the silver oak (*Grevillea robusta* A. Cunn. ex R. Br.) and nerale (*Syzygium jambolana* DC.; syn. *Syzygium jambolanum* (Lam.) DC.) die first, followed by adjacent coffee plants. *Pterocarpus marsupium* Roxb., *Dalbergia latifolia* Roxb., *Cedrela toona* Roxb. ex Rottler & Willd., *Ficus tsiela* Roxb., *Ficus tsjakela* Burm. f., *Ficus glomerata* Roxb., *Ficus retusa* L., *Acrocarpus fraxinifolius* Wight ex Arn., *Artocarpus integrifolia* L., *Erythrina lithosperma* Miq., and wild cinnamon were all found to be collateral hosts. The incubation period of *S. flavipora* on coffee plants was reported to range between 21 and 31 months.

Fig. 27. Affected root exhibiting dark strands of fungal mycelium in an advanced stage of red root disease (scale bar = 0.5 cm). (Courtesy S. Daivasikamani)

Infected roots may be covered with soil, sand, or gravel encrustations, along with the dark red, ribbonlike strands of the fungal mycelium, which can be seen clearly when the root is held against bright sunlight, a good diagnostic symptom of *S. flavipora* infection. In advanced stages of the disease, the reddish mycelium turns black and the disease may be mistaken for brown root disease, caused by *Phellinus noxius* (Corner) G. Cunn. However, washing the roots under running water and vigorously rubbing the surface invariably brings out the deep red color of the *S. flavipora* mycelium (Fig. 27). Infected plants gradually wilt and die, with heavy losses during periods of drought.

Dead or dying plants within the affected area must be uprooted and burned. Isolation of the diseased plant or the affected area by trenching is necessary to control the spread of infection. Replanting in a *S. flavipora*-infected area should not be undertaken immediately after removal of the dead or dying plants. The other control measures recommended for brown root disease serve for red root disease as well.

Santavery Root Disease

Santavery root disease is caused by the fungus *Fusarium oxysporum* Schltdl.:Fr. f. sp. *coffeae* (García Rada) Wellman. The fungus belongs to the class Deuteromycetes, order Moniliales, and family Tuberculariaceae. In general, *Fusarium* spp. are known to cause vascular wilts and root rot of several economic crop plants, including coffee. Santavery root disease of coffee has been reported to be caused by *F. oxysporum* in Puerto Rico, while in El Salvador, the causal organism responsible was identified as a *Fusarium* sp. Tracheomycosis has been reported to be caused by *F. oxysporum* and *F. xylarioides* Steyaert in the former Belgian Congo, Puerto Rico, and Ivory Coast. In India, *F. solani* (Mart.) Sacc. var. *minus* Wollenw. has been reported to cause vascular wilt on 6 month-old arabica coffee seedlings in the nursery and *F. javanicum* Koord. has been reported to cause wilt of coffee plants. The favorable factors for Santavery root disease development are high soil temperature, marginal soil quality, inadequate shade, and wounding of the roots.

Sudden wilting and yellowing of the leaves, followed by defoliation and death of aerial parts of the plant, are symptoms of the disease. Scraping the bark of the infected stem near the ground level reveals internal discoloration. Roots show brown to pinkish discoloration of the vascular system in transverse section. Most arabica selections are susceptible to this disease. Wilting and death of 1.5 to 2-year-old coffee plants in the field was recorded in 1953–1954. Specimens with discoloration at the lower portion of the stem and root that were received from different plantations yielded a *Fusarium* sp. in culture. A similar case of wilting and death of coffee plants caused by a *Fusarium* sp. was reported from Puerto Rico.

Isolations made from infected plants revealed either *F. solani* or *F. javanicum*, which was a new report on coffee in India. This disease was named Santavery root disease, for the state in which it was first reported in the Chikmagalur District, where it was prevalent on arabica coffee. There have been reports of root diseases on certain coffee plantations in the Chikmagalur District of Karnataka and in Yercaud, Tamil Nadu. Isola

tions from infected roots yielded *F. oxysporum*. Pathogenicity of *F. oxysporum* on arabica coffee seedlings has been proven. *F. oxysporum* was described as *F. oxysporum* f. sp. *coffeae*, giving it a *formae speciales* status.

Physiological studies were conducted from leaf and root samples of normal, medium, and severely affected (dead) plants from Santavery State. Total content of phenol and lipids in leaves and roots decreased as the infection levels increased.

In vitro screening of the pathogen *F. oxysporum* f. sp. *coffeae* against *Trichoderma harzianum* Rifai by the dual culture method indicated this antagonist to be an efficient biological control agent, although its effectiveness is not comparable to that of fungicides. Maximum inhibition of fungal mycelial growth was achieved with *T. harzianum* (80%) after 8 days of incubation, and the antagonistic effect of *T. harzianum* against *F. oxysporum* provided 65% inhibition after 4 days of incubation.

Removing and destroying the affected plants, treating the diseased block with well decomposed cattle manure or com post at 10 15 kg per plant, liming the soil to an optimal level of around pH 6.1, maintaining adequate overhead shade, applying balanced nutrients, and following cultural operations to maintain the vigor of the plants are the usual control measures adopted against this disease. Even though these treatments do not offer complete control, they prevent further spread of the disease.

Selected References

Anonymous. 1954. Annual Report of the Research Department of the Indian Coffee Board. Indian Coffee Board, Balehonnur, India.

Berry, P. A., and Abrego, L. 1953. Insects and diseases affecting some crops in El Salvador. FAO Plant Prot. Bull. 1:151 153.

Felix, H. J. 1950. Première action contre la trachéomycose du caféier en Côte d'Ivoire. Publ. Agron. Trop. Nogent sur Marne.

Fraselle, J. 1950. Observations préliminaires sur une trachéomycose de café Robusta. Bull. Agric. Congo Belge 4:361 372.

Fraselle, J., Valleys, G., and Deknnop, U. O. 1953. La lutte contre la trachéomycose du caféier à Yangambi et le problème que pose actuellement cette maladie au Congo Belge. Bull. Inf. INEAC 2:373 394.

Govindarajan, T. S., and Subramanian, S. 1968. Fusarium wilt of coffee in India. Indian Coffee 32:270 271.

Mayne, W. 1931. Annual Report of the Coffee Scientific Officer. My sore Coffee Exp. Stn. Bull. 5:6 9.

Muthappa, B. N. 1977. *Rosellinia bunodes* on coffee spp. J. Coffee Res. 7:79 80.

Muthappa, B. N. 1977. Fusarium root diseases of coffee. J. Coffee Res. 7:109 110.

Nataraj, T. 1973. Preliminary studies on pathogenicity of *Fusarium oxysporum* on arabica coffee plants as a possible cause of coffee root rot. J. Coffee Res. 3:57 59.

Nataraj, T. 1991. Integrated control of red root disease of coffee. J. Plant. Crops 18(Suppl.):248 251.

Nataraj, T. 1996. *Maesopsis eminii*, a resistant shade tree to red root disease of coffee (*Poria hypolateritia*). Indian Coffee 65:9 10.

Petch, T. 1923. Red root disease. Pages 154 158 in: The Diseases of the Tea Bush. MacMillan & Co., London.

Rahman, M. U., and Subramanian, S. 1967. A new *Fusarium* wilt of coffee (*Coffea arabica*) in South India. Plant Dis. Rep. 51:758 759.

Shanmuganathan, N. 1967. Advisory Pamphlet 1166. Tea Research Institute, Ceylon.

Singh Dhaliwal, T., López Rosa, J. H., Steiner, G., Igaravídez, L., and Torres Sepúlveda, A. 1963. Studies on coffee root rot and horti cultural practices for its amelioration. Univ. P.R. Agric. Exp. Stn. Tech. Pap. 36.

Venkataram, C. S. 1974. Red root diseases of tea. Plant. Chron. 69:445 447.

Venkataram, C. S., and Muthappa, B. N. 1975. Red root rot of coffee. Indian Coffee 39:108.

(Prepared by M. Sudha, S. Daivasikamani, and Jayarama)

Coffee Wilt Disease, Fusarium Wilt, or Tracheomycosis

Coffee wilt disease was first reported on excelsa coffee (*Coffea liberica* W. Bull ex Hiern var. *dewevrei* (De Wild. & T. Durand) Lebrun, formerly *C. excelsa* A. Chev.) in the Cen tral African Republic in 1927. Subsequent epidemics in the Central African Republic, Côte d'Ivoire, and the Democratic Republic of the Congo in the mid 1900s caused extensive dam age to *C. liberica* and *C. canephora* Pierre ex A. Froehner (robusta coffee) but were contained by widespread destruction of affected plants and replanting with resistant coffee plants. However, renewed outbreaks on *C. canephora* were reported in the Democratic Republic of the Congo in the 1970s, and the disease has since spread to the neighboring countries of Uganda and Tanzania. Coffee wilt disease has had a major impact on coffee production in the Democratic Republic of the Congo and Uganda, with 90% of *C. canephora* farms currently affected in Uganda. In 1957, coffee wilt disease was found on *C. arab ica* L. in Ethiopia and is causing increasing concern. Coffee wilt disease has not been reported on *C. canephora* in Ethio pia or on *C. arabica* in the Democratic Republic of the Congo, Uganda, or Tanzania, suggesting host specialization within the pathogen and variable resistance within coffee plants. There are no reports of coffee wilt disease outside Africa.

Symptoms

Symptoms are similar to those caused by other vascular wilt pathogens and may develop at any stage of plant development. Leaves of young plants develop brown necrotic lesions, often along the veins and margins initially, that gradually enlarge. Leaves eventually dry, shrivel, and fall, perhaps within weeks after infection and only 3 4 days after the first symptoms ap pear (Fig. 28). Lower leaves tend to be the first to display symp toms. In adult plants, chlorosis, wilting, curling, and drying of the leaves are components of progressive and often unilateral dieback and defoliation. Dark brown discoloration of the leaf

Fig. 28. Advanced symptoms of coffee wilt disease (*Gibberella xylarioides*), more pronounced on the left side of a coffee tree. Leaves become flaccid, dry, and abscise, leaving only the berries. (Re produce, by permission, from Rutherford, 2006; ©M. Rutherford [CABI])

veins may be apparent (Fig. 29), and berries become red as if ripening prematurely. Younger branches develop a dark brown black necrosis that may also be laterally restricted. Symptoms eventually extend to the entire tree.

Great care should be taken when diagnosing chlorosis, wilting of the leaves, and general dieback because these may be caused by other pathogens, including other *Fusarium* spp., or by physiological disorders (e.g., overbearing dieback). However, coffee wilt disease is distinguished by the development of blue black staining of the vascular tissues, visible on the surface of the woody tissues upon removal of the bark (Fig. 30). Discoloration is usually more pronounced toward the stem base but may extend from below the soil level to the apex of the tree and may spiral along stems and branches. Bark on the trunk may become swollen, with spiral cracks appearing along the trunk. Small, black brown perithecioide ascomata (perithecia) form under damp or rainy conditions and may be visible on the surface of the bark and often in the cracks (Fig. 31). Perithecia resembling soil particles are usually more pronounced toward the base of a tree and may also be found on dead and decaying parts of affected plants. The rate of symptom development can vary in mature trees; the plant dies any time between 3 and 15 months after the first appearance of external symptoms. Once coffee wilt disease symptoms appear, death of a tree is inevitable, although older and poorly managed trees appear to be more susceptible to the disease.

Fig. 29. Brown necrosis, caused by coffee wilt disease (*Gibberella xylarioides*), along the veins of a coffee leaf. (Reproduce, by permission, from Rutherford, 2006; ©M. Rutherford [CABI])

Fig. 30. Blue-black staining of coffee wood, caused by coffee wilt disease (*Gibberella xylarioides*), at the base of a coffee tree. (Reproduce, by permission, from Rutherford, 2006; ©M. Rutherford [CABI])

Causal Organism

Coffee wilt disease is attributed to the fungus *Gibberella xylarioides* R. Heim & Saccas (anamorph *Fusarium xylarioides* Steyaert; syn. *F. oxysporum* Schltdl.:Fr. forma *xylarioides* (Steyaert) Delassus). Colony morphology in vitro may vary depending on isolate, culture media, and environmental conditions. Fungal colonies on potato sucrose agar (pH 6.5), a medium routinely used for identifying fusaria, are initially pale beige with sparse white mycelium. Purple discoloration may develop with age and be accompanied by dark bluish black, discrete stromata, some representing ascomatal initials. Microconidia and macroconidia are produced in slimy masses on short, conidiogenous cells on vegetative mycelium. Microconidia, generally abundant, are unicellular, allantoid, curved, and 2.5–3 × 5–10 µm. Macroconidia, which tend to be less abundant, are fusoid and falcate, have two to three septa, and measure 4–5 × 20–25 µm. Chlamydospores, occasionally produced but generally not abundant, are oval to globose, smooth or rough, and 8–10 × 10–15 µm. Perithecia are globose, measure 180–300 × 200–400 µm, have a flattened base, are violaceous in color, and are embedded, singly or in groups, in dark purple stromata. Often observed under natural conditions, and in the absence of chlamydospores, perithecia may provide a way of survival under adverse conditions. Asci are cylindrical and thin walled, have short pedicels, and measure 7 9.5 × 90–110 µm (Fig. 32). Asci contain eight monostichous, hyaline to straw colored ascospores that are fusoid, have one to three septa, are finely roughened, and measure 4.5–6 × 12–14.5 µm.

A number of genetically and biologically distinct forms of the pathogen have been identified. These forms differ in pathogenicity toward particular coffee species (including the widely

Fig. 31. Perithecioide ascomata (perithecia), produced by the coffee wilt disease pathogen, *Gibberella xylarioides*, in the cracks of tree bark. (Reproduced, by permission, from Rutherford et al., 2009; ©J. Flood [CABI])

cultivated commercial species *C. arabica* and *C. canephora*), are sexually incompatible, and are geographically delineated. This has led to the proposal of new species and *formae speciales*. Genetic analyses now place *G. xylarioides* within the African clade of the *Gibberella fujikuroi* species complex (GFC), a clade replacing *Fusarium* section *Liseola*. While the existence of a heterothallic mating system has been demonstrated, doubt exists over earlier descriptions of morphologically distinguishable, sex-linked male and female forms within *G. xylarioides*.

Disease Cycle and Epidemiology

G. xylarioides is considered a soilborne fungus that infects, colonizes, and induces symptoms in a manner similar to that of other vascular wilt pathogens. However, and in comparison to many other fusarial wilt pathogens, the life cycle is unclear and little is known of the underlying processes of host infection and epidemiology. Infection of susceptible coffee plants via the rhizosphere has been confirmed and is probably the primary mode of entry, although isolation of *G. xylarioides* from natural field soil has proven problematic (perhaps because of the presence of competing microorganisms). Precisely how, and for how long, *G. xylarioides* persists in the soil is unclear. The fungus may also enter through wound sites, particularly those in the roots and lower stem that form naturally or result from farming practices. Subsequent colonization of the plant is considered to be primarily via the vascular system (xylem vessels), with the blockage of vessels and toxin production leading to the wiltlike symptoms and discoloration observed in wood.

Vegetative and reproductive parts of the fungus, in particular microconidia, macroconidia, and ascospores, are presumably dispersed by air, water, and human activity. Observations of disease development in on-station and on farm trials suggest the outward spread of infection from focal points to healthy plants in close proximity, possibly via root to root contact. Soil and plant debris, particularly wood, are important sources of inoculum and disease spread. Wood cuttings used for vegetative propagation are exchanged and sold by growers and nurseries to establish new plantings. Coffee berries may provide a source of infection, but efforts to recover the fungus from affected berries have been unsuccessful. Studies suggest that *G. xylarioides* has a very narrow host range, perhaps restricted to *Coffea* spp., but further research is required.

Management

Resistance provides the most effective and sustainable option for managing coffee wilt disease. Geographic delimitation and host specificity of the pathogen offer opportunities for the selection and cultivation of *C. arabica* or *C. canephora* to provide resistance, although contrasting agroecological requirements of the two species must be considered. Resistance is currently being sought among cultivated and wild coffee species to improve *C. canephora* and *C. arabica*. Promising clonal lines and hybrids are also currently being evaluated under field conditions in affected areas of Africa, including "survivor" plants observed in disease hot spots. Although resistance in *C. arabica* is considered to be quantitative in nature and horizontal, the underlying mechanisms of resistance in coffee plants remain unclear.

In the absence of resistance, and given the soilborne nature of coffee wilt disease, it is strongly advisable to avoid the introduction of coffee wilt disease into disease-free areas where susceptible coffee plants are grown. This may be achieved by strict quarantine measures (*cordon sanitaire*) to control the distribution of seeds; vegetative cuttings; clones; seedlings; other plant material, including plantlets produced by nurseries; and soil from affected areas. Care should be taken to ensure that new plantings are established with disease-free material. Maintaining a vigorous and well-managed crop, in which physical damage to trees is minimized, may reduce the risk of infection and delay subsequent disease development.

In infected areas, further spread and crop loss may be reduced or prevented by adopting phytosanitary measures, including in situ removal and destruction, by burning or burying, of affected trees that provide foci of infection. Land may be replanted with resistant coffee or an alternate crop or left fallow for at least a year, preferably longer.

The removal of trees to leave gaps of 100 m or more between plantings has previously been shown to be effective in containing the disease. Coffee plant material and soil should not be moved within or from affected farms, and coffee wood should not be stored as a source of firewood. Farm implements, particularly those used for pruning and weeding around coffee trees, should be routinely cleansed with a suitable disinfectant or fungicide or by heat (e.g., in a fire). Care should be taken not to damage trees because wounds may provide entry points for the fungus. Although disinfecting wounds and spraying with copper have been suggested as possible management measures, fungicidal management of coffee wilt disease is problematic given the soilborne and vascular nature of the pathogen and the difficulties associated with acquiring and using suitable chemicals in affected regions. As such, it is not common practice. There are no recognized biological control agents for coffee wilt disease.

Vigilance is required by growers and local and national authorities to monitor disease progress, identify and tackle new outbreaks at an early stage of development, and enforce required management measures.

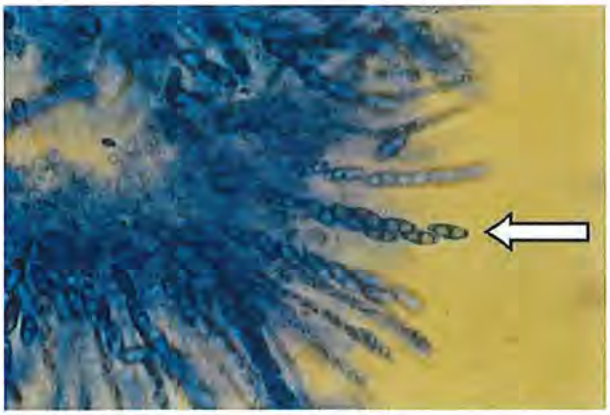


Fig. 32. Asci of *Gibberella xylarioides*, the coffee wilt disease pathogen, with two celled ascospores (arrow). (Reproduced, by permission, from Rutherford et al., 2009; ©J. Flood [CABI])

Selected References

Adugna, G., Hindorf, H., Steiner, U., Nirenberg, H. I., Dehne, H. W., and Schellander, K. 2005. Genetic diversity in the coffee wilt pathogen (*Gibberella xylarioides*) populations: Differentiation by host specialization and RAPD analysis. Z. Pflanzenkrankh. Pflanzenschutz 112:134 145.

Booth, C. 1971. The Genus *Fusarium*. Commonwealth Mycological Institute, Kew, United Kingdom.

Booth, C., and Waterston, W. M. 1964. CMI Descriptions of Plant Pathogenic Fungi and Bacteria No. 24. CAB International, Wallingford, United Kingdom.

Geiser, D. M., Lewis Ivey, M. L., Hakiza, G., Juba, J. H., and Miller, S. A. 2005. *Gibberella xylarioides* (anamorph: *Fusarium xylarioides*), a causative agent of coffee wilt disease in Africa, is a previously unrecognized member of the *G. fujikuroi* species complex. Mycologia 97:191-201.

Lepoint, P. C. E., Munaut, F. T. J., and Maraite, H. M. M. 2005. *Gibberella xylarioides* sensu lato from *Coffea canephora*: A new mating population in the *Gibberella fujikuroi* species complex. Appl. Environ. Microbiol. 71:8466 8471.

Muller, R. A. 1997. Some aspects of past studies conducted in western and central francophone Africa on coffee tracheomycosis (Côte

d'Ivoire, Cameroon and Central African Republic). Pages 15 26 in: Proc. Regional Workshop Coffee Wilt Disease [Tracheomycosis], 1st. National Agricultural Research Organisation (NARO) and the European Union.

O'Donnell, K., Cigelnik, E., and Nirenberg, H. I. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90:465 493.

Pieters, R., and van der Graaff, N. A. 1980. Resistance to *Gibberella xylarioides* in *Coffea arabica*: Evaluation of screening methods and evidence for the horizontal nature of the resistance. Neth. J. Plant Pathol. 86:37 43.

Rutherford, M. A. 2006. Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa. Phytopathology 96:663 666.

Rutherford, M. A., Bieysse, D., Lepoint, P., and Maraite, H. M. M. 2009. Biology, taxonomy and epidemiology of the coffee wilt pathogen *Gibberella xylarioides* sensu lato. Pages 99-119 in: Coffee Wilt Disease. J. Flood, ed. CAB International, Wallingford, United Kingdom.

Tshilenge Djim, P., Munaut, F., Kalonji-Mbuyi, A., and Maraite, H. 2004. Caractérisation des *Fusarium* spp. associées au dépérissement du caféier Robusta en République Démocratique du Congo. Parasitica 60:19 34.

Waller, J. M., Bigger, M., and Hillocks, R. J. 2007. Coffee Pests, Diseases and Their Management. CAB International, Wallingford, United Kingdom.

(Prepared by M. Rutherford)

Ceratocystis Canker Stain

Canker stain, caused by the complex *Ceratocystis fimbriata sensu lato*, is one of the most important diseases of coffee. The disease was first reported in 1900 from Indonesia, where it was discovered on the island of Java. Canker stain of coffee subsequently appeared in Colombia in 1932. Canker stain of coffee is now found in various other parts of Central and South America, as well as in Indian coffee-growing areas. The disease is best known and most important in Colombia, where the disease is locally referred to as *llaga macana* or *mal de machete* and it is found in all coffee-growing areas, causing substantial losses.

Symptoms

Infected coffee trees are usually randomly scattered in the fields, without any clear pattern of distribution. The first symptom of canker stain on coffee plants is chlorosis of the foliage. This is followed by wilting of the leaves, dieback, and tree death (Fig. 33). The primary site of infection, as seen by vascular streaking, is the root collar or other parts of the stem, where dark sunken lesions can be found on the outer bark. When the bark is removed, darkly colored lesions with a streaked pattern can be found in the infected wood, which can also extend into the roots (Fig. 34). In advanced stages of the disease, lesions girdle the trunks, resulting in tree death (Fig. 35). Common

Fig. 34. Canker stain, caused by *Ceratocystis fimbriata*, in the vascular tissue beneath the bark of a coffee tree. (Courtesy M. J. Wingfield)

Fig. 33. General chlorosis and wilting of leaves caused by the canker stain pathogen, *Ceratocystis fimbriata*. (Courtesy M. J. Wingfield)

Fig. 35. Advanced stage of canker stain, with lesions girdling the trunk, resulting in tree death. (Courtesy M. J. Wingfield)

sites for infection are the pruning wounds created when plants are rejuvenated by stem stumping; here, new shoots become infected, wilt, and die rapidly.

Causal Organism

Ceratocystis spp. are ascomycetes that represent some of the most important plant pathogens, causing canker stain and wilt diseases, especially in fruit, forest, and ornamental trees. Structures of the sexual states of *Ceratocystis* spp. are perithecia with globose bases and diameters of 138 185 µm, giving rise to long necks of 408 627 µm, typically terminating in distinct ostiolar hyphae (Fig. 36). Asci are evanescent and seldom seen in the case of *Ceratocystis fimbriata* Ellis & Halst. Ascospores are hat shaped, 2.4 2.7 × 5.7 6.5 µm, and borne in sticky masses at the apices of the ascomatal necks. The asexual state of these fungi is in the genus *Thielaviopsis*, typified by distinct tubular conidiophores and rectangular endoconidia that are 2.4 × 19.4 µm and produced in chains (Fig. 37). These fungi also produce distinct, darkly colored chlamydospores that facilitate survival in the soil.

The causal organism of canker stain of coffee is broadly referred to as *C. fimbriata sensu lato*. Contemporary studies on *C. fimbriata* have shown that this fungus represents a complex of cryptic species that have been described during the last decade. Recent studies on isolates of the pathogen in Colombia have shown that these isolates reside in two distinct phylogenetic clades other than *C. fimbriata sensu stricto*. Isolates in these clades have been given the names *C. colombiana* M. van Wyk & M. J. Wingf., sp. nov. and *C. papillata* M. van Wyk & M. J. Wingf., sp. nov. It is not known whether the same *Ceratocystis* species in the *C. fimbriata sensu lato* species complex cause canker stain of coffee elsewhere in the world.

There are two methods to isolate the fungus: baiting in the soil with freshly cut coffee twigs or placing pieces of infected stems in between slices of fresh carrots, as if in a sandwich, in a humid chamber. Once ascomata perithecia arise, ascospore masses are transferred to V8-juice agar medium containing thiamine and chloramphenicol, pH 5.5, at 22 24°C under dark or light conditions. The colonies are initially white and become olive green, stone gray, or bronze and produce fruity odors. The perithecia are formed in concentric rings or grouped in central clumps.

Fig. 36. Perithecium of *Ceratocystis fimbriata* (×200). (Courtesy M. J. Wingfield)

Disease Cycle and Epidemiology

Mechanical injuries, especially pruning wounds, constitute the principal mode of entry for *C. fimbriata* into coffee tissues. The fungus is easily transported on agricultural tools, with soil particles on the shoes of farmers, or with splash during the rainy season. It has been shown that the principal factor associated with the spread of canker stain of coffee in Colombia is infection through damage to the stems of trees made by the shoes of farmers needing to secure themselves on the steep slopes, typical of the terrain where coffee is cultivated. More than half of the coffee plantations in Colombia are established on land with slopes steeper than 70%. Under these conditions, it is almost impossible for farmers to cultivate coffee without damaging the trees and spreading *C. fimbriata*-infested soil.

Ceratocystis spp. in the *C. fimbriata sensu lato* species complex have close associations with causal insects, such as picnic beetles (Coleoptera: Nitidulidae) and flies (Diptera), that feed on wounded plant tissue. *Ceratocystis* spp. produce fruity odors that are attractive to these insects, and when the fungi are present on the surface of lesions, their spores attach to the insect that carries them to fresh wounds. There have been no detailed studies of the insect associations with *Ceratocystis* spp. that cause canker stain of coffee, but it is most likely that these fungi are transmitted by insects. Soil represents another major source of inoculum for the *Ceratocystis* spp. causing canker stain of coffee. Although no detailed studies have been made regarding this inoculum source, these fungi can easily be isolated from the soil taken from infected lands by baiting with freshly cut coffee twigs.

Management

The most important strategy that can be applied to manage canker stain is to avoid any form of injury to the tree trunks. In addition, at the sites where trees are rejuvenated through pruning or stem stumping, it is necessary to protect the wounds with fungicides, such as benomyl, thiabendazole, and carbendazim. The major long-term strategy to control canker stain of coffee is to plant resistant varieties. *Coffea canephora* Pierre ex A. Froehner is reported as resistant, and in *Coffea arabica* L. just one selection, *Coffea arabica* var. Bourbon, has a known resistance to *C. fimbriata sensu lato*. In recent years, lineages resistant to Ceratocystic canker stain have been bred from either *Coffea arabica* var. Bourbon or *Coffea canephora*, crossed with *Coffea arabica* var. Caturra. Further breeding using genotypes from commercially available coffee leaf rust resistant varieties provides simultaneous genetic control for both diseases.

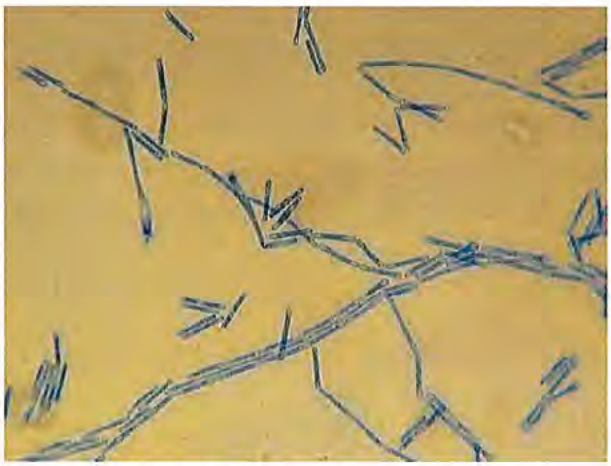


Fig. 37. *Thielaviopsis* rectangular endoconidia produced in chains. (Courtesy M. J. Wingfield)

Selected References

Baker, C. J., Harrington, T. C., Krauss, U., and Alfenas, A. C. 2003. Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata*. Phytopathology 93:1274 1284.

Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B. D., and Wingfield, M. J. 2001. Microsatellite markers reflect intra specific relationships between isolates of the vascular wilt pathogen *Cerato cystis fimbriata*. Mol. Plant Pathol. 2:319 325.

Bath, S. S., Rajamani, R., and Nandagopal, N. 2002. Manifestation of *Ceratocystis fimbriata* on coffee in relation to age of the bark wound and season. J. Coffee Res. 30:8 13.

Castillo Z., J. 1982. Producción de una selección resistente a llaga macana *Ceratocystis fimbriata* Ell Halst. Hunt. con relación a las variedades Típica y Borbón. Cenicafé 33:53 66.

Castro C., B. L., and Cortina G., H. A. 2007. Selección de progenies de café resistentes a llaga macana (*Ceratocystis fimbriata* Ellis & Halst.). Fitotec. Colomb. 7:51 62.

Castro Caicedo, B. L., Cortina Guerrero, H. A., Roux, J., and Wingfield, M. J. 2013. New coffee (*Coffea arabica*) genotypes derived from *Coffea canephora* exhibiting high levels of resistance to leaf rust and Ceratocystis canker. Trop. Plant Pathol. 38:485 494.

Fernández B., O. 1964. Patogenicidad de *Ceratocystis fimbriata* y po sible resistencia en café var. Borbón. Cenicafé 15:3-17.

Izquierdo B., J. E. 1988. Comportamiento de genotipos de cafetos ante *Ceratocystis fimbriata*. Cienc. Téc. Agric. Café Cacao 10:53 59.

Kile, G. A. 1993. Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. Pages 173 183 in: *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology, and Pathogenicity. M. J. Wingfield, K. A. Seifert, and J. A. Webber, eds. American Phytopathological Society, St. Paul, MN.

Marin, M., Castro, B., Gaitán, A., Preisig, O., Wingfield, B. D., and Wingfield, M. J. 2003. Relationships of *Ceratocystis fimbriata* iso lates from Colombian coffee growing regions based on molecular data and pathogenicity. J. Phytopathol. 151:395 405.

Moller, W. J., and DeVay, J. E. 1968. Insect transmission of *Cera tocystis fimbriata* in deciduous fruit orchards. Phytopathology 58:1499 1508.

Van Wyk, M., Wingfield, B. D., Marin, M., and Wingfield, M. J. 2010. New *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia. Fungal Divers. 40:103 117.

(Prepared by M. J. Wingfield and B. L. Castro Caicedo)

Fusarium Bark Disease

A bark disease on coffee was first observed in 1930 in the Usambara Mountains of Tanganyika (now mainland Tanzania) in a form called Storey's bark disease. In 1934, the disease was reported in Nyasaland (now Malawi) in a form called scaly bark disease. A third form of the disease, called collar rot, was described in Nyasaland in 1958. The bark diseases were subsequently reported in 1960 in Madagascar and in 1964 in southern Kenya (but farmers had observed symptoms since 1956). Disease forms have also been observed in Zimbabwe and Ethiopia.

Symptoms

There are three main forms of the disease, resulting from infection of different tissues of the coffee plant. In all cases, the infected trees gradually decline and finally die when the main stem becomes girdled at the base.

Unlike normal *Fusarium* vascular wilts, the fungus does not penetrate the xylem and mycelium and is limited to the outer bark, phloem, cambium, and some parenchyma cells of medullary rays. Purplish to dark brown dead tissues appear below the dead bark, and calli may develop at the edges of the lesions.

Storey's bark disease. Storey's bark disease attacks the bases of young suckers and starts as slightly depressed, dark brown lesions surrounded by yellow halos that enlarge and girdle the stems, causing constriction (Fig. 38). On very young sappy suckers, pink sporodochia appear on the dark brown tissue. The leaves dry on the sucker as it rapidly wilts and usually dies 8 days after the onset of symptoms. Similar symptoms are observed in young coffee seedlings. On older suckers, the initial infections are masked by the bark, but the constriction becomes noticeable and the whole cycle takes longer. Infected suckers easily break off.

Scaly bark disease. Scaly bark disease starts as depressed cankerous lesions on the main stem. The lesions elongate from the point where primary branches have been pruned and the bark roughens to appear like scales, hence the name (Fig. 39). The bark easily rubs off and the lesion may girdle the stem. Le sions from the main stem can infect the lateral branches, result ing in dieback. This form of the disease is the most difficult to recognize, although it may be the most common.

Collar rot. Collar rot is normally fatal, starting with general weakening and then yellowing and death of the plant. Symptoms are the same as those of scaly bark disease but they occur at the soil level and the tree dies rapidly after the trunk is girdled.

Leaf spot and berry diseases. Infection of leaves and berries causes dark brown lesions that are covered by pinkish sporodo chia. These forms of the disease are of negligible importance.

Fig. 38. Storey's bark disease on a sucker. (Courtesy E. K. Gichuru)

Fig. 39. Scaly bark disease around the pruning cut point of a lateral branch. (Courtesy E. K. Gichuru)

Causal Organism

Initial reports in East and Central Africa associated all forms of the disease with *Fusarium lateritium* Nees var. *longum* Wollenw. The cause of the disease reported in Madagascar was suspected to be a strain of the *F. lateritium* group that was different from the one causing the disease in East and Central Africa. Two strains of the fungus, A.1 (also called *F. stilboides* Wollenw.) and A.2, cause Storey's bark disease, but only A.1 causes scaly bark disease. Both strains are described as mutants of the fungus *F. stilboides* and its perfect stage *Gibberella stilboides* W. L. Gordon ex C. Booth. The sexual stage has not been observed in the field and its role in epidemiology is unknown. All three main forms of the disease are caused by the same fungus. *F. stilboides* is very common on coffee trees, even in regions where the disease is not observed, and this raises the question of variation in pathogenicity. The fungus has a deep pinkish red pigment (carmine red) in culture that may vary on different common laboratory media. Cultures of *F. stilboides* on potato sucrose agar have a white or pink floccose mycelium that becomes reddish brown with age. Sporulation is initially on aerial mycelium, giving rise to colonies that are powdery in appearance. Small, scattered sporodochia are later formed on the agar surface. The fungus produces only macroconidia, which are three to seven celled, thin walled, and straight or slightly curved. It is suggested that there are different types of the fungus associated with the disease in Kenya. More studies need to be done to further characterize the different types of the fungus in terms of pathogenicity and molecular traits.

Disease Cycle and Epidemiology

F. stilboides is commonly found on the coffee canopy and was first isolated from coffee cherries in Kenya in 1940. The reservoir for spores is infected bark, where large pink spores can be observed under warm, wet weather conditions, especially on poorly managed trees. Poor plant-growing conditions, such as poor nutrition and unbalanced soil conditions, especially pH, excessive weed growth, and overbearing, weaken the plants, making them more susceptible to infection. Wounds introduced by stem borers (which are also prevalent in warm areas), mechanical damage (e.g., during mechanical weeding), and pruning injuries provide the entry points for the fungus. Pruning cuts and the presence of infected materials or soil on the cut surfaces increase the risk of infection. Some practices, such as rotating communal working groups from farm to farm in the villages and sharing working tools, promote spread of the disease.

Macrospores are borne in sporodochia that develop below the cuticle before rupturing it. The spores are spread by rain splash, insects, and men with working tools. The fungus is rarely found in the soil, except where a plant has died of collar rot, but it can remain viable in infected bark for up to 1 year. The latency period depends on the weather and the form of the disease. The latency period of Storey's bark disease varies from 1 week to 11 months, and symptoms may take up to 4 months to appear. In scaly bark disease, the first symptoms appear about 4 weeks after infection, but girdling and death of the stems can take years. The first visible symptoms of collar rot appear up to 15 weeks after infection.

Management

So far, there is no practical cure for the disease, and cultural practices are the best management options. The disease develops slowly and, consequently, farmers may delay taking action. There are a number of recommended disease management practices. Proper fertilization should be determined through regular leaf and soil sampling. Integrated approaches should be used for proper weed control. Pruning tools should be cleansed with disinfectants (e.g., hypochlorite and spirit). Diseased trees should be uprooted and burned, especially in cases of collar rot. Trees with Storey's bark disease and scaly bark disease symptoms high above the soil may be cut back way below the infection point to allow new suckers to develop. The cut surfaces are protected when painted with a fungicide (e.g., captan) in agricultural/vegetable oil. Once an infected tree is uprooted, the hole should be left open for at least 3 months or treated with the soil fumigant dazomet at 150 g per hole. Wood-boring insects should be controlled. Weed debris that is difficult to dry should not be piled.

In infested fields, the cut surfaces after pruning can be protected by painting them with copper fungicides in mineral oil. An earlier recommendation of spraying young suckers weekly with captan was found to be too expensive.

No biological control agents have been developed yet, but this is an area of interest for research. Although many trials have been done to assess the resistance of *Coffea arabica* L. to the pathogen, no resistant varieties have been distinguished, indicating the rarity of such resistance. An option that could be explored is the use of robusta coffee root stock in areas where the disease is prevalent.

Selected References

Anonymous. 2005. Recognition and management of Fusarium bark disease and Fusarium root disease in coffee. Tech. Circ. No. 801. Coffee Research Foundation, Ruiru, Kenya.

Corbett, D. C. M. 1959. Fusarium bark diseases of coffee in Nyasa land. Nyasal. Farmer For. 4:3 7.

Firman, I. D. 1964. Fusarium diseases of coffee in Kenya. Kenya Cof fee 29:353 355.

Gordon, W. L. 1961. Sex and mating types in relation to the produc tion of perithecia by certain species of *Fusarium*. Proc. Can. Phy topathol. Soc. 28:11.

King'ori, P. N. 2001. Some cultural characteristics and pathogenic ity of *Fusarium* isolates from Fusarium bark disease on coffee in Kenya. Paper PA612 in: Proc. Int. Conf. Coffee Sci. (ASIC), 19th. Association for Science and Information on Coffee, Bussigny, Switzerland.

Siddiqi, M. A., and Corbett, D. C. M. 1963. Coffee bark diseases in Nyasaland. I. Pathogenicity, description and identity of the causal organism. Trans. Br. Mycol. Soc. 46:91 101.

Siddiqi, M. A., and Corbett, D. C. M. 1968. Coffee bark diseases in Nyasaland. I. Properties of the causal organism and conditions fa vouring the disease. Trans. Br. Mycol. Soc. 51:129 135.

Storey, H. H. 1932. A bark disease of coffee in East Africa. Ann. Appl. Biol. 19:173 184.

Wallace, G., and Wallace, M. M. 1955. The bark diseases of coffee. East Afr. Agric. J. 21:25 27.

(Prepared by E. K. Gichuru)

Black Rot Disease or Koleroga

Koleroga is considered an important disease affecting both arabica and robusta coffee during the monsoon season. This disease is common in all the coffee growing areas in India that come under the influence of heavy southwest monsoon rains. In severely affected areas, there have been recorded crop losses of up to 10–20% on coffee-growing estates and of 70–80% in affected individual plants. The disease has been detected sporadically in the Americas (i.e., Costa Rica, Brazil, and Colombia) with no economic impact. Black rot disease was first fully described by Cooke in 1876. Control measures combine cultural methods and a chemical spray of a 1% Bordeaux mixture. This disease is generally noticed in higher elevations and in the valley areas of plantations.

Symptoms

The pathogen *Corticium koleroga* infects leaves, developing berries, and young shoots. The most striking symptoms are blackening and rotting of the infected leaves, developing

berries, and young twigs. Affected leaves become detached from the branches and hang down by means of slimy fungal strands (Fig. 40). On green berries, the characteristic blacken ing starts from a side and spreads gradually in a narrow band. Close examination reveals the presence of characteristic threads of mycelium running along the twig (Fig. 41) and petioles and spreading mostly on the lower surface of the leaves. When the affected leaves and berries become dry, a white web consisting of closely interwoven mycelium is visible (Fig. 42). Infected branches defoliate and infected berries drop during advanced stages of the disease. The dropped berries and infected young shoots affect the next year's crop.

Fig. 40. Coffee leaf detached from the branch and hanging down by means of *Koleroga noxia* strands. (Courtesy A. Gaitán, Cenicafé)

Fig. 41. Threads of *Koleroga noxia* mycelium running along a twig. (Courtesy A. Gaitán, Cenicafé)

Fig. 42. White interwoven mycelium of *Koleroga noxia* on a dry leaf. (Courtesy A. Gaitán, Cenicafé)

Causal Organism

Black rot disease is caused by the fungus *Corticium koleroga* (Cooke) Höhn., which has also been known as *Koleroga noxia* Donk, *Ceratobasidium anceps* (Bres. & Syd.) H. S. Jacks., and *Pellicularia koleroga* Cooke.

Disease Cycle and Epidemiology

Continuous rains during the monsoon season without a long dry spell, a saturated atmosphere with 95 100% relative hu midity, hanging mist, thick shade, and shelter from sunlight and wind in the valley areas all favor disease development. The *K. noxia* disease cycle has pellicle and sclerotial stages. Dur ing the pellicle stage, the fungus forms a continuous mycelial mat on the leaf surface and dense, whitish, powdery, spore bearing patches. The pellicle, which is entirely superficial, can be peeled off. Basidiospores produced in this stage spread with the high humidity during the monsoon season. During the scle rotial stage, the leaves are initially brownish and later become black. During the course of development, the fluffy, pulveru lent appearance of the film changes into dotlike hyphal clumps with intervening thin space. The microsclerotia formed on the affected parts remain dormant during the 8–10 months of the unfavorable nonmonsoon season.

Management

Cultural methods of control aim to reduce the inoculum po tential of the pathogen by minimizing the conditions favorable for pathogen development and encouraging the healthy growth of the host plant.

The amount of shade should be reduced in black rot endemic areas before the onset of the monsoon season. Planting a mono culture of shade trees, such as silver oak (*Grevillea robusta* A. Cunn. ex R. Br.), in disease prone areas should be avoided.

Centering and handling plants by removing crisscross branches, dry branches, and suckers prior to the onset of the monsoon season are advocated. To minimize the disease dur ing the monsoon season, it is essential to remove shade tree leaves, particularly silver oak leaves, that fall on the canopy of coffee plants. Affected leaves, berries, and young shoots need to be removed and destroyed during the monsoon wet weather period. It is essential to remove the affected plant parts along with the mycelial web to reduce the inoculum and disease inci dence during the following season.

The entire leaf area and the developing berries should be properly covered with a 1% Bordeaux mixture as a prophy lactic spray before the onset of the monsoon season and after handling and pruning.

If black rot disease is detected even after the 1% Bordeaux mixture spray and during the monsoon rains, carbendazim 50% wettable powder (WP) (120 g per 200 L of water) has to be foliarly applied during the break in the monsoon (end of July or early August). Carbendazim spray should only be applied more than 45 days after the Bordeaux mixture spray.

Selected References

Coleman, L. C., Venkata Rao, K., and Narasimhan, M. J. 1923. Black rot or koleroga of coffee in Mysore. Dep. Agric. Mysore State Mycol. Ser. Bull. 5:1 12.

Cooke, M. C. 1900. Two coffee diseases. Pop. Sci. Rev. 15(59):161. Cited after G. Delacroix, 1900, Les Maladies des Cafeiers. Pages 715-726 in: VI Congrès International D'Agriculture.

Mathew, K. T. 1954. Studies on the black rot of coffee. I. The disease in South India and some general considerations. Indian Acad. Sci. Proc. Sect. B 39:131 170.

Mayne, W. W., Narasimhan, M. J., and Sreenivasan, K. H. 1933. Spraying of coffee in South India. Mysore Coffee Exp. Stn. Bull. 9:1 63.

(Prepared by M. Sudha, S. Daivasikamani, and Jayarama)

Coffee Berry Disease

Coffee berry disease is confined to date to the African continent and is the major economic threat to the production of arabica coffee normally growing at high altitudes (1,000 2,000 m). Coffee berry disease was detected for the first time in 1922 near Soy and Turbo in western Kenya, close to the Uganda border. The disease was reported in the former Zaire in 1938, Cameroon in 1955–1957, Uganda in 1959, northern Tanzania in 1964, Ethiopia in 1971, southern Tanzania in the early 1980s, Zimbabwe in 1984, and northern Malawi in 1985. Crop losses in arabica growing countries of Africa reach 20 30% but can exceed 80% in extremely wet years. Strict quarantine precautions should be undertaken when introducing new seedlings to prevent the distribution of the disease to other coffee-growing regions.

Symptoms

Colletotrichum kahawae attacks all stages of the crop, including flowers, leaves, unopened inflorescences, and ripe berries. The infection on leaves is rare and not important. The white tissue of infected flowers develop dark brown blotches or streaks that become black and the flowers die. The most susceptible stage of berry development is 4–14 weeks after flowering (Fig. 43).

There are two distinct symptoms on berries, "active" and "scab" lesions. When the berry is attacked during the green stage, the first sign of an active lesion is a dark brown, slightly sunken spot that can be on any part of the berry (Fig. 44). Under suitable conditions, the spot enlarges to cover the whole berry, and under conditions of high humidity, a pinkish mass of spores is produced on the lesion surface. Scab lesions are normally pale in color, are only slightly sunken, and often form concentric rings of incipient black acervuli (Fig. 45). These fungal structures develop slowly, becoming corky and stagnate, and they sporulate sparsely, if at all. The scab lesions remain latent following berry development, becoming active only during the ripening stages, during which the pulp tissue changes into a more favorable substrate for fungal growth. Scab lesions have no detectable effect on yield.

Causal Organism

Colletotrichum kahawae J. M. Waller & Bridge is in the phylum Ascomycota, class Ascomycetes, subclass Sordariomycetidae, order Phyllachorales, and family Phyllachoraceae. Several strains of the genus *Colletotrichum* (including *C. gloeosporioides* (Penz.) Penz. & Sacc. and *C. acutatum* J. H. Simmonds) occur on coffee, but only *C. kahawae*, which is indistinguishable under the microscope from the other species, has the ability to attack green fruits. This strain (fresh culture) is, however, distinguishable morphologically in vitro, having green to dark mycelium and growing slowly between 25 and 30°C. The inability of *C. kahawae* to utilize either citrate or tartrate as a sole carbon source also provides a useful test to distinguish the pathogen from other *Colletotrichum* isolates. In addition, pathogenicity tests can be used for diagnosis.

Conidia are one celled, hyaline, straight, rounded or pointed at both ends, and borne singly on hyphae or phialides produced in black acervuli (Fig. 46). Conidia shapes, sizes (3.4 4.7 × 10.8 23.0 µm), and frequency vary widely. Some isolates of *C. kahawae* produce pinkish conidial mucilage in culture. The pinkish mucilaginous masses are more pronounced on the host, e.g., on green berries. There are no records of the perfect state of *C. kahawae*. Molecular studies carried out on populations

Fig. 43. Coffee branch with coffee berry disease symptoms. (Courtesy E. K. Gichuru)

Fig. 44. Green berries with active *Colletotrichum kahawae*-sporulated lesions. (Courtesy E. K. Gichuru)

Fig. 45. Scab lesions caused by *Colletotrichum kahawae* on a green coffee berry. (Courtesy V. Varzea)

of *C. kahawae* from different geographic origins did not show the existence of polymorphism within the species. However, iso enzymatic characterizations using nondenaturing polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) tech niques showed polymorphism among some *C. kahawae* isolates.

Disease Cycle and Epidemiology

With coffee berry disease, water plays a central role in spore production, dispersal, and germination and in the infection process. Conidia of *C. kahawae* are produced on the bark of young twigs and on infected berries. Conidia are covered with a thin gelatinous coat, which under wet conditions swells so that the spores are exuded as pinkish mucilaginous masses that are readily dispersed by rain. Spores are liberated into water films and are either washed down the branches or dripped onto the surface of berries. Spore dispersal is primarily downward through the tree canopy, and from this it can be inferred that tree crowns are of paramount importance as sources of in oculum in coffee berry disease epidemics. Spores are laterally dispersed between trees by splashing droplets blown around in strong winds. Natural dispersal therefore tends to be very local ized. Medium and long distance dispersal depends on other vectors, such as coffee harvesters, birds, and possibly insects. Conidia produced on green berries represent the most impor tant inoculum source for infection. Under relatively dry condi tions, lesions become inactive (scab lesions). Scab development is due to the formation of a cork barrier. A phellogen is formed in a layer of cells below the site of infection and the fungal inva sion is effectively blocked by a barrier of suberized cells. Such barriers are absent or incompletely developed in coffee berry disease susceptible varieties.

The conidia of *C. kahawae* germinate and differentiate mela nized appressoria that penetrate different coffee tissues directly through the epidermal cell walls. The turgor pressure of the melanized appressoria of *C. kahawae* seems to play a major role in coffee cuticle penetration. This fungus is a hemibiotroph because it exhibits a transient postpenetrative asymptomatic biotrophy that is rapidly succeeded by a phase of destructive necrotrophy, culminating in the appearance of disease symp toms and pathogen reproduction. During the symptomless bio trophic phase, the pathogen invades host cells without killing them and feeds on living cells. This period may last for 48 or 72 h after inoculation, depending on the aggressiveness of the fungal isolates. The pathogen then switches to a necrotrophic mode of nutrition, feeding on dead host tissue. During the necrotrophic phase, the fungus colonization is associated with severe cell wall alterations and death of the host protoplast.

Management

Management of coffee berry disease has been largely carried out by the use of fungicides. Different copper-based fungicides (e.g., cuprous oxide, cupric chloride, cupric hydroxide, and cop per sulfate) and organic fungicides (e.g., chlorothalonil, fluazi nam, dithianon, and azoxystrobin), as well as mixtures (e.g., anilazine/copper and chlorothalonil/copper), are recommended to control coffee berry disease in Kenya, where this disease has been intensively studied. Copper is frequently used because of its low cost and also because it provides effective control of other major diseases, such as coffee leaf rust. Cultural prac tices, which are the most effective, reduce the favorable condi tions for disease development. These practices include pruning methods to avoid overlapping crop cycles and minimizing mi croclimatic conditions optimal for the fungus.

Competitive and antagonistic microorganisms in the coffee phyllosphere, including the fungi *Fusarium stilboides* Wollenw. and *Epicoccum nigrum* Link and also some yeasts, could play a decisive role in limiting coffee berry disease development. Circumstantial evidence for natural biological control comes from observations that fungicide applications can result in in creased levels of coffee berry disease, suggesting that the re moval of indigenous fungal antagonists is responsible for this phenomenon.

Coffea arabica L. var. Rume Sudan is identified as having a high resistance level. The varieties Blue Mountain, K7, Java, and some lines of Local Bronze are reported as moderately sus ceptible and the varieties Caturra, Catuai, Harar, Moka, Kent, and SL selections are reported as very susceptible. Interspecific tetraploid hybrids, such as Timor Hybrid, a spontaneous hybrid between *Coffea canephora* Pierre ex A. Froehner and *Coffea arabica*, manifest a high level of resistance to coffee berry dis ease. Derivatives of 'Timor Hybrid' as well as 'Rume Sudan' have been used in different breeding programs as a source of resistance to this disease in African countries, such as Kenya (varieties Catimor 128, Catimor 129, and Ruiru 11), Tanzania, and Malawi. Coffee-growing countries where this disease does not exist, such as Colombia and Brazil, are developing new cof fee varieties or increasing the level of resistance of their com mercial varieties to coffee berry disease as a precaution for the possible introduction of the pathogen into other continents. In stead of natural screening for resistance to this disease, which requires a long time, artificial methods of screening have been developed to detect coffee berry disease resistance in the early stages of coffee development, notably the inoculation of seed ling hypocotyls (Fig. 47).

Fig. 46. Acervuli of *Colletotrichum kahawae* with setae. (Courtesy V. Varzea)

Fig. 47. Coffee hypocotyls affected by *Colletotrichum kahawae*. (Courtesy E. K. Gichuru)

Selected References

Beynon, S. M., Coddington, A., Lewis, B. G., and Varzea, V. 1995. Genetic variation in the coffee berry disease pathogen, *Colletotrichum kahawae*. Physiol. Mol. Plant Pathol. 46:457 470.

CAB International. 1996. *Colletotrichum kahawae* J. M. Waller and Bridge. Distribution Maps of Plant Diseases No. 716. CAB International, Wallingford, United Kingdom.

Chen, Z., Nunes, M. A., Silva, M. C., and Rodrigues, C. J., Jr. 2004. Appressorium turgor pressure of *Colletotrichum kahawae* might have a role in coffee cuticle penetration. Mycologia 96:1199 1208.

Firman, I. D., and Waller, J. M. 1977. Coffee berry disease and other *Colletotrichum* diseases of coffee. Phytopathological Paper No. 20. Commonwealth Mycological Institute, Kew, Surrey, United Kingdom.

Guichuru, E. K. 1997. Resistance mechanisms in arabica coffee to coffee berry disease (*Colletotrichum kahawae* sp. nov.) A review. Kenya Coffee 62:2441-2444.

Hindorf, H. 1970. *Colletotrichum* species isolated from *Coffea arabica* in Kenya. Z. Pflanzenkrankh. Pflanzenschutz 77:328-331.

Kirl, P. M., Cannon, J. C., David, J. C., and Stalpers, J. A. 2001. Ainsworth & Bisby's Dictionary of the Fungi, 9th ed. CAB International, Wallingford, United Kingdom.

Loureiro, A., Várzea, V., Guerra Guimarães, L., Ribeiro, A., Silva, M. C., and Bertrand, B. 2006. Characterization of *Colletotrichum kahawae* diversity. Pages 1277 1283 in: Proc. Int. Conf. Coffee Sci. (ASIC), 21st. Association for Science and Information on Coffee, Bussigny, Switzerland.

Masaba, D. M., and Waller, J. M. 1992. Coffee berry disease The current status. Pages 237 249 in: *Colletotrichum*: Biology, Pathology and Control. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, United Kingdom.

Omondi, C. O., Ayiecho, P. O., Mwang'ombre, A. W., and Hindorf, H. 2000. Reaction of some *Coffea arabica* genotypes to strains of *Colletotrichum kahawae*, the cause of coffee berry disease. J. Phytopathol. 148:61 63.

Silva, M. C., Várzea, V., Guerra-Guimarães, L., Azinheira, H. G., Fernandez, D., Petitot, A. S., Bertrand, B., Lashermes, P., and Nicole, M. 2006. Coffee resistance to the main diseases: Leaf rust and coffee berry disease. Braz. J. Plant Physiol. 18:119 147.

Sreenivaprasad, S., Brown, A. E., and Mills, R. R. 1993. Coffee berry disease pathogen in Africa: Genetic structure and relationship to the group species *Colletotrichum gloeosporioides*. Mycol. Res. 97:995 1000.

van der Vossen, H. A. M. 2006. State of-the art of developing cultivars of arabica coffee with durable resistance to coffee berry disease (*Colletotrichum kahawae*). In: Proc. Int. Conf. Coffee Sci. (ASIC), 21st. Association for Science and Information on Coffee, Bussigny, Switzerland.

van der Vossen, H. A. M., and Walyaro, D. J. 1980. Breeding for resistance to coffee berry disease in *Coffea arabica* L. 2. Inheritance of the resistance. Euphytica 29:777 791.

van der Vossen, H. A. M., Cook, R. T. A., and Murakura, G. N. W. 1976. Breeding for resistance to coffee berry disease caused by *Colletotrichum coffeanum* Noack (*sensu* Hindorf) in *Coffea arabica* L. I. Methods of preselection for resistance. Euphytica 25:733-745.

Várzea, V. M. P., Rodrigues, C. J., Jr., and Lewis, B. G. 2002. Distinguishing characteristics and vegetative compatibility of *Colletotrichum kahawae* in comparison with other related species from coffee. Plant Pathol. 51:202-207.

Waller, J. M., Bridge, P. D., Black, R., and Hakiza, G. 1993. Characterization of the coffee berry disease pathogen *Colletotrichum kahawae* sp. nov. Mycol. Res. 97:989 994.

(Prepared by V. Varzea)

Berry Blotch or Iron Spot

Although characteristically on coffee leaves, where it is also known as brown eye spot, *molestia dos olhos pardos*, and *chasparria*, iron spot is a very important disease when present on green berries (berry blotch) because it results in significant losses in yield quantity and quality. A high incidence of iron spot on plantations can reduce harvest values to less than 30%. The disease causes some berries to dry in the tree and fall off and others to be reduced in weight. It also causes the berries to become deformed when the dried pulp sticks to the beans, staining the parchment. Farmers see their income diminish when they harvest less coffee per hectare and when they sell parchment coffee with defects, resulting in price penalties. Threshing factors are affected directly by the disease, going from the expected 1.3 kg of dry parchment coffee to obtain 1 kg of green coffee to needing 1.6 or even 2.3 kg to obtain the same 1 kg of green coffee. The disease was reported by Morris in Jamaica at the end of the nineteenth century and described later by Berkeley and Cooke in 1881. It has become more evident since the 1960s, when fertilization was mandatory for high producing systems.

Symptoms

The disease is initially observed as small, chlorotic, brown or necrotic spots that reach 1–3 mm in diameter on either surface of the leaves (Fig. 48). Lesions develop a grayish center surrounded by a uniform brown ring. The injury develops a reddish brown color that can vary to dark brown and even black, with a diffuse external yellow circle. Concentric rings without a delimited edge can also appear. When growing, the spots eventually coalesce and form irregular shapes. All stages of leaf development are susceptible. Close observation of the spots reveals the presence of a feltlike mycelium with superficial gray ramifications.

On green and ripe fruits, lesions begin with small, isolated, reddish spots that become larger and deeper as the fruit develops, especially if it is protected from direct sunlight (Fig. 49). Infection of green fruits causes premature ripeness and they drop from the branch. Mature lesions are brown with a red ring that later turns into a depressed necrotic spot. The disease can cover half or even the entire coffee berry, which then looks dry and dark brown.

Causal Organism

Cercospora coffeicola Berk. & Cooke is the imperfect stage of the ascomycete *Mycosphaerella coffeicola* (Cooke) J. A. Stev. & Wellman, a fungus in the class Dothideomycetes, order

Fig. 48. Iron spot on a nursery plantlet leaf. (Courtesy C. A. Rivillas, Cenicafé)

Capnodiales, and family Mycosphaerellaceae. *C. coffeicola* has fasciculate conidiophores with multiple septa, which are clustered at their bases and insinuated on the leaf mesophyll. The conidiophores can measure up to 4 × 200 µm. Conidia are abundant and subhyaline, have 3–12 septa, and measure 2.5 3.5 × 30–210 µm. Sporulation is difficult to obtain in vitro. For controlled inoculations, mycelium has been successfully used, reproducing symptoms 14–17 days after inoculation. Isolates can attack leaves and fruits without distinction, and their fungicide resistance, especially against benzimidazoles, can be related to their high genetic variability.

Disease Cycle and Epidemiology

In the field, *C. coffeicola* sporulates at 24 30°C and a relative humidity equal to or greater than 98%, conditions that can be complemented with the presence of moisture on the susceptible tissues. Conidia and ascospores are produced throughout the year and dispersed mainly by rain and wind. Sustained inoculum production guarantees infection of the leaves and fruits at different ages. From 24 to 72 h after inoculation, the fungus penetrates either through the stomata on the underside of the leaf or directly through the epidermal cuticle on the upper surface, preferably during the day. The incubation period is 14 days in plants exposed to sunlight and 17 days in those with 50% shade. After intra- and intercellular hyphal invasion, the plant tissue collapses. As with several *Cercospora* spp., *C. coffeicola* produces cercosporin, a light-activated toxin that causes cell death. In fruits, the first symptoms appear 90 days after flowering, the most critical time for significant losses. Disease severity increases in 4 month old fruits, which ripen prematurely. The berries either fall off the tree or have to be harvested early. Later infections (in fruits older than 6 months) are less serious because the fruits have completed the maturation process and the mesocarp damage is removed after pulping.

Iron spot is a disease closely related to plant nutrition and is favored by all the factors that influence a proper supply of nutrients. Adequate nitrogen levels reduce the incidence and severity of iron spot in leaves and fruits, but nitrogen excess affects the plant and favors disease development. Applications of phosphorus and potassium, either alone or in combination, do not significantly affect the disease. A balanced nitrogen potassium interaction reduces the effect of the pathogen, as do repeated applications of complete fertilizers (N P K) throughout the year. Coffee plants with arbuscular mycorrhizal fungal colonization in their roots showed minor rates of iron spot development. Nematode attacks, poor root development, and marginal soil quality are factors that increase the incidence of iron spot.

Fig. 49. Reddish coloration of Cercospora berry blotch on coffee. (Courtesy G. Hoyos, Cenicafé)

Management

To prevent the disease under nursery conditions, care must be taken with the factors that affect coffee plant health. These factors include using certified seed; protecting coffee seedlings against *Rhizoctonia solani* Kühn; allowing proper root growth in large nursery bags (17 × 23 cm); transplanting seedlings into substrates with organic matter; preventing nematode infestation; using shade at the beginning of the nursery production cycle; managing weeds; providing additional soil fertilization with nitrogen and phosphorus, when required; and applying fungicides either systemically (e.g., triazoles and benzimidazoles) or protectively (e.g., dithiocarbamates).

On productive plantations, adequate, balanced, and well timed fertilizer applications based on soil analysis are sufficient to maintain a low incidence of iron spot. In plots where the disease is endemic, chemical control may be required for fruit protection during the first stages of development. For this, a mixture of copper oxychloride (3 kg/ha) + triazole (triadimefon 1 L of 50% wettable powder [WP]/ha) + vegetable oil spray carrier (0.4 L/ha) should be sprayed 90–120 days following the main flowering event. In spite of the reduced susceptibility of some *Coffea canephora* Pierre ex A. Froehner genotypes reported in Costa Rica, all the species and varieties of coffee are considered similarly susceptible to iron spot.

Selected References

Lopez, S., and Fernandez, O. 1969. Epidemiología de la mancha de hierro del cafeto *Cercospora coffeicola* Berk y Cook. Cenicafé 20:3 19.

Martinez, A., and Echeverri, J. 1981. Resultado de la evaluación de los cultivares de café del Banco de Germoplasma del CATIE, a la afección de *Cercospora coffeicola* (Berk Cooke). Instituto Interamericano de Cooperación para la Agricultura Centro Agronómico Tropical de Investigación y Enseñanza (IICA CATIE), Turrialba, Costa Rica.

Nataraj, T. 1986. Screening of resistance against browneye-spot disease. Indian Coffee 50:7 16.

Nataraj, T., and Subramanian, S. 1975. Effect of shade and exposure on the incidence of brown eye spot of coffee. Indian Coffee 39:179 180.

Pozza, A., Martinez, P., Pozza, A., Caixeta, L., and Zambolim, L. 2000. Intensidade da mancha de olho pardo em mudas de cafeeiro em funcao de doses de N e de K em solucao nutritiva. Summa Phytopathol. 26:29 34.

Rengifo G., H. G., Leguizamon C., J. E., and Riaño H., N. M. 2006. Incidencia y severidad de la mancha de hierro en plántulas de *Coffea arabica* en diferentes condiciones de nutrición. Cenicafé 57:232 242.

Soto, C. A., and Rodríguez, R. A. 1972. Efecto del tratamiento del cobre y nutrientes foliares en la calidad del café. (Abstr.) Phytopathology 62:790 791.

Sridhar, T. S., and Subramanian, S. 1966. Studies on the brown eyespot disease *Cercospora coffeicola* B. Cke. of coffee *Coffea arabica* L. Riv. Patol. Veg. 2:141 146.

van der Vossen, H., and Cook, R. 1975. Incidence and control of berry blotch caused by *Cercospora coffeicola* on arabica coffee in Kenya. Kenya Coffee 40:58 61.

(Prepared by C. A. Rivillas)

Pink Disease

Pink disease is widespread and can affect more than 200 hosts besides coffee, including citrus, mango, avocado, rubber trees, cocoa, tea, and eucalyptus. The disease was first observed in Java in 1873, but reports are mainly from Central and South America from as early as 1933. Effective cross-inoculations using coffee and citrus isolates provide evidence of a wide host range for the pathogen and of the important role of alternate hosts in epidemiology and control.

Symptoms

Trees affected by pink disease can be seen at a distance be cause the tips of the branches are yellow and all the leaves along the branch are wilted or dead (Fig. 50). In Central America, the name *brasa* or burnt is used to describe the disease because of the wilting on the branches. Berries become brown, dry, and mummified (Fig. 51), exhibiting pink dots on their surface and later falling off. The underside of affected branches is covered with a continuous pink fungal crust (Fig. 52). A heavy fall of berries and leaves can lead to a skeletal appearance of the trees. In very advanced attacks, the trunk exhibits long, deep necrotic areas, forming a callous-looking ring that girdles the stem and ultimately results in plant death.

Fig. 50. *Coffea arabica* branches affected by pink disease. (Courtesy G. Hoyos, Cenicafé)

Fig. 51. Coffee berries attacked by *Corticium salmonicolor*. (Courtesy C. A. Rivillas, Cenicafé)

Causal Organism

Pink disease is caused by the basidiomycete *Corticium salmonicolor* Berk. & Broome (synonym: *Erythricium salmonicolor* (Berk. & Broome) Burds.), which belongs to the class Agaricomycetes and family Corticiaceae. The basidiomata are not highly organized and are produced on dead wood. Basidia are simple and club shaped, have an average size of 7.6 × 23.6 µm, and hold four sterigmata (Fig. 53). Basidiospores are colorless, globose, smooth, round, and 4.1 × 5.4 µm. Basidiospores germinate in water and produce a thick, extensive mycelium in less than 24 h.

Disease Cycle and Epidemiology

C. salmonicolor survives well as a saprophyte in soil, dry twigs, and plant debris. Mycelium can grow unnoticed on the branches for long periods of time. When the rainy season arrives with high temperatures, conditions are conducive for further proliferation of the fungus and for host attack. Low terrains with poor drainage, water-retentive soils, high-density planta tions, and heavy shade cover all promote humid conditions that favor disease onset. Hyphae develop on the branches, leaves, and fruits as thin, white-silver threads that can form weblike structures. Mycelia then start to aggregate and infections begin at multiple points, with necrosis of the tissues underneath. The infection period lasts 40–45 days. The disease advances from the branches toward the main trunk, with the fungus block ing the vascular system (Fig. 54). After 30 days, an intricate

Fig. 52. Pink crust of *Corticium salmonicolor* on the underside of branches. (Courtesy C. A. Rivillas, Cenicafé)

Fig. 53. Basidia and basidiospores of *Corticium salmonicolor* (×400). (Courtesy G. Cadena Gómez, Cenicafé)

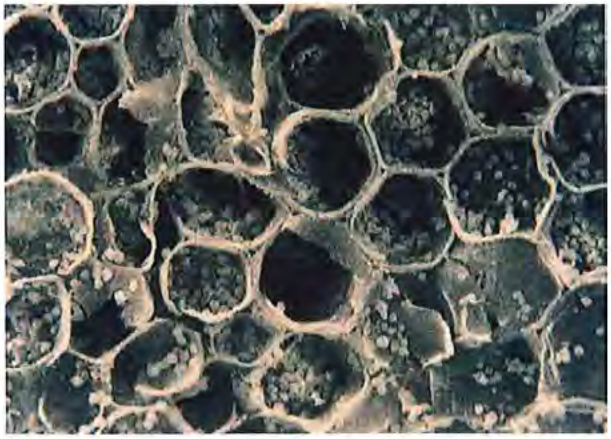


Fig. 54. Transmission electron microscopy (TEM) image of vascular tissue invaded by *Corticium salmonicolor.* (Courtesy M. Ortiz, Cenicafé)

mycelial mass (the crust) forms away from direct sun exposure, where naked basidia emerge from the hymenium. Up to 150,000 basidiospores can disperse from every square centimeter of pink crust, mostly through wind and water splash, to initiate another saprophytic phase.

Management

Preventive measures must be taken to increase aeration and light infiltration into those places where humidity and temperature conditions are optimal for disease development. Coffee trees must be free of plant debris, which can accumulate after the harvest. Similarly, plant density and shade can be reduced. It is important to make frequent, direct observations of the plots, especially during the rainy season, to detect early outbreaks. Pruning dry tree branches is recommended to reduce inoculum and avoid further dispersal of the basidiospores. Berries in the initial developmental stages must be chemically protected in areas of high susceptibility. Because of its toxicity to humans, lead arsenate has been replaced by sprays of cupric oxide at 4 g/L. However, applications to the pink crust stage of the disease have no effect. No genetic resistance has been detected among *Coffea arabica* L. accessions. Although antimicrobial activity has been observed under laboratory conditions with *Trichoderma*, *Verticillium*, and *Gliocladium* isolates, the effectiveness of biological control agents in the field remains to be tested. Susceptible host species growing nearby must be considered potential inoculum sources.

Selected References

Cadena, G. 1982. Estructuras reproductivas de *Corticium salmonicolor* Berk. y Br. agente causal del mal rosado del café. Cenicafé 33:35 37.

Galvis, C. A. 2003. Mal rosado *Corticium salmonicolor* Berk. y Br. Pages 121 127 in: Enfermedades del Cafeto en Colombia. L. F. Gil V., B. L. Castro C., and G. Cadena G., eds. Cenicafé, Chinchiná, Colombia.

Ramirez, C. J., and Cadena, G. 1982. Estudio biológico de *Corticium salmonicolor* Berk. y Br. agente causal del mal rosado del cafeto. Cenicafé 33:40 52.

Rodriguez M., R. A. 1964. Estudios sobre la enfermedad rosada del café. Bol. Téc. 46. Ministerio de Agricultura y Ganadería, San José, Costa Rica.

Rodriguez M., R. A. 1983. Patogenicidad de *Corticium salmonicolor* en cítricos y en el cafeto. El mal rosado. Agron. Costarric. 7:35 41.

(Prepared by G. Cadena Gómez and A. Gaitán)

Dieback

The agricultural border of coffee has moved toward the highlands to avoid coffee rust and to use new land for production. This has caused the onset of new diseases and disorders because of the departure from the optimal temperatures and altitudes for maximum production. Dieback is one of these problems. It was initially reported in 1951 in Colombia, more than 1,600 m above sea level. Dieback is associated with long rainy seasons, average temperatures below 20°C, and coffee plantations that have full sun exposure and are vulnerable to incursions of cold air.

Symptoms

Dieback attacks growing tissue in the nursery, after stumping, and adult plants in the field. Meristematic buds and leaves, on either plagiotropic or orthotropic branches, initially exhibit small, irregular, chlorotic spots that coalesce and become necrotic in about 10 days (Fig. 55). Because the leaves are growing, these spots result in malformation of the foliar area, giving the leaf a crescent shape as the healthy tissue grows around the spot (Fig. 56). Younger leaves and buds are completely necrotic. The plant then responds with lignification in the nodes and new buds that sprout below the infection site, generating proliferations that provide a rosette appearance (Fig. 57). In the absence of control measures, these new buds also become infected, resulting in stunting in the medium term. Similarly, stems proliferate after stumping. New plants are attacked as

Fig. 55. Necrosis of meristematic buds caused by a *Phoma* sp. (Courtesy G. Hoyos, Cenicafé)

Fig. 56. Malformations of the foliar area caused by *Phoma* attack. (Courtesy G. Hoyos, Cenicafé)

well. Therefore, coffee plots exhibit a variety of plant heights, yields are very irregular, and harvesting becomes inefficient.

Symptoms may resemble boron deficiency, yellowing from the apex to the bottom of the leaf and suberization of the leaf veins. An exception is that veinal necrosis is also observed.

Causal Organism

Dieback is produced by a complex of *Phoma* spp., which includes *Phoma costaricensis* Echandi in Central America and *Phoma tarda* (R. B. Stewart) H. Verm. *Phoma* spp. are deuteromycetes without a known teleomorph connection. The fungus is characterized by the presence of dark pycnidia, and it is ostiolated, lenticular and globoid, and 25 × 280 µm. It produces abundant unicellular, hyaline, elongated pycnidiospores (1 × 6 µm). Mycelium is also hyaline, septate, and branched.

Disease Cycle and Epidemiology

Pycnidiospores carried by the wind land on susceptible tissue and the germ tubes penetrate through either natural openings or wounds, forming an appressorium. From this structure, colonizing hyphae move through intercellular spaces in the mesophyll and colonize the palisade tissue, producing the first symptoms 4 9 days after infection. Affected cells are plasmolyzed, causing tissue collapse. Pycnidia develop within 48 72 days after tissue disruption, embedded in the collapsed palisade tissue, and emerge from the upper and lower surfaces of the leaf, releasing their spores.

Dieback is common on those plantations above 1,600 m above sea level having extended rainy periods, cloudy skies (low solar radiation), and especially low temperatures. Free standing water and 3 to 6 h light periods are necessary for germination. Average temperatures must be between 18 and 22°C. Plant exposure to cold winds, which are associated with river basins, is a critical factor.

Management

Where there are environmental conditions conducive to disease onset, the first control measures are to produce healthy plants in the nursery and to avoid planting symptomatic stock in the field. On established plantations, it is necessary to prune affected branches and buds and immediately follow with fungicide sprays. If the problem is endemic, a preventive spray is recommended before the rainy season, with continuing applications at biweekly or monthly intervals. Where there are cold air currents, establishing a windbreak reduces disease incidence. This can be done with plantain or corn in the short term, but shade trees can also be used in the medium and long term. There are many fungicides that are effective against the fungus, including dichlofluanid, captan, cyproconazole, anilazine, and iminoctadine.

Fig. 57. New buds sprouting in response to dieback, caused by a *Phoma* sp. (Courtesy M. Ortiz, Cenicafé)

Selected References

Echandi, E. 1957. La quema de los cafetos causada por *Phoma costaricensis*. Rev. Biol. Trop. 5:81-102.

Gomez Q., R., and Bustamante R., E. 1977. Influencia de la luz y la temperatura en el desarrollo de la muerte descendente del cafeto, causado por *Phoma* sp. Fitopatol. Colomb. 6:73-80.

Kannan, N., Puttaswamy, P., and Ramaiah, P. 1985. Studies on the control of coffee blight in India. J. Coffee Res. 15:56 59.

Sanchez de L., A. 1975. Comparación de distintos fungicidas para el control de *Phoma* en el cafeto. Rev. Café. (Guatem.) 147:17 26.

(Prepared by A. Gaitán)

Coffee Powdery Rust

Coffee powdery rust is a disease confined to the African continent, and although it can attack several *Coffea* spp., it is most common on *C. canephora* Pierre ex A. Froehner. The disease also affects coffee relatives, including *Psilanthus* spp. Coffee powdery rust causes economic losses due to severe defoliation of the trees, especially during berry filling. The disease is a threat to coffee-producing countries outside of Africa, so quarantine measures should be taken for its early detection.

Symptoms

The fungus affects mainly old leaves, and unlike coffee yellow rust, lesions are not of a defined shape; instead, abundant orange powder formed by the urediniospores is diffused all over the undersides of the leaves. This sporulation feature gives the disease its name.

During the initial stages of the disease, leaves retain their normal color and symptoms are not visible. The first symptoms appear, with backlighting, as small chlorotic spots in the lower leaf surface after sporulation. Slight yellowing of the leaves, similar to the initial stages of nitrogen deficiency, also appears. Leaves eventually fall off. The disease starts in the internal lower canopy of the tree and then spreads to the rest of the plant. The disease develops much faster than the infection caused by *Hemileia vastatrix* Berk. & Broome and causes the loss of all the mature leaves of the plant.

Causal Organism

Hemileia coffeicola Maubl. & Roger (teleomorph) is an obligate biotroph in the order Pucciniales. It was formerly known as *Uredo coffeicola* Maubl. & Roger, before the telial stage was discovered. Urediniospores are kidney shaped, have different ornamentations than those of *H. vastatrix*, and seem to be the only ones with epidemiological significance. Haustoria also have a different shape compared with that of the haustoria formed by *H. vastatrix*. The completion of the telial stage is common, unlike *H. vastatrix*, and teliospores emerge especially in chlorotic leaves and during dry seasons. Teliospores are rounded, cap shaped, and not involved in the dispersal of the pathogen. It has not been proven that there are races of the pathogen, although there is evidence of their existence. Although the disease has also been known as gray coffee rust, this is not an appropriate name because the grayish color is due to the *H. coffeicola* hyperparasites *Paranectria hemileiae* Hansf. and *Paranectria carrissiana* Sousa da Câmara & Luz.

Disease Cycle and Epidemiology

Urediniospores of *H. coffeicola* germinate 6–8 h after reach ing the leaves. Germ tubes enter through stomata and penetrate the substomatal cells, where they form an appressorium. My celium develops from the appressoria and invades new cells. Haustoria are formed 12 14 days after the start of the infec tion and the first spores grow 28–30 days after penetration by the fungus. Teliospores may frequently accompany uredinio- spores, depending on the conditions.

The main factor for disseminating urediniospores is wind, followed by men, animals, and insects. The disease occurs at the start of the wet season and its incidence depends on the precipitation patterns each season. *H. coffeicola* can exist at the same time on the same plant as *H. vastatrix*. *H. coffeicola* may arise later but grows much faster. In Cameroon, coffee powdery rust epidemics start between April and June, with a maximum incidence in November; coffee yellow rust epidemics are in October. The severe defoliation caused by coffee powdery rust compromises the harvest of the following year. Resistance to coffee powdery rust has not been intensively investigated and the only species with resistance to the disease is *Coffea race mosa* Lour. Cultivars resistant to *H. vastatrix* show no resis tance to *H. coffeicola*.

Management

There are no commercial varieties resistant to *H. coffeicola*. Chemical control is recommended through the use of copper fungicides. Triazole fungicides, applied three times during the productive cycle, effectively control the disease.

Selected References

Goujon, M. 1979. Un exemple d'interactions entre populations na turelles: Les *Coj'ea* et les rouilles: *Hemileia vastatrix* Berk. et Br. et *H. coffeicola* Maubl. et Rog. Bull. Soc. Bot. Fr. Actual. Bot. 126:7 19.

Kushalappa, A. C., and Eskes, A. B. 1989. Coffee Rust: Epidemiology, Resistance, and Management. CRC Press, Boca Raton, FL.

Partiot, M., Amefia, Y. K., Djiekpor, E. K., and Segbor, A. 1979. Une nouvelle maladie du caféier au Togo: La rouille farineuse du *Hemileia coffeicola* Maub. et Rog. Café Cacao Thé 23:43 48.

Saccas, A. M. 1972. La rouille "farineuse" des caféiers due à *Hemileia coffeicola* Maub. et Rog. Inst. Fr. Café Cacao Bull. No. 11.

Tarjot, M., and Lotode, R. 1979. Contribution a l'étude des rouilles oran gée et farineuse du caféier au Cameroun. Café Cacao Thé 23:103 118.

(Prepared by M. A. Cristancho)

Coffee Leaf Rust

Coffee leaf rust is the most famous and probably most dam aging coffee disease, producing average crop losses of 30% if no control measures are performed but reaching up to 100% when attacks are severe. Coffee leaf rust was responsible for the destruction of the crop in Sri Lanka in the late nineteenth century and for the subsequent switch to tea cultivation within the country. The disease is present in almost every coffee producing country in the world; Hawaii being one of the few places where the pathogen has not yet been reported. As a leaf disease, coffee leaf rust reduces the photosynthetic capacity, which in turn affects the quality of the coffee, especially when the epidemic is early during bean formation and filling, result- ing in high proportions of empty and dry beans during the harvest. In addition, extreme defoliation changes the normal development of the plant over the years, which is observed later as declining yields. Because of the economic losses and the capacity of the pathogen to overcome resistance in cultivars, coffee leaf rust continues to pose a serious threat to the coffee industry worldwide.

Symptoms

The fungus affects the leaves of coffee plants of all ages. The distinct symptom is the formation of yellow-orange lesions on the lower surface of the leaves (Fig. 58). These orange spots have an initial diameter of approximately 2–4 mm, increasing in size and becoming more irregular, reaching up to 5 cm wide. Lesions begin to sporulate and are surrounded by a nonsporu lating, pale orange, chlorotic halo (Fig. 59). Unlike cereal and other rusts, spores emerge through the stomata of the lower sur face of the leaf. Rust lesions commonly cover 25% of the leaf area. At a later stage, before the leaves fall, sporulation stops and the lesions become senescent; the central region becomes brownish and then turns necrotic. In old lesions, often a white cottony growth can be found (Fig. 60), caused by the develop- ment of the hyperparasite *Lecanicillium lecanii* (Zimmerman) Zare & W. Gams (formerly *Verticillium lecanii* (Zimmerman) Viégas). The plant can be partially to severely defoliated, de pending on the simultaneous combination of factors such as presence of compatible inoculum, adequate weather conditions, deficient agricultural practices, and the coffee species and va- rieties used (Fig. 61).

Causal Organism

Coffee leaf rust is caused by the obligate pathogen *Hemileia vastatrix* Berk. & Broome, a basidiomycete fungus of the order Uredinales and family Pucciniaceae. Besides coffee, other hosts have not been found. Although completion of the telial and

Fig. 58. *Hemileia vastatrix* lesions on the underside of coffee leaves. (Courtesy G. Hoyos, Cenicafé)

Fig. 59. Macroscopic details of *Hemileia vastatrix* lesion sori. (Courtesy G. Hoyos, Cenicafé)

basidial stages has been reported, only the uredinial stage has epidemiological impact. Urediniospores are reniform (kidney like shape) and 12–28 × 25 35 µm, with an echinulated dorsal side and a smooth ventral side, hence its name (*Hemi* = half, *leios* = smooth). The mycelium is completely located within the leaf tissue and is composed of hyaline hyphae, 5 6 µm in diameter, with irregular ramifications that penetrate the meso phyll cells by means of haustoria, oval expansions of 4–4.5 × 7–8 µm. Urediniospores found on the lower surface of the leaf are formed from interwoven masses of hyphae within sori lo cated in substomatal cavities.

Gene for-gene interactions between coffee and the coffee rust can result in complete resistance reactions. More than 35 physio logical races of the pathogen have been identified at the Center for Coffee Rust Research (CIFC) in Portugal. However, several new races have emerged in coffee cultivated in India, Brazil, Colombia, and other coffee-producing countries, and they re main unidentified by the set differential plants used at CIFC.

Disease Cycle and Epidemiology

Rain, wind, and worker activities are the main pathways for disseminating the disease inside the plant canopy and across coffee plots. However, transportation of planting material or contaminated goods are important modes for long distance movement of the disease. Once contact is made with the lower surface of the leaf, urediniospores germinate in the dark (Fig. 62), producing three to four germ tubes and giving rise to ap pressoria on or near a stoma 6 h later. These appressoria pro-duce infection hyphae that penetrate through the stomata into the substomatal cavity. After 15 h, the hyphae branch inside the leaf cells and produce the first haustorial mother cells, which give rise to one or two haustoria, the structures that mediate nutrient exchange between the plant and the pathogen. The role of these haustoria in the suppression of host defense responses has not yet been proven in coffee leaf rust as has been done for other rusts. An uredinial thallus derives from a single in fection, which produces more than one sorus. Each sorus is a pustule and each visible orange lesion on a leaf contains a large number of pustules. Every lesion can disseminate 300–400,000 urediniospores in its lifetime, a 3 to 5 month period. Sporula tion is always outside the host cells. The three subprocesses of infection, sporulation, and dissemination constitute a monocy clic process.

A coffee leaf rust epidemic consists of a succession of mono cyclic processes, and disease development depends greatly on the initial inoculum level. Residual inoculum, which comes from the low internal zone of the coffee plant, infects the leaves associated with the productive branches of the tree, where fruit develops. Rainfall distribution and amount are fundamental for the germination, infective process, and dissemination of the pathogen. Peaks in rust progress overlap rainy seasons. Coffee leaf rust develops well at 16 28°C, with an average of 22°C. Plantations located at higher altitudes usually do not need controls because conditions are too cool for epidemic develop ment. However, when the difference between daily maximum and minimum temperatures (the thermal amplitude) narrows due to continuous cloud coverage during the bean filling pe riod, those places can become susceptible to significant disease levels that deserve monitoring and eventually control measures. Increased precipitation favors fungal dispersal and might result in elevated incidence. Other important factors are related to crop conditions, such as exceeded plant density, deficient fer tilization, acid or compacted soils, weak root development, and insufficient weed management. High-yielding cultivars seem to have a higher predisposition to the disease. Similarly, years of high production are associated with severe epidemics, and for the following year, both production and disease diminish. De foliation is less severe under shady conditions than under sunny conditions. Disease behavior, therefore, varies depending on the cultivation system, the local weather, the varieties planted, and the presence of compatible rust races.

Management

On susceptible varieties, disease control must begin 16 months after field planting. For chemical control, protective fungicides that inhibit pathogen germination and systemic fun gicides with additional curative effects to stop the infection process are recommended. Among the protective fungicides, copper based products, such as copper oxychloride, cuprous oxide, copper hydroxide, and copper sulfate (as a Bordeaux mixture), are frequently used and have no apparent differences in efficacy. The maximum deposit recommended is 30 mg of

Fig. 61. Defoliation caused by severe coffee leaf rust attack. (Cour tesy M. A. Cristancho, Cenicafé)

Fig. 60. Hyperparasitism of rust pustules by *Lecanicillium lecanii*. (Courtesy M. A. Cristancho, Cenicafé)

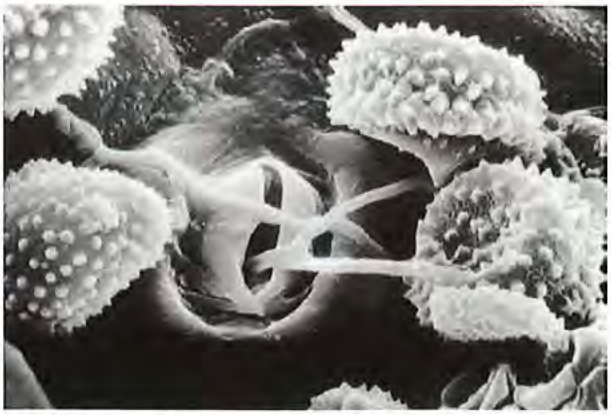


Fig. 62. Transmission electron microscopy (TEM) image of *Hemileia vastatrix* urediniospores penetrating through stomata on the lower side of a coffee leaf. (Courtesy J. E. Leguizamón, Cenicafé)

metallic copper per square meter of leaf surface area, without exceeding 3 kg/ha. Foliar applications of systemic fungicides, mainly the azole group (e.g., cyproconazole, triadimefon, hexaconazole, and flutriafol), but also strobilurins (e.g., azoxystrobin and pyraclostrobin), or mixed preparations of both, provide economically effective disease control. Applications on soil of cyproconazole combined with subsequent foliar sprays of systemic triazoles have been equally useful for rust management. Doses of systemic fungicides vary between 250 ml (cyproconazole) and 1 L (hexaconazole and triadimefon) of commercial product per hectare per application.

Chemical control follows either a fixed calendar spraying program or applications based on infection level criteria. Fixed calendar programs consist of a constant number of applications throughout the year, depending on the product used (protectant, systemic, or both) and the environmental conditions of the production zone. Applications based on infection levels vary and are a more rational use of fungicides, reducing the costs of control but requiring more field disease evaluations. Sprayings applied on a timely calendar schedule or from clear criteria of disease management based on infection levels, together with good foliage coverage, a suitable fertilizer program, and proper weed control, significantly reduce the negative effects of coffee leaf rust on coffee production.

To reduce the economic impact of *H. vastatrix* on coffee plantations, several breeding programs around the world have made use of the complete resistance in some *Coffea* spp., especially in a self-fertile, spontaneous, tetraploid hybrid between *C. arabica* L. and *C. canephora* Pierre ex A. Froehner, known as Timor Hybrid. CIFC researchers have identified nine genes (SH1 to SH9) involved in complete resistance interactions. Genes SH1, SH2, SH4, and SH5 are in *C. arabica*; gene SH3 seems to come from *C. liberica* W. Bull ex Hiern, and genes SH6 to SH9 are found in materials such as 'Timor Hybrid'. Using the 'Timor Hybrid' accessions 832/1 and 832/2 from CIFC, the genotypes designated 'Catimor' ('Timor Hybrid' × yellow 'Caturra'), 'Sarchimor' ('Timor Hybrid' × 'Villa Sarchi'), 'Cavimor' ('Catimor' × 'Catuai'), and others have been obtained in Brazil and Central America. 'Timor Hybrid' accession 1343 crossed with 'Caturra' resulted in the varieties Colombia and Castillo, widely used on Colombian plantations. Selection pressure and the high capacity for fungal change have caused the breakdown of resistance in most of the breeding materials commercially cultivated. Incomplete resistance has been demonstrated in varieties of *C. arabica*; interspecific hybrids, such as Icatu (*C. canephora* × *C. arabica*); and progenies from 'Timor Hybrid', *C. canephora*, and other diploid species, such as *C. racemosa* Lour. and *C. eugenioides* S. Moore. This type of resistance, governed polygenically and characterized by a low number of sporulating lesions, fast recovery from strong attacks, and rapid fall of affected leaves, results in a reduced rate of disease development and a delaying effect on the epidemic. Durable resistance to coffee leaf rust by conventional breeding methods is currently based on both complete and incomplete interactions and on the combination of multiple resistance genes either in a single plant (gene pyramiding strategy) or in several lines in the plot (composite variety strategy).

Biological control of *H. vastatrix* is becoming a large field of research for the future because of the increased interest in the specialty coffee market, sustainable agriculture, and fungicide residue limits. However, the inducible resistance observed in nursery plantlets using dead urediniospores, *Bacillus thuringiensis* Berliner, *Pseudomonas* spp., yeasts, and chemical inducers has yet to prove its practical utility under field conditions.

Selected References

Avelino, J., Zelaya, H., Merlo, A., Pineda, A., Ordoñez, M., and Savary, S. 2006. The intensity of a coffee rust epidemic is dependent on production situations. Ecol. Modell. 197:431 447.

Capucho, A. S., Zambolim, L., Lopes, U. N., and Milagres, N. S. 2013. Chemical control of coffee leaf rust in *Coffea canephora* cv. *conilon*. Australas. Plant Pathol. 42:667-673.

de Souza, A. F., Zambolim, L., de Jesus, V. C., Jr., and Cecon, P. R. 2011. Chemical approaches to manage coffee leaf rust in drip irrigated trees. Australas. Plant Pathol. 40:293-300.

Fulton, R. H., ed. 1984. Coffee Rust in the Americas. American Phytopathological Society, St. Paul, MN.

Kushalappa, A. C., and Eskes, A. B. 1989. Coffee Rust: Epidemiology, Resistance, and Management. CRC Press, Boca Raton, FL.

Kushalappa, A. C., and Eskes, A. B. 1989. Advances in coffee rust research. Annu. Rev. Phytopathol. 27:503-531.

Rodrigues, C. J., Jr., Bettencourt, A. J., and Rijo, L. 1975 Races of the pathogen and resistance to coffee rust. Annu. Rev. Phytopathol. 14:49-70.

Rozo, Y., Escobar, C., Gaitán, A., and Cristancho, M. 2012. Aggressiveness and genetic diversity of *Hemileia vastatrix* during an epidemic in Colombia. J. Phytopathol. 160:732-740.

Silva, M. do C., Várzea, V., Guerra-Guimarães, L., Azinheira, H. G., Fernandez, D., Petitot, A.-S., Bertrand, B., Lashermes, P., and Nicole, M. 2006. Coffee resistance to the main diseases: Leaf rust and coffee berry disease. Braz. J. Plant Physiol. 18:119 147.

(Prepared by C. A. Rivillas and M. A. Cristancho)

American Leaf Spot

American leaf spot of coffee was the very first disease reported in American plantations, where it is known as *ojo de gallo* or *gotera*, after the introduction of coffee at the end of the eighteenth century. It received this name to differentiate it from the leaf spot caused by coffee leaf rust in Asia and Africa. It is a very important disease on shady plantations, but it is completely absent under sunny planting conditions.

Symptoms

The disease starts as brown to reddish dots that turn gray as they grow into circular lesions 6 10 mm in diameter (Fig. 63). The lesions have red borders. Leaves of any age can be attacked, and in severe cases, up to 50 lesions per leaf can be observed. The inner part of the lesions can fall from the leaf, which results in holes, a very characteristic symptom. On the berries, the disease spots are round and deep but not necrotic. There can be extensive defoliation.

Causal Organism

The basidiomycete *Mycena citricolor* (Berk. & M. A. Curtis) Sacc. is the causal organism of American leaf spot. Its ba

Fig. 63. Leaf lesions caused by *Mycena citricolor*. (Courtesy C. A. Rivillas, Cenicafé)

Fig. 64. Pin-shaped coremia of *Mycena citricolor* that are present during rainy seasons. (Courtesy C. A. Rivillas, Cenicafé)

sidiocarp is difficult to find in the field. On the contrary, the asexual stage (*Omphalia flavida* Maubl. & Rangel) produces a coremium that has a pin shape and is easy to find during rainy seasons and in the morning (Fig. 64). There are reports of these structures being phosphorescent at night, resulting in the disease being called *candelilla* (little light) in some places.

Disease Cycle and Epidemiology

Asexual conidia, produced from the coremia on the leaves and berries, are the main source of inoculum. They are dispersed by rain and wind, and once on plant tissue, they germinate in about 1 h. The fungus produces oxalic acid that captures calcium from the cell walls of the host, weakening the tissues and facilitating penetration. A relative humidity of more than 80% and abundant precipitation favor disease development. These conditions are common under trees or on coffee plantations with excessive shade. During dry seasons, the fungus can survive as a saprophyte in the soil among plant debris. Besides coffee, *M. citricolor* has more than 500 host species, including cocoa and shade trees, that can be important sources of inoculum.

Management

There are no sources of genetic resistance reported in coffee. Reducing the density as well as the shade (via tree species and density) can regulate the relative humidity inside a coffee plot. If required, copper-based fungicides (e.g., copper oxychloride, cuprous oxide, and a Bordeaux mixture), as well as systemic chemicals based on cyproconazole or triadimefon, have been effective. Calcium hydroxide has been successfully used, and *Trichoderma* spp. can act as a biological control agent.

Selected References

Alvarado, J. A. 1933. La gotera en los cafetales. Rev. Café. Colomb. 5:1726-1728.

Avelino, J., Toledo, J. C., and Medina, B. 1992. El caldo bordelés y la recepa en el control del ojo de gallo. Pages 123 129 in: Memoria Técnica de Investigaciones en Café. Asociación Nacional del Café (ANACAFÉ), Guatemala.

Barriga, O. R. 1957. Ensayo comparativo de fungicidas para control de la gotera del café, *Mycena citricolor* (Berk. & Curt.) Sacc. Agric. Trop. 13:1991 1996.

Castaño A , J. J. 1951. Principales causas predisponentes para la enfermedad de la "gotera" en nuestros cafetales. Rev. Café. Colomb. 10:3750 3756.

Leandro, G., and Soto, C. A. 1980. Evaluación de fungicidas para el combate de *Mycena citricolor* y *Cercospora coffeicola* en café. Agron. Costarric. 4:41 45.

Rao, D. V., and Tewari, J. P. 1987. Production of oxalic acid by *Mycena citricolor*, causal agent of the American leaf spot of coffee. Phyto pathology 77:780 785.

Rao, D. V., and Tewari, J. P. 1988. Suppression of the symptoms of American leaf spot of coffee with calcium hydroxide. Plant Dis. 72:688 690.

Rodríguez, F., and Chaves, C. D. 1994. Dosis y épocas de aplicación de Atemi/Alto 100 SL. Cyproconazol en el control de Ojo de Gallo *Mycena citricolor* en Costa Rica. Pages 125-130 in: Simposio sobre Caficultura Latinoamericana, 14th. Ciudad de Panamá, 1991. Instituto Interamericano de Cooperación para la Agricultura (IICA) PROMECAFE, Tegucigalpa, Honduras.

Sequeira, L. 1958. The host range of *Mycena citricolor*. Turrialba 8:136 147.

Vargas, E. 1984. Interacción de tratamiento biológico y químico en el combate del ojo de gallo (*Mycena citricolor*) en el cafeto. Agron. Costarric. 8:91 97.

Wang, A., and Avelino, J. 1999. El ojo de gallo del cafeto (*Mycena citricolor*). Pages 243 260 in: Desafíos de la caficultura en Centro américa. B. Bertrand and B. Repidel, eds. Centro de Cooperación Internacional de Investigación Agrícola para el Desarrollo (CIRAD)/Instituto Interamericano de Cooperación para la Agricultura (IICA) PROMECAFE, San José, Costa Rica.

(Prepared by A. Gaitán)

Greasy Spot

Greasy spot, also known as oily spot or buttery spot, has been reported on coffee plants in Costa Rica, Colombia, and Brazil and has a limited distribution and occurs occasionally in commercial areas. A very low percentage of the plants shows susceptibility, which is transmitted to their progeny.

Symptoms

Circular spots are easily observed on the upper surface of the leaves of seedlings as well as adult plants. These spots are about 2 4 mm in diameter, depressed, green pale, and oily in appearance (Fig. 65) and may cause defoliation. Fruits are affected at all stages of development. Small, yellowish, circular lesions appear on green berries. These lesions coalesce, forming sunken, rough spots. Attacks during the early stage of fruit development can cause fruit fall or loss of bean quality.

Fig. 65. Greasy spot on a coffee leaf. (Courtesy G. Cadena Gómez, Cenicafé)

Causal Organism

The fungus associated with greasy spot is *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc., which shows pathogenicity only on plants derived from seeds of affected plants. In culture, conidia are ovoid (4.8 6.4 × 12–19 µm) and abundantly produced. There is no seed transmission. It is well known that specific isolates can be associated with attacks on different plant tissues.

Management

Because the disease does not cause economic losses and its distribution is limited, no control measures are recommended, except for the eradication and replacement of affected plants. Seeds from susceptible plants should not be used for propagation.

Selected References

Chagas, C. M., and Sousa, A. C. 1973. Desfolhamento de cafeeiros associado a mancha de óleo em Urania, Estado de São Paulo. Biológico 39:225 228.

Leguizamon C., J. E., and Baeza A., C. A. 1973. La "Mancha mantecosa" una nueva enfermedad del cafeto en Colombia. Av. Téc. Cenicafé 27:1 4.

Lins, S. R. de O. 2006. Estudos histopatologicos da mancha manteigosa em cafeeiro (*Coffea arabica* L.) e comportamento de isolados de *Colletotrichum* spp. em plantas obtidas por cultura de embriao. Thesis Mestre. Universidade Federal de Lavras, Lavras, Brazil.

Vargas, E., and Gonzalez, C. 1972. La Mancha mantecosa del café causada por *Colletotrichum* spp. Turrialba 22:129-135.

(Prepared by B. L. Castro Caicedo)

Anthracnose

Anthracnose on coffee plants is caused by *Colletotrichum* spp. that have been associated with necrosis on branches, leaves, and floral buds (Figs. 66 and 67). Fruits can also be affected when fully ripe, causing difficulties in processing because the epicarp sticks to the bean. Isolates of the species *C. gloeosporioides* (Penz.) Penz & Sacc. (Fig. 68) and *C. acutatum* J. H. Simmonds have been identified as causing anthracnose under favorable weather conditions of high humidity followed by elevated temperatures. Because of the sporadic nature of this disease, coffee plantations displaying anthracnose must attempt to reduce humidity by increasing planting distances, controlling weeds, and reducing or removing shade.

Fig. 66. Branch anthracnose, caused by *Colletotrichum gloeosporioides*. (Courtesy G. Hoyos, Cenicafé)

Fig. 67. Stages of floral bud attack by a *Colletotrichum* sp. (Courtesy G. Hoyos, Cenicafé)

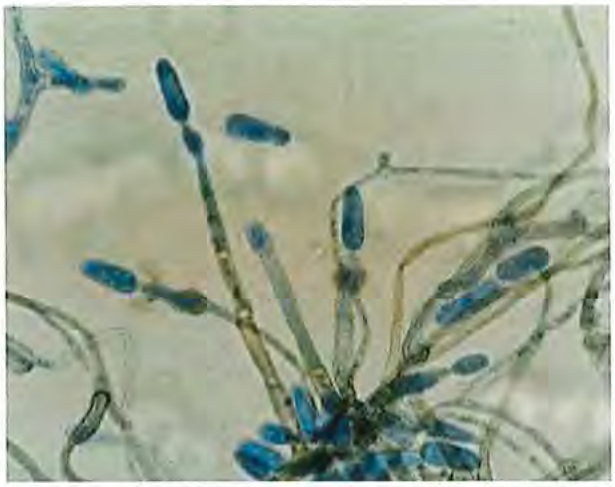


Fig. 68. Conidia of *Colletotrichum gloeosporioides*. (Courtesy A. Gaitán, Cenicafé)

Selected Reference

Gil V., L. F. 2003. Descripción de daños por *Colletotrichum* en flores y frutos de café en Colombia. Pages 164 168 in: Enfermedades del cafeto en Colombia. L. F. Gil V., B. L. Castro C., and G. Cadena G., eds. Cenicafé, Chinchiná, Colombia.

(Prepared by A. Gaitán)

Sooty Mold

Sooty mold on coffee plants is a superficial black, thin film that covers the leaves and fruits (Figs. 69 and 70) and is easily removed by rubbing. Sooty mold is caused by fungi of the genera *Capnodium* and *Fumago* and is usually associated with insects such as *Planococcus citri* (Risso), whose sweetened secretions promote fungal growth. Sooty mold is especially abundant in tropical climates, under shady and humid conditions, and where abundant insecticide applications have been made. It is not considered a serious economic problem. Therefore, no control measures are recommended, apart from removing some shade and increasing planting distances in plots where it persistently occurs.

(Prepared by B. L. Castro Caicedo)

Fig. 69. Sooty mold on a coffee leaf. (Courtesy B. L. Castro Caicedo, Cenicafé)

Fig. 70. Berries covered by sooty mold. (Courtesy B. L. Castro Caicedo, Cenicafé)

Diseases Caused by Nematodes

Root-Knot Nematodes

Nematodes are multicellular animals, the great majority microscopic, that are present in a wide variety of habitats. Nematode attacks have been recognized for many years, in particular, root knot caused by *Meloidogyne* spp., although they are not always considered a limiting condition for coffee production. The large demand for nursery plants in intensive coffee farming, together with new market forces affecting producers, low international prices, specialty coffee requirements, and diversification of farm use, have created opportune conditions for nematode proliferation and damage. Calculations indicate that for every 1% of root tissue infected while at the nursery there is a yield loss of 78 g of cherry coffee under field conditions, causing a serious economic impact when disease incidence is high.

Symptoms

When infections are very advanced, symptoms of *Meloidogyne* attack appear as slow growth and defoliation of coffee plants, in isolation or in groups; lack of response to fertilizer applications; and increased susceptibility to foliar pathogens, such as *Cercospora coffeicola* Berk. & Cooke, the causal organism of iron spot. The confirmatory sign is the presence of knots in the roots (Fig. 71). The knots caused by *M. exigua* tend to be intact and without ruptures, have the same color as the root, and be generally located in the lateral roots. *M. incognita* and *M. javanica*, individually or as a complex, establish themselves on the neck and the main and lateral roots, causing smaller galls than the ones produced by *M. exigua* (Fig. 72). When the galls break through the tissue, partial or total necrosis by other soil microorganisms occurs. The neck cortex and the upper part of the root increase in size, developing a corky and cracked appearance. Secondary roots proliferate, growing parallel to the main root and branching heavily. As a consequence of root damage, coffee plants exhibit mineral deficiencies, greater damage under prolonged droughts, and yield reductions and the potential for renovation by stumping is seriously affected.

Causal Organisms

Several *Meloidogyne* spp. have been associated with coffee root attacks around the world, including *M. incognita* (Kofoid & White) Chitwood, *M. javanica* (Treub) Chitwood, *M. exigua* Goeldi, *M. megadora* Whitehead, *M. hapla* Chitwood,

Fig. 71. Infestation of root-knot nematodes in a nursery plantlet. (Courtesy C. A. Rivillas, Cenicafé)

M. arenaria (Neal) Chitwood, *M. coffeicola* Lordello & Zamith, *M. africana* Whitehead, *M. decalineata* Whitehead, *M. kikuyensis* de Grisse, *M. inornata* Lordello, *M. oteifae* El miligy, *M. thamesi* Chitwood in Chitwood, Specht & Havis, *M. arabicida* Lopez & Salazar, *M. konaensis* Eisenback, Bernard & Schmitt, *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, *M. graminicola* Golden & Birchfield, *M. naasi* Franklin, *M. acronea* Coetzee, and *M. artiellia* Franklin. The genus *Meloidogyne* belongs to the phylum Nematoda, order Tylenchida, and family Heteroderi dae. Adult females have rounded, lemon shaped bodies that are 0.4 1.3 mm in length and 0.27–0.75 mm in diameter. They are morphologically different from the males, which are ver- miform, 1.2 1.5 mm long, and 30–36 µm wide. Species iden tification is based on inoculation tests using differential hosts, observation of perineal patterns in adult females, and morpho logical features of second stage juveniles. Molecular markers have now been increasingly effective for delineating *Meloido- gyne* species.

Disease Cycle and Epidemiology

The life cycle starts with an egg mass, laid by a *Meloido- gyne* female, that contains approximately 500–3,000 eggs and can be partially (*M. incognita* and *M. javanica*) or completely (*M. exigua*) immersed inside the root tissue of the host plant (Fig. 73). Egg development begins a few hours after its deposi tion, forming two cells (in a period of 8 days) that duplicate consecutively until a completely developed infective juvenile is formed (J1) with a visible stylet. The first molting takes place still inside the egg, and then the egg hatches to release the second infective stage (J2). The time between the J1 and J2 stages takes 8 12 days. The J2 stage penetrates the host roots and initiates parasitism. Stages J3 and J4 and adult females develop within the host tissue over approximately 29 days. The total cycle of *M. incognita* in *Coffea arabica* L. cv. Caturra is 48–52 days at 20 ± 2°C, whereas that of *M. exigua* is 70 days. Location of the eggs masses is relevant in *Meloidogyne* population dynamics. Colonization of new roots by *M. exigua* juveniles is internal because the mass is surrounded by pa renchymatous tissue; while for *M. javanica* and *M. incognita*, dispersion is facilitated by the external position of the masses.

Fig. 72. *Meloidogyne incognita or Meloido- gyne javanica* on *Coffea arabica*. (Courtesy C. A. Rivillas, Cenicafé)

Once released, infective stages penetrate between the root cap and the beginning of the vascular tissue, a root zone that has no lignification. Infested coffee plantlets are the most com mon way to spread root knot nematodes into new or renovated coffee production zones. Once in the field, *Meloidogyne* popu lations survive in coffee roots but also in almost every weed present on coffee plantations because most species have a very wide host range.

Management

Proper management of nematodes in coffee begins with the production of disease-free plantlets in the nursery. Soil should be nematode free and from the same farm to avoid the risk of inoculum introduction and, therefore, increasing the presence of nematodes in areas with low populations. One application of a nematicide with systemic action (e.g., carbofuran), 1 g of commercial product for every 2 kg of soil, just before the seed ling is sowed or during the first week after planting is recom mended. On plantations, once the nematode is established in the root system and alters the xylem vessels, coffee plants do not recover an appropriate level of water and nutrient exchange between the root and the aerial parts. Under field conditions, nematicide treatments are not profitable because the yields of treated and untreated plants are similar. (Solarization of nurs ery soil to destroy nematodes is a useful practice.)

Restrictions on the use of chemical controls have stimulated the use of biological agents as an alternative for nematode man- agement. In the nursery, commercial formulations of *Beau veria bassiana* (Bals. Criv.) Vuill. and *Metarhizium anisopliae* (Metschn.) Sorokin offer control levels similar to those found with chemical treatment, causing losses in egg turgor and lysis of J2 stages. Arbuscular mycorrhizal fungi, such as *Glomus manihotis* R. H. Howeler, Sieverd. & N. C. Schenck and *Glo mus fistulosum* Skou & I. Jakobsen, are also beneficial against root knot nematodes if associations are formed with the coffee roots well before the nematode attack and later complement the use of other biological control agents. To recover plot areas af- fected by root-knot nematodes, alternate hosts must be elimi nated and plants that benefit native microbiota, such as corn and legumes, can be intercropped.

Genetic resistance has been observed in *Coffea dewevrei* De Wild. & T. Durand, *C. canephora* Pierre ex A. Froehner, and *C. congensis* A. Froehner. Nematodes penetrate the roots but their reproduction is affected. In Central America, the variety Nemayá (*C. canephora*) was developed and used successfully as root stock for *C. arabica* graftings, offering a promis ing alternative for the management of both *Pratylenchus* and *Meloidogyne* spp.

Fig. 73. Egg masses from adult *Meloidogyne* females embedded in roots. (Courtesy C. A. Rivillas, Cenicafé)

Selected References

Baeza A., C. A., Benavides G., M., and Leguizamon C., J. E. 1978. Plantas de la zona cafetera colombiana hospedante de *Meloidogyne* Goldi. Cenicafé 29:35 45.

Bertrand, B., Peña D., M. X., Anzueto, F., Cilas, C., Etienne, H., Anthony, F., and Eskes, A. B. 2000. Genetic study of *Coffea canephora* coffee tree resistance to *Meloidogyne incognita* nematodes in Guatemala and *Meloidogyne* sp. nematodes in El Salvador for selection of rootstock varieties in Central America. Euphytica 113:79 86.

Brand, D., Roussos, S., Pandey, A., Zilioli, P., Pohl, J., and Soccol, C. 2004. Development of a bionematicide with *Paecilomyces lilacinus* to control *Meloidogyne incognita*. Appl. Biochem. Biotechnol. 118:81 88.

Di Vito, M., Crozzoli, R., and Vovlas, N. 2000. Pathogenicity of *Meloidogyne exigua* on coffee (*Coffea arabica* L.) in pots. Nematropica 30:55 61.

Leguizamon, J. E. 1994. Efecto de *Meloidogyne* app. en plantaciones establecidas de café variedad Caturra. In: Informe Anual de Actividades Diciplina de Fitopatología 1993 1994. Cenicafé, Chinchiná, Colombia.

(Prepared by C. A. Rivillas)

Root Lesion Nematodes

The first *Pratylenchus* sp. reported and also described on coffee by Zimmermann in 1898 in Java was *P. coffeae* (Zimmerman) Filipjev & Schuurmans Stekhoven. This species still remains the most widely reported root lesion nematode on coffee, with a large pantropic distribution (Southeast Asia, Latin America including the Caribbean, and Africa). Other root lesion nematode species have been reported on coffee: *P. brachyurus* (Godfrey) Filipjev & Schuurmans Stekhoven, *P. vulnus* Allen & Jensen, and *P. zeae* Graham in Brazil (*P. brachyurus* is the most frequent root lesion nematode in Brazil); *P. goodeyi* Sher & Allen in Tanzania; and *P. pratensis* (de Man) Filipjev and *P. loosi* Loof in India. More recently, another species has been described on coffee: *P. panamaensis* Siddiqi, Dabur & Bajaj in Panama (syn. *P. gutierrezi* (Golden, Lopez & Vilchez) Siddiqi, reported on coffee in Costa Rica). Recent studies have questioned the taxonomic position of several root lesion nematode isolates that are morphologically close to *P. coffeae* and collected from coffee in Central America and Brazil. It is possible that many reports of *P. coffeae* on coffee should probably be questioned. Root lesion nematodes, especially *P. coffeae*, are reported as major pests on *Coffea arabica* L. plantations in some countries, such as Guatemala, El Salvador, and India, and are responsible more locally for serious damages in some areas in Brazil. *P. coffeae* has also been reported as very destructive on *C. canephora* Pierre ex A. Froehner cv. Robusta plantations in Indonesia, with losses up to 79%.

Symptoms

Unlike root knot nematodes (*Meloidogyne* spp.), root lesion nematodes induce noncharacteristic root symptoms, such as necrosis of the root cortex and destruction of the secondary roots. These can easily be confused with physiological root death or with consequences of unfavorable abiotic telluric conditions (e.g., water saturation and physical and chemical soil factors). For this reason, the presence of *Pratylenchus* spp. can frequently pass unnoticed without laboratory analyses of nematode extractions from soil samples, particularly if root lesion nematodes are present in association with *Meloidogyne* spp. responsible for root knots, which could easily mask *Pratylenchus* attacks.

Aerial plant symptoms are similar to those caused by *Meloidogyne* infestations. The foliage turns yellow, followed by defoliation and, in cases of severe attacks, the plant's progressive decay and death. On new plantations, plants can be stunted and decay is accelerated when coffee trees begin to produce, 2 or 3 years after field planting, depending on crop conditions. Different degrees of pathogenicity on *C. arabica* have been observed between different *Pratylenchus* spp. in Guatemala as well as between *P. coffeae* and *P. brachyurus* in Brazil.

Causal Organism

The genus *Pratylenchus* belongs to the phylum Nematoda, order Tylenchida, suborder Tylenchina, superfamily Hoplolaimoidea, and family Pratylenchidae. *Pratylenchus* is considered a stenomorphic genus since species of this taxon are difficult to separate because they differ by only a few metric or secondary characters. There are no reliable morphological characters that can be easily observed by light microscopy because of the intraspecific variability in many of the characters classically used (e.g., female tail shape). No routine molecular diagnostic tools have been developed for these tropical or subtropical root lesion nematodes that parasitize coffee. As already mentioned, uncertainty remains regarding the taxonomic position of root lesion nematode populations that belong to a complex of species morphologically close to *P. coffeae*. Intraspecific differences of pathogenicity in *P. coffeae* have been shown between populations coming from coffee and those from *Musa* AAA and AAB by cross inoculations on these two hosts. No molecular support has been found in relation to these physiological differences.

Disease Cycle and Epidemiology

All *Pratylenchus* spp. are migratory endoparasites, so all stages from second stage juveniles to adults are free and able to penetrate the plant roots. They can leave the roots at any time and live temporarily in the soil before infesting new roots. These parasites live on parenchyma cells, causing lesions or cavities that can be colonized by secondary pathogens, such as bacteria or fungi, including *Fusarium* spp. (Fig. 74). This pathogenic complex leads to necrosis of the root cortex. Root lesion nematode population fluctuations are more closely related to the physiological cycle of coffee trees than directly to rain patterns. In Guatemala, higher population levels were observed during root-growing periods (mostly throughout dry periods), while low population levels were observed during the

Fig. 74. Root tissue infested with a *Pratylenchus* sp. (Courtesy L. Villain)

fruit maturation period when coffee berries create physiological sinks, drawing on a large portion of the assimilates produced by the plant. Even for sexually propagating species, root lesion nematode populations can develop very quickly and cause plant mortality over very short periods on plantations.

Knowledge of the life cycle of most of these species is still limited. *P. coffeae* is a bisexual species (as many males as fe males) with obligatory amphimictic reproduction (sexual repro duction). *P. goodeyi*, *P. vulnus*, *P. loosi*, and *P. panamaensis* (syn. *P. gutierrezi*) are also bisexual species. On the contrary, *P. brachyurus* and *P. zeae* are monosexual species (males ab sent or very rare) with mitotic parthenogenetic reproduction (asexual reproduction). Eggs are laid in the roots and hatch to produce second stage juveniles. After three molts, these free juveniles transform into adults. For *P. coffeae*, studies showed that eggs hatch in 6 8 days at 28–30°C, while adults emerge about 2 weeks after hatching at 25–30°C. Little information is available on the optimal temperatures for these different root lesion nematode species. *P. coffeae* and other species, such as *P. vulnus* and *P. brachyurus*, seem to have a temperature optimum just below or around 30°C. Above these temperatures, reproductive capacity of these root lesion nematodes decreases with each degree increase in temperature. In contrast, *P. loosi* seems to have a optimal temperature for development around 18–20°C.

Management

Laboratory diagnosis based on nematode extraction from roots is necessary to confirm the presence of root lesion nematodes, but population levels should not be the primary information when making control decisions. It is not possible to establish standard thresholds because of the extreme vari ability of agronomic and ecological conditions found on cof fee plantations (e.g., varietal tolerance levels, plant age, soil fertility, temperature and water conditions, and sun exposure) that can influence the amount of damage caused. Moreover, population levels depend on the sampling date. Studies show drastic changes in population levels over short periods. Popula tion level estimation also depends on root sampling methodolo gies. Nematode distribution, in general, is highly aggregated (heterogeneous) and root lesion nematode populations can be low on the most affected plants because their root systems are already seriously damaged. Finally, population estimation de pends on the efficiency of the extraction methodologies used by the diagnostic laboratory.

Root lesion nematode species parasitizing coffee can present different degrees of pathogenicity, and in regions where more than one species is present, diagnostic laboratories are not al ways able to identify the nematodes to the species level. The simple presence of pathogenic nematodes on the plantation or in the region should be enough to recommend control measures that emphasize preventive measures.

The most important method of nematode distribution is human activities, i.e., the transport of infested nursery seed lings or infested soil. Therefore, special precautionary mea sures should be taken at this stage of the crop. The potting substrate used in nursery bags should be disinfested to produce nematode-free nursery plants.

On established coffee plantations, the efficiency of chemical control, mainly provided by granular nematicides, is limited. An efficient and durable chemical control of nematodes would require doses and frequencies that are unsustainable on eco nomical and ecological bases.

On the contrary, genetic control via grafting *C. arabica* cul tivars on resistant *C. canephora* rootstocks provides effective control of root lesion nematode populations. This practice has been implemented since 1965 in Guatemala with very good results, and it has also been recommended for controlling *P. coffeae* in Indonesia. In Indonesia, a wide range of resis tance levels to *P. coffeae* among *C. canephora* clones has been observed. Grafting offers an advantage when *C. canephora* rootstock cultivars have also been developed for resistance to *Meloidogyne* spp., as in Central America ('Nemayá') or in Brazil ('Apoatã'). A wide range of semiwild lines of *C. arab ica* from Ethiopia and Yemen have also been tested, but no source of resistance has been found among this germplasm. Because various nematode genera and species complexes are frequently present on coffee plantations, the ability to control all communities of pathogenic nematodes is necessary to avoid an imbalance that could result in an epidemic of a particular population.

Selected References

Campos, V. P., and Villain, L. 2005. Nematode parasites of coffee and cocoa. Pages 529 579 in: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture, 2nd ed. M. Luc, R. Sikora, and J. Bridge, eds. CAB International, Wallingford, United Kingdom.

Duncan, L. W., Inserra, R. N., Thomas, W. K., Dunn, D., Mustika, I., Frisse, L. M., Mendes, M. L., Morris K., and Kaplan, D. T. 1999. Molecular and morphological analysis of isolates of *Pratylenchus coffeae* and closely related species. Nematropica 29:61 80.

Loof, P. A. A. 1978. The Genus *Pratylenchus* Filipjev, 1936 (Nematoda: Pratylenchidae): A review of its anatomy, morphology, distribution, systematics and identification. Vaxtskyddsrapporter Jordbruk 5:1 50.

Souza, R. M., ed. 2008. Plant Parasitic Nematodes of Coffee. Springer, Dordrecht, Netherlands.

Toruan Mathius, N., Pancoro, A., Sudarmadji, D., Mawardi, S., and Hutabarat, T. 1995. Root characteristics and molecular poly morphisms associated with resistance to *Pratylenchus coffeae* in Robusta coffee. Menara Perkebunan 63:43 51.

Villain, L., Anzueto, F., Hernández, A., and Sarah, J. L. 1999. Los nematodos parásitos del cafeto. Pages 327 368 in: Desafíos de la caficultura en Centroamérica. B. Bertrand and B. Rapidel, eds. In stituto Interamericano de Cooperación para la Agricultura (IICA) PROMECAFE, San José, Costa Rica.

Villain, L., Molina, A., Sierra, S., Decazy, B., and Sarah, J. L. 2000. Effect of grafting and nematicide treatments on damage by root-lesion nematodes (*Pratylenchus* sp.) to *Coffea arabica* L. in Guatemala. Nematropica 30:87 100.

Villain, L., Anzueto, F., and Sarah, J. L. 2004. Resistance to root lesion nematodes on *Coffea canephora*. Pages 289 302 in: Nematology Monographs and Perspectives 2. Proceedings of the Fourth International Congress of Nematology. Brill, Leiden, The Netherlands.

Villain, L., Baujard, P., Anzueto, F., Hernandez, A., and Sarah, J. L. 2005. Manejo integrado de nematodos en plantaciones de café de Centroamerica. Bol. Promecafé 102:11 19.

Willmott, S., Gooch, P. S., Siddiqi, M. R., and Franklin, M. 1972. Descriptions of plant parasitic nematodes. Eight sets. Commonwealth Institute of Helminthology, St. Albans, Herts., England.

(Prepared by L. Villain)

Coffee Corky-Root Syndrome

Root knot nematodes of the genus *Meloidogyne* are fre quently and abundantly found on arabica coffee plantations in Latin America. In certain areas, the prevailing nematodes are highly destructive, leading to coffee tree death. In 1982, a syn drome, locally called *corchosis*, was detected on coffee for the first time at the Hacienda Juan Viñas in the Cartago Province of Costa Rica. The syndrome was attributed to a root knot nema tode, *Meloidogyne arabicida*. Later, the interaction between *Fusarium oxysporum* and *Meloidogyne incognita* (Kofoid & White) Chitwood was demonstrated to be responsible for the corchosis symptoms on coffee in Puerto Rico. In Mexico in 1993, *Meloidogyne*, *Pratylenchus*, *Fusarium*, and *Trichoderma* spp. were isolated from coffee trees displaying corky roots, suggesting a disease complex as in Puerto Rico. Finally, it was concluded that the corky-root syndrome discovered in the Ha-

cienda Juan Viñas in Costa Rica had a complex etiology involving both *M. arabicida* and *F. oxysporum*.

Symptoms

The syndrome is characterized by a reduction in vigor and production. Diseased plants show a gradual decline, starting with leaf chlorosis, followed by flower and fruit fall, and leading to plant death in 2–4 years (Fig. 75). The root systems have reduced growth and many galls evolving to the extensive development of corky tissue on the main and secondary roots (Fig. 76), leading to considerable cracking of the cortical tissues. These symptoms are very similar to those on coffee trees in Brazil or Guatemala infected by *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida.

Fig. 75. Poor vigor and defoliation of coffee trees caused by corky-root disease. (Courtesy B. Bertrand)

Fig. 76. Corky tissue on coffee roots. (Courtesy B. Bertrand)

Causal Organisms

In Costa Rica, *Meloidogyne exigua* Goeldi is the root knot nematode most commonly found on coffee roots, but it is unable to cause severe damage to coffee trees under field conditions. This nematode was frequently found in the Juan Viñas fields (Costa Rica) but was ruled out as a causal organism of the corky root symptom. *Meloidogyne arabicida* Lopez & Salazar was described from the same locality as a new coffee pathogen (Fig. 77), which was confirmed by the expression of a specific esterase phenotype (M1F1; relative mobility [Rm] 1.20, 1.40) and an original perineal pattern. Using Booth's determination keys, *Fusarium oxysporum* Schltdl.:Fr. was identified in corky root samples (Fig. 78). Inoculations under controlled conditions showed that *F. oxysporum* alone was nonpathogenic and that *M. exigua* or *M. arabicida* by itself induced galls and a reduction in shoot height but did not reproduce any corky root symptoms unless the combination of *M. arabicida* with *F. oxysporum* was present. These observations led to the conclusion that corky root syndrome has a complex etiology and emphasized the dominant role of *M. arabicida* as a predisposing agent for subsequent *F. oxysporum* invasions.

Fig. 77. *Meloidogyne arabicida* male on coffee roots. (Courtesy B. Bertrand)

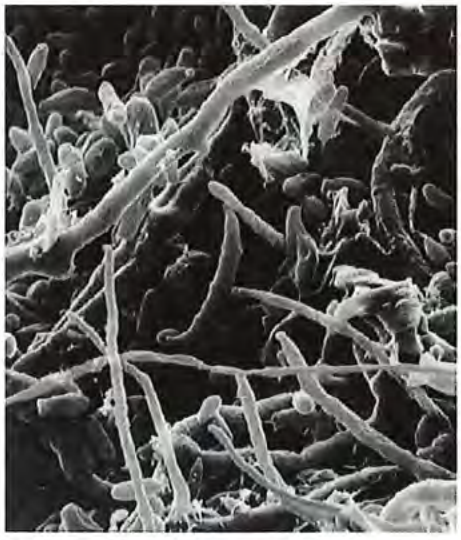


Fig. 78. Macroconidiophores, macroconidia, and microconidia of *Fusarium oxysporum* isolated from coffee roots. (Courtesy B. Bertrand)

Disease Cycle and Epidemiology

Corchosis is a complex syndrome involving a nematode and a soilborne fungus. The polyphagous nematode *M. arabicida* has been detected on tomato (*Solanum lycopersicon* L.), a *Erythrina* sp., and *Impatiens balsamina* L. As with *M. paranaensis*, development of the *M. arabicida* female in *Coffea arabica* L. var. Caturra leads to root peridermal disruptions with exterior egg masses (Fig. 79). Whether a special form or one already described is involved remains to be determined. The root knot nematode *Fusarium* interaction has received considerable attention on several host crops. In coffee, no study has been undertaken on the pathogen penetration mechanisms or on the ultrastructural modifications induced by these two pathogens.

Management

It is important to point out that *M. arabicida* has so far only been reported in Costa Rica. Until 2002, its presence was limited to the Juan Viñas region only (Cartago Province). Since then, corchosis foci have been reported near Pérez Zeledón. However, seedlings of those coffee plantations came from Juan Viñas region nurseries. As with most nematodes, it is important to take restrictive measures on transporting soil or vegetative material from farm to farm. Such measures are usually not taken in coffee producing countries (except for Colombia).

Several attempts have been made by the Costa Rican Coffee Institute and Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) to control the syndrome by ecologically acceptable growing methods: liming, applying organic matter, planting cover crops, and applying plastic mulches. None of these measures showed any marked effect on syndrome development. For many years, the producers in the region applied nematicides without any notable effect on the syndrome. Genetic resistance is an essential component in the integrated control of plant parasitic nematodes. The resistance of *C. arabica* and *C. canephora* Pierre ex A. Froehner accessions was tested against *M. arabicida* alone and against the field population under controlled and field conditions. Introgression lines derived from a natural interspecific hybrid (*C. arabica* × *C. canephora*) and F1 hybrids between cultivars and Ethiopian wild coffee accessions were tested for a characterization of their resistance to corky root syndrome. Three subspontaneous-derived accessions resistant to *M. arabicida* proved to be resistant to corky root syndrome under controlled conditions. Resistance to *M. arabicida* consequently afforded a high level of resistance to corky-root syndrome. The F1 hybrid derived from a crossing between 'Caturra' and one of the resistant accessions (T16693) proved to be resistant under field parasitic conditions. These results show that resistance to corky root syndrome is heritable and that genetic resistance to *M. arabicida* is an effective strategy against corky root syndrome. Such resistance can be exploited by creating vigorous F1 hybrids resistant to corky root syndrome. A comparison between 'Caturra' grafted on rootstock resistant to *M. arabicida* and nongrafted material revealed the advantage offered by grafted plants in terms of mortality and number of galls on roots 4 years after planting. Approximately 20% of the rootstocks were affected by corky roots. The proportion of *C. canephora* plants affected in the field was similar to that of plants developing small galls after inoculation of *M. arabicida* alone and to that of plants presenting corky roots after inoculation of a field population. Use of tolerant rootstocks, therefore, seems to be an alternative to the development of resistant cultivars. This practice needs to be accompanied by phytosanitary measures in the nursery in order to prevent gall formation during the seedling early stages of development.

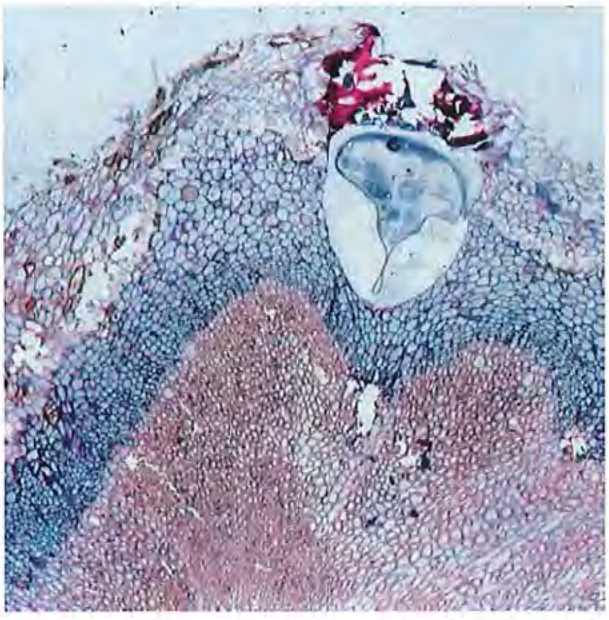


Fig. 79. Root peridermal disruption from an exterior egg mass laid by a *Meloidogyne arabicida* female. (Courtesy B. Bertrand)

Selected References

Bertrand, B., Nuñez, C., and Sarah, J. L. 2000. Disease complex in coffee involving *Meloidogyne arabicida* and *Fusarium oxysporum*. Plant Pathol. 49:383 388.

Bertrand, B., Ramirez, G., Topart, P., and Anthony, F. 2002. Resistance of cultivated coffee (*Coffea arabica* and *Coffea canephora*) trees to corky root caused by *Meloidogyne arabicida* and *Fusarium oxysporum*, under controlled and field conditions. Crop Prot. 21:713 719.

Booth, C. 1971. The Genus *Fusarium*. Commonwealth Mycological Institute, Kew, United Kingdom.

Campos, V., and Villain, L. 2005. Plant parasitic nematodes in subtropical and tropical agriculture. Pages 529 579 in: Nematode Parasites of Coffee and Cocoa. M. Luc, R. Sikora, and J. Bridge, eds. CAB International, Wallingford, United Kingdom.

Hernández, A. M., Fargette, M., Molinier, V., Ramanason, B., Decazy, B., and Sarah, J. L. 1996. Enzymatic characterization and reproductive fitness on coffee of root knot nematode populations from Central America. Nematropica 26:274 275.

Hernández, A. M., Fargette, M., and Sarah, J. L. 2004. Characterisation of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) from coffee plantations in Central America and Brazil. Nematology 6:193 204.

López, R., and Salazar, L. 1989. *Meloidogyne arabicida* sp. n. (Nematoda: Heteroderidae) nativo de Costa Rica: Un nuevo y severo patoâgeno del cafeto. Turrialba 39:313 323.

Negrón, J. A., and Acosta, N. 1989. The *Fusarium oxysporum* f. sp. *coffeae Meloidogyne incognita* complex in 'Bourbon' coffee. Nematropica 19:161 168.

Powell, N. T. 1971. Interactions between nematodes and fungi in disease complexes. Annu. Rev. Phytopathol. 9:253-274.

Teliz Ortiz, D., Castillo Ponce, G., and Nieto Angel, D. 1993. La corchosis del cafeto en México. Rev. Mex. Fitopatol. 11:5 12.

(Prepared by B. Bertrand)

Disease Caused by *Phytomonas* spp.

Phloem Necrosis

Phytomonas spp. have been ascribed as plant pathogens only in a handful of cases. In coffee, they have been reported attacking mainly *Coffea liberica* W. Bull ex Hiern in Surinam, Guyana, and northern Brazil, but they can also attack *C. excelsa* A. Chev. and *C. arabica* L. In Surinam, the disease is known as phloem necrosis of coffee. The last report of this disease was in 1977 in Trinidad, and it was still present in Surinam in the 1960s.

Symptoms

Phloem necrosis was first described as causing a reduction in the starch reserves of the plant. A more detailed examination showed a deposit of callose in the sieve tubes and the consequent necroses that give the disease its name. The evidence shows that the disease moves between neighboring trees. The disease seems to only attack trees that are more than 3 years old, since younger plants are not infected.

Phloem necrosis has an acute form and a chronic form, but the latter is more common. In the chronic form, the initial stage of infection turns the older leaves yellowish and they fall prematurely. The affected coffee trees produce fewer and smaller leaves that rapidly become pale yellow and then fall prematurely, leaving bare branches. Diseased trees die in 3–12 months. Given the genetic variability of *C. liberica*, symptoms and severity differ between varieties. In the acute form, only some of the older leaves fall prematurely. Young leaves turn yellow slowly, but eventually they too turn brown, necrotic, and fall within 2–3 weeks. In the chronic and acute forms, roots become brown and die. Microscopic examination of tissues shows hyperplasia of the phloem and sieve tubes that are much smaller than normal, become necrotic, and exhibit a deposit of callose.

Causal Organisms

Phloem necrosis of coffee is caused by intraphloemic flagellate protozoa, similar to those found in plants of the family Euphorbiaceae. The unique attributes of *Phytomonas* spp. that attack coffee are that they are smaller and their kinetoplast does not have the same shape. The vector of the disease is not known, although some scale insects, such as *Rhizoecus coffeae* Laing and some heteropteran insects of the family Pentatomidae, are considered candidates as vectors. Eradication of diseased trees is recommended.

Selected References

Dollet, M. 1984. Plant diseases caused by flagellate protozoa (*Phytomonas*). Annu. Rev. Phytopathol. 22:115 132.

Vermeulen, H. 1968. Investigations into the cause of the phloem necrosis disease of *Coffea liberica* in Surinam, South America. Annu. Rev. Phytopathol. 22:115 132.

(Prepared by M. A. Cristancho)

Disease Caused by an Alga

Algal Red Leaf Spot

Parasitic algae are only found in the class Chlorophyta. The disease known as algal red leaf spot has been reported in a large number of angiosperms, including apple, gardenia, citrus, banana, and raspberry. Parasitic algae have been detected mainly in tropical and subtropical countries. There are reports of algal red leaf spot presence in most coffee growing countries since the beginning of the nineteenth century, although it has not caused important economic losses on coffee plantations.

The initial attribution of this disease to fungi led to the unfortunate name "red rust". Tea is the plant most seriously affected by this pathogen, and in many provinces of India and Indonesia, red rust is considered the most important disease of the crop.

Symptoms

Leaf spots develop as pale green or pale red, rough, superficial, netlike, circular spots with wavy or feathered margins. Occasionally, the alga may infect twigs and branches, causing girdling lesions. Algal infections of twigs often cause superficial cell layers to become slightly swollen and cracked. This cracking causes the twigs to be more susceptible to fungal infection. When algal spore structures, or sporangia, are produced, the lesions become reddish. When sporangia are not produced, the spots remain light green. Small, necrotic, brown spots of irregular shape, 1–5 mm in diameter, develop on the upper leaf surface. On the lower leaf surface, spots are similar but have a velvet aspect and a pale color. The alga grows superficially on the leaf cells.

Causal Organism

The green alga *Cephaleuros virescens* Kunze is the causal organism of algal red leaf spot. Algal red leaf spot has been confused with a fungal disease, but detailed microscope examination of leaf lesions has shown the presence of the alga, usually readily recognized by the presence of stalked gemmae, the vegetative dispersal stage of the alga.

Disease Cycle and Epidemiology

Frequent rains favor disease spread in coffee. The disease is very common in old leaves during wet weather, when water droplets or wind driven rain spreads spores to leaves or twigs, which are then colonized by the alga. *C. virescens* survives adverse conditions in spots on leaves and branches. It is more common in shaded coffee crops located in hollow valleys and under conditions of high relative humidity and low levels of sunlight. Weakened plants are the most susceptible to attack. The interference of the growing alga on leaf photosynthesis might cause losses in production.

Management

When a severe attack is detected, algal red leaf spot can be controlled by the one of the following strategies. Overhanging trees can be pruned around diseased plants to help lower humidity levels and speed the drying of leaf surfaces. Soil drainage can be improved if this is diagnosed as a problem. A

Bordeaux mixture (8 tablespoons per gallon) can be applied as a protective spray on heavily spotted plants.

Selected References

Cadena G., G. 1982. Sintomatología de la mancha algácea del cafeto *Cephaleuros virescens* Kunze. Cenicafé 33:67-73.

Chapman, R. L. 1976. Ultrastructure of *Cephaleuros virescens* (Chro olepidaceae; Chlorophyta). I. Scanning electron microscopy of zoo sporangia. Am. J. Bot. 63:1060 1070.

Holcomb, G. E. 1986. Hosts of the parasitic alga *Cephaleuros virescens* in Louisiana and new host records for the Continental United States. Plant Dis. 70:1080-1083.

Joubert, J. J., and Rijkenberg, F. H. J. 1971. Parasitic green algae. Annu. Rev. Phytopathol. 9:45-64.

Wellman, F. L. 1965. Pathogenicity of *Cephaleuros virescens* in the Neotropics. (Abstr.) Phytopathology 55:1082.

(Prepared by M. A. Cristancho)

Screening for detection of covert and endophytic bacteria

Adapted from P. Thomas. 2004. A three-step screening procedure for detection of covert and endophytic bacteria in plant tissue cultures. Current Sci. 87:67-72.

Media

Nutrient Broth

	Per L
Peptone	5 g
Beef Extract	3 g
Water to	1L

pH 6.8

dispense to tubes and autoclave

523 Medium (Phytotechnology Lab – B129)

	Per L
Sucrose	10 g
Casein hydrolysate	8 g
Yeast Extract	4 g
KH2PO4	2 g
MgSO4 7H20	0.15 g
Water to	1L

pH 6.9

dispense to tubes and autoclave

Procedure

1. During transfer under laminar flow or Biological Safety Cabinet, cut a 2-5 cm piece of stem from the plant.
2. Place stem into media 1 tube with Nutrient broth and 1 tube with 523 medium.
3. Also using sterile forceps or scapel, take plant growth media where plant was growing and place into 1 tube with Nutrient broth and 1 tube with 523 medium.
4. Place on shaker incubator at 30 C for 3 days to 1 week.
5. Note any cloudy growth and trace back to corresponding plant line and report to supervisor.
6. Dispose of line by autoclaving and keep temperature logs for records.

World Journal of Microbiology and Biotechnology 7, 452–469

Review

Contaminants of plant-tissue and cell cultures

C. Leifert, J.Y. Ritchie and W.M. Waites

This review describes (1) bacteria, yeasts, fungi, thrips and mites found as contaminants and their effect on plants growing *in vitro*; (2) methods used for detecting such contaminants and the sources of contamination; and (3) methods for their prevention and/or elimination.

C. Leifert is with the Department of Cell and Structural Biology, Manchester University, Manchester M13 9PT, UK. Fax: (061) 275 3915. J.Y. Ritchie was formerly with the Department of Physiology and Environmental Science, University of Nottingham, Sutton Bonington LE12 5RD, UK. W.M. Waites is with the Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington LE12 5RD, UK. C Leifert is the corresponding author.

Plant-tissue culture can be defined as the growing of 'sterile' plant cells or tissues separate from the mother plant on artificial media *in vitro*. It is an important method in the study of plant metabolism, plant genetics, plant morphogenesis and plant physiology, in the genetic transformation of plants, in elimination of plant pathogens, in preservation of important plant species in limited space, and in multiplication of plant tissues *in vitro* (for reviews see George & Sherrington 1984; Grierson & Covey 1984). Although aseptic conditions are usually implied, many plant cultures are not or do not stay aseptic *in vitro* and contamination with micro-organisms is considered to be the single most important reason for losses during *in vitro* culture of plants (Cassells 1986; Boxus & Terzi 1987; Leifert & Waites 1990a,b).

The list of organisms described as contaminants in plant-tissue cultures includes viruses, bacteria, yeasts, fungi, mites and thrips (Blake 1988; Enjalric *et al.* 1988; Leggatt *et al.* 1988; Leifert 1990). Contamination with bacteria is thought to be the most serious and has been described extensively in the literature (for example, Knauss & Miller 1978; Horsch & King 1983; Trick & Lingens 1985; Leifert *et al.* 1989a,b; 1991a,b). Fewer publications describe yeast and fungal contaminants and their effect on plantlets grown *in vitro* (Boxus & Terzi 1988; Enjalric *et al.* 1988, Leifert *et al.* 1990). Mites and thrips found in tissue cultures do not usually harm the plants directly but introduce other contaminants such as fungi, yeast and bacteria into sterile plant cultures (Debergh & Maene 1984; Klocke & Myers 1984; Blake 1988).

Contaminants Isolated from Plant-Tissue Cultures and Their Effect on Plants

This section describes the different contaminants found in plant-tissue cultures. Identification of these organisms is an important first step in determining the source of contamination and the methods for treatment and prevention of future contamination.

Bacterial Contaminants

A variety of bacterial genera and species have been described as contaminants in plant tissue or cell cultures. Bacteria repeatedly isolated by different workers and

from different plant species include *Acinetobacter, Agrobacterium, Bacillus, Corynebacterium, Enterobacter/Erwinia, Flavobacterium, Micrococcus, Pseudomonas, Staphylococcus* and *Xanthomonas* (Tables 1 and 2).

After identification of 293 bacterial strains from two commercial micropropagation laboratories, we found that 26% were *Staphylococcus* or *Micrococcus*, 19% *Pseudomonas*, 13% *Bacillus*, 12% *Enterobacter* or *Erwinia*, 11% *Lactobacillus*, 3% *Agrobacterium* and 3% *Acinetobacter* species (Table 3). The quantitative importance of different bacteria in other laboratories is more difficult to assess because many workers have only isolated small numbers of contaminants or not reported the number of times different bacterial species were isolated (Tables 1 and 2). Comparison of bacterial contaminants described by different workers is difficult, because the identification method and/or test results are either not described or authors have used different identification methods (see Leifert & Waites 1990a for a description of identification methods).

Different bacterial communities were isolated from disinfected stock plant cuttings, used to initiate plant-tissue cultures, and from established plant cultures, which had been *in vitro* for longer than 12 months (Table 3). All 58 bacteria isolated from stock plant cuttings of eight different plant species after growth for 4

Table 1. Gram-positive bacterial species isolated as contaminants in plant tissue and cell cultures.

Bacterial genera/species	Plant genus	Reference
Actinomyces spp.	*Malus*	6
Bacillus spp.	*Gerbera, Hevea, Nephrolepsis*	5, 6, 8
	Nauclea, Malus, Pteris,	10, 12
	Saxifraga, Viola	13
Bacillus circulans	*Begonia, Fragaria, Primula*	10, 14
Bacillus cereus	*Fragaria, Begonia*	13
Bacillus polymyxa	*Gerbera*	13
Bacillus pumilus	*Astilbe, Arunchus, Cotinus,*	10
	Pulmonaria, Primula	
Bacillus subtilis	*Astilbe, Cotinus, Delphinium,*	10, 16
	Hemerocallis, Malus, Thalictrum,	
	Viola	
Bordetella branchiseptica	*Hevea*	5
Coryneforms	*Fragaria, Fremontodendron,*	1, 2, 6
	Geranium,	
	Gerbera, Hemerocallis, Malus,	9, 10
	Prunus, Solanum, "fruit trees"	11, 13
Lactobacillus plantarum	*Hemerocallis*	10
Lactobacillus acidophilus	*Delphinium*	10
Propionibacterium	*Hevea brasiliensis*	5
Staphylococcus spp.	*Choisya, Hemerocallis, Paeony*	9, 10
Staphylococcus capitis	*Paeony, Hosta*	10
Staphylococcus epidermidis	*Choysia, Delphinium, Hemerocallis,*	10
	Hosta,	
Staphylococcus saprophyticus	*Choysia*	10
Staphylococcus warneri	*Delphinium, Hosta, Paeony*	10
Micrococcus spp.	*Choysia, Delphinium, Paeony, Prunus*	2, 10
Micrococcus kristinae	*Hemerocallis, Hosta*	10
Micrococcus varians/roseus	*Delphinium, Hosta*	10

1 Boxus & Terzi 1988
2 Cornu & Michel 1988
3 Cassells *et al.* 1988
4 Duhem *et al.* 1988
5 Enjalric *et al.* 1988
6 Hennerty *et al.* 1988
7 Horsch & King 1983
8 Knauss & Miller 1978
9 Leggatt *et al.* 1988
10 Leifert *et al.* 1989a
11 Long *et al.* 1988
12 Mathias *et al.* 1987
13 Podwyszynska & Hempel 1987
14 Trick & Lingens 1985
15 Reuther 1988
16 Rossini & Standardi 1990

Table 2. Gram-negative bacterial species isolated as contaminants in plant tissue and cell cultures.

Bacterial genera/species	Plant genus	Reference
Acinetobacter calcoaceticus	*Astilbe, Delphinium, Fragaria, Gerbera, Hevea, Hosta,* 'Fruit trees'	1, 5, 10
Achromobacter spp.	*Choysia*	10
Alcaligenes denitrificans	*Iris*	10
Agrobacterium radiobacter	*Hevea, Choysia, Paeony, Gerbera, Viola*	5, 10
Enterobacter/Erwinia spp.	*Choysia, Coffea, Fragaria, Gerbera, Hevea, Hosta, Prunus, Viola,* 'woody species'	1, 2, 4 5, 10
Erwinia carotovora	*Iris, Nephrolepsis, Saxifraga, Pteris*	8, 10
Enterobacter cloacae	*Coffea, Iris, Hevea, Hemerocallis,*	4, 5, 10
Klebsiella pneumonieae	*Coffea*	4
Klebsiella oxytoca	*Delphinium*	10
Rhanella aquatilis	*Iris, Hemerocallis*	10
Flavobacterium spp.	*Fragaria, Gerbera, Hosta,*	1, 10
Pseudomonas spp.	*Coffea, Daphne, Delphinium, Fragaria, Hevea, Hosta, Nauclea, Nephrolepsis, Prunus, Pteris, Saxifraga, Solanum,* 'fruit trees'	1, 2, 4 5, 8, 9 10, 11 12
Pseudomonas cepacia	*Hevea, Hosta*	5, 10
Pseudomonas diminuta	*Hevea*	5
Pseudomonas fluorescens	*Delphinium, Gerbera, Hosta, Iris, Viola*	10,13
Pseudomonas maltophilia	*Delphinium*	10
Pseudomonas paucimobilis	*Choysia, Delphinium, Hevea, Hosta*	5, 10
Pseudomonas putida	*Hevea, Gerbera*	5, 13
'Pseudomonas typhiflavum'	*Hevea*	5
Xanthomonas pelargonii	*Pelargonium*	4
Xanthomonas spp.	*Prunus*	2, 15
Budding bacteria		
Hyphomicrobium spp.	*Datura*	7

1 Boxus & Terzi 1988
2 Cornu & Michel 1988
3 Cassells *et al.* 1988
4 Duhem *et al.* 1988
5 Enjalric *et al.* 1988
6 Hennerty *et al.* 1988
7 Horsch & King 1983
8 Knauss & Miller 1978
9 Leggatt *et al.* 1988
10 Leifert *et al.* 1989a
11 Long *et al.* 1988
12 Mathias *et al.* 1987
13 Podwyszynska & Hempel 1987
14 Trick & Lingens 1985
15 Reuther 1988
16 Rossini & Standardi 1990

weeks *in vitro* were found to be motile Gram-negative bacteria, except for one isolate which was *Bacillus subtilis* (Leifert *et al.* 1989a). Nearly all isolates belonged to genera and species which are known as common plant pathogens or inhabitants of aerial plant surfaces or the rhizosphere, such as *Alcaligenes, Achromobacter, Agrobacterium, Erwinia* and other Enterobacteriaceae and fluorescent pseudomonads (Krieg & Holt 1984; Sneath *et al.* 1986). Similar populations of plant saprophytic and pathogenic bacteria were also isolated from contaminated stock plant cuttings of *Coffea, Theobroma* and *Hevea* (Duhem *et al.* 1988; Enjalric *et al.* 1988). Some plant pathogenic bacteria found as contaminants, for example *Agrobacterium tumefaciens* and *Erwinia carotovora*, produce similar symptons on *in vivo* and *in vitro* plants (Reustle *et al.* 1988; Leifert 1990). Many of the 'non-pathogenic' plant-inhabiting bacteria found on explants (Tables 1 to 3) are adapted to utilize dead or stressed plant tissue and the nutrients of the plant medium for their metabolism (Krieg & Holt 1984; Sneath *et al.* 1986) and usually overgrow the explant after transfer onto initiation media (Knauss & Miller 1978; Duhem *et al.* 1988; Enjalric *et al.* 1988; Leifert 1990).

In comparison, of the 240 bacterial isolates found in 12-month-old cultures of

Table 3. Bacterial contaminants isolated from micropropagated plants.

Plant species	Time for which plant material was cultured *in vitro*: 1 month (explants)		>12 month (shoots/calli)	
	No. of strains	Identification	No. of strains	Identification
Laboratory 1				
Astilbe	—	—	1	*B. subtilis*
			1	*A. calcoaceticus*
			1	*M. kristinae*
Arunchus	—	—	4	*B. pumilus*
Choisya	3	*Agrobacterium radiobacter*	21	*S. saprophyticus*
	2	*Achromobacter* group VD	2	*S. epidermidis*
	2	*P. paucimobilis*	1	*E. agglomerans/Erwinia*
	1	*P. luteola/paucimobilis*	1	*Micrococcus* spp.
Cotinus	—	—	5	*B. subtilis*
			3	*B. pumilus*
Delphinium	2	*P. fluorescens*	25	*P. maltophila*
	3	*K. oxytoca*	6	*P. paucimobilis*
	1	*A. calcoaceticus*	2	*L. acidophilus*
	1	*B. subtilis*	3	*S. epidermidis*
			3	*S. warneri*
			2	*Staphylococcus* spp.
			1	*M. varians/roseus*
			3	*Micrococcus* spp.
Hemerocallis	2	*P. paucimobilis*	29	*L. plantarum*
	1	*Rhanella aquatilis*	24	Coryneforms
			11	*S. epidermidis*
			6	*E. cloacae*
			4	*M. kristinae*
			2	*Staphylococcus* spp.
			1	*B. subtilis*
Hosta	1	*Erwinia/E. agglomerans*	7	*Erwina/E. agglomerans*
	2	*P. fluorescens*	4	*M. kristinae*
	1	*P. paucimobilis*	1	*M. varians/roseus*
			11	*Micrococcus* spp.
			6	*S. epidermidis*
			3	*A. calcoaceticus*
			3	*S. warneri* or *S. capitis*
			1	*S. intermedius/epidermidis*
			2	*P. cepacia* or *P. luteola*
			1	*P. paucimobilis*
			1	*Flavobacterium* spp.
Iris	7	*Erwinia carotovora*	—	—
	2	*E. cloacae*		
	1	*R. aquatilis*		
	1	*Alcaligenes denitrificans*		
	1	*Serratia plymuthica*		
	1	*P. fluorescens*		
Paeony	5	*Agrobacterium radiobacter*	2	*P. maltophilia*
			2	*S. capitis*
			1	*S. warneri*
			1	*Staphylococcus* spp.
			1	*Micrococcus* spp.
			1	*P. diminuta*
Pulmonaria	—	—	4	*B. pumilus*
	—	—	3	*Enterobacter* spp.
			3	*B. subtilis*
Thalictrum	—	—		
Laboratory 2				
Gerbera	7	*P. fluorescens/putida*	—	—
	2	*A. calcoaceticus*		

(*continued on next page*)

Table 3. Bacterial contaminants isolated from micropropagated plants.

Plant species	Time for which plant material was cultured *in vitro*: 1 month (explants)		>12 month (shoots/calli)	
	No. of strains	Identification	No. of strains	Identification
	2	*Erwinia/E. agglomerans*		
	2	*Agrobacterium radiobacter*		
	1	*Flavobacterium* spp.		
Viola	1	*P. fluorescens*	8	*B. subtilis*
	1	*Erwinia/E. agglomerans*	6	*Bacillus* spp.
	1	*Agrobacterium radiobacter*		
	1	*Flavobacterium* spp.		
Primula	—	—	6	*B. circulans*
			2	*B. pumilus*
Total	58		240	

12 different plant species, 75% were Gram-positive and only 25% Gram-negative (Table 3). Sixty five percent of the bacterial strains were non-motile (Leifert *et al.* 1989a; Leifert 1990) and more than half of the bacteria isolated were *Staphylococcus, Micrococcus*, or *Lactobacillus* species (Table 3), which are usually inhabitants of the skin or other tissues of humans and other mammals (Kloos & Schleifer 1986; Kocur 1986). This clearly indicates that a large proportion of contaminants found in old plant tissue cultures has been introduced due to the poor aseptic technique of operators in the laboratory. Other genera found included *Bacillus, Corynebacterium, Acinetobacter, Pseudomonas*, Enterobacteriaceae and *Acinetobacter* species. Of the 35 different bacterial species isolated only four (*Pseudomonas paucimobilis, Enterobacter agglomerans/Erwinia, Acinetobacter calcoaceticus* and *Bacillus subtilis*) were found on both stock plant cuttings and 12 month old *in vitro* cultures (Table 3) indicating that:

(1) detection of bacteria on stock plant cuttings is inefficient; and/or

(2) sources in the laboratory are responsible for contamination of old cultures.

Many bacteria found in old plant tissue cultures have been shown not to produce symptoms on the plant or visible growth on the propagation medium *in vitro* and these are usually described as being 'latent', 'internal', 'endophytic' or 'endogenous' (Bastiaens 1983; Fisse *et al.* 1987; Leifert & Waites 1990a; Cassells 1991). In this review we will use the term 'latent' to describe these organisms, since 'endogenous', 'endophytic' or 'internal' imply that these bacteria inhabit internal plant tissues while no investigations describing the exact location of bacterial contaminants in plant tissue cultures are available. Many latent bacteria have been shown to require additional nutrients for growth on plant-tissue culture media in the absence of plants (Trick & Lingens 1985; C. Leifert, W.M. Waites and H. Camotta, unpublished work). In the presence of plants the growth of these bacteria is still limited and extremely dependent on the changes in the medium induced by the plant (Leifert 1990). Such bacteria are not detected by visual assessment of plant cultures and are propagated together with the plant material. Multiplication rates of plant cultures contaminated with such bacteria were, however, shown to decrease (Long *et al.* 1988; Leifert 1990). Certain latent bacteria such as *Lactobacillus plantarum* have been also found to kill plants after many subcultures when a sufficiently high level of contamination has been reached (Leifert *et al.* 1989b). For most latent bacterial contaminants competition for the mineral and carbohydrate nutrients in the medium is less likely to be an important reason for reduction in plant growth. Bacterial contaminants were, however,

shown to reduce plant growth and/or to kill plants by producing phytotoxic metabolites such as lactic acid (Leifert *et al.* 1989b), and bacterial metabolites such as organic acids, antibiotics, plant-growth regulators and/or cyanide have been linked to reductions of plant growth *in vivo* (Lynch 1976, 1977 & 1978; Bakker & Schippers 1987; see Schippers *et al.* 1987 for a recent review).

Some plant-pathogenic bacteria, including *Agrobacterium tumefaciens, Corynebacterium sepedonicum, Erwinia carotovora, Pseudomonas syringae, Xanthomonas campestris* and *Xanthomonas pellargonii*, also stay latent on certain plant species and do not induce symptoms on host plants *in vitro* (Cassells *et al.* 1988; Deimling & Möllers 1988; Cooke *et al.* 1991). Successfully micropropagated plants infected with these latent plant-pathogenic bacteria can result in the spread of diseases, if plants are distributed subsequent to weaning (Cooke *et al.* 1991).

Bacterial communities found on diffferent plant species were also found to differ considerably when 12-month-old cultures were examined: for example *Lactobacillus plantarum* and coryneforms were repeatedly and exclusively isolated from different *Hemerocallis* species whereas pseudomonads were the predominant contaminants in *Delphinium*, suggesting that different plant species have different bacterial floras *in vitro* (Table 3). This view is further supported by the finding that plant species such as *Hemerocallis* have resistance mechanisms *in vitro* which prevent a range of bacterial contaminants from growing and persisting in their cultures (Leifert 1990; C. Leifert, W.M. Waites and H. Camotta, unpublished work).

Different bacterial communities were also isolated from 12-month-old *in vitro* cultures in the two laboratories investigated; only *Bacillus* spp. were isolated in one laboratory, whereas *Bacillus* spp. accounted for as little as 10% of bacterial contamination in the other laboratory (Table 3), which indicates that the problems found in different laboratories are due to different organisms and/or sources. Distinct approaches for prevention or treatment of contamination might therefore be needed in different laboratories.

Fungal and Yeast Contaminants

Yeasts have been described as contaminants in plant-tissue cultures and the identification results are summarized in Table 4. The most frequently isolated yeast species are *Rhodotorula* (red yeasts) and *Candida* species. They belong to the group of osmophilic yeasts which show a tolerance to high concentrations of sugar and salt (Tilbury 1980) and hence are well adapted to grow in plant-growth media (Leifert *et al.* 1990).

Although fungi are repeatedly found in plant tissue cultures very few have been described. Fungi isolated from plant-tissue cultures include *Fusarium, Neurospora, Aspergillus, Microsporium, Cladosporium*, and *Philalophora*, and rapid increases in fungal contamination in some laboratories were found to be caused by mite and thrip vectors (Blake 1988; Enjalric *et al.* 1988; J. Ritchie, J.R. Nicholas & C. Leifert, unpublished work).

Most fungi and yeast contaminants grow well on plant media even in the absence of plant material (Enjalric *et al.* 1988; Leifert 1990; Leifert *et al.* 1990). By reducing the medium pH to below 3, by metabolizing much of the carbohydrate in the medium and by producing phytotoxic fermentation products such as ethanol and acetic acid, *Candida* and *Rhodotorula* yeasts can create an extremely unfavourable environment for plant growth (Leifert *et al.* 1990). Yeast contamination usually results in plant death within 1 to 3 subcultures after introduction into plant-tissue cultures (Leifert & Waites 1990a,b).

Mite and Thrip Contaminants

Few mites and thrips found in plant tissue cultures have been identified (Table 5). Many mites and thrips, such as *Sideroptes graminis, Stemeotarfomemus palidus* or *Thrips tabaci*, were found to be species which are known to be plant inhabitants

Table 4. Yeast species described as contaminants in plant tissue and cell cultures.

Yeast genera/species	Number of isolates	Plant species	Reference
*Candida albicans**	4	*Astilbe, Hosta*	3
*Candida famata**	1	*Hosta*	3
*Candida guilliermondii**	16	*Astilbe, Doronicum, Hemerocallis, Hosta*	3
*Candida parapsilosis**	4	*Doronicum, Hosta, Hemerocallis*	3
*Candida pelliculosa**	3	*Delphinium, Hemerocallis*	3
Candida tropicalis†	ND‡	*Hevea*	2
*Cryptoc. laurentii**	1	*Hemerocallis*	3
*Rhodotorula glutinis**	3	*Astilbe, Rose*	3
*Rhodotorula minuta**	1	*Delphinium*	3
*Rhodotorula rubra**	3	*Astilbe, Hosta*	3
*Torulopsis glabrata**	ND	ND	1

Cryptoc. = *Cryptococcus.*
ND: not described.
* Isolated from plant material which had been *in vitro* for longer than 12 months.
† Isolated from infected stock plant cuttings.
‡ 5% of all contaminants found.
1 Boxus & Terzi 1987.
2 Enjalric *et al.* 1988.
3 Leifert *et al.* 1990.

and/or pests *in vivo* (Scopes 1979; Fennemore 1984) while others, such as *Tyrophagus putrescentiae*, are more ubiquitous. It is interesting to note that common house-dust mites have so far not been described as a contaminant in plant-tissue cultures (Blake 1988).

Detection of Contaminants

The ability of deleterious contaminants (especially bacteria) to stay latent for long periods when introduced into plant-tissue cultures and the finding that certain

Table 5. Mite and thrip contaminants found in plant tissue cultures.

Genus/species	Common name	Plant species found on *In vitro*	vector for:	References
Mites				
Stemeotarfomemus palidus†	Strawberry of Cyclamen mite	*Geranium*	—*	3
Sideroptes‡ *graminum* (= *avenae*)	—	various	*Fusarium poe*	1, 3,
Sideroptes spp.	—	various	Fungi	1, 3,
Tyrophagus putrescentiae	—	various	Fungi	1
Thrips				
Thrips tabaci †	—	various	ND	1
Allothrips spp.	—	*Simmondsia*	ND	2

* Plants were visibly uncontaminated with other microorganisms.
† Feeds on plants.
‡ Feeds on fungus.
ND: not described.
1 Blake 1988.
2 Klocke & Myers 1984.
3 Ritchie J. unpublished.

bacteria, pathogenic to plants *in vivo*, can stay latent *in vitro* must be considered as the biggest threat to plant-tissue culture. Early detection of latent contaminants is, therefore, essential in preventing losses due to such contaminants and the spread of plant pathogens through micropropagation.

Detection of Bacterial Contaminants

Various 'sterility test' or 'indexing' methods have been developed to detect latent bacterial contaminants on stock explants and plant material growing *in vitro*.

(1) Knauss (1976) transferred sections cut from the base of sterilized shoot-tip explants into two different liquid sterility-test media and assessed the turbidity developing in the medium after incubation for 3 weeks at 26°C as an indication of contamination.

(2) Cassells (1986) plated sap from surface-sterilized *Pelargonium* on to five different solidified sterility-test media and assessed plates for growth of contaminants, but did not describe incubation time or temperature.

(3) Menard *et al.* (1985) and Boxus & Terzi (1987) detected contamination by subculturing plants onto plant growth medium containing additional nutrients such as peptone and yeast extracts.

Transfer of plant material or plant-growth medium to liquid or solidified sterility-test or indexing media has also been described by other authors (Cassells 1986; Debergh & Vanderschaeghe 1988; Fisse *et al.* 1987; Reuther 1983; Leifert 1990; Cooke *et al.* 1991). The media described are usually based on meat or plant extracts and are similar to those used as general bacterial, yeast or fungal-growth media or for sterility testing in other environments (Anonymous 1978 & 1980; Mersch-Sundermann 1989). Since no studies have been published which describe the minimal inoculum needed for growth and the growth characteristics of contaminants on different media, it is difficult to describe the effectiveness and limitations of these indexing methods. In our laboratory we have found that most bacteria tested (*Bacillus, Clavibacter, Micrococcus, Staphylococcus, Acinetobacter, Agrobacterium, Enterobacter/Erwinia, Pseudomonas*, and *Xanthomonas* species) grow well on a variety of different indexing media even when the inoculum is as low as 10^1 to 10^2 c.f.u./10 ml of indexing medium. The extent of growth, however, varied greatly between different bacterial species. Certain fastidious bacteria, such as *Lactobacillus plantarum*, did not grow on nutrient agar, although they were able to grow on more complex sterility-test media (Leifert 1990). In comparison, *Hyphomicrobium* spp. have been shown not to grow or only to grow poorly on most complex media, although they grow on media containing mineral nutrients and one or two carbon compounds as energy and carbon sources (Horsch & King 1983).

The concentration of nutrients in indexing medium and the amount of plant material transferred to it were also found to affect the reliability of the indexing test (C. Leifert and D.L. Cooke, unpublished work). Plants are known to produce antibacterial substances (Reuther 1985; Deans & Svoboda 1988a,b; for a review see Darvill & Albersheim 1984) and the production of such substances by plant material inoculated into indexing medium might stop or reduce the growth of contaminants and therefore reduce the reliability of the indexing test (Cassells 1986).

Bacteria such as the plant-pathogenic 'bacteria of uncertain affiliation', which include *Rickettsia, Actinomycete, Spiroplasma*, and *Mycoplasma*-like organisms are known not to grow on common bacteriological media (Schaad 1980) and are therefore unlikely to be detected by the indexing methods described above. Some *Spiroplasma* and *Rickettsia*-like bacteria grow on media

containing serum (see Davis 1980 and Hopkins 1980 for a description of the media used) and such media have also been recommended as general indexing media (Debergh & Vanderschaeghe 1988). Other bacteria of uncertain affiliation cannot be cultured *in vitro* and are only detectable by using electron microscopy, DNA/RNA fluorochrome staining and serological and genetic methods (see Schaad 1980, Bové 1988 and Möllers & Sarkar 1989 for a detailed description of the methods used).

Serological and genetic detection methods can also be used to detect specific contaminants in micropropagated plant cultures and *Xanthomonas* specific Elisa tests and cDNA probes have been used to detect *Xanthomonas pelargonii* in *Pelargonium* cultures (Cassells *et al.* 1988, Reuther 1988).

Detection of Fungi and Yeasts

Most fungi and yeasts grow well on plant-tissue culture media and can be detected by visual assessment of cultures (Cassells 1990; Leifert 1990). Yeasts, such as *Candida* and *Rhodotorula*, found as contaminants in plant-tissue cultures, also grow on the sterility-test or indexing media used to detect latent bacteria (Leifert 1990).

Detection of Thrips and Mites

Thrips and mites are not usually visible to the naked eye and are therefore only detectable by microscopic examination of plant media, culture vessels and plant tissue. Migrating mites and thrips can act as vectors for other contaminants, especially fungi. Sudden increases in fungal and/or yeast contamination in certain crops and/or patches of fungal contamination in the growthroom can, therefore, indicate mite and/or thrip infestation (Debergh & Maene 1984; Blake 1988; J.R. Nicholas, C. Leifert & J. Ritchie, unpublished work).

Sources of Contamination and Methods for Preventing and Treating Contamination

This section summarizes the work carried out to determine the sources of the different contaminants isolated and identified from plant tissue cultures. It also describes methods for prevention and/or treatment of contamination.

Explants Taken From In Vivo *Plants*

If the initial surface sterilization is inefficient, fungi, yeasts and bacteria can be introduced into *in vitro* cultures with the plant material (for example see Cornu & Michel 1987; Enjalric *et al.* 1988). High numbers of bacterial and fungal saprophytes on plant tissues taken as explants will reduce the success of the initial sterilization. Explants which have been taken from: (1) plant tissues exposed to or near to soil; (2) plants grown in tropical climates in the field; or (3) overhead irrigated stock plants, are more difficult, and sometimes impossible, to sterilize (Knauss & Miller 1978; Duhem *et al.* 1988; Enjalric *et al.* 1988; De Fossard 1990; Leifert 1990). Growing stock plants under glass in a dry atmosphere without overhead irrigation was, however, found to improve the success rate of sterilization (Enjalric *et al.* 1988; Leifert 1990).

Most laboratories use sodium or calcium hypochlorite or various commercial bleaches routinely for surface sterilization (more exactly 'disinfection') of explants (Abdul-Baki 1974; Mathias *et al.* 1987; Wainwright & England 1987; De Fossard 1990; for a review see George & Sherrington 1984). The active chemical in household bleach is hypochlorous acid (HOCl) which is a strong oxidizing agent. Since the undissociated form of hypochlorous acid has a 100-fold higher anti-

microbial activity than the dissociated hypochlorite ion (OCl^+) Brazis *et al.* 1958), the antimicrobial activity of hypochlorite solution is dependent on the pH. Different commercially available bleaches include differing concentrations of sodium hypochlorite and the content of hypochlorous acid decreases with time, particularly at low pH and high temperature (Hoffman *et al.* 1981). It is usual, therefore, for bleach to be stored in the refrigerator for only a limited time and for bleach from different manufacturers to be tested for their active chlorine content (see Hoffman *et al.* 1981 for the test method).

Other chemicals such as antibiotics (see Table 6), mercuric chloride, alcohols and fungicides have also been used for surface treatment of explants. Concentrations and exposure times for the different chemicals depend greatly on the type and size of the explant and can be found in the appropriate literature (for example, Mathias *et al.* 1987; Wainwright & England 1987; Wilson & Power 1989; De Fossard 1990; Leifert 1990). Although most published work (for example, Mathias *et al.* 1987) suggests that the initial treatment of explants sterilizes all the plant tissue, only outer tissues of the explant, which come in contact with the chemicals, are disinfected. It is apparent, therefore, that a successful treatment is only possible if the internal tissues which are not reached by the chemical are free of contaminants.

The sudden appearance of visible growth of bacterial contamination at later *in vitro* stages (after many subcultures or during rooting) has often been attributed to contaminants which had been introduced with the initial plant material. The apparent lag period between introduction and appearance of visible growth of the contaminants has been explained by the presence of latent bacteria, which need either to adapt to the *in vitro* environment or require a change in the *in vitro* environment for growth (for example, transfer to rooting media and/or a change in growth-room temperature) (Bastiaens 1983; Cassells 1986; Cornu & Michel 1987; Fisse *et al*, 1987). Losses due to latent bacteria introduced with the initial explant can be avoided by regular indexing of cultures during the initial stages of plant-tissue culture and discarding infected plants.

If cultures cannot be discarded (for example if no clean plant material is available or if the contaminant is only detected after large quantities of plants have been produced), it may be possible to eliminate the contaminant by incorporating antibiotics into the plant-growth medium. Various authors have described the antibiotic sensitivity of bacteria isolated from plant-tissue cultures (for example Cornu & Michel 1987; Leggatt *et al.* 1988; Poulsen 1988) or have included antibiotics in the plant-growth medium to suppress or eliminate bacterial contaminants (Phillips *et al.* 1981; Cornu & Michel 1987; Fisse *et al.* 1987; Mathias *et al.* 1987; Podwyzynska & Hempel 1987). Many of these workers reported bactericidal or bacteriostatic effects of their antibiotic treatments on contaminants (Table 6), but were subsequently criticized for not assessing plant cultures for long enough to make certain of the success of their treatments (Debergh & Vanderschaeghe 1988). In addition, in many of these studies identification and/or sensitivity testing of the bacterial contaminants did not precede incorporation of antibiotics into tissue culture medium, while some authors did not describe the concentrations of antibiotics used. Because of these limitations, many studies cannot be repeated by other workers and are therefore of little use.

Other authors have reported that certain antibiotic treatments had no effect on contamination (for example, Phillips *et al.* 1981; Bastiaens *et al.* 1983; Horsch & King 1983; Reustle *et al.* 1988; Leifert *et al.* 1991a). In addition, many antibiotics have been found to be phytotoxic to plants *in vivo* and *in vitro* and can therefore only be incorporated into plant-growth media for limited periods of time (Brian 1957; Owens 1979; Thurston *et al.* 1979; Bastiaens *et al.* 1983; Pollock *et al.* 1983; Horsch & King 1983; Cornu & Michel 1987; Fisse *et al.* 1988; Mathews 1988; Falkiner 1990; Leifert *et al.* 1991a,b). However, a range of different bacteria (*Lactobacillus*

Table 6. Antibiotics used to suppress or eliminate contaminants from plant tissue cultures.

Antibiotics used	Conc. (mg/l)	Length of exposure	Contaminants identified as	Success of treatment (length of time bacteria were not detected after treatment)	Plant species	Reference
Individual antibiotic treatments						
Cephaloridine	ns	30 d	ns	(+)(ns)	*Syngonium*	2
Streptomycin	100–400	30 d	ns	+ (ns)*	*Syngonium, Ficus Philodendron*	2
Streptomycin	10–50	ns	*P. fluorescens*	(+)(44 h)	*Helianthus*	8
Rifampicin	ns	ns	ns	(+)(ns)	*Gerbera*	6
Rifampicin	10–100	2–14 d	ns	+ (14 d)	*Nauclea*	7
Rifampicin	10–50	ns	*P. fluorescens*	+ (44 h)	*Helianthus*	8
Rifampicin	10–50	ns	ns	+ (44 h)	*Helianthus*	8
Doxycycline	ns	ns	ns	(+)(ns)	*Gerbera*	6
Polymyxin	25–50	2–14 d	ns	(+)(14 d)	*Nauclea*	7
Chloramphenicol	10, 50	ns	ns	(+)(44 h)	*Helianthus*	8
Chloramphenicol	25–50	72 d	*L. plantarum*	(+)(72 d)*	*Hemerocallis*	5
Phosphomycin	10–50	ns	*P. fluorescens*	(+)(44 h)	*Helianthus*	8
Phosphomycin	10–50	ns	ns	(+)(44 h)	*Helianthus*	8
Combinations of antibiotics						
Streptomycin	100	1–3	*Hyphomicrobium*	+ (2 years)	*Datura*	3
+ Carbenicillin	100	subcultures				
Chlortetracycline	100	7d	*Pseudomonas* spp.	+ (1 year)*	*Prunus*	1
+ Gentomycine	25					
+ Colistin	25					
Streptomycin	50–100	72d	*L. plantarum*	+ (2 years)	*Hemerocallis*	5, 6
+ Carbenicillin	50–100		*S. saprophyticus*	+ (6 months)	*Choysia*	5, 6
+ Rifampicin	50–100					
Gentomycin	200	72d	*Corynebacterium*	+ (2years)	*Hemerocallis*	5, 6
+ Carbenicillin	200					
+ Rifampicin	100					
Gentomycin	50	72d	*P. paucimobilis*	+ (1 year)	*Delphinium*	6
+ Carbenicillin	200					
+ Rifampicin	100					
Penicillin	10000 units	ns	ns	+ (ns)	*Solanum*	4
+ Streptomycin	10					
+ Amphotericin	25					
Erythromycin	16	14d	ns	+ (11d)	*Hevea*	10
+ Nystatin	8					
+ Streptomycin	8					

ns: not specified.
d: days.
h: hours.
* Treatment found to be very phytotoxic.
† Plant cell cultures.
— no suppression or elimination.
(+) bacteriostatic effect.
+ bacteriocidal effect (bacterium eliminated).

1 Cornu & Michel 1987.
2 Fisse *et al.* 1987.
3 Horsch & King 1983.
4 Gilbert *et al.* 1990.
5 Leifert 1990.
6 Leifert *et al.* 1991.
7 Mathias *et al.* 1987.
8 Phillips *et al.* 1981.
9 Podwyszynska & Hempel 1987.
10 Wilson & Power 1989.

plantarum, *Staphylococcus saprophyticus*, a *Corynebacterium* species, *Pseudomonas paucimobilis* and *Hyphomicrobium*) were eliminated from contaminated plant-tissue cultures by incorporating combinations of antibiotics involving an aminoglycoside, a penicillin and/or cephalosporin and rifampicin or polymyxin into the growth medium, and the cultures stayed free of the contaminant when assessed for up to 2 years after antibiotic treatment (Horsch & King 1983; Leifert *et al.* 1991a). Plant tissue or cell cultures which became free of the contaminant usually showed an increase in multiplication rate of between 50 and 300%, but treatment with antibiotics often resulted in only a proportion (between 98 and 20%) of the plants becoming free of the contaminant. Putting plants individually on to fresh media after antibiotic treatment was, therefore, found to be essential to separate non-contaminated from contaminated plants (Leifert *et al.* 1991a).

Because the success of antibiotic treatments can only be predicted reliably after bacteria isolated from contaminated plant cultures have been identified and sensitivity tested, and because of the phytotoxicity and high cost of the treatment, antibiotics should only be used to eliminate specific identified contaminants from valuable plant stocks. It is most important that the prophylactic use of antibiotics and treatment of mixed populations of micro-organisms on stock plants and/or established plant cultures should be avoided since it is unlikely to be successful and may lead to the development of resistant strains of micro-organisms.

Mites and Thrips as Contaminants and Vectors

Mites and thrips are also likely to be introduced with the initial plant material, since many found in plant-tissue cultures are known to be plant inhabitants and/or plant pests *in vivo* and some are restricted to specific plant species. To avoid their introduction into the growth rooms, new cultures should be kept in quarantine and regularly examined for contamination. All infected cultures should be discarded.

Mites and thrips are also easily transported by dust, insects, and by humans (Blake 1988). Laboratory hygiene and physical barriers between plant-tissue culture laboratories and the glasshouses used for weaning plants are therefore essential to prevent mite or thrip infestation of laboratories and growth rooms. The use of acaricides for cleaning work surfaces, fumigating growth rooms and as an additive to plant-tissue culture medium has been reported as a method to control mites in plant tissue (Klocke & Myers 1984) and fungal cultures which are also frequently infested with mites (Onions 1990). Many mites and thrips are, however, resistant to a wide range of acaricides and insecticides (Scopes 1979; Fennemore 1984) and many insecticides/acaricides, such as endosulphane, which are active against mites and thrips, are highly phytotoxic when present in plant tissue culture medium (J. Ritchie, & C. Leifert, unpublished work).

Fungicides can be incorporated into the medium to suppress fungi introduced by mites but have been found to reduce growth rates and/or rooting of plants (J.R. Nicholas & C. Leifert unpublished work).

Aseptic Handling of Plant Material

Identification of bacteria found in plant cultures, which had been in culture for longer than 12 months, showed that at least 50% of those bacteria found after many subcultures were likely to have originated from sources within the laboratory. The introduction of fungal, yeast and bacterial contaminants during handling of clean plant cultures has been described by various authors (Boxus & Terzi 1987, 1988; Kunneman and Faaij-Groenen 1988; Leifert *et al.* 1989a; De Fossard 1990) and introduction of contaminants at a rate of between 5 and 15% per subculture have been reported (Leifert 1990).

Infection with bacteria of the genus *Bacillus* (which form alcohol and heat-resistant endospores: Claus & Berkeley 1986; Sneath 1986), due to inefficient

sterilization of instruments used for handling plants, has been reported by various authors. Spread of bacteria which are 'resistant to instrument flaming and able to survive in alcohol for a few hours' from infected to non-infected plant culture via the alcohol used to sterilize instruments has been reported by Boxus & Terzi (1987). Kunneman & Faaij-Groenen (1988) described the survival of alcohol-resistant contaminants in Dettol and sodium hypochlorite solutions. *Bacillus* strains, isolated from plant-tissue cultures and tentatively identified as *Bacillus macerans*, survived alcohol dips (75 and 95% for up to 5 min) and flaming with an alcohol burner (16 s) on contaminated forceps, and were only killed by flaming over a Bunsen burner (12 s) (Singha *et al.* 1987). Survival of *Bacillus pumilus* on flamed forceps was reported by Constantine *et al.* (1980), and *Bacillus circulans* strains isolated from alcohol used as a dip prior, and subsequent, to flaming of instruments were found to persist in the alcohol for more than 1 week (Leifert 1990). Sterilization of instruments in a hot flame (Bunsen burner or hot-bead sterilizers, AGROGEN PROMOTION, PO Box 21, 1701 Freiburg CH.) and regular autoclaving of instruments is therefore essential to prevent the spread of this type of contaminant. *Bacillus* spp. were found to be sensitive to a wide range of antibiotics and can therefore be suppressed by incorporating antibiotics into the medium (Leifert 1991a). However, due to their ability to form dormant spores, which are not killed by antibiotics, it is unlikely that they can be totally eliminated from cultures by antibiotics.

The presence of bacteria from the genera *Staphylococcus* and *Micrococcus* and the yeast *Candida albicans* clearly indicates inefficient aseptic technique of operators as the source of contamination (Leggatt *et al.* 1988; Leifert *et al.* 1989a, 1990), since these contaminants are known to be obligate inhabitants of humans and other animals (Hurley 1980; Kloos & Schleifer 1986; Kocur 1986). The number of contaminants introduced from human skin can be reduced by using surgical soap or scrubs, by wearing gloves and by protective clothing.

The laboratory environment (especially the cleanliness of floors, work surface inside the laminar flow cabinets and the laboratory air) has also been shown to affect the rate of contamination with fungi, yeasts and bacteria in plant-tissue culture laboratories (see De Fossard 1990 and Leifert 1990). Repeated indexing of all cultures and discarding plants found to be contaminated has been suggested, but is very time-consuming and expensive. Detailed training of operators and improvements in laboratory design have, however, been shown greatly to reduce the overall contamination rate (Leifert 1990). Disinfection of cultures which have been recontaminated in the laboratory (due to poor conditions or aseptic technique) is extremely difficult because of the mixture of different organisms (fungi, yeasts and bacteria) introduced. Incorporation of different antibiotics and/or fungicide combinations is either unlikely to eliminate all contaminants or may be phytotoxic. Disinfection of plants with the same chemicals used to disinfect stock plant cuttings has been described in the literature (see for example De Fossard 1990) but is very time-consuming. Using chemicals such as hypochlorite on the tissue of established plant cultures is also likely to be more phytotoxic than using the same treatment on stock plant cuttings.

Sterilization and Preparation of Media

Heat-resistant *Baccilus* species have been reported to survive autoclaving of growth media. Autoclaving for 20 min at 110°C was reported to allow the survival of 'a more thermostable germ', which could be killed by doubling the autoclave time (Boxus & Terzi 1987). Trick & Lingens (1985) reported that *Bacillus cereus* and *Bacillus circulans* spores can survive autoclaving for 20 min at 120°C. When the survival of spores of the thermophile, *Bacillus stearothermophilus*, was tested during autoclaving (20 min at 120°C), they survived when media were autoclaved in large plastic (15 × 10 × 7.5 cm) culture vessels (Leifert 1990).

Sterility testing will show inefficient aseptic technique during media preparation. For example, poor technique during pouring into γ-irradiated plastic containers subsequent to autoclaving and media preparation was identified as a major source of contamination with *Candida guilliermondii* and *Candida parapsilosis* (Leifert *et al.* 1990). Other micro-organisms introduced during pouring of media included *Staphylococcus* and *Micrococcus* species. Improvements in the aseptic technique of operators and reduction in micro-organisms in the laboratory environment have been shown to result in decreased contamination rates during media pouring (Leifert 1990).

Conclusions

Most of the problems caused by contamination of plant-tissue and cell cultures are due to the use of inefficient methods for:

(1) sterilizing explants taken from *in vivo* plants;

(2) detecting contaminants in *in vitro* plant cultures;

(3) aseptic handling of plant material; or

(4) the sterilization of culture vessels, instruments and media.

Determining the source of contamination and the relative importance of different sources can yield vital information about methods of prevention of contamination. In this review, the identification of contaminants has been shown to provide valuable information about the sources of contamination. Identification of contaminants is also essential when plant cultures infected with latent contaminants are propagated and distributed in order to ensure that the organisms are not pathogenic to the plant *in vivo*. If contaminants were isolated and identified on a regular basis and different laboratories published identification methods and test results, together with information on methods of aseptic technique and media preparation, a list of 'indicator bacteria' for the different sources of contamination would be developed. Such regular isolation could also be the basis for successful production control in commercial tissue-culture laboratories.

The variety of contaminants found on plants, the ability of some contaminants to stay latent *in vitro* and the relative unreliability of the detection methods available suggest that screening for contaminants should, therefore, be carried out at all stages of micropropagation as follows:

(1) Stock plants should be examined for symptoms and tested for the presence of plant pests (especially mites and thrips, since they are difficult to detect when present in small numbers on sterilized explants) and known diseases of the plant species (especially viruses and plant-pathogenic bacteria because of their ability to stay latent *in vitro*). Infected stock plants should only be used if no other plant material is available (see for example Agrios 1978, Hoffmann & Schmutterer 1983, and Smith *et al.* 1986 for symptoms and the methods used to detect and identify diseases and pests on plants).

(2) After disinfection, stock-plant cuttings used to initiate plant-tissue and cell cultures should be tested for the presence of viruses and non-culturable micro-organisms (see Hill 1984, and Cassells 1991 for the methods used) and indexed for contaminating micro-organisms (see above for the methods used) and all infected plant material discarded.

(3) After initiation of growth, cultures should be repeatedly tested for the presence of viruses and non-culturable micro-organisms and indexed to detect latent bacteria. Indexing should be carried out with different indexing media, because high numbers of contaminants are needed for detection by some media and each indexing medium detects only a certain range of organisms. For the first 4 to 8 months, new cultures should also be physically separated (for

example by incubation in a separate growth cabinet or growth room) from already established plant cultures and regularly examined for mites and thrips.

(4) All stock cultures of plants should be examined for mites and thrips, tested for the presence of viruses and non-culturable micro-organisms and indexed to detect latent bacteria at regular intervals.

(5) After weaning (transferring back to soil) plants should again be assessed for disease symptoms and tested for known pathogens of the plant species.

Although 'endogenous' or 'internal' bacteria have often been described as the reason for failure to grow certain plant species in tissue culture, very little is known about the source of such bacteria and of their location in the plant. Detailed investigations of bacterial contaminants of internal and external plant tissues are, therefore, needed to evaluate whether contaminants are 'endogenous', to understand their importance and to improve the methods used to detect and eradicate them.

References

ABDUL-BAKI, A.A. Hypochlorite and tissue sterilisation. *Planta* **115**, 373–376.

AGRIOS, G.N. 1978 *Plant pathology*. New York/London: Academic Press.

ANONYMOUS 1978 *Standard methods for the examination of Dairy Products*, 14th ed. Washington, DC: American Public Health Association Inc.

ANONYMOUS 1980 *Standard methods for the examination of water and waste water*, 15th ed. Washington, DC: American Public Health Association Inc.

BAKKER, A.W. & SCHIPPERS, B. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp.—mediated plant growth stimulation. *Soil Biology and Biochemistry* **19**, 451–457.

BASTIAENS, L. 1983 Endogenous bacteria in plants and their implications in tissue culture—a review. *Mededelingen van de Faculteit Landbouwettenschappen Rijksuniversiteit Gent* **48**, 1–11.

BASTIAENS, L., MAENE, L., HARBAOUI, Y., VAN SUMERE, C., VAN DE CASTEELE, K.L. & DEBERGH, P.C. 1983 The influence of antibacterial products on plant tissue cultures. *Mededelingen van de Faculteit Landbouwettenschappen Rijksuniversiteit Gent* **48**, 13–24.

BLAKE, J. 1988 Mites and thrips as bacterial and fungal vectors between plant tissue cultures. *Acta Horticulturae* **225**, 163–166.

BOVÉ, J.M. 1988 Plant mollicutes: phloem-restricted agents and surface contaminants. *Acta Horticulturae* **225**, 215–223.

BOXUS, PH. & TERZI, J.M. 1987 Big losses due to bacterial contamination can be avoided in mass propagation scheme. *Acta Horticulturae* **212**, 91–93.

BOXUS, PH. & TERZI, J.M. 1988 Control of accidental contamination during mass propagation. *Acta Horticulturae* **225**, 189–193.

BRAZIS, A.R., LESLIE, J.E., KABLER, P.W. & WOODWARD, B.L. 1958 The inactivation of spores of *Bacillus globigii* and *Bacillus anthracis* by free available chlorine. *Applied Microbiology* **6**, 338–342.

BRIAN, P.W., 1957 Effects of antibiotics on plants. *Annual Review of Plant Pathology* **8**, 413–426.

CASSELLS, A.C. 1983 Chemical control of virus diseases of plants. *Progress in Medical Chemistry* **21**, 119–155.

CASSELLS, A.C. 1986 Production of healthy plants. In *Proceedings of the Institute of Horticulture Symposium: Micropropagation in Horticulture*, ed. Alderson, P.G. & Dullforce, W.M. pp. 53–71. Nottingham: University of Nottingham Trent Print Unit.

CASSELLS, A.C., HARMEY, M.A., CARNEY, B.F., MCCARTHY, E. & MCHUGH, A. 1988 Problems posed by cultivable bacterial endophytes in the establishment of axenic cultures of *Pelargonium domesticum*: the use of *Xanthomonas pelargonii*-specific ELISA, DNA probes and culture indexing in the screening of antibiotic treated and untreated donor plants. *Acta Horticulturae* **225**, 153–161.

CASSELLS, A.C. 1990 Problems in tissue culture: culture contamination. In *Micropropagation*, ed. Debergh P. & Zimmerman R.H. (in press). Dordrecht: Kluwer Academic Publishers.

CASSELLS, A.C. 1991 Screening for pathogens and contaminating micro-organisms in micropropagation. In *Techniques for Detection and Diagnosis in Plant Pathology*, ed. Duncan J.M. & Torrance L. (in press). London: Butterworth.

CLAUS, D. & BERKELEY, R.C.W. 1986 Description of the Genus *Bacillus*. In *Bergey's Manual of Systematic Bacteriology*, 9th Edition, Vol. 2. ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. pp. 1105–1139. Baltimore/London: Williams and Wilkins.

CONSTANTINE, D.R., WILTSHIRE, S. & BEDDOWS, C. 1980 Contamination of cultures (abstract). *Long Ashton Research Station Reports* **1979**, 74.

COOKE, D.L., LEIFERT, C., WAITES, B., KEETLEY, J.W., NICHOLAS, J.R. & WAITES, W.M. 1991 Effect of plant pathogenic bacteria on micropropagated *Aster, Cherianthus, Delphinium Iris* and *Rose. Plant Cell Tissue and Organ Culture* (submitted).

CORNU, D. & MICHEL, M.F. 1987 Bacteria contaminants in shoot cultures of *Prunus avium* L. choice and phytotoxicity of antibiotics. *Acta Horticulturae* **212**, 83–86.

DARVILL, A.G. & ALBERSHEIM, P. 1984 Phytoalexins and their elicitors—A defence against microbial infection in plants. *Annual Review of Plant Physiology* **35**, 243–275.

DAVIS, R.E. 1980 Spiroplasma and mycoplasma-like organisms. In *Identification of Plant Pathogenic Bacteria*, ed. Schaad, N.W. pp. 57–66. St Paul: American Phytopathological Society.

DEANS, S.G. & SVOBODA, K.P. 1988a Antibacterial activity of French tarragon (*Artemisia dracunculus* Linn.) essential oil and its constituents during ontogeny. *Journal of Horticultural Science* **63**, 503–508.

DEANS, S.G. & SVOBODA, K.P. 1988b Antibacterial activity of Summer savory (*Satureja hortensis* L.) essential oil and its constituents during ontogeny. *Journal of Horticultural Science* **64**, 205–210.

DEBERGH, P. & MAENE, L. 1984 Pathological and physiological problems related to the *in vitro* culture of plants. *Parasitica* **40**, 69–75.

DEBERGH, P.C. & VANDERSCHAEGHE, A.M. 1988 Some symptoms indicating the presence of bacterial contaminants in plant tissue cultures. *Acta Horticulturae* **225**, 77–83.

DE FOSSARD, R.A. 1990 *Micropropagation.* Queensland: Xarma Pty Ltd.

DEIMLING, G. & MÖLLERS, C. 1988 Aseptic handling of potato material during protoplast isolation and regeneration. *Acta Horticulturae* **225**, 209–215.

DUHEM, K., LE MERCIER, N. & BOXUS, P.H. 1988 Difficulties in the establishment of axenic *in vitro* cultures of field collected coffee and cacao germ plasm. *Acta Horticulturae* **225**, 67–77.

ENJALRIC, F., CARRON, M.P. & LARDET, L. 1988 Contamination of primary cultures in tropical areas: The case of *Hevea brasiliensis*. *Acta Horticulturae* **225**, 57–65.

FALKINER, F.R. 1990 The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. *International Association for Plant Tissue Culture Newsletter* **60**, 13–23.

FENNEMORE, P.G. 1984 *Plant Pests and their Control.* London/Boston: Butterworth.

FISSE, J., BATALLE, A. & PERA, J. 1987 Endogenous bacteria elimination in ornamental plants. *Acta Horticulturae* **212**, 87–90.

GEORGE, E.F. & SHERRINGTON, P.D. 1984 *Plant propagation by tissue culture* pp. 88–93. Eversley, Basingstoke: Exegetics Ltd.

GILBERT, J.E., SHOHET, S., CALIGARI, P.D.S. & DUNWELL, J.M. 1990 Detection and elimination of latent bacteria in potato cultures. In *Abstracts VIIth International Congress on Plant Tissue and Cell culture*, ed. Nijkamp, H.J.J., van der Plas, L.H.W. & van Aartrijk, J. p. 380. Dordrecht: Kluwer Academic Publishers.

GRIERSON, D. & COVEY, S. 1984 *Plant Molecular Biology.* Glasgow/London: Blackie.

HENNERTY, M.J., UPTON, M.E., FURLONG, P.A., HARRIS, D.P., JAMES, D.J. & EATON, R.A. 1988 Microbial contamination of *in vitro* cultures of apple rootstocks M26 and M9. *Acta Horticulturae* **225**, 129–139.

HILL, S.A. 1984 *Methods in Plant Virology.* Oxford/London: Blackwell Scientific Publications.

HOFFMAN, P.N., DEATH, J.E. & COATES, D. 1981 The stability of sodium hypochlorite solutions. In *Disinfectants: Their use and evaluation of effectiveness*, pp. 77–83. London/New York: Academic Press.

HOFFMAN, G.M. & SCHMUTTERER, H. 1983 *Parasitäre Krankheiten und Schädlinge an landwirtschaftlichen Kulturpflanzen.* Stuttgart: Eugen Ulmer Verlag.

HOPKINS, D.L. 1980 Rickettsia-like Bacteria (RLB). In *Identification of Plant Pathogenic Bacteria*, ed. Schaad, N.W. pp. 55–56. St. Paul: American Phytopathological Society.

HORSCH, R.B. & KING, J. 1983 A covert contaminant of cultured plant cells: elimination of a *Hyphomicrobium* spp. from cultures of *Datura innoxia* (Mill.). *Plant Cell Tissue Organ Culture* **2**, 21–28.

HURLEY, R. 1980 The pathogenic *Candida* species and diseases caused by Candidas in man. In *Biology and Activities of Yeasts*, ed. Skinner, F.S., Passmore, S.M. & Davenport, R.R. London/New York: Academic Press.

KLOCKE, J.A. & MYERS, P. 1984 Chemical control of thrips on cultured *Simmondsia chinensis* (Jojoba) shoots. *Horticultural Science* **19**, 400.

KLOOS, W.E. & SCHLEIFER, K.H. 1986 Description of the genus *Staphylococcus*. In *Bergey's*

Manual of Systematic Bacteriology, 9th Edition, Vol. 2, ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. pp. 1209–1235. Baltimore/London: Williams and Wilkins.

KNAUSS, J.F. 1976 A tissue culture method for producing *Dieffenbachia picta* CV. 'Perfection' free of fungi and bacteria. *Proceedings of the Florida State Horticultural Society* **89**, 293–295.

KNAUSS, J.F. & MILLER, J.M. 1978 A contaminant, *Erwinia carotovora*, affecting commercial plant tissue cultures. *In Vitro* **14**, 754–756.

KOCUR, M. 1986 Description of the species of the genus *Micrococcus*. In *Bergey's Manual of Systematic Bacteriology*, 9th Edition, Vol. 2, ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. pp. 1004–1008. Baltimore/London: Williams and Wilkins.

KRIEG, N.R. & HOLT, J.G. 1984 *Bergey's Manual of Systematic Bacteriology* 9th Edition, Vol. 1. Baltimore/London: Williams and Wilkins.

KUNNEMAN, B.P.A.M. & FAAIJ-GROENEN, G.P.M. 1988 Elimination of bacterial contaminants: a matter of detection and transplanting procedures. *Acta Horticulturae* **225**, 183–189.

LEGGATT, I., WAITES, W.M., LEIFERT, C. & NICHOLAS J.R. 1988 Characterisation of micro-organisms isolated from plants during micropropagation. *Acta Horticulturae* **225**, 93–102.

LEIFERT, C., WAITES, W.M. & NICHOLAS, J.R. 1989a Bacterial contaminants of micropropagated plant cultures. *Journal of Applied Bacteriology* **67**, 353–361.

LEIFERT, C., WAITES, W.M., CAMOTTA, H. & NICHOLAS, J.R. 1989b *Lactobacillus plantarum*; a deleterious contaminant of plant tissue culture. *Journal of Applied Bacteriology* **67**, 363-370.

LEIFERT, C. 1990 *Contaminants of plant tissue cultures*. PhD thesis, Nottingham University, School of Agriculture.

LEIFERT, C. & WAITES, W.M. 1990a Contaminants of plant tissue cultures. *International Association for Plant Tissue Culture Newsletter* **60**, 2–13.

LEIFERT, C. & WAITES, W.M. 1990b Bacteria and yeasts; important contaminants in micropropagated plant cultures. *Abstracts VIIth International Congress on Plant Tissue and Cell Culture* ed. Nijkamp, H.J.J., van der Plas, L.H.W. & van Aartrijk, J. p. 112. Dordrecht: Kluwer Academic Publishers.

LEIFERT, C., WAITES, W.M., NICHOLAS, J.R. & KEETLEY, J.W. 1990 Yeast contaminants of micropropagated plant cultures. *Journal of Applied Bacteriology* **69**, 471–476.

LEIFERT, C., CAMOTTA, H., WRIGHT, S.M., WAITES, B., CHEYNE, V.A. & WAITES, W.M. 1991a Elimination of bacteria from micropropagated plant cultures using antibiotics. *Journal of Applied Bacteriology* (submitted).

LEIFERT, C., KEETLEY, J.W., CAMOTTA, H. & WAITES, W.M. 1991b Effect of antibiotics on micropropagated plants. *Plant Cell Tissue and Organ Culture* (submitted).

LONG, R.D., CURTIN, T.F. & CASSELLS, A.C. 1988 An investigation of the effects of bacterial contaminants on potato nodal cultures. *Acta Horticulturae* **225**, 83–91.

LYNCH, J.M. 1976 Products of soil microorganisms in relation to plant growth. *CRC Critical Review of Microbiology* **5**, 67–107.

LYNCH, J.M. 1977 Phytotoxicity of acetic acid produced in the anaerobic decomposition of wheat straw. *Journal of Applied Bacteriology* **42**, 81–87.

LYNCH, J.M. 1978 Production and phytotoxicity of acetic acid in anaerobic soils containing plant residues. *Soil Biology and Biochemistry* **10**, 131–3.

MATHEWS, H. 1988 *In vitro* responses of *Brassica juncea* and *Vigna radiata* to the antibiotic Kanamycin. *Annals of Botany* **62**, 671–675.

MATHIAS, P.J., ALDERSON, P.G. & LEAKEY, R.R.B. 1987 Bacterial contamination in tropical hardwood cultures. *Acta Horticulturae* **212**, 43–49.

MENARD, D., COUMANS, M. & GASPAR, TH. 1985 Micropropagation du *Pelargonium* a partir de meristemes. *Mededelingen van de Faculteit Landbouwettenschappen Rijksuniversiteit Gent* **50**, 327–331.

MENGEL, K. 1984 *Ernährung und Stoffwechsel der Pflanze*. Stuttgart: Gustav Fischer Verlag.

MERSCH-SUNDERMANN, V. 1889 *Medizinische Microbiologie für MTA*. Stuttgart/New York: Georg Thieme Verlag.

MÖLLERS, C. & SARKAR, S. 1989 Regeneration of healthy plants from *Cataranthus roseus* infected with mycoplasma-like organisms through callus culture. *Plant Science* **60**, 83–89.

MURASHIGE, T. and SKOOG, F. 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497.

ONIONS, A.H.S. 1990 Prevention of mites in cultures. *World Journal of Microbiology and Biotechnology* **6**, 219–221.

OWENS, L.D. 1979 Kanamycin promotes morphogenesis of plant tissues. *Plant Science Letters* **16**, 225–230.

PALLERONI, N.J. 1984 Description of the species of the genus *Pseudomonas*. In *Bergey's Manual of Systematic Bacteriology*, 9th Edition, Vol. 1, ed. Krieg, N.R. & Holt, J.G. pp. 141–199. Baltimore/London: Williams and Wilkins.

PHILLIPS, R., ARNOTT, S.N. & KAPTAN, S.E. 1981 Antibiotics in plant tissue culture: Rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of *Helianthus tuberosus*. *Plant Science Letters* **21**, 235–240.

PODWYSZYNSKA, M. & HEMPEL, M. 1987 Identification and elimination of 'slowly growing' bacteria from a micropropagated *Gerbera*. *Acta Horticulturae* **212**, 112.

POLLOCK, K., BARFIELD, D.G. & SHIELDS, R. 1983 The toxicity of antibiotics to plant cell cultures. *Plant Cell Reports* **2**, 36–39.

POULSEN, G.B. 1988 Elimination of contaminating micro-organisms from meristem culture of apple rootstock M26. *Acta Horticulturae* **225**, 193–197.

REUSTLE, G., MANN, M. & HEINTZ, C. 1988 Experience and problems with infections in tissue cultures of grapevine. *Acta Horticulturae* **225**, 119–129.

REUTHER, G. 1983 Propagation of disease-free *Pelargonium* cultivars by tissue culture. *Acta Horticulturae* **131**, 311–319.

REUTHER, G. 1985 Principles and application of the micropropagation of ornamental plants. In *In Vitro Techniques: Propagation and Longterm Storage* ed. Schafer-Menuhr, A. pp. 1–14. Dordrecht: Martinus Nijhoff/Dr W. Junk.

REUTHER, G. 1988 Problems of transmission and identification of bacteria in tissue culture propagated geraniums. *Acta Horticulturae* **225**, 139–152.

ROSSINI, G. & STANDARDI, A. 1990 Studies on the elimination of contaminating micro-organisms from tissue cultures of apple rootstock M27 by antiseptic treatment. In *Abstracts VIIth International Congress on Plant Tissue and Cell Culture*, ed. Nijkamp, H.J.J., van der Plas, L.H.W. & van Aartrijk, J. p. 380. Dordrecht: Kluwer Academic Publishers.

SCHAAD, N.W. 1980 Initial identification of common genera. In *Identification of Plant Pathogenic Bacteria* ed. Schaad, N.W. pp. 1–11. St Paul: American Phytopathological Society.

SCOPES, N. 1979 *Pests and Disease Control Handbook*. London: BCPC Publications.

SCHIPPERS, B., BAKKER, A.W. & BAKKER, P.A.H.M. 1987 Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annual Review of Phytopathology* **25**, 339–358.

SINGHA, S., BISSONNETTE, G.K. & DOUBLE, M.L. 1987 Methods for sterilising instruments contaminated with *Bacillus* sp. from plant tissue cultures. *Horticultural Science* **22**, 659.

SMITH, I.M., DUNEZ, J., LELLIOTT, R.A., PHILLIPS. D.H. & ARCHER, S.A. 1986 *European Handbook of Plant Diseases*. Oxford/London: Blackwell Scientific Publications.

SEATH, P.H.A. 1986 Description of the genus *Bacillus*. In *Bergey's Manual of Systematic Bacteriology*, 9th Edition, Vol. 2, ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. pp. 1004–1008. Baltimore/London: Williams and Wilkins.

SNEATH, P.H.A., MAIR, N.S., SHARPE, M.E. & HOLT, J.G. 1986 *Bergey's Manual of Systematic Bacteriology*, 9th Edition, Vol. 2, Baltimore/London: Williams and Wilkins.

THURSTON, K.C., SPENCER, S.J. & ARDITTI, J. 1979 Phytotoxicity of fungicides and bactericides in orchid culture media. *American Journal of Botany* **66**, 825–835.

TILBURY, R.H. 1980 Xerotolerant (osmophilic) yeasts. In *Biology and Activities of Yeasts*, ed. Skinner, F., Passmore, S.M. & Davenport, R.R. Academic Press: London/New York.

TRICK, I. & LINGENS, F. 1985 Aerobic spore-forming bacteria as detrimental infectants in plant tissue cultures. *Applied Microbiology and Biotechnology* **21**, 245–249.

WAINWRIGHT, H. & ENGLAND, N. 1987 The micropropagation of *Prosopis juliflora*: Establishment *in vitro*. *Acta Horticulturae* **212**, 49–54.

WILSON, Z.A. & POWER, J.B. 1989 Elimination of systemic contamination in explant and protoplast cultures of Rubber (*Hevea brasiliensis*). *Plant Cell Reports* **7**, 622–625.

(Received 13 November 1990; revised 15 February 1991; accepted 23 February 1991).

A three-step screening procedure for detection of covert and endophytic bacteria in plant tissue cultures

Pious Thomas

Division of Biotechnology, Indian Institute of Horticultural Research, Hessarghatta Lake, Bangalore 560 089, India

A sequential three-step screening procedure to detect any covert or endophytic bacteria in plant tissue cultures was developed consequent to the observation that conventional detection methods were inconsistent and undependable. This procedure involved diligent visual examination of cultures for any inconspicuous growth (step-1), indexing the medium of visually clean cultures using bacteriological media (step-2), and subsequent tissue-indexing (step-3) using split segments from different plant parts. Step-2 indexing of grape, watermelon, papaya, capsicum, eggplant and gerbera cultures revealed bacteria in 0–100% cultures in different batches. Varying proportions of cultures that passed step-2 indexing turned positive during step-3 indexing, suggesting the essentiality of tissue-indexing. Use of two bacteriological indexing media (BIM), namely nutrient agar (BIM_1) and 523 medium (BIM_2) differing in nutrient constituents, pH (6.4 and 7.0 respectively) and gel strength (10 and 20 g l^{-1} agar), pre-incubation of nutrient plates at 30–37°C to ensure freedom from incidental contaminants, post-indexing incubation at two different temperatures (25–30°C and 37°C respectively) and sterility testing of tools prior to use were other considerations during indexing. This screening procedure practised for two–four cycles allowed reliable scrutiny of plant tissue cultures for freedom from cultivable bacteria at culture initiation or while sanitizing contaminated cultures; this would find application for certification of *in vitro* cultures and gene banks.

PLANT tissue culture offers an important tool for rapid clonal multiplication of elite plants, crop improvement, genetic transformation, basic morphogenesis studies, and conservation and exchange of germplasm. To attain these goals, cultures should essentially be free from all microorganisms. Bacteria, which may be introduced in cultures as epiphytes and endophytes or later during culture handling, sometimes remain covert or latent[1–3] and go unnoticed in the absence of specific indexing[2,4,5]. Presence of covert bacteria in the cultures is highly undesirable due to obvious adverse effects on growth[6,7], lack of reproducibility of tissue-culture protocols[7], possible hormone-mediated growth effects[8], ramifications in cell cultures[9], possibility of carrying pathogens[10], potential risk to *in vitro* gene banks[5] and safe exchange of germplasm[11]. All these reduce the reliability of plant cell/tissue-culture systems[3,7].

Availability of a reliable screening method is the primary requirement for tackling the covert contamination problem. Many studies have addressed the isolation and identification of common bacteria in tissue cultures[5,6,12], but reliable detection which is essential to ascertain asepsis remains neglected. Testing the cultures using bacteriological indexing medium (BIM) is the simplest and best method as it allows non-specific detection of a wide range of bacteria even when present in low numbers[2,13]. Molecular and serological methods allow detection of specific organisms but they are expensive, expertise-demanding and of limited use for general indexing[2,14]. Conventional indexing methods include incorporation of bacterial growth-enhancing constituents in the tissue culture medium[15], placing the tissue in enriched liquid medium and assessing its turbidity[12,16], or streaking the base of plantlets on BIM[4,5]. Placing pieces of tissue on BIM[2] or testing the tissue homogenate on BIM[17] has been suggested to detect any endophytic bacteria that survived initial decontamination treatment.

While handling long-term micropropagated cultures of seedless watermelon and grape, covert bacteria were found rampant in them, which emerged as the cause of the *in vitro* decline manifested in the form of poor growth response, severe drop in propagation rate, lack of rooting and root growth in them[7,18]. During the efforts to sanitize the cultures, use of the above-mentioned conventional indexing methods resulted in inconsistent and inconclusive results. Often bacteria resurfaced during subsequent cycles similar to other reports on freeing the cultures from bacteria employing antibiotic treatment[19,20]. Based on the observations over three years using established as well as fresh cultures of various plant species, a step-wise screening involving visual examination, medium-indexing and tissue-indexing was found necessary for reliable detection of covert and endophytic bacteria. This article outlines the procedure and precautions to be taken during the indexing of plant cultures.

e-mail: pioust@iihr.res.in

Materials and methods

The studies were carried out mainly using long-term micropropagated cultures of grape (*Vitis vinifera* L.) cv. Arka Neelamani[21] and triploid watermelon (*Citrullus lanatus* Thunb. [Matsum. & Nakai]) cv. Arka Manik[22] during their 5–9-year period *in vitro*. Grape was raised on a medium gelled with phytagel (Sigma Chemical Co, St. Louis) as described elsewhere[21], which gave a clear medium, while watermelon was raised on a medium gelled with agar (Sigma) owing to hyperhydricity[23] that gave a semi-translucent medium. Both the cultures showed single shoot growth, and shoot-tip and nodal microcuttings from these stocks were used for propagation[21,23,24]. Fresh culture of diploid watermelon (cv. Arka Manik) was initiated from seeds and gerbera (cv. Pink Elegans) from capitulum explants[25], while cultures of papaya (cv. Surya), grape (cv. Thompson Seedless), capsicum (cv. Arka Gaurav) and eggplant (cv. Arka Keshav) were obtained from colleagues. Grape and watermelon cultures in this study refer to long-term maintained stocks, unless mentioned otherwise.

The cultures were grown in bottles (120 mm height × 65 mm diameter) with a screw cap providing near air-tight sealing, unless mentioned differently. Grape stock was subcultured at 6–8 weeks interval and watermelon at 4–6 weeks using four microcuttings per vessel in 50 ml medium. All the cultures were incubated at 26 ± 2°C under 16 h photoperiod (30–40 $\propto$E m^{-2} s^{-1}) provided by cool-white fluorescent tubes.

The optimized screening procedure evolved based on observations from different trials consisted of three steps, including diligent visual examination of cultures (step-1), medium-indexing of visually clean cultures (step-2), and tissue-indexing of medium-index-negative cultures (step-3). The cultures were first observed at eye level, from above and from underneath with and without background light for any inconspicuous bacterial growth. Two BIMs were identified based on the preliminary trials towards medium- and tissue-indexing. These included nutrient agar (NA) containing 5 g l^{-1} each peptone and NaCl, 3 g l^{-1} beef extract and 20 g l^{-1} agar (BIM_1) and 523 medium of Viss *et al.*[4] containing 10 g l^{-1} sucrose, 8 g l^{-1} casein hydrolysate, 4 g l^{-1} yeast extract, 2 g l^{-1} KH_2PO_4, 0.15 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 10 g l^{-1} agar (BIM_2). Medium-indexing was done by inserting into the culture medium a sterile 200 $\propto$l disposable tip attached to a bleach-swabbed pipette (or using a flamed inoculation needle for cultures growing in culture tubes and those with vigorous shoot growth) and bringing the same in contact with the two BIMs. Before tissue-indexing, the forceps were first indexed on BIM to ascertain their sterility with their results known along with tissue-indexing results. In case of micropropagated cultures, shoot part (one plant per vessel) was excised using a pair of sterile scissors. After sub-culturing the microcuttings, left-over tissue segments from upper/middle part and lower half of stem (5–8 mm) were split longitudinally and one segment was placed on each BIM. The stump was lifted and split-segments from basal swollen part and roots (if available) were placed on BIM. Other plantlets in the same vessel were tested using pieces of basal swelling or root after culturing the microcuttings. Finally the medium was indexed to confirm the step-2 screening results. Cultures other than the micropropagated ones were indexed using shoot, root, regenerating tissue and/or callus according to the availability.

Step-1 and step-2 screening was carried out mostly using the long-term cultures of grape and watermelon (40–100 bottles at any given time), while step-3 screening was undertaken using visually clean and medium-index-negative cultures that were subjected to $HgCl_2$ (0.05 or 0.1%) or sodium hypochlorite (4% available chlorine; Sd-fine Chemicals, Mumbai, India) treatment as discussed later and/or to antibiotics for sanitizing them[7,18]. Cultures of other plant species were used for validation of the results. Fresh, sterile petri dishes were used for handling each vessel of the culture and the tools were autoclaved before use on any day, and sterilized in a glass-bead sterilizer (250°C; 3–5 min) or over a gas flame (30–40 s) between cultures after wiping-off any adhering medium using ethanol-drenched cotton. Alcohol dip and flaming were shunned owing to the possibility of transmission of some contaminants through flamed tools[26]. BIM in sterile single-use plates (10 mm × 15 mm; Hi-Media, Mumbai, India) was pre-incubated (upside-down) at 37°C for 2–3 days followed by 2–4 days at room temperature (approx. 25°C) before use to ensure freedom from incidental contaminants and to evaporate away any free water on the surface so as to avoid spreading colony growth.

During medium-indexing, 20–100 cultures were used per batch, accommodating 20–32 cultures per plate and 4–12 cultures during tissue-indexing. Post-indexing, BIM_1 plates were incubated in the dark at 37°C and BIM_2 at 25 or 30°C for 2–7 days upside-down followed by another 3–4 weeks at room temperature. The plates were observed on days 1, 2, 3 and 4 and thereafter weekly to detect any slow-growing bacteria. The cultures were classified as 'index-positive' based on visible bacterial growth from one or more indexed spots or tissue samples on one or more BIMs. Application of statistical treatments did not appear pertinent because of the unique situation in a study of this kind.

Results

Any culture showing obvious microbial growth in the medium could easily be picked up and discarded, and this was encountered to the tune of 0–5% in different batches. Visibly clean cultures showing any faint growth on the medium surface or 'halo' at the base of plantlets could be picked up if examined carefully before the roots grew extensively. Occasionally hazy streaks delusive of preci-

pitated medium constituents were seen within the gelled medium and such cultures often tested positive during medium-indexing. Dry grainy tracts on the surface which mimicked dried-up water/medium turned out to be faint bacterial patches on the surface when observed from below. Loss of clarity of medium, general pale growth, partial drying of leaves (Figure 1 *a*) and emission of a foul odour upon opening the vessels were other indicators of covert bacteria. Thus, a certain proportion of covertly contaminated cultures could be identified based on diligent visual examination. Indexing of medium, however, made it easier to detect unambiguously such cultures.

Medium-indexing revealed covert bacteria in the range of 0–100% in various plant cultures with consistent results. Use of disposable tips or inoculation needle gave confined colony growth around the point of inoculation, unlike with explant base-streaking[4] and accommodated several cultures per plate (Figure 1 *b*). Testing the culture medium at several points and spotting at 4–5 points on the BIM using the same tip offered better or faster contaminant detection. The medium collected in the pipette tips also formed a good sample for indexing, but it demanded careful dispensing onto the BIM to avoid its spattering and scattered colony growth. Provision of a 2 ml overlay of half-strength liquid BIM in the cultures a week before and testing 1–2 µl of this medium allowed detection of bacteria that were not evenly spread on the medium.

In the preliminary trial where BIM_1 and BIM_2 were tested with other BIMs which included NA with 10 g l^{-1} sucrose (BIM_3), half-strength Murashige and Skoog medium[27] with 10 g l^{-1} each sucrose and dextrose, 8 g l^{-1} casein hydrolysate, 4 g l^{-1} yeast extract, 2 g l^{-1} peptone (BIM_4), MS constituents in BIM_4 substituted with half-strength Rugini medium[28] (BIM_5), 4 g l^{-1} each casein hydrolysate and peptone, 8 g l^{-1} NaCl and 10 g l^{-1} dextrose (BIM_6), casein hydrolysate in BIM_6 substituted with beef extract (BIM_7), BIM_7 supplemented with fresh extract from 10 g l^{-1} potato (BIM_8), Lauria broth agar (BIM_9), potato dextrose agar (BIM_{10}) and BIM_{10} containing 10 g l^{-1} dextrose (BIM_{11}), the extent of detection and speed of colony growth differed with various BIMs (data not presented). Use of potato extract in the medium did not contribute to better or faster detection. Use of dextrose, higher pH (7.0) and low gel strength in general favoured earlier and faster colony growth, but lower pH (6.4 or 5.8) and stronger gelling offered confined colonies and clearer results. Similarly, incubation of plates at 37°C enhanced the speed and rate of colony growth, while overall detection after one week was relatively better at 25–30°C (data not presented).

Tissue-indexing of visually clean and medium-index-negative cultures showed bacterial growth on one or both media within 2–4 days in 0–100% of the cultures depending on the pre-treatments given or the extent of screening at steps 1 and 2. Medium-embedded basal swelling which formed a part of the original microcutting and roots showed more frequent and early bacterial growth. Some specific examples are presented below to demonstrate covert bacterial survival in the tissue without detectable presence in the medium.

Medium-indexing of a batch of 16 grape cultures four weeks after $HgCl_2$ treatment (0.1%; 5 min) showed six of them as index-negative on three BIMs, i.e. $BIM_{1,2,3}$. Tissue-indexing a week later revealed bacteria in all the indexed tissue parts of one culture (17%), while the medium still tested negative (Table 1). However, the medium turned index-positive in the next passage indicating gradual inoculum build-up with time. The other five cultures remained index-negative for the next three cycles, confirming freedom from cultivable bacteria.

In an experiment aimed at cleansing watermelon cultures through NaOCl treatment (5 min), six of 16 apparently clean cultures on multiplication medium tested positive when indexed on four different BIMs four weeks after the previous culturing (Figure 2 *a*). Tissue-indexing a week later using $BIM_{1,2}$ revealed bacteria in the upper or lower stem segments in just one or both media, while the basal segment did not show any bacterial growth on either medium (Figure 2 *b*). This indicated that covert bacteria would not always be brought out through streak test and the need for using more than one indexing medium.

In another study on sanitizing watermelon cultures, ten out of 20 shoot tips that were treated with $HgCl_2$ (0.05%; 10 min) and planted singly on filter paper bridges in liquid medium (Figure 3 *a*) showed low to moderate turbidity of medium 2 weeks later. Upon testing the ten visibly clean cultures along with eight cultures with low visual turbidity by transferring 2 µl liquid medium, six of the former (60%) and whole of the latter revealed bacteria in them (Figure 3 *b*(a)). Next 100 µl of medium from the four index-negative cultures was plated on BIM_1 at 3 and again 4 weeks from the original culturing which revealed no bacteria in the medium (Figure 3 *b*(b)). When these cultures were subjected to tissue-indexing a week later, endogenous bacteria were found in all four of them (Figure 3 *b*(c)), while the medium remained index-negative.

Eggplant cotyledon and watermelon shoot-tip cultures initiated from aseptically raised seedlings tested index-negative during medium-indexing at 4 weeks after culture

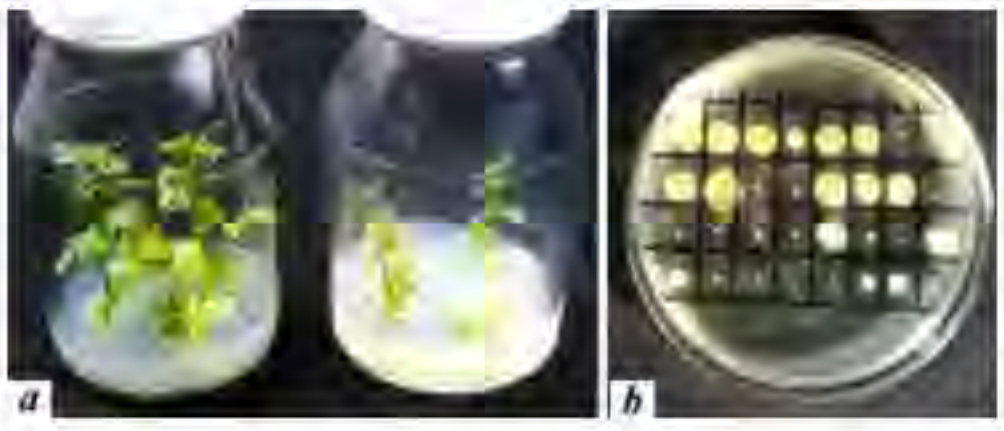


Figure 1. *a*, Healthy bacteria-index-negative (left) and visibly clean but index-positive (right) cultures of triploid watermelon. *b*, Medium-indexed plate using disposable pipette tips showing different bacterial types one week post-indexing.

Table 1. Screening of micropropagated grape cultures for covert and endophytic bacteria planted after treatment with $HgCl_2$ (0.1%) for 5 min

Culture no.	Step-2: Indexing of culture medium (4 weeks)[a] Index + ve/− ve and BIM	Step-3: Tissue-indexing with sterility testing of forceps (Using BIM_{1-3}) (5 weeks)[a]					
		Forceps: Pre-use	Upper stem	Lower stem	Basal swelling	Main roots	Medium
1[b]	− ve	− ve	− ve	− ve	− ve	− ve	− ve
2[b]	− ve	− ve	− ve	− ve	− ve	− ve	− ve
3	− ve (BIM_{1-3})	ND	ND	ND	ND	ND	ND
4	− ve	BIM_1: − ve	+ ve d_1	+ ve d_1	+ ve d_1	+ ve d_1	− ve
		BIM_2: − ve	+ ve d_1	+ ve d_1	+ ve d_2	− ve	− ve
		BIM_3: − ve	+ ve d_2	+ ve d_2	+ ve d_1	+ ve d_1	− ve
5[b]	− ve	− ve	− ve	− ve	− ve	− ve	− ve
6[b]	− ve	− ve	− ve	− ve	− ve	− ve	− ve
7	− ve ($BIM_{1,3}$)	ND	ND	ND	ND	ND	ND
8	− ve (BIM_{1-3})	ND	ND	ND	ND	ND	ND
9	+ ve (BIM_{1-3})	ND	ND	ND	ND	ND	ND
10[b]	− ve	− ve	− ve	− ve	− ve	− ve	− ve
11	− ve ($BIM_{1,3}$)	ND	ND	ND	ND	ND	ND
12	− ve ($BIM_{1,3}$)	ND	ND	ND	ND	ND	ND
13	+ ve (BIM_{1-3})	ND	ND	ND	ND	ND	ND
14	− ve (BIM_{1-3})	ND	ND	ND	ND	ND	ND
15	− ve (BIM_{1-3})	ND	ND	ND	ND	ND	ND
16	− ve (BIM_{1-3})	ND	ND	ND	ND	ND	ND

[a]Time in weeks after decontamination treatment; [b]Cultures that remained index-negative at all steps.
BIM_1, NA; BIM_2, 523 medium[4]; BIM_3, NA with 10 g l^{-1} each agar and sucrose.
+ ve, Index positive; − ve, Index negative; ND, Indexing not done; d_n, Days taken for visible bacterial growth on BIM.

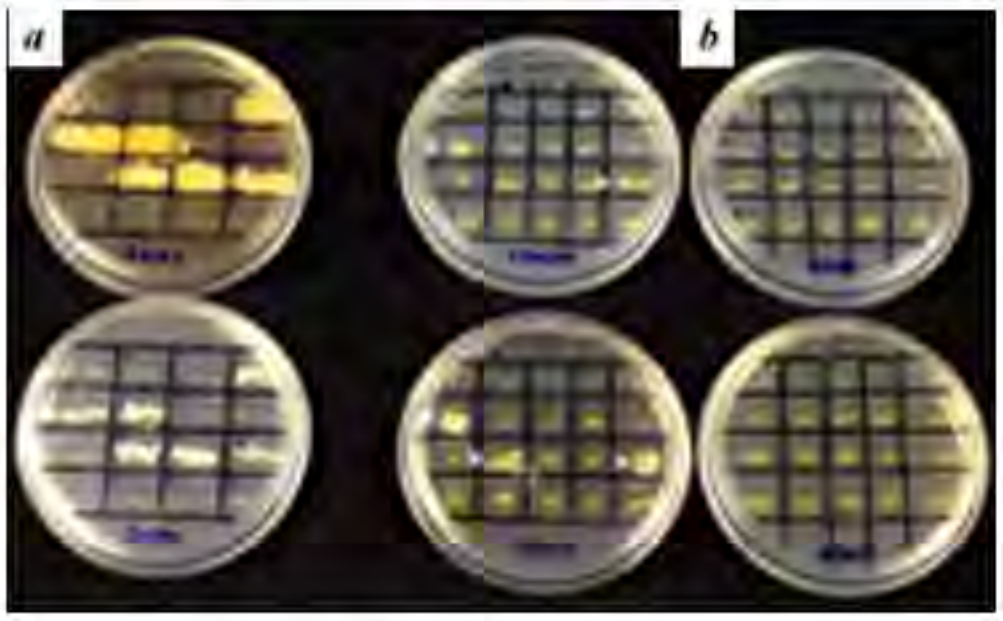


Figure 2. Indexing the medium of visibly clean watermelon cultures on two modified BIMs using disposable tips (*a*) and tissue-indexing of 'medium-index negative' cultures (ID no. 1, 2, 3, 7, 8, 9, 13, 14, 15 and 16) using BIM_1 and BIM_2 (*b*: row 1, Sterility testing of forceps; row 2, Stem from upper part; row 3, Lower stem and row 4, Basal swelling). Arrowhead indicates bacterial growth from tissue.

Figure 3. *a*, Watermelon shoot tips cultured on filter paper bridges showing clear medium as in control tubes. *b*, (a) Indexing shoot tip cultures by spotting 2 ∝l medium on BIM_1; (b) testing of 'index negative' culture by plating 100 ∝l medium, and (c) tissue-indexing of four index-negative cultures using split stem segments from upper (row 2), middle (row 3) or lower part of the shoot (row 4). Forceps before use were indexed in row 1.

initiation but showed 0–10% index-positives in different batches during tissue-indexing a week later. Eleven per cent of a batch of 18 visibly clean papaya cultures turned index-positive for medium and 19% of the remaining cultures tested positive during tissue-indexing. In grapes, 17% of the 18 visibly clean new cultures tested both medium- and tissue-index positive. In gerbera, 10 and 25% of the capitulum cultures (80 nos) tested index-positive for medium and tissue respectively, in the first cycle.

In a trial where capsicum cotyledon cultures growing on medium supplied with cefotaxime (500 ppm) and kanamycin (75 ppm) were subjected to medium-indexing, 25% of the 16 cultures were index-positive, which took as much as 4 weeks for visible growth in some instances. However, only 50% of these cultures tested positive during tissue-indexing.

Discussion

The present study confirms earlier reports on the possible survival of bacteria in covert or endophytic form in plant tissue cultures[1,4,6,9,17] and demonstrates the essentiality of a sequential screening of cultures involving visual examination, indexing of medium followed by tissue-indexing to detect such bacteria. Four types of bacterial contamination/association were found to be prevalent in tissue cultures. This included obviously visible growth which could easily be picked up, inconspicuous growth which might be identified after careful visual examination, covert bacteria in the medium which might be brought out through medium-indexing and endophytic bacteria detection of which needed indexing of tissue. The last three types may

go undetected and gradually spread to more cultures, contributing to culture degeneration.

Phytagel-gelled medium facilitated better visual detection of inconspicuous bacterial growth compared with cloudy agar-gelled medium, but also allowed accumulation of some bacteria that were not visible in such medium. Eight or more distinct bacterial types were found associated with agar-gelled watermelon medium[7] compared with about six types isolated from phytagel-gelled grape medium[18]. On the other hand, some bacteria (e.g. *Bacillus pumilus*) showed visible growth on the former medium but survived in subdued form on the latter[26]. This indicated that the transparent medium would not ensure visual detection of bacterial contaminants and the omnipresence of endophytic bacteria which may get easily introduced in the cultures[2,17,29,30].

Various bacterial species differ in their ability to grow on a particular medium and no single bacteriological medium is able to detect all the contaminants[2]. On the other hand, economic considerations and operational feasibility do not permit the use of a wide array of media or incubating conditions. Thus, use of two indexing media differing in major constituents, pH and gel strength, and two different incubating temperatures was found necessary during step-2 and step-3 indexing. Our choice for general detection of contaminants prevalent under tropical and sub-tropical environments included BIM_1 (pH 7.0; 20 g l^{-1} agar) with 37°C incubation and BIM_2 (pH 6.4; 10 g l^{-1} agar) with 25 or 30°C incubation.

Indexing of medium is preferably done 1–2 weeks prior to the intended date of sub-culturing, while tissue-indexing is best done at sub-culturing. Most of the contaminants (> 90%) showed up on BIM within 2–7 days, while some showed delayed growth. Use of sterile disposable tips for medium-indexing avoided the chances of cross-contamination and simplified the operation. The tips could be recycled after autoclaving twice, the first one submerged in water and the second after arranging them in tip-boxes.

The original explant part that comes in direct contact with the medium formed the best candidate for tissue indexing in general. Splitting the stem/root facilitates direct contact of endophytic bacteria with the BIM. Distribution of endophytes may be uneven[13,18] as observed in this study too, and it is desirable to use representative tissue from as many different parts of plantlets as possible[18]. Detection of bacteria in some plant parts following chemical or antibiotic treatment indicated cells trapped or surviving in isolated pockets which would multiply slowly and appear during subsequent cycles. Testing of tissue from various parts facilitated the early detection of such bacteria.

Tissue-indexing should be carried out for the whole lot of primary cultures or chemical/antibiotic treated cultures. Down the line, a small proportion of cultures may be kept as certified stocks, indexed regularly and used to supplement the routine production cultures[2]. Tissue-indexing is more tedious and time-consuming than medium-indexing. It needs proper record-keeping to track individual cultures. While step-1 and step-2 screening would suffice for general production cultures in a commercial set-up, step-3 indexing is essential for certifying stocks and while cleansing the cultures. Plating the tissue homogenate on BIM for detecting endophytic bacteria[17] is not feasible for regular indexing besides the danger of introduction of contaminants during handling.

In the case of antibiotic-treated cultures, tissue-indexing could give false results due to the residual effect of chemicals imbibed by the tissue and bacteria may reappear months later because of the transient bacteriostatic activity of antibiotics[19,20]. In such instances, indexing for two–four cycles after withdrawing the antibiotics would be needed. It may be noted that one surviving cell or spore is enough to cause bacterial reemergence later. Besides ensuring freedom from cultivable bacteria, this indexing approach also helped in detecting any lateral entry of contaminants and in differentiating them from residual endophytes. In this study, we were only concerned about detection of bacterial contaminants but not with the effect of covert bacteria on the growth which can be found elsewhere[7,18], or their isolation and identification[7,26]. The grape and watermelon cultures have now been sanitized of covert and endophytic bacteria through chemical and antibiotic treatments, and this screening procedure has been instrumental to attain this goal[18].

The screening procedure described here would help in ensuring the safety and reliability of *in vitro* gene banks, exchange of clean germplasm, checking the multiplication of pathogenic bacteria-harbouring cultures and in preventing the escape of *Agrobacterium*-mediated vector systems to the environment[3]. Bacteria harbouring apparently clean cultures shipped across sometimes show up contamination by the time they reach the destination due to fluctuating temperature conditions during transit[30] (personal experience), affecting the supply and reputation of the supplier besides inviting legal issues[1]. The BIMs identified in this study will detect most of the cultivable bacteria but probably not the ones which have specific growth requirements or those that are non-amenable to culturing, detection of which needs molecular-based approach[31].

It is suggested that the term 'covert bacteria' as described by Holland and Polacco[8] or Horsch and King[9] is more appropriate than the frequently used term 'latent bacteria' to describe such bacteria which are not normally visible on tissue culture medium. 'Latent' is a common term used in plant pathology to describe infections with no obvious adverse effects. Bacterial contaminants in tissue cultures may be latent to the extent that they are not normally visible on tissue culture medium but their effects on plant growth – whether inhibitory, null or promotive – are not clearly known. Thus it has a different meaning from its usage in plant pathology.

In conclusion, plant tissue cultures could harbour bacteria in a totally unsuspecting manner, either externally in

the medium/plant or endophytically. A sequential three-step screening involving visual examination of cultures, indexing of medium and indexing of tissue from various parts of the culture using two BIMs and elevated incubation temperature is suggested for reliable detection of covert and endophytic bacteria-harbouring cultures and to ensure freedom from such bacteria.

1. Cassells, A. C., Problems in tissue culture: culture contamination. In *Micropropagation* (eds Debergh, P. C. and Zimmerman, R. H.), Kluwer Academic Publishers, Dordrecht, 1991, pp. 31–44.
2. Leifert, C. and Woodward, S., Laboratory contamination management: the requirement for microbiological quality assurance. *Plant Cell Tiss. Org. Cult.*, 1998, **52**, 83–88.
3. Leifert, C. and Cassells, A. C., Microbial hazards in plant tissue and cell cultures. *In Vitro Cell. Dev. Biol. Plant*, 2001, **37**, 133–138.
4. Viss, P. R., Brooks, E. M. and Driver, J. A., A simplified method for the control of bacterial contamination in woody plant tissue culture. *In Vitro Cell. Dev. Biol. Plant*, 1991, **27P**, 42.
5. Van den houwe, I. and Swennen, R., Characterization and control of bacterial contaminants in *in vitro* cultures of banana (*Musa* spp.). *Acta Hortic.*, 2000, **530**, 69–79.
6. Leifert, C. and Waites, W. M., Bacterial growth in plant tissue culture media. *J. Appl. Bacteriol.*, 1992, **72**, 460–466.
7. Thomas, P., *In vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause for degeneration of long-term micropropagated triploid watermelon cultures. *Plant Cell Tiss. Org. Cult.*, 2004, **77**, 173–179.
8. Holland, M. A. and Polacco, J. C., PPFMs and other covert contaminants: Is there more to plant physiology than just plant? *Annu. Rev. Plant Physiol.*, 1994, **45**, 197–209.
9. Horsch, R. B. and King, J., A covert contaminant of cultured plant cells: elimination of a *Hyphomicrobium* sp. from cultures of *Datura innoxia* (Mill.). *Plant Cell Tiss. Org. Cult.*, 1983, **2**, 21–28.
10. Cooke, D. L., Waites, W. M. and Leifert, C., Effect of *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* on plant tissue cultures of *Aster*, *Cheiranthus*, *Delphinium*, *Iris* and *Rosa*, disease development as a result of latent infection *in vitro*. *J. Plant Dis. Protect.*, 1992, **99**, 469–481.
11. Salih, S., Waterworth, H. and Thompson, D. A., Role of plant tissue culture in international exchange and quarantine of germplasm in the United States and Canada. *HortScience*, 2001, **36**, 1015–1021.
12. Tanprasert, P. and Reed, B. M., Detection and identification of bacterial contaminants from strawberry runner explants. *In Vitro Cell. Dev. Biol. Plant*, 1997, **33**, 221–226.
13. Cassells, A. C., Contamination detection and elimination in plant cell culture. In *Encyclopedia of Cell Technology* (ed. Spier, R. E.), John Wiley, New York, 2000, vol. 2, pp. 577–586.
14. Stead, D. E., Elphinstone, J. G., Weller, S., Smith, N. and Hennessy, J., Modern methods for characterizing, identifying and detecting bacteria associated with plants. *Acta Hortic.*, 2000, **530**, 45–57.
15. Boxus, Ph. and Terzi, J-M., Big losses due to bacterial contaminations can be avoided in mass propagation scheme. *Acta Hortic.*, 1987, **212**, 91–93.
16. Niedz, R. P. and Bausher, M. G., Control of *in vitro* contamination of explants from greenhouse and field-grown trees. *In Vitro Cell. Dev. Biol. Plant*, 2002, **38**, 468–471.
17. Kamoun, R., Lepoivre, P. and Boxus, P., Evidence for the occurrence of endophytic prokaryotic contaminants in micropropagated plantlets of *Prunus cerasus* cv. 'Montmorency'. *Plant Cell Tiss. Org. Cult.*, 1998, **52**, 57–59.
18. Thomas, P. and Prakash, G. S., Sanitizing long-term micropropagated *in vitro* grapes from covert and endophytic bacteria and preliminary field testing of plants for fertility after eight years *in vitro*. *In vitro Cell. Dev. Biol. Plant* (in press).
19. Tanprasert, P. and Reed, B. M., Determination of minimal bactericidal and effective antibiotic treatment concentrations for bacterial contaminants from micropropagated strawberries. *In Vitro Cell. Dev. Biol. Plant*, 1997, **33**, 227–230.
20. Reed, B. M., Mentzer, J., Tanprasert, P. and Yu, X., Internal bacterial contamination of micropropagated hazelnut: identification and antibiotic treatment. *Plant Cell Tiss. Org. Cult.*, 1998, **52**, 67–70.
21. Thomas, P., Increase in clonal propagation of 'Arka Neelamani' grape (*Vitis vinifera* L.) through induction of axillaries in *in vitro* layering technique. *Indian J. Agric. Sci.*, 1997, **67**, 594–596.
22. Thomas, P., Pitchaimuthu, M., Mythili, J. B. and Srinivas, M., Salvaging of abortive embryos from mature tetraploid × diploid watermelon fruits through *in vitro* culturing and realization of a triploid seedless watermelon. *Curr. Sci.*, 2003, **84**, 813–816.
23. Thomas P., Mythili, J. B. and Shivashankara, K. S., Explant, medium and vessel aeration affect the incidence of hyperhydricity and recovery of normal plantlets in triploid watermelon. *J. Hortic. Sci. Biotechnol.*, 2000, **75**, 19–25.
24. Thomas P., Mythili, J. B. and Shivashankara, K. S., Effects of photooxidative loss of Fe-EDTA and of higher iron supply on chlorophyll content, growth and propagation rate in triploid watermelon cultures. *In Vitro Cell. Dev. Biol. Plant*, 2000, **36**, 537–542.
25. Laliberté, S., Chrétien, L. and Veith, J., *In vitro* plantlet production from young capitulum explants of *Gerbera jamesonii*. *HortScience*, 1985, **20**, 137–139.
26. Thomas, P., Isolation of *Bacillus pumilus* from *in vitro* grapes as a long-term alcohol-surviving and rhizogenesis inducing covert endophyte. *J. Appl. Microbiol.*, 2004, **97**, 114–123.
27. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 1962, **15**, 473–497.
28. Rugini, M., *In vitro* propagation of some olive (*Olea europea sativa* L.) cultivars with different root-ability and medium development using analytical data from developing shoots and embryos. *Sci. Hortic.*, 1984, **24**, 123–134.
29. Hallmann, J., Plant interactions with endophytic bacteria. In *Biotic Interactions in Plant–Pathogen Associations* (eds Jeger, M. J. and Spence, N. J.), CABI Publishing, Wallingford, Oxon, 2000, pp. 87–119.
30. Mantell, S. H., Microbes intimately associated with tissue and cell cultures of tropical Dioscorea yams. *Plant Cell Tiss. Org. Cult.*, 1998, **52**, 47–52.
31. Thomas, P., Detection of covert and endophytic bacteria in plant tissue cultures through indexing and molecular approach. 26th Meeting of the Plant Tissue Culture Association of India and National Seminar on Biotechnology for Better Future, Mangalore, (Abstr.), 15–17 January 2004, p. 19.

ACKNOWLEDGEMENTS. The supply of cultures of papaya, grape, *Capsicum* and eggplant by Prakash Patil, B. N. S. Murthy, J. B. Mythili and Vageesh Babu is acknowledged. I thank P. Patil and M. B. Ravindra for critical reading of the manuscript. Technical help by N. Shivrudraiah is acknowledged. This publication bears IIHR contribution no. 17/2003.

Received 18 October 2003; revised accepted 24 February 2004

Techniques

Development of a Polymerase Chain Reaction Protocol for Detection of *Xylella fastidiosa* in Plant Tissue

G. V. Minsavage, C. M. Thompson, D. L. Hopkins, R. M. V. B. C. Leite, and R. E. Stall

First and fifth authors: Department of Plant Pathology, University of Florida, Gainesville 32611; second and third authors: Central Florida Research and Education Center, University of Florida, Leesburg 34748; fourth author: Instituto Agronômico do Paraná, Londrina, PR, 86001, Brazil.

Florida Agricultural Experiment Station Journal Series paper R-03484.
This research was supported by USDA-ARS/IFAS cooperative agreement 58-6617-0-102.
Accepted for publication 22 February 1994.

ABSTRACT

Minsavage, G. V., Thompson, C. M., Hopkins, D. L., Leite, R. M. V. B. C., and Stall, R. E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84:456-461.

A 7.4-kb *Eco*RI fragment of genomic DNA of *Xylella fastidiosa* strain PCE-RR (ATCC 35879) was used as a probe and was conserved in 18 strains of *Xylella*. The nucleotide sequence of a 1.0-kb internal *Eco*RV portion of the fragment was determined, and oligonucleotides were selected for primers that amplified genomic DNA specific to *X. fastidiosa* in 33 strains tested by the polymerase chain reaction (PCR). Plant extracts for PCR and enzyme-linked immunosorbent assay (ELISA) were obtained by maceration of grape petioles and by vacuum extraction of citrus stems. Known cell numbers of *X. fastidiosa* were added to the plant extracts contained in a succinate-citrate-phosphate buffer prior to assay. Amplification of DNA by PCR was inhibited in the presence of plant extracts unless sodium ascorbate and acid-washed polyvinylpyrrolidone were added to the extraction buffer. Detection of *Xylella* by PCR was 100-fold more sensitive than by ELISA; the limits of detection were 1×10^2 cfu/ml for PCR and 2×10^4 cfu/ml for ELISA. Restriction endonuclease digestion of PCR amplification products with *Rsa*I differentiated two pathotypes of *X. fastidiosa*.

Additional keywords: citrus variegated chlorosis, Pierce's disease of grapevine, plum leaf scald.

Xylella fastidiosa Wells et al (21), a gram negative, xylem-limited bacterium, is responsible for economic losses in many agriculturally important plants, including grapevine, peach, plum, and citrus (9). Pierce's disease of grapevine, for example, limits the commercial production of bunch grapes in the southeastern United States (7). *X. fastidiosa* also causes leaf scorch and declines in many urban shade trees, including elm, sycamore, oak, and maple (18). It also could be involved in some of the current forest decline problems that are of unknown etiology.

Certain strains of *X. fastidiosa* appear to have very wide host ranges; for example, strains that cause Pierce's disease of grapevine were shown to infect at least 28 families of monocotyledonous and dicotyledonous plants (3). However, many of the hosts are symptomless, and in some of these hosts, the populations of *X. fastidiosa* were low and difficult to detect. Strains of *X. fastidiosa* can be grouped into two or more pathotypes (2,12). Strains that cause Pierce's disease are genetically uniform, whereas those from various tree hosts are diverse. Sensitive detection methods and techniques to distinguish the pathotypes are needed for epidemiological and ecological studies of this bacterium.

Current methods of detection used in surveys for *X. fastidiosa* include electron, phase contrast, and fluorescence microscopy; culture of the bacterium; and enzyme-linked immunosorbent assay (ELISA) (4,10,18). However, microscopy is slow and inefficient and limited with respect to the amounts of host tissue that can be observed; culturing is very slow and inconsistent with some hosts and strains of the bacterium; and the limit of sensitivity of ELISA prohibits the detection of low numbers of cells. Recently, amplification of pathogen-specific DNA sequences by the polymerase chain reaction (PCR) has been used in highly sensitive methods for detection of various pathogens in their plant hosts (5).

Our objectives were to develop a sensitive and specific detection protocol for *X. fastidiosa* by using the PCR amplification of specific DNA sequences and to compare the sensitivity and utility of this detection system with ELISA. Restriction endonuclease digestion of the PCR products was also evaluated for use in the differentiation of pathotypes of the bacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *X. fastidiosa* belonging to the Pierce's disease (PD) group, PD-1r, PD-1FD, PD-4, PD88-1M, PD91-1, PD92-4, PCE-FG (ATCC 35881), Grape H, PD92-3, PD92-9, PD88-5A, PCE-RR (ATCC 35879), and CB-9 (isolated from citrus), were maintained in PD3 or buffered charcoal-yeast extract (BCYE) medium (8) at 28 C. Other strains, PLM-G83 (ATCC 35871, plum), 83-G1 (goldenrod), O88-9 (oak), OAK (ATCC 35874, red oak), SYC86-1 (sycamore), and SYC89-A (sycamore), were maintained in BCYE or periwinkle wilt (PW) medium (8) at 28 C. An additional group of Brazilian strains that had been isolated from citrus trees with citrus variegated chlorosis (CVC) (IAPAR [Instituto Agronômico do Paraná, Londrina, PR, Brazil] 9712, IAPAR 9713, IAPAR 9714, IAPAR 9766, IAPAR 10437, and IAPAR 10438), from plums with leaf scald (IAPAR 9746, IAPAR 9748, IAPAR 9765, IAPAR Coeur de Lion, IAPAR Santa Rita, IAPAR Santa Rosa, and IAPAR Rosada de Camelati), and from grapevine with Pierce's disease (Instituto Biologico Seção Bactérias Fitopatogenicas [IBSBF] 755) were maintained in BCYE at IAPAR. The DNA of these strains was extracted in Brazil and used in tests in Florida. Strains were routinely subcultured every 14 days. Long-term storage was at −70 C in PD3 broth plus 30% glycerol for PD strains and in PW broth plus 30% glycerol for all other strains. Strains of *X. fastidiosa* used for DNA extractions were grown in PW broth for 7 days on a rotary shaker at 28 C. Various bacteria used in probe and primer specificity tests (Figs. 1 and 2) were grown in nutrient broth for 12–16 h on a rotary shaker at 30 C for DNA extraction.

Cloning, Southern hybridization, and DNA sequencing. Genomic DNA was obtained from broth cultures by the CTAB (cetyltrimethylammonium bromide) extraction method (1). Restriction endonuclease digestions, agarose gel electrophoresis, and cloning of DNA fragments were performed by standard procedures (14). DNA fragments that were conserved in strains of *X. fastidiosa* were purified from agarose gels by the freeze-squeeze method (20). These fragments were cloned into pLAFR3 (19) or pBluescript 11 KS +/− (Stratagene, La Jolla, CA) for labeling or sequencing experiments. Plasmid clones were transformed into *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) for routine maintenance.

Southern hybridizations (14) were performed by labeling linearized plasmid DNA or PCR product DNA with the Genius nonradioactive DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and probing against target DNA immobilized on Nytran membranes (Schleicher and Schuell, Keene, NH). DNA fragments cloned into the polylinker of pBluescript were sequenced by the dideoxy method with Sequenase T7 DNA polymerase (United States Biochemicals, Cleveland,

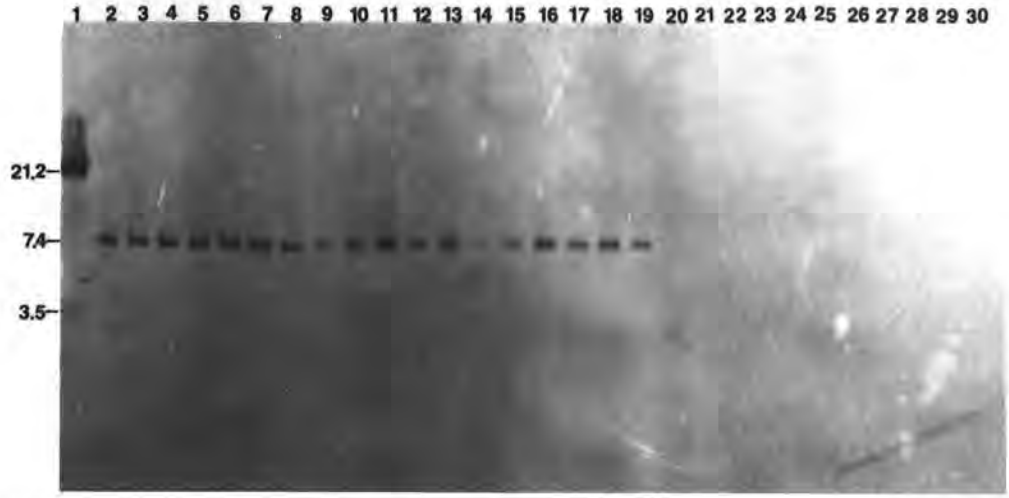


Fig. 1. Southern hybridization assay in which labeled DNA of plasmid pX10-2 was used as a probe against *Eco*RI-digested genomic DNA from various strains of *Xylella fastidiosa* and other bacteria. Lane 1, *Eco*RI + *Hin*dIII-digested λ DNA; lanes 2–19, *X. fastidiosa* strains PD-4, PD-1r, PD-1FD, PD88-1M, PD91-1, PD92-4, PCE-FG (ATCC 35881), Grape H, PD92-3, PD88-5A, PCE-RR (ATCC 35879), CB-9, PLM-G83 (ATCC 35871), 83-G1, 088-9, OAK (ATCC 35874), SYC86-1, and SYC89-A, respectively; lane 20, *Xanthomonas campestris* pv. *vesicatoria* strain XV56; lane 21, *X. c. pruni* strain FLA-1; lane 22, *X. c. phaseoli* strain 85-6; lane 23, *X. fragariae* strain GC6265; lane 24, *X. maltophilia* (=*Stenotrophomonas maltophilia* [16]) strain ASM-1; lane 25, *Xanthomonas* sp. (saprophyte) strain T-55; lane 26, *Pseudomonas syringae* pv. *syringae* strain INB; lane 27, *P. s. tomato* strain 987; lane 28, *P. solanacearum* strain GMI 1000; lane 29, *Erwinia stewartii* strain SW2; and lane 30, *Agrobacterium tumefaciens* strain 1050. Molecular weights of markers are given in kilobase pairs.

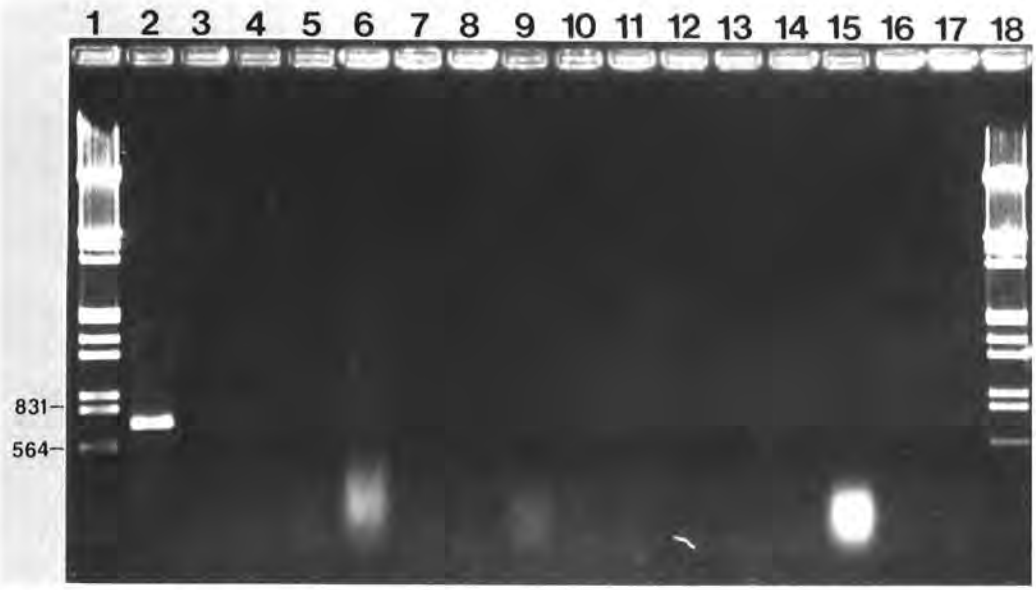


Fig. 2. Agarose gel electrophoresis of the products from the polymerase chain reaction amplification (primers RST31/RST33) of DNA from various bacteria (R. E. Stall collection). Target DNA (100 ng) sources: lane 2, *Xylella fastidiosa* strain PCE-RR (ATCC 35879); lane 3, *Xanthomonas campestris* pv. *vesicatoria* strain 75-3; lane 4, *X. c. campestris* strain ATCC 33913; lane 5, *X. maltophilia* (=*Stenotrophomonas maltophilia* [16]) strain ASM-1; lane 6, *Xanthomonas* sp. (saprophyte) strain T-55; lane 7, *Acidovorax avenae* subsp. *citrulli* strain W3; lane 8, *Pseudomonas pseudoalcaligenes* strain ATCC 29625; lane 9, *P. syringae* pv. *syringae* strain INB; lane 10, *P. solanacearum* strain K60; lane 11, *P. putida* strain PR300; lane 12, *Erwinia stewartii* strain SW2; lane 13, *E. carotovora* strain KSR347; lane 14, *E. herbicola* strain NF33; lane 15, *Escherichia coli* strain HB101; lane 16, *Agrobacterium tumefaciens* strain 1050; and lane 17, *Clavibacter michiganense* strain 75-1. Lanes 1 and 18 contain 0.5 μg of *Eco*RI + *Hin*dIII-digested λ DNA. Molecular weights of markers are given in base pairs.

OH). Oligonucleotide primers used in PCR amplification tests were synthesized with a 394 DNA synthesizer (Applied Biosystems, Foster City, CA) at the University of Florida, ICBR Facility, Gainesville. Primers synthesized were RST29 (5′-GCAACAAACAACGAACAAAGGCAATGC-3′), RST30 (5′-GTGTAAGTTTGAACACCGTCGTGG-3′), RST31 (5′-GCGTTAATTTTTCGAAGTGATTCGATTGC-3′), RST32 (5′-CGAACAAAGGCAATGCCGC-3′), and RST33 (5′-CACCATTCGTATCCCGGTG-3′).

Preparation of plant samples for ELISA and PCR. Rooted cuttings of grapevine, *Vitis vinifera* 'Carignane,' were grown in the greenhouse at 28–33 C during the day and 20–25 C at night. Plants with symptoms of leaf marginal necrosis were produced by needle inoculation of stems (8) with a PD strain of *X. fastidiosa*. Petioles and leaf veins (1.0 g) from PD-inoculated, naturally infected, or noninfected plants were ground with a mortar and pestle in 5 ml of sterile distilled water or sample buffers and filtered through cheesecloth to remove plant debris. Sample buffers were either succinate-citrate-phosphate (SCP) (disodium succinate, 1.0 g/L; trisodium citrate, 1.0 g/L; K_2HPO_4, 1.5 g/L; and KH_2PO_4, 1.0 g/L; pH 7.0) or SCPAP (SCP, 0.02 M sodium ascorbate, and 5% acid-washed insoluble polyvinylpyrrolidone [PVPP; Sigma Chemical Company, St. Louis, MO]) (6,17).

In some experiments, known concentrations of *X. fastidiosa* were added to the sample buffer at an early stage in the extraction procedure. Suspensions of bacteria in buffer from 4- to 6-day-old cultures were standardized to an optical density of 0.25 (10^7–10^8 cfu/ml) at A_{600} nm with a spectrophotometer. Suspensions were added to the mortar containing petioles and leaf veins from noninfected plants and then ground with a pestle.

Xylem fluid samples were extracted from naturally infected or noninfected rough lemon (*Citrus jambhiri* Lush.) citrus rootstock seedlings. Stem segments (4–12 mm in diameter and 2–3 cm long) were vacuum extracted (4) with SCP buffer or SCP buffer containing sodium ascorbate. In some instances, standardized bacterial suspensions were added to the extract before the samples were assayed by ELISA or PCR.

For ELISA, the plant sample extracts in SCP or SCPAP were diluted serially (10-fold) in the respective buffers containing noninfected plant tissue prior to dilution (1:1) in a 2× concentration of ELISA extraction buffer (15). For PCR, DNA was extracted from aliquots of the samples diluted for ELISA. The actual concentrations of *X. fastidiosa* in the samples were determined by dilution plating on PD3 medium.

ELISA. The ELISA procedure was similar to that previously described (10,15) with slight modifications. Flat-bottom microtiter plates were coated with gamma globulin by incubation for 4 h at room temperature (25 C). The plant extracts were added to the wells, and the plates were incubated overnight at 6 C. The wells were washed, alkaline phosphatase conjugated antibody was added, and the plates were then incubated at room temperature for 6 h. The enzyme substrate (*p*-nitrophenyl phosphate, 1 mg/ml) was allowed to react for 30 min, and then the reactions were terminated by adding 50 μl of 3 M NaOH per well. Plates were read at $A_{405\ nm}$ in a microplate auto reader (Bio-Tek Instruments Inc., Winooski, VT). A suspension of 10^5 cfu/ml of a PD strain of *X. fastidiosa* in ELISA extraction buffer was used as a positive control. A mean absorbance greater than the mean of the negative control wells plus four times the standard deviation was determined to be a positive reaction.

PCR amplification. PCR amplification of DNA was done in a DNA thermocycler (M. J. Research, Watertown, MA) with *Taq* DNA polymerase (Promega, Madison, WI). Individual PCR samples (50 μl) contained 1× amplification buffer (supplied by the manufacturer with the enzyme), 100 μM of each dNTP, 50 μM of each primer, 1.25 U *Taq* DNA polymerase, and either 100 ng of purified *X. fastidiosa* genomic DNA in 4 μl of TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) or 4 μl of plant sample extract. Oligonucleotide primers, dNTPs, 10× amplification buffer, and enzyme were diluted in sterile distilled water on ice to prepare a mastermix solution for each PCR experiment. Individual reactions were assembled on ice by the addition of 46 μl of mastermix solution to 4 μl of sample in sterile 500-μl microcentrifuge tubes. Each reaction was overlayed with 50 μl of molecular biology-grade mineral oil (Sigma) before PCR.

In preliminary experiments, attempts were made to extract DNA suitable for PCR amplification from plant samples by direct boiling in sterile water, by lyse and boil methods (11,13), and by standard DNA isolation procedures (*1*). Subsequently, DNA was extracted from aliquots of the plant samples prepared for ELISA by the CTAB method with slight modification. Briefly, 400 μl of lysing solution (TAE buffer containing 0.75% sodium dodecyl sulfate and 0.15 mg of freshly dissolved Proteinase K per milliliter) was gently mixed into 200 μl of ELISA sample in 1.5-ml microcentrifuge tubes. The tubes were incubated at 37 C for 1.5 h. Next, 100 μl of 5 M NaCl and 80 μl of CTAB-NaCl solution (4.1% NaCl and 10% CTAB) prewarmed to 65 C were added. The tubes were inverted several times and incubated at 65 C for 10 min. Samples were extracted once with an equal volume of chloroform-isoamyl alcohol (24:1) followed by one extraction with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous solution by the addition of 0.6 volume of isopropanol and incubation of the tubes at −70 C for 15 min. The tubes were centrifuged for 20 min at room temperature, and the pellets were washed in 70% ethanol and recentrifuged for 15 min. The DNA pellets were redissolved in 50 μl of 1× PCR buffer containing 0.5% Tween 20 (Sigma), and the solutions were boiled for 10 min before samples were removed for PCR. DNA preparations were stable when stored at 4 C.

For PCR amplification experiments, a 733-bp region of *X. fastidiosa* genome was amplified with the primer set RST31/RST33. Template DNA was initially denatured in the thermocycler at 95 C for 1 min. This was followed by 40 PCR cycles of denaturation at 95 C for 30 s, primer annealing at 55 C for 30 s, and DNA extension at 72 C for 45 s. A final cycle included the denaturation and annealing steps followed by extension at 72 C for 5 min before cooling to 4 C. The PCR products were extracted once with 25 μl of chloroform-isoamyl alcohol before aliquots (typically 15 μl) containing gel tracking dye were added to wells of 1% agarose gels in TAE buffer (14). The gels were run at 5 V/cm for 1.5 h (5 cm) and then stained with ethidium bromide (10 mg/ml) for 20–30 min. The PCR products were photographed in gels on a UV transilluminator with Polaroid type 55 film (Polaroid Corp., Cambridge, MA).

Restriction endonuclease digestion of PCR products. Aliquots (5 μl) of individual PCR products were digested in 10 μl total volume reactions containing 3 μl of TAE buffer, 1 μl of enzyme (8–12 U), and 1 μl of 10× restriction buffer (supplied by the manufacturer, Promega or Sigma). Digestions were incubated at 25 or 37 C, dependent on the enzyme, for 2–15 h. The digestions were terminated by the addition of 1 μl each of 250 mM EDTA, pH 8.0, and gel tracking dye. Samples were heated to 65 C for 5 min and placed in ice before being loaded into wells in 3:1% NuSieve-GTG agarose gels (FMC BioProducts, Rockland, ME). Gels were run in TAE buffer at 5 V/cm for 2 h. The gels were stained for 30 min in ethidium bromide, destained in 1 mM $MgSO_4$ for 30 min, and then photographed. Enzymes used were *Alu*I, *Alw*I, *Cfo*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Hpa*II, *Pst*I, *Rsa*I, *Sau*3AI, *Spe*I, *Taq*I, *Tru*9I, and *Xba*I.

RESULTS

***X. fastidiosa*-specific probes.** Individual random *Bam*HI, *Eco*RI, and *Hin*dIII DNA digestion fragments were visually assessed as being present in genomic digests of 18 strains of *X. fastidiosa*. Several conserved fragments were cloned from Pierce's disease strain PCE-RR (ATCC 35879) and individually labeled for use as probes. A 7.4-kb *Eco*RI fragment (clone pX10-2) hybridized to all strains tested and was selected for further evaluation (Fig. 1). This fragment did not hybridize to genomic digests of *Agrobacterium tumefaciens, Erwinia stewartii, Pseudomonas solanacearum,* several *P. syringae* pathovars, saprophytic *Xanthomonas* sp., *X. maltophilia* (=*Stenotrophomonas maltophilia* [16]), *X. fragariae*, or various *X. campestris* pathovars.

Digestion of pX10-2 with *Eco*RV generated an internal 1-kb DNA fragment, which when labeled and used as a probe against *Eco*RV genomic digests of all strains of *X. fastidiosa,* hybridized without polymorphisms. On the basis of the conserved nature and suitable size for PCR amplification, this 1-kb fragment was sequenced to obtain potential primer oligonucleotide sequences.

Selection of PCR primers specific for *X. fastidiosa.* Initially, five oligonucleotide primers were synthesized, which would, in pair combinations, amplify three DNA regions of 600, 720, or 733 bp from the genome of *X. fastidiosa* (Fig. 3). The expected fragment sizes for each primer set were successfully amplified by PCR from 33 diverse strains of *X. fastidiosa.*

In analyses of the PCR products by restriction endonuclease digestion with 17 enzymes, only primer set RST31/RST33 (733 bp) generated products that allowed for the differentiation of the two pathotypes of *X. fastidiosa.* Digestion of the 733-bp PCR products with *Rsa*I followed by electrophoresis through NuSieve agarose gel differentiated strains of the Pierce's disease and CVC groups from strains of *X. fastidiosa* from other hosts (Fig. 4). Because of the possible usefulness of this differentiation in future epidemiological studies, primer set RST31/RST33 was used in plant sample detection experiments. No apparent PCR amplification of DNAs from a collection of several plant-pathogenic or saprophytic bacteria, as well as from noninfected plant extracts, occurred with these primers (Fig. 2).

PCR detection of *X. fastidiosa* added to plant extracts. In preliminary tests, known concentrations of viable *X. fastidiosa* cells were added to plant tissues, and several simple methods for preparing plant extracts for PCR amplification were evaluated. Direct boiling in water, lyse and boil methods, and standard DNA extraction methods were not successful. Dilution of the samples to concentrations of 1:100 or 1:1,000 in buffer or water prior

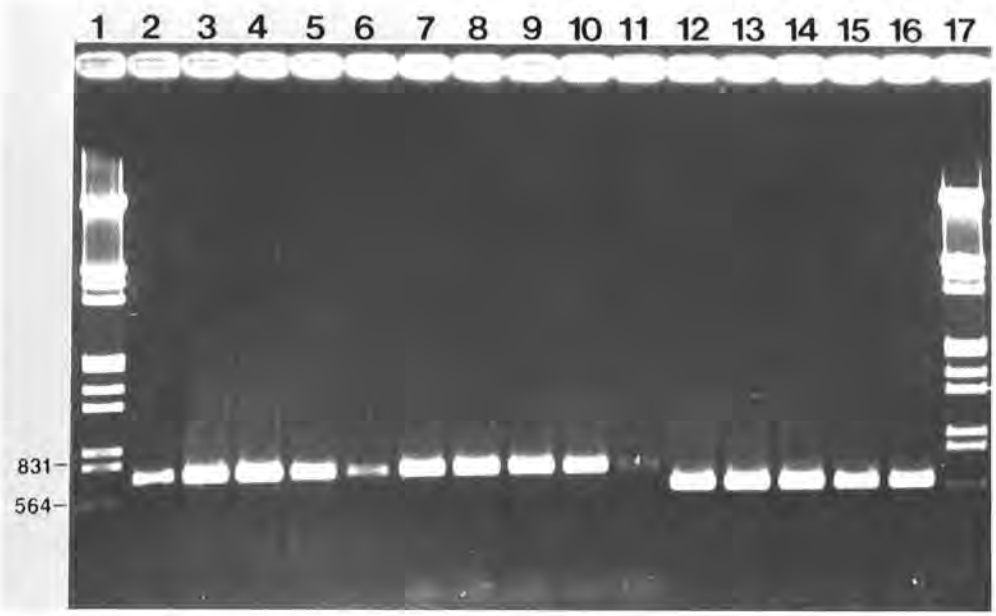


Fig. 3. Agarose gel electrophoresis of the products of the polymerase chain reaction amplification of DNA from various strains of *Xylella fastidiosa* with three primer sets. Lanes 2–6, primers RST29/RST30; lanes 7–11, primers RST31/RST33; and lanes 12–16, primers RST32/RST33. Target DNA (100 ng) sources: lanes 2, 7, and 12, Pierce's disease strain PCE-RR (ATCC 35879); lanes 3, 8, and 13, plum leaf scald strain PLM-G83 (ATCC 35871); lanes 4, 9, and 14, citrus variegated chlorosis strain IAPAR 10437; lanes 5, 10, and 15, oak leaf scorch strain OAK (ATCC 35874); and lanes 6, 11, and 16, sycamore leaf scorch strain SYC86-1. Lanes 1 and 17 contain 0.5 μg of *Eco*RI + *Hin*dIII-digested λ DNA. Molecular weights of markers are given in base pairs.

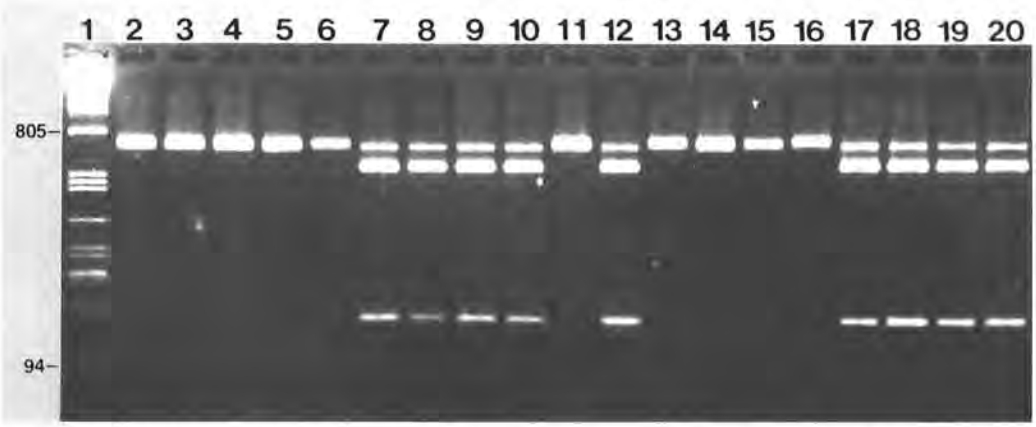


Fig. 4. NuSieve agarose gel electrophoresis of *Rsa*I restriction digestion fragments of the polymerase chain reaction products (primers RST31/RST33) from the DNA of strains of *Xylella fastidiosa* representing pathotype groups. Target DNA (100 ng) sources: lanes 2–6, Pierce's disease strains PCE-RR (ATCC 35879), PCE-FG (ATCC 35881), PD-4, PD92-4, and IBSBF 755; lanes 7 and 8, oak strains OAK (ATCC 35874) and 088-9; lanes 9 and 10, sycamore strains SYC86-1 and SYC89-A; lane 11, citrus blight strain CB-9; lane 12, goldenrod strain 83-G1; lanes 13–16, citrus variegated chlorosis strains IAPAR 9712, IAPAR 9713, IAPAR 10437, and IAPAR 10438; and lanes 17–20, plum strains PLM-G83 (ATCC 35871), IAPAR 9746, IAPAR 9748, and IAPAR Coeur de Lion. Lane 1 contains 3 μg of *Pst*I-digested λ DNA. Molecular weights of markers are given in base pairs.

to the DNA extraction procedure allowed for detection of *X. fastidiosa* by PCR.

To avoid the necessity for sample dilution, inhibitors of PCR were inactivated by the addition of acid-washed PVPP and sodium ascorbate to the SCP buffer (SCPAP) in which the tissue was ground as previously described (6,17). PCR amplification was achieved in the presence of undiluted plant tissue extracts (Fig. 5). This method was adopted in all further experiments.

In the preparation of vacuum extracts from trees, the most consistent PCR results were obtained when sodium ascorbate was added to the SCP buffer passed through the xylem vessels. PVPP was added to the extracts after passage of the buffer through the plant.

Sensitivity of PCR compared with ELISA for detection of *X. fastidiosa*. Replicated dilutions of suspensions of *X. fastidiosa* added to grape petiole extracts or citrus xylem extracts were tested for detection of the pathogen by ELISA and PCR amplification of DNA (Table 1 and Fig. 6). PCR amplification of DNA consistently detected populations of *X. fastidiosa* 100-fold lower than those detected by ELISA. The limit of detection with ELISA ranged from 2×10^4 to 1×10^5 cfu/ml, whereas 2×10^2 to 1×10^3 cfu/ml were detected by PCR amplification. The PCR method consistently detected *X. fastidiosa* in extracts from three naturally infected grapevine tissue sources as well as from artificially contaminated samples. In addition, the PCR method detected *X. fastidiosa* in seven of seven ELISA-positive, naturally infected citrus rootstocks sampled. Noninfected tissue samples used as negative controls during PCR experiments revealed no false positive results in our tests.

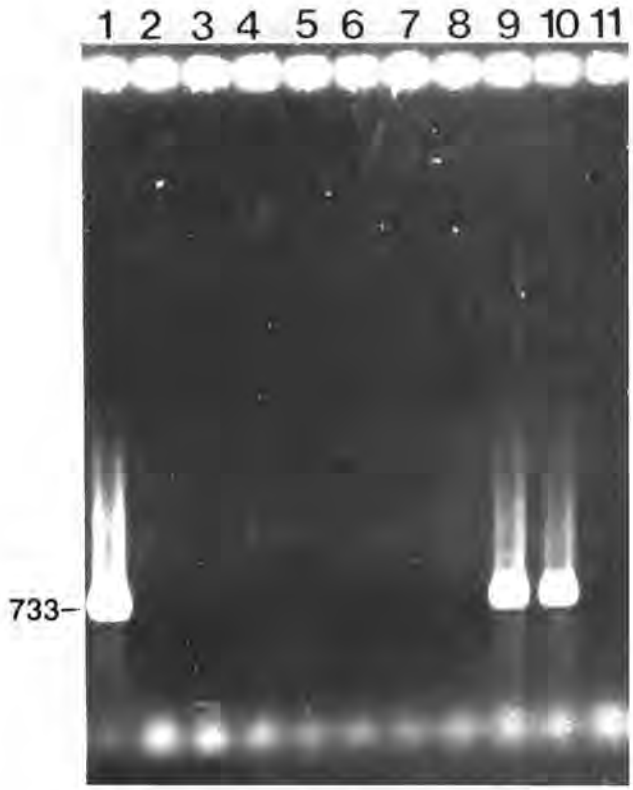


Fig. 5. Agarose gel electrophoresis of the products of the polymerase chain reaction (PCR) amplification (primers RST31/RST33) of DNA extracted from dilutions of *Xylella fastidiosa* present in undiluted noninfected grape tissue extracts. Target DNA sources: lane 1, 100 ng of genomic DNA from *X. fastidiosa* strain PCE-RR (ATCC 35879); lanes 2, 3, 7, and 8, noninfected plant extract without (lanes 2 and 3) or with (lanes 7 and 8) polyvinylpyrrolidone (PVPP) and sodium ascorbate; lanes 4–6, 10-fold dilutions of *X. fastidiosa* added to plant extract in succinate-citrate-phosphate (SCP) buffer without PVPP and sodium ascorbate; and lanes 9–11, 10-fold dilutions of *X. fastidiosa* added to plant extract in SCP buffer containing PVPP and sodium ascorbate. Lanes 4 and 9 correspond to a concentration of 1.8×10^4 cfu/ml as determined by dilution plating on PD3 medium. Molecular weight of PCR products is given in base pairs.

DISCUSSION

Oligonucleotide primers specific for *X. fastidiosa* and conserved in 33 strains isolated from various hosts were used in a PCR detection procedure that was more sensitive than ELISA. The procedure detected as few as 200 cfu/ml of sample. However, since DNA was extracted from only 200 μl of sample containing approximately 40 cfu and less than one-tenth of the extracted DNA was added to the PCR reaction mixture, the procedure actually allowed for the detection of 3–4 cfu. By comparison, a minimum of 20,000 cfu/ml of sample was required for a positive ELISA test with 200 μl of sample per well, which is equivalent to 4,000 cfu.

DNA from *X. fastidiosa* was not amplified from grape petiole extracts unless the plant extract was diluted 100-fold or more with buffer or water, evidence that phenolic compounds or polysaccharides from the grapevine tissue inhibited the PCR reaction (17). Since diluting the plant samples with water or buffer decreased sensitivity of the method, a way to inactivate or remove PCR inhibitors was needed. The addition of acid-washed PVPP and sodium ascorbate to the plant sample buffer was compatible with both methods of tissue extraction (tissue maceration and vacuum extraction of xylem vessels) commonly used for detection of *X. fastidiosa*. Insoluble PVPP present in the sample extracts was centrifuged from the aqueous phase to the interface layer at the chloroform extraction step during DNA isolation or was removed from microtiter plate wells in washing steps during ELISA and, therefore, did not pose any special handling problems during PCR and ELISA assays.

Restriction endonuclease digestion of the PCR amplification products with *Rsa*I allowed for the differentiation of two pathotypes of *X. fastidiosa*. This correlates with other studies that have shown that there are at least two pathotype groups of *X. fastidiosa*, the Pierce's disease group and the group causing other diseases, often referred to as the phony peach group (2,12). The phony peach group may consist of several pathotypes, although research has not conclusively differentiated them. The ability to distinguish the Pierce's disease strains by restriction enzyme digestion of PCR products will be a valuable tool in epidemiological studies of Pierce's disease of grapevine. Since the Pierce's disease strain is reported to have a very wide natural host range (3), the PCR procedure will be a convenient and rapid method of determining whether a strain of *X. fastidiosa* discovered in a new host is a Pierce's disease strain or possibly a new strain. Further research is needed in the evaluation of new primers that will allow for finer differentiation within and between the pathotypes of *X.*

TABLE 1. Comparison of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) amplification of DNA for the detection of *Xylella fastidiosa* in plant extracts

Sample	Minimum concentration detected (cfu/ml)[a]	
	ELISA	PCR
Experiment 1		
Noninfected grape petiole extract + *X. fastidiosa*	2.8×10^4	2.8×10^2
Infected grape petiole extract	6.1×10^4	6.1×10^2
Experiment 2		
Noninfected grape petiole extract + *X. fastidiosa*	1.8×10^5	1.8×10^3
Noninfected citrus xylem extract + *X. fastidiosa*	1.3×10^5	1.3×10^3

[a] Actual numbers of live bacteria in the samples were determined by dilution plating on PD3 medium. Data represent the lowest concentrations of bacteria in the 10-fold dilutions that were positive by the respective assays in replicated experiments.

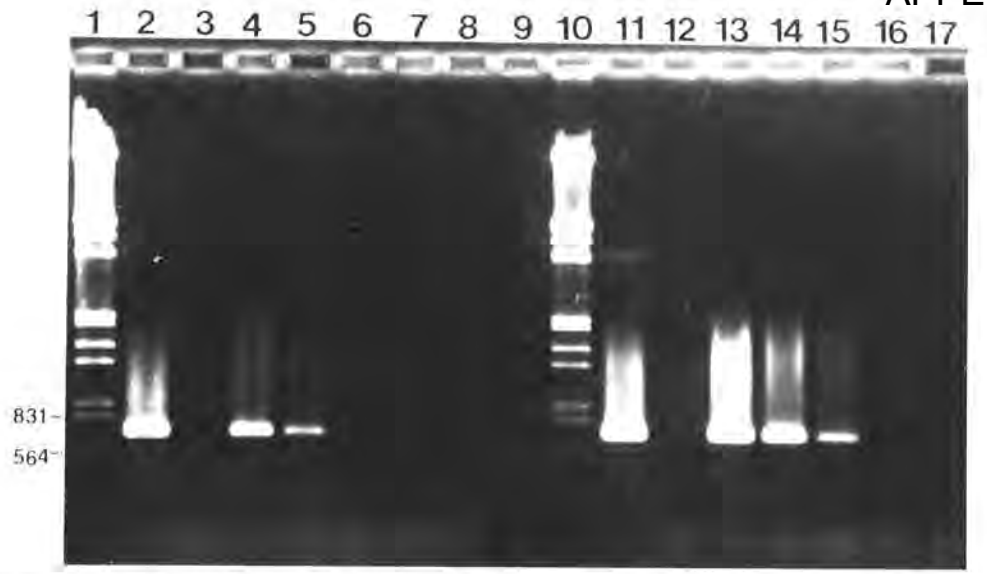


Fig. 6. Agarose gel electrophoresis of the products of the polymerase chain reaction amplification (primers RST31/RST33) of DNA extracted from dilutions of *Xylella fastidiosa* added to undiluted noninfected grape petiole extracts and citrus xylem vacuum extracts in succinate-citrate-phosphate buffer containing polyvinylpyrrolidone and sodium ascorbate. Target DNA sources: lanes 2 and 11, 100 ng of genomic DNA from *X. fastidiosa* strain PCE-RR (ATCC 35879); lane 3, noninfected grape petiole extract; lanes 4–8, 10-fold dilutions of *X. fastidiosa* added to grape petiole extract; lane 12, noninfected citrus xylem extract; and lanes 13–17, 10-fold dilutions of *X. fastidiosa* added to citrus xylem extracts. Lanes 4 and 13 correspond to 1.8×10^6 and 1.3×10^5 cfu/ml, respectively, as determined by dilution plating on PD3 medium. Lane 9 is empty. Lanes 1 and 10 contain 0.5 μg of *Eco*RI + *Hin*dIII-digested λ DNA. Molecular weights of markers are given in base pairs.

fastidiosa by PCR amplification or by restriction digestion of PCR amplification products.

Currently, there are no rapid, accurate diagnostic techniques available to determine whether or not a strain of the bacterium in a newly discovered host is a Pierce's disease strain, one of the other host strains, or an entirely new strain. The sensitivity of the PCR method for detecting *X. fastidiosa* in host tissue should make it useful for both research and disease diagnostic programs. The current method of choice for surveys or epidemiological studies involving large numbers of samples is ELISA. The concentration of bacteria in many hosts may be very low and at the limit, or below the limit, of detection with ELISA. Since PCR is at least 100-fold more sensitive than ELISA, the PCR protocol could be useful in research to identify alternate hosts of the Pierce's disease pathogen. In addition, the PCR protocol may be adapted for detection of *X. fastidiosa* in insect vectors.

LITERATURE CITED

1. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. Current Protocols in Molecular Biology. J. Wiley & Sons, New York.
2. Chen, J., Chang, C. J., Jarret, R. L., and Gawel, N. 1992. Genetic variation among *Xylella fastidiosa* strains. Phytopathology 82:973-977.
3. Freitag, J. H. 1951. Host range of the Pierce's disease virus of grapes as determined by insect transmission. Phytopathology 41:920-934.
4. French, W. J., Christie, R. G., and Stassi, D. L. 1977. Recovery of rickettsialike bacteria by vacuum infiltration of peach tissues affected with phony disease. Phytopathology 67:945-948.
5. Henson, J. M., and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol. 31:81-109.
6. Holben, W. E., Jansson, J. K., Chelm, B. K., and Tiedje, J. M. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. 54:703-711.
7. Hopkins, D. L. 1977. Disease caused by leafhopper-borne rickettsialike bacteria. Annu. Rev. Phytopathol. 17:277-294.
8. Hopkins, D. L. 1988. *Xylella fastidiosa* and other fastidious bacteria of uncertain affiliation. Pages 95-103 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN.
9. Hopkins, D. L. 1989. *Xylella fastidiosa:* Xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.
10. Hopkins, D. L., and Adlerz, W. C. 1988. Natural hosts of *Xylella fastidiosa* in Florida. Plant Dis. 72:429-431.
11. Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. 1990. PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.
12. Kamper, S. M., French, W. J., and DeKloet, S. R. 1985. Genetic relationships of some fastidious xylem-limited bacteria. Int. J. Syst. Bacteriol. 35:185-188.
13. Klimyuk, V. I., Carroll, B. J., Thomas, C. M., and Jones, J. D. G. 1993. Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant J. 3:493-494.
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. Nomé, S. F., Raju, B. C., Goheen, A. C., Nyland, G., and Docampo, D. 1980. Enzyme-linked immunosorbent assay for Pierce's disease bacteria in plant tissues. Phytopathology 70:746-749.
16. Palleroni, N. J., and Bradbury, J. F. 1993. *Stenotrophomonas,* a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings et al. 1983. Int. J. Syst. Bacteriol. 43:606-609.
17. Rowhani, A., Chay, C., Golino, D. A., and Falk, B. W. 1993. Development of a polymerase chain reaction technique for the detection of grapevine fanleaf virus in grapevine tissue. Phytopathology 83:749-753.
18. Sherald, J. L., and Kostka, S. J. 1992. Bacterial leaf scorch of landscape trees caused by *Xylella fastidiosa.* J. Arboric. 18:57-63.
19. Staskawicz, B. J., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea.* J. Bacteriol. 169:5789-5794.
20. Thuring, R. W. J., Sanders, J. P., and Borst, P. 1975. A freeze-squeeze method for recovering long DNA from agarose gels. Anal. Biochem. 66:213-220.
21. Wells, J. M., Raju, B. C., Hung, H-Y., Weisburg, W. G., Mandelco-Paul, L., and Brenner, D. J. 1987. *Xylella fastidiosa* gen. nov., sp. nov.: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Syst. Bacteriol. 37:136-143.

Techniques

Development of LAMP and Real-Time PCR Methods for the Rapid Detection of *Xylella fastidiosa* for Quarantine and Field Applications

S. J. Harper, L. I. Ward, and G. R. G. Clover

Plant Health and Environment Laboratory, Investigation and Diagnostic Centre, MAF Biosecurity New Zealand, P.O. Box 2095, Auckland 1140, New Zealand.
Accepted for publication 18 August 2010.

ABSTRACT

Harper, S. J., Ward, L. I., and Clover, G. R. G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. Phytopathology 100:1282-1288.

Xylella fastidiosa is a regulated plant pathogen in many parts of the world. To increase diagnostic capability of *X. fastidiosa* in the field, a loop-mediated isothermal amplification (LAMP) and real-time polymerase chain reaction (PCR) assay were developed to the *rimM* gene of *X. fastidiosa* and evaluated for specificity and sensitivity. Both assays were more robust than existing published assays for detection of *X. fastidiosa* when screened against 20 isolates representing the four major subgroups of the bacterium from a range of host species. No cross-reaction was observed with DNA from healthy hosts or other bacterial species. The LAMP and real-time assays could detect 250 and 10 copies of the *rimM* gene, respectively, and real-time sensitivity was comparable with an existing published real-time PCR assay. Hydroxynapthol blue was evaluated as an endpoint detection method for LAMP. When at least 500 copies of target template were present, there was a noticeable color change indicating the presence of the bacterium. Techniques suitable for DNA extraction from plant tissue in situ were compared with a standard silica-column-based laboratory extraction method. A portable PickPen and magnetic bead system could be used to successfully extract DNA from infected tissue and could be used in conjunction with LAMP in the field.

Additional keywords: diagnostics.

Xylella fastidiosa (39) is a bacterial plant pathogen that causes several economically important diseases, including Pierce's disease of grapevine, citrus veinal chlorosis, almond leaf scorch, phony peach, and leaf scorch on a range of ornamental plants and shade trees (10–12). *X. fastidiosa* is a regulated organism in many parts of the world. Leafhoppers of the subfamily *Cicadellinae* (*Hemiptera: Cicadellidae*) and spittle bugs or frog hoppers of the family *Cercopidae* (*Hemiptera*) are the most common known vectors (27). The distribution of *X. fastidiosa* is generally limited to the Americas (27), with two exceptions, in *Vitis vinifera* in Kosovo (1) and pear in Taiwan (18). It is thought that *X. fastidiosa* is sensitive to low temperatures, which has restricted its movement into regions with temperate climates and, in particular, cold winters (27). However, many colder parts of the world do possess one or more vector species, such as the spittlebug (*Philaenus spumarius*); therefore, the potential does exist for *X. fastidiosa* to spread into such areas should cold-tolerant strains, such as almond leaf scorch, become established (27). From a quarantine perspective, rapid detection and diagnosis is the key feature of any exclusion strategy.

Current assays for *X. fastidiosa* diagnosis include bacterial cell culture, conventional polymerase chain reaction (PCR) (12,13, 20,26,28), and real-time PCR (7,29). Although many of these methods have been used routinely in the laboratory, most of these methods are not easily transferable to the field. In addition, the PCR assay of Minsavage et al. (20) was developed over 15 years ago when there was little DNA sequence of *X. fastidiosa* available. This assay is commonly used for quarantine screening and, therefore, it is particularly important to verify that it detects all isolates of the bacterium reliably. In view of these factors, alternative methods of detection were considered.

One method that has been recently adopted for plant pathogen diagnostics is loop-mediated isothermal amplification (LAMP). Because the LAMP reaction is isothermal, it can be performed in a heat block or water bath, thereby removing the need for specialized equipment. In addition, positive amplification can be observed by colorimetric or fluorescent dyes (9,33), removing the need to run gels. Both of these factors contribute to transferability to the field.

Here, we present the development and evaluation of a LAMP assay for *X. fastidiosa* to improve diagnostic capability by enabling surveillance activities, improving response times during incursions, and allowing testing of imported commodities at the border. During the development of the LAMP assay, the potential arose to develop an alternative real-time TaqMan (Applied Biosystems, Foster City, CA) PCR based on detection of the same region used for the LAMP primer design. The new TaqMan (Applied Biosystems) real-time assay was also evaluated alongside the LAMP method.

MATERIALS AND METHODS

Samples. *X. fastidiosa* cultures were obtained from commercial (DSMZ, Mannheim, Germany) and academic (Landcare Research, Auckland, New Zealand) sources. Freeze-dried *X. fastidiosa*-infected samples of *V. vinifera*, *V. rotundifolia*, and *Quercus rubra* leaves and infected blue-green sharpshooters (*Graphocephala atropunctata*) were obtained from Dr. R. Almeida (University of California, Berkley) and C. Chang (University of Georgia, Griffin). DNA samples of *X. fastidiosa*, extracted from a range of host species, were obtained either on FTA cards (Whatman Inc., Florham Park, NJ) or lyophilized, from Dr. L. Nunney (University of California, Riverside), Dr. C. Su (Taiwan Agricul-

Corresponding author: L. I. Ward; E-mail address: lisa.ward@maf.govt nz

doi:10.1094/PHYTO-06-10-0168

tural Chemicals Toxic Substances Research Institute, Taichung, Taiwan), and Dr. H. Coletta Filho, (Centro de Citricultura, Cordieropolis, Brazil). *Spiroplasma citri* DNA was obtained from Dr. R. Yokomi (United States Department of Agriculture, Parlier, CA). Finally, DNA extracts of healthy host-plant species and nontarget bacterial species were obtained from the MAF Biosecurity New Zealand nucleic acid collection.

Sample DNA extraction. Plant samples (200 mg of leaf midrib and petiole) and whole insects were ground to a fine powder in liquid nitrogen prior to extraction using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) as per published protocols (13). Samples on FTA cards (Whatman Inc.) were eluted using the Sigma Extract-N-Amp Kit (Sigma-Aldrich, St. Louis) using the manufacturer's protocol. Lyophilized cell cultures were disrupted with a Roche MagNA Lyser instrument (Roche Applied Science, Auckland, New Zealand), then extracted using DNeasy Plant Mini Kit (Qiagen) as above.

Testing alternative DNA extraction methods for field use. Two alternative DNA extraction methods that could potentially be used in the field with minimal specialized equipment were tested: the Extract-N-Amp Kit (Sigma-Aldrich) using the manufacturer's protocol and the Bio-Nobile 8-M PickPen (Bio-Nobile, Turku, Finland) using Invimag Plant DNA KFmL Mini Kit reagents (Invitek, Berlin, Germany). Samples (200 mg) of infected lyophilized petiole and leaf midrib tissue were homogenized in 2 ml of lysis buffer P (Invitek) in sample extraction bags (BioReba, Basel, Switzerland) using a hand-roller. DNA extraction was then performed in a Nunc 96 DeepWell plate (Thermo-Fisher Scientific, Waltham, MA) using the PickPen to manipulate the magnetic beads. Briefly, 420 µl of homogenized plant sap was added to the first well with 20 µl of magnetic beads and 200 µl of binding buffer and mixed using the PickPen (Bio-Nobile) for 3 min. Beads were collected and transferred into 800 µl of wash buffer 1 for 2 min, followed by two washes of 2 min with 800 µl of wash buffer 2. DNA was eluted for 3 min in 100 µl of nuclease-free H_2O. DNA was stored at –80°C prior to use.

Gene target selection and primer design. Using the work of Doddapaneni et al. (5) as a starting point, potential gene targets within the *X. fastidiosa* genome were assessed on the basis of sequence conservation between isolates with an arbitrary threshold of >98% nucleotide identity, and significant sequence difference from related species in the family *Xanthomonadaceae*. In total, four candidate genes were selected for primer design from the data of Doddapaneni et al. (5) and an alignment of the extant genomes 9a5C, Temecula, M12, and M23 (National Center for Biotechnology Information GenBank accession numbers NC_002488, NC_004556, NC_010513, and NC_010577, respectively). These genes were annotated as per the genome of the 9a5c isolate: disulfide isomerase (XF_1834), the 16S rRNA processing protein *rimM* (XF_0108), a HicB-related protein (XF_1668), and a cell division protein (XF_0095). Two additional regions, citrate synthase *gltA* (XF_1535) (2) and a hypothetical protein (7) used in published assays for *X. fastidiosa* detection and diagnosis, were also selected.

All potential target regions were examined for primer design using the alignment of the *X. fastidiosa* genomes described above and the online PrimerExplorer V4 software (Eiken Chemical Co., Tokyo) with default program parameters. Viable primer sets were generated for three targets—disulfide isomerase, *rimM,* and *gltA*—initial testing of which (data not shown) led to the adoption of the *rimM* primer set (Table 1) for further development and testing. The two inner primers designed, XF-FIP and XF-BIP, were modified with a TTTT linker sequence between the sense and antisense sequences to ensure loop formation, and two complimentary loop primers, XF-LF and XF-LB, were designed to accelerate strand displacement and amplification (23).

Following the selection of the LAMP primers, a set of real-time PCR primers and the associated 6′-carboxyfluorescein/Black Hole Quencher-1-labeled (6′FAM/BHQ) TaqMan (Applied Biosystems) probe (Table 1) were also designed to the *rimM* open reading frame (ORF) using the online RealTimeDesign software (BioSearch Technologies, Novato, CA) with the default parameters.

Optimization of the rimM LAMP assay. The LAMP protocol was developed from the method described by Varga and James (37). To optimize the LAMP reaction, the concentrations of core reagents were tested as follows: $MgSO_4$ at 4 to 8 mM and betaine at 0.6 to 1 M. Trehalose was examined as an alternative to betaine (30) at a concentration of 0.2 to 1 M. Reaction temperatures of 62 to 65°C were tested, as were optimal reaction times of 45 to 90 min. The optimized *rimM* LAMP reaction was performed in a 25 µl reaction volume containing 1× ThermoPol buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100, pH 8.8) (New England Biolabs, Beverly, MA) with additional $MgSO_4$ to a total final concentration of 8 mM, 0.8 M Betaine, 1.4 mM each dNTP, 0.2 µM outer (XF-F3/XF-B3) primers, 0.8 µM loop (XF-LF/XF-LB) primers, and 1.6 µM inner primers (XF-FIP/XF-BIP), with eight units of *Bst* DNA polymerase (New England Biolabs) and 2 µl of total DNA extract (of 100 to 300 ng of total DNA, depending on sample type). The reaction was incubated at 65°C for 60 min, followed by a 2 min enzyme inactivation step at 80°C in an ABI 9700 thermocycler (Applied Biosystems). Successful amplification of *X. fastidiosa* DNA was confirmed by agarose gel electrophoresis of a 15 µl aliquot of the LAMP reaction. Target specificity of the LAMP assay was confirmed by sequencing of the major amplification product using the XF-LF and XF-LB primers using the Sanger method; sequencing was performed by Ecogene (Auckland, New Zealand).

Evaluation of hydroxynapthol blue. The addition of hydroxynapthol blue (HNB) (Sigma-Aldrich) as a colorimetric means of indicating positive reactions (9) was assessed. HNB was added at

TABLE 1. Primers designed for the amplification of the partial *rimM* open reading frame of *Xylella fastidiosa* by loop-mediated isothermal amplification (LAMP)[a] and real-time polymerase chain reaction[b]

Assay, primer	Sequence (5′–3′)	Binding site
qRT-PCR		
XF-F	CACGGCTGGTAACGGAAGA	106,620–106,602
XF-R	GGGTTGCGTGGTGAAATCAAG	106,550–106,570
XF-P	TCGCATCCCGTGGCTCAGTCC	106,601–106,584
LAMP		
XF-F3	CCGTTGGAAAACAGATGGGA	106,884–106,865
XF-B3	GAGACTGGCAAGCGTTTGA	106,676–106,694
XF-FIP	ACCCCGACGAGTATTACTGGGTTTTTCGCTACCGAGAACCACAC	106,788–106,862
XF-BIP	GCGCTGCGTGGCACATAGATTTTTGCAACCTTTCCTGGCATCAA	106,773–106,695
XF-LF	TGCAAGTACACACCCTTGAAG	106,824–106,844
XF-LB	TTCCGTACCACAGATCGCT	106,753–106,735

[a] LAMP primer binding sites are given for the genome of isolate 9a5C (GenBank accession no. NC_002488).
[b] Quantitative reverse-transcription polymerase chain reaction.

a concentration of 150 μM as described by Goto et al. (9) in a final reaction volume of 25 μl. The rate of color change from purple to blue was assessed from 45 to 75 min at 15-min intervals at 65°C using a dilution series of *X. fastidiosa* DNA extracted from lyophilized cultured cells (DSMZ), of 1,000, 500, 250, 125, and 10 copies per reaction diluted in clean (uninfected) *V. vinifera* total DNA extract; concentration was calculated using an estimated genome size of 2.5 MB with the formula copies per microliter = (concentration in nanograms × 6.023 × 10^{23})/(genome length × 1 × 10^9 × 650).

Real-time PCR optimization. The *X. fastidiosa rimM* real-time PCR assay designed in this study was optimized for primer and TaqMan (Applied Biosystems) probe concentration, Mg^{2+} and thermocycling conditions using Invitrogen quantitative (q)PCR Supermix-UDG (Invitrogen, Carlsbad CA), and a Bio-Rad CFX-96 gradient real-time thermocycler (Bio-Rad Laboratories, Hercules, CA). Final optimized reaction conditions were as follows: real-time PCR reactions were done in 20-μl reaction volumes containing 10 μl of 2× qPCR Supermix-UDG (Invitrogen) with a final concentration of 4 mM $MgCl_2$, 300 nM *X. fastidiosa* sense (XF-F) and antisense (XF-R) primers, 100 nM 6′FAM/BHQ-1-labeled XF-P probe, bovine serum albumin (BSA) at 300 ng/μl (Sigma-Aldrich), and 2 μl of total DNA template. Optimal thermocycling conditions were as follows: 50°C for 2 min and 94°C for 2 min, then 40 cycles of 94°C for 10 s and 62°C for 40 s. All samples were amplified in triplicate. Threshold values were applied automatically by the CFX Manager V1.6 software (Bio-Rad Laboratories). Reaction efficiency was calculated using the dilution series described above for HNB evaluation, with the formula $E = 10^{(-1/\text{slope})}$.

Comparison to extant methods. The sensitivity, specificity, and reliability of the *rimM* LAMP and real-time PCR assays were compared with published *X. fastidiosa* conventional PCR (20) and real-time PCR (7) assays. DNA from 20 isolates representing the four major subgroups of *X. fastidiosa* and the phylogenetically distinct pear leaf scorch isolate was tested with all assays, and positive amplification was determined by either the presence of a band of the expected size for LAMP and conventional PCR or a crossing threshold (Ct) value of <38 cycles for the real-time PCR assays. In addition, infected blue-green sharpshooters were also tested to determine whether the designed assays would amplify *X. fastidiosa* from the vector; healthy sharpshooters could not be sourced. Finally, DNA was extracted from cultures and plant and insect tissue as described above. DNA from nontarget bacterial species and healthy plant hosts were tested to check for cross-reactivity (Table 2). The sensitivity of each assay was determined using the dilution series described earlier. Confirmation of the copy numbers of the dilution series and an estimation of target concentration in the *X. fastidiosa*-positive sample extracts were obtained by absolute real-time PCR using the assay of Francis et al. (7) against a cloned DNA standard. Real-time PCR and LAMP assays were performed in triplicate, while conventional PCR was performed in duplicate.

Conventional PCR reactions were done in 20-μl reaction volumes containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 250 nM forward and reverse primers, 200 nM dNTPs, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen), and 2 μl of DNA template in an ABI 9700 thermocycler (Applied Biosystems). Cycling conditions consisted of 3 min at 94°C; followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s; with a final extension step of 5 min at 68°C. PCR products were visualized on a 1.5% agarose gel using a 15-μl aliquot of PCR reaction.

The real-time PCR assay of Francis et al. (7) was performed with a Bio-Rad CFX-96 thermocycler (Bio-Rad Laboratories), using a reaction volume of 20 μl containing 2× qPCR Supermix-UDG (Invitrogen) with a final concentration of 4.5 mM $MgCl_2$, 300 nM *X. fastidiosa* sense and antisense primers, 100 nM 6′FAM-labeled probe, BSA at 300 ng/μl, and 2 μl of DNA template. Thermocycling conditions were 50°C for 2 min and 94°C for 2 min, then 40 cycles of 94°C for 10 s and 60°C for 45 s. The optimized *rimM*-specific LAMP and real-time PCR assays were performed as described above.

RESULTS

Primer design. A conserved region of the *rimM* gene was selected for primer design to ensure consistent and reproducible amplification of *X. fastidiosa*. The other genes considered had unacceptable homology to non-target organisms in silico or were unable to support LAMP primer design. During the initial primer screening, LAMP primer sets for the disulfide isomerase and *gltA* failed to amplify DNA from many of the *X. fastidiosa* isolates tested and were therefore discarded (data not shown).

Optimization of the rimM LAMP assay. Optimization of the *rimM* LAMP assay indicated that high-performance liquid chromatography (HPLC) purification of the two large inner primers was necessary for reaction efficacy and sensitivity, as previously observed by Varga and James (37). The remaining outer and loop primers functioned with standard desalted purification. Trehalose was examined as an alternative to betaine in the reaction; however, it proved unable to support LAMP amplification and, therefore, was discarded (data not shown). Optimized reaction conditions are given in the Methods section.

Using a serial dilution of *X. fastidiosa* DNA (diluted in *V. vinifera* DNA extract) and varying the incubation time from 45 to 90 min, an incubation time of 60 min at 65°C was found to be sufficient to amplify all isolates tested and consistently amplify a dilution of 500 copies of template per reaction. Inconsistent amplification was sometimes observed with a starting dilution of 250 copies; however, increasing incubation to 75 min improved the reliability of DNA amplification (Table 3). Extending the incubation time past 75 min gave no further increase in sensitivity. Agarose gel electrophoresis of the *rimM* LAMP products produced a ladder pattern with a high-titer band of the major amplification product (180 bp) and several faint, larger products (Fig. 1). This banding pattern is typical when using HPLC-purified primers; use of non-HPLC-purified primers results in a lower titer of the main amplification product and more non-specific laddering (data not shown). Direct sequencing of the major amplification product using the loop primers indicated that the primers were specific to bases 106,752 to 106,862 of the *X. fastidiosa* isolate 9a5C genome (NC_002488), within the *rimM* gene as designed.

Evaluation of hydroxynapthol blue. A color change caused by successful amplification of *X. fastidiosa* in the presence of HNB dye was readily distinguishable, with a clear shift from purple to a light blue (Fig. 2). PCR products from the HNB reaction were also run out on a gel for comparison (data not shown). There was no noticeable inhibition caused by the presence of the dye for samples >500 copies of template per reaction, with a color shift being observed within 60 min. Lower concentrations of template, although producing the typical ladder pattern of successful amplification when examined by gel electrophoresis, did not trigger an identifiable color shift. The results showed that a minimum amount of template (250 to 500 copies) was required to trigger the color change.

Real-time PCR optimization. The real-time PCR assay targeting the *rimM* ORF was found to be robust over a range of annealing temperatures of 58 to 66°C, although the highest reaction efficiency (94.7%, $r^2 = 0.993$) was obtained with an annealing or extension stage of 62°C. The addition of BSA was necessary to reduce inhibition from grapevine leaf samples (data not shown). The detection limit was observed to be ≈10 copies/reaction but there was considerable variation (standard deviation > 1 cycle) between replicate Ct values for samples at this concen-

tration. A concentration of 125 copies per reaction was the minimum titer needed for consistent amplification (standard deviation < 0.5 cycle), with an average Ct value of 31.67 ± 0.39 cycles. The *X. fastidiosa rimM* real-time assay was comparable with the assay of Francis et al. (7) with regard to copy number detection but there was a decrease in Ct of 0.4 to 5.54 cycles for the *rimM* assay for many samples, suggesting that this target region amplifies more readily or that it is less susceptible to inhibitors present in extracts. The *rimM* assay also amplified two samples (Table 2) not detected by the assay of Francis et al. (7).

Comparison to extant methods. The specificity and sensitivity of the *rimM* LAMP and real-time assays compared favorably with the existing conventional (20) and real-time PCR (7) assays. Both the LAMP and *rimM* real-time assay amplified DNA from all 20 *X. fastidiosa* isolates tested. The pear leaf scorch isolate was not amplified by either assay. In comparison, the conventional PCR and Francis et al. (7) real-time assay amplified only 12 and 18 of the 20 *X. fastidiosa* isolates, respectively (Table 2). The pear leaf scorch isolate was amplified by the conventional PCR but not by the Francis et al. (7) real-time PCR. *X. fastidiosa* was successfully detected from infected insect vectors by all assays (Table 2). No amplification was detected from nontarget bacterial species or healthy host species by either the LAMP or real-time PCR assays developed in this study.

For the LAMP and *rimM* real-time assay, amplification of *X. fastidiosa* DNA was successfully achieved from total DNA extracted from a range of infected plant hosts, from infected insect vectors, and from cultured bacterial cells. Although there was no identifiable inhibition from host tissue, the target titer in these samples was well above the limit of detection, with between 4×10^3 and 2.4×10^5 copies/µl as determined by real-time PCR. For the LAMP assay (in the absence of the HNB dye), the limit of detection was ≈250 copies of template per reaction, compared with 10 copies for both real-time PCR assays and 500 copies for conventional PCR (Table 3); the conventional PCR results indicated a sensitivity to inhibitors present in *V. vinifera* extracts in particular, because it successfully amplified similar isolates from culture extracts but not from grape.

TABLE 2. Detection of *Xylella fastidiosa* from infected and healthy host plants, infected insects, and non-target bacterial species using the loop-mediated isothermal amplification (LAMP) and real-time and conventional polymerase chain reaction (PCR)

Bacterial species	Host plant or insect species	Country of origin	Isolate	Source[a]	*rimM*[b] LAMP	*rimM*[b] PCR[c]	*rimM* PCR[d]	Real-time PCR[e]
Xylella fastidiosa								
subsp. *fastidiosa*	*Vitis vinifera*	United States	DSMZ-10026	DSMZ, Germany	+	+	14.50 ± 0.05	15.03 ± 0.07
	V. vinifera	United States	PD0001	L. Nunney, UC Riverside	+	–	24.05 ± 0.05	25.76 ± 0.22
	V. vinifera	United States	PD0004	L. Nunney, UC Riverside	+	+	26.95 ± 0.21	25.76 ± 0.15
	V. vinifera	United States		R. Almeida, UC Berkley	+	–	19.84 ± 0.05	19.25 ± 0.05
	V. vinifera	United States		R. Almeida, UC Berkley	+	–	20.86 ± 0.01	20.46 ± 0.18
	Prunus dulcis	United States	ALS0005	L. Nunney, UC Riverside	+	+	26.20 ± 0.03	28.69 ± 0.05
	P. dulcis	United States	ALS0095	L. Nunney, UC Riverside	+	+	22.32 ± 0.08	25.54 ± 0.57
	P. dulcis	United States	ALS0096	L. Nunney, UC Riverside	+	+	23.26 ± 0.01	25.39 ± 0.00
	V. rotundifolia	United States		C. Chang, UG	+	-	24.54 ± 0.18	26.29 ± 0.67
	Graphocephala atropunctata	United States		R. Almeida, UC Berkley	+	+	30.52 ± 0.23	28.38 ± 0.10
subsp. *multiplex*	*P. salicina*	United States	ICMP-8375	ICMP, Auckland, New Zealand	+	+	17.34 ± 0.14	13.46 ± 0.25
	P. dulcis	United States	ICMP-6575	ICMP, Auckland, New Zealand	+	+	14.92 ± 0.17	14.98 ± 0.19
	P. dulcis	United States	ALS0003	L. Nunney, UC Riverside	+	+	25.51 ± 0.75	27.71 ± 0.06
	Quercus laevis	United States	OAK0023	L. Nunney, UC Riverside	+	–	25.92 ± 0.23	30.18 ± 0.27
	Q. rubra	United States	OAK0024	L. Nunney, UC Riverside	+	–	29.57 ± 0.11	…
	Q. rubra	United States		C. Chang, UG	+	–	17.82 ± 0.29	20.49 ± 0.00
	Liquidambar styraciflua	United States	LIQ0063	L. Nunney, UC Riverside	+	–	29.45 ± 0.06	…
subsp. s*andyi*	*Nerium oleander*	United States	OLS002	L. Nunney, UC Riverside	+	+	19.35 ± 0.04	22.63 ± 0.27
	N. oleander	United States	OLS008	L. Nunney, UC Riverside	+	+	21.45 ± 0.02	25.21 ± 0.63
	N. oleander	United States	OLS009	L. Nunney, UC Riverside	+	+	27.17 ± 0.22	33.01 ± 0.40
subsp. *pauca*	*Citrus* sp.	Brazil	9a5C	H. Coletta Filho, CDC Brazil	+	+	15.86 ± 0.19	16.04 ± 0.07
Unspecified subsp.	*Pyrus* sp.	Taiwan		C. Su, TACTRI, Taiwan	–	+	…	…
Xanthomonas axonopodis pv. *aurantifolii*	…	Brazil	ICMP 14285	ICMP, Auckland, New Zealand	–	–	…	…
X. campestris pv. *citri*	…	United States	ICMP 10012	ICMP, Auckland, New Zealand	–	–	…	…
	…	New Zealand	ICMP 24	ICMP, Auckland, New Zealand	–	–	…	…
X. arboricola pv. *fragariae*	…	Italy	ICMP 17064	ICMP, Auckland, New Zealand	–	–	…	…
Pseudomonas syringae pv. *persicae*	…	New Zealand	ICMP 7090	ICMP, Auckland, New Zealand	–	–	…	…
Pantoea agglomerans	…	New Zealand	…	MAF Collection, New Zealand	–	–	…	…
Agrobacterium tumefaciens	…	New Zealand	…	M. Pearson, Univ. Auckland	–	–	…	…
Spiroplasma citri	…	United States	…	R. Yokomi, USDA-ARS	–	–	…	…
Healthy host species	*V. vinifera*	New Zealand	…	MAF Collection, New Zealand	–	–	…	…
	V. rotundifolia	United States	…	C. Chang, UG	–	–	…	…
	P. persica	New Zealand	…	MAF Collection, New Zealand	–	–	…	…
	Citrus latifolia	New Zealand	…	MAF Collection	–	–	…	…

[a] UC = University of California, UG = University of Georgia, USDA-ARS = United States Department of Agriculture–Agricultural Research Service.
[b] LAMP detection: + = positive and – = negative.
[c] Minsavage et al. (20). Conventional PCR: + = positive and – = negative.
[d] *rimM* real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.
[e] Francis et al. (7) real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.

Testing alternative DNA extraction methods for field use. Two alternative DNA extraction methods for plant tissue were compared with the standard DNeasy (Qiagen) column-based method to establish whether extractions could be done in the field. DNA extracted from infected lyophilized tissue of *V. vinifera*, *V. rotundifolia*, and *Q. rubra* was tested using the LAMP and the *rimM* real-time PCR assays developed in this study. PCR competency was checked using a real-time internal control assay for the plant cytochrome oxidase (COX) gene (38); results are presented in Table 4. Neither the COX internal control or *X. fastidiosa* were amplified from DNA extracted using the Extract-N-Amp method (Sigma-Aldrich), whereas the PickPen/Invimag (Bio-Nobile/Invitek) protocol worked effectively. Samples amplified an average of 0.40 cycles earlier for COX and 6.45 cycles earlier for *X. fastidiosa* using the DNeasy (Qiagen) compared with the PickPen (Bio-Nobile) method, suggesting that, although field extraction by PickPen is possible, it may not be as effective as the column-based technique for recovering low titers of *X. fastidiosa* DNA.

DISCUSSION

From a quarantine perspective, the ability to exclude important regulated plant pathogens or undertake surveillance depends on rapid and reliable methods of detection. These methods must be easily transferable between laboratories and, if possible, be suitable for use in the field. The objective of this work was to develop such a diagnostic method for *X. fastidiosa*. LAMP (23, 24) seemed to be an appropriate method, because it has been used to amplify and detect plant-pathogenic bacteria, fungi, viruses, and nematodes (8,15,16,25,35,37) as well as human and animal pathogens (6,14). The reaction can potentially be performed in the field because minimal equipment is needed to run the reaction and positive reactions can be identified visually using colorimetric dyes (9).

Assay design made use of the recent advances in *X. fastidiosa* genomic information (4,5,36). The two assays developed, LAMP and real-time PCR, both target the 16S rRNA processing protein gene (*rimM*) that is conserved between all genomic sequences of *X. fastidiosa* but is sufficiently distant from related xanthomonad species. Many other regions targeted by extant PCR-based detection methods are strain or subspecies specific (2–4,12). In contrast to existing PCR assays, both the LAMP and the new real-time assay detected all 20 *X. fastidiosa* isolates tested, representing the four major subspecies. In addition, the assays were able to detect the bacterium from infected insect vectors. The pear leaf scorch isolate was not amplified by either real-time or LAMP assays, which is unsurprising given the genetic divergence between this and other extant *X. fastidiosa* strains (19) but, curiously, was amplified by the conventional PCR.

The conventional PCR (20) is still used routinely for quarantine purposes in several countries. However, the assay does not detect all isolates of *X. fastidiosa* and the sequences that have been published since its development should be incorporated into the design of new primers (5,36).

The LAMP assay is highly specific and shows greater sensitivity than conventional PCR. However, it is not as sensitive as real-time PCR, which is consistent among LAMP assays designed for plant bacterial and viral pathogens (8,16,25,33,37). The level of sensitivity (≈500 copies of template per reaction) obtained by LAMP is acceptable for first-instance screening, although with the caveat that samples of marginal titer or of poor DNA quality may be missed. Application of an internal control such as COX would reduce the likelihood of false-negative results caused by the latter. It was noted during assay development using *X. fastidiosa* DNA diluted in water versus dilution in healthy grapevine DNA that the LAMP assay was less sensitive to inhibition than conventional and real-time PCR (S. J. Harper, *unpublished*).

Both the LAMP and real-time PCR assays are rapid, being able to detect *X. fastidiosa* extracted from infected tissue using a simple magnetic-bead based method in ≈1 h, similar to that described previously for real-time PCR (29,32). The assays diverge considerably in equipment requirements. LAMP can be conducted in a water-bath or heat-block; although real-time PCR can be used in the field (34), this method requires an expensive specialized portable thermocycler. The LAMP method may be assessed using a range of endpoint detection methods, including magnesium pyrophosphate accumulation (22), colorimetric hydroxynapthol blue dye (9), fluorescent intercalating dyes such as SYBR Green (Molecular Probes, Inc., Eugene, OR) or PicoGreen (Molecular Probes, Inc.) (32), precipitation with cationic

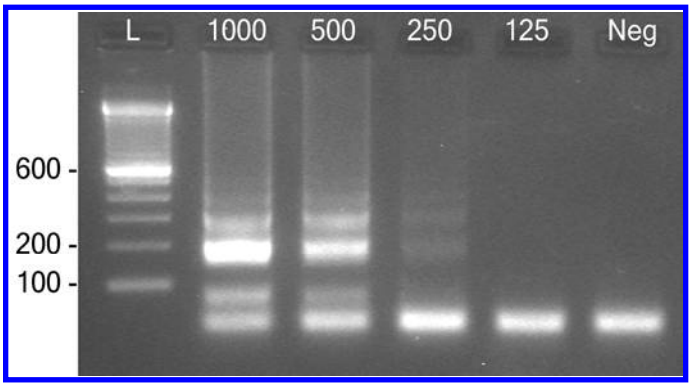


Fig. 1. Results of *rimM* loop-mediated isothermal amplification of serially diluted *Xylella fastidiosa* DNA (copy numbers of 1,000 to 125, diluted in healthy *Vitis vinifera* DNA extract) after 60 min of incubation at 65 C. Neg = healthy *V. vinifera* DNA extract and L = Invitrogen 100-bp ladder.

TABLE 3. Comparison of the sensitivity of the *Xylella fastidiosa rimM* loop-mediated isothermal amplification (LAMP) and real-time polymerase chain reaction (PCR) assays against published real-time and conventional PCR assays

	rimM LAMP[b]								
	45 min		60 min		75 min				
Copies[a]	Gel	HNB	Gel	HNB	Gel	HNB	*rimM* PCR[c]	Real-time PCR[d]	PCR[e]
1,000	+ – –	+ + –	+ + +	+ + +	+ + +	+ + +	28.61 ± 0.09	29.82 ± 0.13	+ +
500	–	–	+ + +	+ + –	+ + +	+ + +	29.64 ± 0.05	30.93 ± 0.07	+ +
250	–	–	+ + –	? – –	+ + +	+ + +	30.52 ± 0.19	31.84 ± 0.11	? –
125	–	–	–	–	+ ? ?	+ – –	31.67 ± 0.39	32.82 ± 0.14	–
10	–	–	–	–	–	–	35.77 ± 1.62	37.84 ± 0.32	–
Negative	–	–	–	–	–	–	–	–	–

[a] Copies per reaction.
[b] LAMP sensitivity: + = positive; – = negative; ? = weakly positive for hydroxynapthol blue (HNB).
[c] *rimM* real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.
[d] Francis et al. (7) real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.
[e] Minsavage et al. (20). Conventional PCR: + = positive; – = negative; and ? = weakly positive by gel electrophoresis.

polymers (21), lateral flow devices with labeled primers (35), and gel electrophoresis. Magnesium pyrophosphate detection can be difficult to determine visually (14), while the latter four methods require the tubes to be opened post amplification, yet opening the tubes leads to a risk of aerosol contamination due to the high titer of the LAMP amplicon (31). HNB allows visual detection and is added prior to amplification, which allows the reaction to be performed as a closed-tube system. During this study, HNB required at least 500 copies of target template to trigger color change within an hour; therefore, there is a risk that very low titers of *X. fastidiosa* may not be detected. Increasing the reaction time to 75 min did improve the likelihood of detecting lower concentrations of target DNA; however, reaction times >75 min did not improve the sensitivity of the assay.

A consideration for field-based detection with any assay is DNA extraction. Standard laboratory-based methods are not easily applied in the field due to the need for specialized equipment, and many of the field-based methods proposed are specific to each host–pathogen system (17,34). Being xylem-limited, *X. fastidiosa* presents a particular difficulty for field-based extraction because physical disruption of the tissue or extraction of sap is required (29). In this study, the Extract-N-Amp method which relies on thermal and chemical degradation failed to extract viable DNA, whereas homogenization with a hand-roller and DNA extraction using magnetic beads with a hand-held device (PickPen; Bio-Nobile) was sufficient to extract *X. fastidiosa* DNA from lyophilized samples. Only lyophilized tissue was available for this study; therefore, a comparison could not be made with the sap extraction protocol of Schaad et al. (29). Using the PickPen extraction, the titer of extracted *X. fastidiosa* DNA was, on average, 100-fold lower than in samples extracted using the Qiagen DNeasy system, as evidenced by lower Ct values for the Qiagen DNeasy extracts, although Ct values for the COX internal control were not markedly different between the extraction types. Such a loss of sensitivity may cause false-negative results for samples of marginal titer. Further extractions using fresh tissue may give a better indication of the performance of the PickPen method, and the use of additives to reduce the presence of inhibitors should be investigated.

Finally, the cost of each of the assays tested may be considered. The LAMP assay, with its requirement for specialized enzymes and reagents (especially dNTP usage and the need for HPLC-purified inner primers) costs ≈$5.30 USD (at time of writing) compared with ≈$1.00 USD for conventional and real-time PCR. However, this does not include the cost of specialized equipment such as real-time thermocyclers. The cost of LAMP may be a limitation for large-scale surveys and, for such applications, real-time PCR may be more cost effective. Despite this, LAMP offers a time-saving advantage if reactions are to be carried out in the field, and using LAMP in situ may reduce the need to move infected tissue across country for laboratory testing.

Both the LAMP and the *rimM* real-time PCR assays have a high level of specificity for the detection and diagnosis of the major subspecies of *X. fastidiosa*. Provided that care is taken to avoid contamination by using LAMP as a closed-tube assay (31) with colorimetric reporter dyes such as HNB, LAMP has the

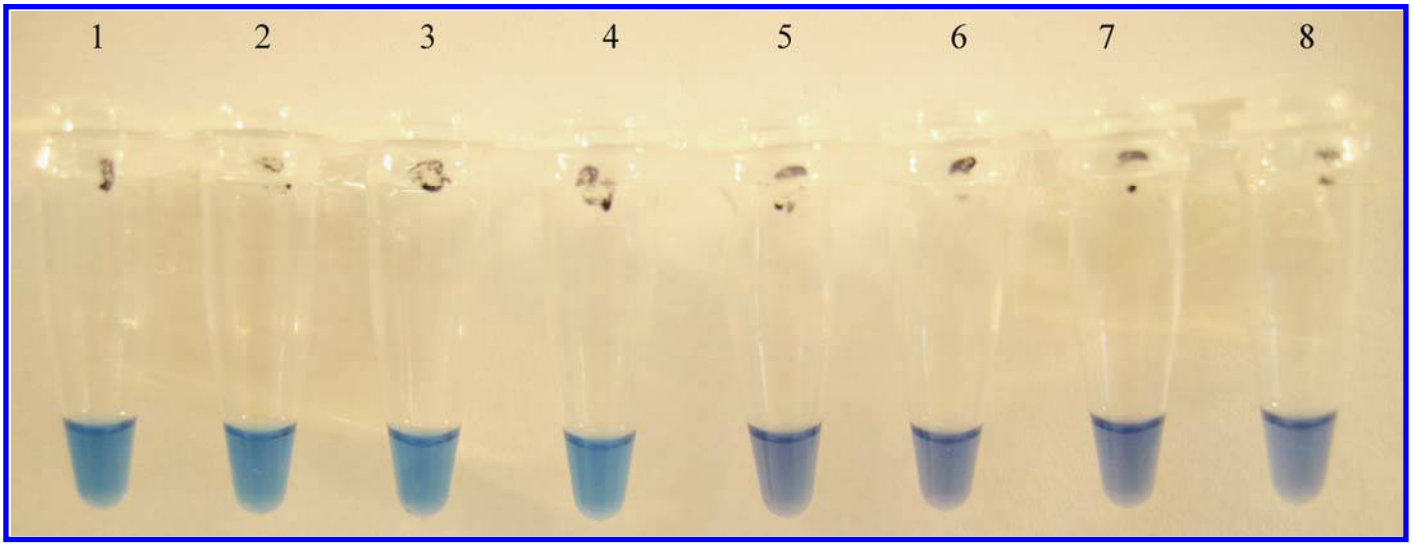


Fig. 2. Successful *rimM* loop-mediated isothermal amplification visualized using hydroxynapthal blue dye showing the sky-blue color change (tubes 1 to 4) observed with *Xylella fastidiosa*-positive samples. Negative samples in which no amplification occurred remain violet (tubes 5 to 8).

TABLE 4. Comparison of field-based extraction methods (BioNoble PickPen or Invimag DNA reagents and Sigma-Aldrich Extract-N-Amp) to the Qiagen DNeasy method, assessed using *Xylella fastidiosa rimM* loop-mediated isothermal amplification (LAMP) (+ = positive and – = negative) and real-time polymerase chain reaction (PCR) assays and a cytochrome oxidase (COX) internal control assay to show PCR competency

		Test		
Source of material	Extraction method	LAMP	Real-time PCR[a]	COX[a]
Vitis vinifera cv. Cabernet Sauvignon	DNeasy	+	20.86 ± 0.01	16.63 ± 0.16
	PickPen w/Invimag Kit	+	28.45 ± 0.11	20.00 ± 0.49
	Sigma Extract n' Amp	–	…	…
V. rotundifolia	DNeasy	+	24.54 ± 0.18	21.23 ± 0.22
	PickPen w/Invimag Kit	+	30.19 ± 0.03	20.83 ± 0.03
	Sigma Extract n' Amp	–	…	…
Quercus rubra	DNeasy	+	17.82 ± 0.29	17.59 ± 0.03
	PickPen w/Invimag Kit	+	22.39 ± 0.22	16.17 ± 0.53
	Sigma Extract n' Amp	–	…	…

[a] Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.

potential to be used in the field. Initial work comparing extraction methods suggests that there is a suitable extraction method that can be used alongside LAMP in situ. The method described here should be readily transferable to other laboratories due to the fact that expensive specialized equipment is not required. It is estimated that a reasonable number of samples (>50) could be processed or screened within 2 h in the field.

ACKNOWLEDGMENTS

We thank C. Chang, R. Almeida, and L. Nunney for providing *X. fastidiosa* samples; N. Boonham and J. Tomlinson (Food and Environment Research Agency, York, UK) for help and assistance in developing the LAMP assay and the PickPen extraction method; and MAF Biosecurity New Zealand Operational Research for funding this work.

LITERATURE CITED

1. Berisha, B., Chen, Y. D., Zhang, G. Y., Xu, B. Y., and Chen, T. A. 1998. Isolation of Pierce's disease bacteria from grapevines in Europe. Eur. J. Plant Pathol. 104:427-433.
2. Blexine, B., and Child, B. 2007. *Xylella fastidiosa* genotype differentiation by SYBR green-based qRT-PCR. FEMS Microbiol. Lett.276:48-54.
3. Chen, J., Banks, D., Jarret, R. L., Chang, C. J., and Smith, B. J. 2000. Use of 16S rDNA sequences as signature characters to identify *Xylella fastidiosa*. Curr. Microbiol. 40:29-33.
4. Chen, J., Civolero, E., Tubajika, K., Livingston, S., and Higbee, B. 2008. Hypervariations of a protease-encoding gene, PD0218 (*pspB*), in *Xylella fastidiosa* strains causing Almond leaf scorch and Pierce's disease in California. Appl. Environ. Microbiol. 74:3652-3657.
5. Doddapaneni, H., Yao, J., Lin, H., Walker, M. A., and Civolero, E. L. 2006. Analysis of the genome-wide variations among multiple strains of the plant pathogenic bacterium *Xylella fastidiosa*. BMC Genomics 7:225-240.
6. Dukes, J. P., King, D. P., and Alexandersen, S. 2006. Novel reverse-transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Arch. Virol. 151:1093-1106.
7. Francis, M., Lin H., Cabrera-La Rosa J., Doddapaneni H., and E. L., C. 2006. Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. Eur. J. Plant Pathol. 115:203-213.
8. Fukuta, S., Mizikami, Y., Ishida, A., Ueda, J., Kanabe, M., and Ishimoto, Y. 2003. Detection of *Japanese yam mosaic virus* by RT-LAMP. Arch. Virol. 148:1713-1720.
9. Goto, M., Honda, E., Ogura, A., Nomoto, A., and Hanaki, K. 2009. Colormetric detection of loop mediated isothermal amplification reaction by using hydroxy napthol blue. Biotechnqiues 46:167-172.
10. Hendson, M., Purcell, A. H., Chen, D., Smart, C., Guilhabert, M., and Kirkpatrick, B. 2001. Genetic diversity of Pierce's disease strain and other pathotypes of *Xylella fastidiosa*. Appl. Environ. Microbiol. 67:895-903.
11. Hopkins, D. L., and Purcell, A. H. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis. 86:1056-1066.
12. Huang, Q. 2009. Specific detection and identification of *Xylella fastidiosa* strains causing oleander leaf scorch using polymerase chain reaction. Curr. Microbiol. 58:393-398.
13. Huang, Q., Bentz, J., and Sherald, J. L. 2006. Fast, easy and efficient DNA extraction and one-step polymerase chain reaction for the detection of *Xylella fastidiosa* in potential insect vectors. J. Plant Pathol. 88:77-81.
14. Iwamoto, T., Sonobe, T., and Hayashi, K. 2003. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin. Microbiol. 41:2616-2622.
15. Kikuchi, T., Aikawa, T., Oeda, Y., Karim, N., and Kanzaki, N. 2009. A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. Phytopathology 99:1365-1369.
16. Kubota, R., Vine, B. G., Alvarez, A. M., and Jenkins, D. M. 2008. Detection of *Ralstonia solanacaerum* by loop-mediated isothermal amplification. Phytopathology 98:1045-1051.
17. Kuske, C. R., Banton, K. L., Adorada, D. L., Stark, P. C., Hill, K. K., and Jackson, P. J. 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol. 64:2463-2472.
18. Leu, L. S., and Su, C. C. 1993. Isolation, cultivation, and pathogenicity of *Xylella fastidiosa*, the causal bacterium of pear leaf scorch disease in Taiwan. Plant Dis. 77:642-646.
19. Mehta, A., and Rosato, Y. 2001. Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts, based on 16S rDNA and 16S–23S intergenic spacer sequences. Int. J. Syst. Evol. Microbiol. 51:311-318.
20. Minsavage, G. V., Thompson, C. M., Hopkins, D. L., Leite, R. M. V. B. C., and Stall, R. E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84:456-461.
21. Mori, Y., Hirano, T., and Notomi, T. 2006. Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnol. 6:3.
22. Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289:150-154.
23. Nagamine, K., Hase, T., and Notomi, T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16:223-229.
24. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28:E63.
25. Okuda, M., Matsumoto, M., Takana, Y., Subandiah, S., and Iwanami, T. 2005. Characterization of the *tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*B gene cluster of the citrus greening organism and detection by loop-mediated isothermal amplification. Plant Dis. 89:705-711.
26. Pooler, M. R., and Hartung, J. S. 1995. Genetic relationships among strains of *Xylella fastidiosa* from RAPD-PCR data. Curr. Microbiol. 31:134-137.
27. Purcell, A. H. 1997. *Xylella fastidiosa*, a regional problem or global threat? J. Plant Pathol. 79:99-105.
28. Rodrigues, J. L. M., Silva-Stenico, M. E., Gomes, J. E., Lopes, J. R. S., and Tsai, S. M. 2003. Detection and diversity assessment of *Xylella fastidiosa* in field-collected plant and insect samples by using 16S rRNA and *gyrB* sequences. Appl. Environ. Microbiol. 69:4249-4255.
29. Schaad, N. W., Opgenorth, D., and Gaush, P. 2002. Real-time polymerase chain reaction for one-hour on-site diagnosis of Pierce's disease of grape in early season asymptomatic vines. Phytopathology 92:721-728.
30. Speiss, A. N., Mueller, N., and Ivell, R. 2004. Trehalose is a potent PCR enhancer: Lowering of DNA melting temperature and thermal stabilization of *Taq* polymerase by the disaccharide trehalose. Clin. Chem. 50:1256-1259.
31. Tomita, N., Mori, Y., Kanda, H., and Notomi, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protocols 3:877-882.
32. Tomlinson, J., Barker, I., and Boonham, N. 2007. Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. Appl. Environ. Microbiol. 73:4040-4047.
33. Tomlinson, J., and Boonham, N. 2008. Potential of LAMP for detection of plant pathogens. Commonw. Agric. Bur. Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 3:1-7.
34. Tomlinson, J. A., Boonham, N., Hughes, K. J. D., Griffin, R. L., and Barker, I. 2005. On-site DNA extraction and real-time PCR for detection of *Phytophthora ramorum* in the field. Appl. Environ. Microbiol. 71:6702-6710.
35. Tomlinson, J. A., Dickinson, M. J., and Boonham, N. 2010. Rapid Detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. Phytopathology 100:143-149.
36. Van Sluys, M. A., de Oliveira, M. C., Monteiro-Vitorello, C. B., Miyaki, C. Y., Furlan, I. R., Camargo, L. E. A., da Silva, A. C. R., Moon, D. H., Takita, M. A., Lemos, E. G. M., Machado, M. A., Ferro, M. I. T., da Silva, F. R., Goldman, M. H. S., Goldman, G. H., Lemos, M. V. F., El-Dorry, H., Tsai, S. M., Carrer, H., Carraro, D. M., de Oliveira, R. C., Nunes, L. R., Siqueira, W. J., Coutinho, L. L., Kimura, E. T., Ferro, E. S., Harakava, R., Kuramae, E. E., Marino, C. L., Giglioti, E., Aberu, I. L., Alves, L. M. C., do Amaral, A. M., Baia, G. S., Blanco, S. R., Brito, M. S., Cannavan, S., Celestino, A. V., da Cunha, A. F., Fenille, R. C., Ferro, J. A., Formighieri, E. F., Kishi, L. T., Leoni, S. G., Oliveira, A. R., Rosa, V. E., Sassaki, F. T., Sena, J. A. D., de Souza, A. A., Truffi, D., Tsukumo, F., Yanai, G. M., Zaros, L. G., Civolero, E. L , Simpson, A. J. G., Alameida, N. F., Setubal, J. C., and Kitajima, J. P. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa.* J. Bacteriol. 185:1018-1026.
37. Varga, A., and James, D. 2006. Use of reverse transcription loop-mediated isothermal amplification for the detection of *Plum pox virus*. J. Virol. Methods 138:184-190.
38. Weller, S. A., Elphinstone, J. G., Smith, N. C., Boonham, N., and Stead, D. E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl. Environ. Microbiol. 66:2853-2858.
39. Wells, J. M., Raju, B. C., Hung, H. Y., Weisberg, W. G., Mandelco-Paul, L., and Brenner, D. J. 1987. *Xylella fastidiosa* gen. nov., sp. nov: gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Syst. Bacteriol. 37:136-143.

Erratum

The probe sequence was corrected on page 1283, Table 1, to TCGCATCCCGTGGCTCAGTCC. Changes to this article were made on May 16, 2013.

State of Hawai'i
Department of Agriculture
Plant Industry Division
Pesticide Branch

March 15, 2021

Board of Agriculture
Honolulu, Hawai'i

Subject: Request that the Final Order for In the Matter of SAFEWAY, INC., Docket No. 19-PE-029, be Referred to the Attorney General for Collection.

PROCEDURAL BACKGROUND:
On July 13, 2020, the Pesticides Branch of the Hawai'i Department of Agriculture ("HDOA"), Plant Industry Division issued a Notice of Finding of Violation and Proposed Order to Pay Civil Penalty ("NOV") under Docket No. 19-PE-029 to SAFEWAY, INC. ("Respondent"). The NOV was based upon inspections conducted on April 30, 2019 and May 1, 2019, pursuant to the authority granted in Hawai'i Revised Statutes ("HRS") section 149A-36 for purposes of determining compliance with state and federal pesticide laws. The NOV stipulated a civil penalty of five hundred dollars ($500.00). On July 20, 2020, the NOV was served on the agent for Respondent. A copy of the NOV is attached hereto as "Exhibit A".

Pursuant to HRS section 149A-41(b)(3), Respondent was entitled to a hearing to contest the NOV if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the NOV. The NOV clearly advised Respondent that the NOV would become a FINAL ORDER unless Respondent filed a written request for hearing within twenty (20) calendar days.

The twenty (20) day time period expired August 9, 2020, and no request for a hearing was received by the Office of the Chairperson of the Department of Agriculture by that time. Accordingly, Respondent waived the opportunity to challenge the finding of violation and the NOV became a FINAL ORDER.

On October 7, 2020, HDOA issued the Final Order to Respondent. The Final Order required the Respondent to submit the civil penalty of five hundred dollars ($500.00) within twenty (20) calendar days from the receipt of the Final Order, or HDOA would refer the matter to the Hawai'i Department of the Attorney General for collection. The agent for

C1

C2

Request that the Final Order for In the Matter of SAFEWAY, INC., Docket No. 19-PE-029, be Referred to the Attorney General for Collection

Respondent was served with the Final Order on October 14, 2020. A copy of the Final Order is attached hereto as "Exhibit B".

On December 8, 2020, HDOA issued a Demand Letter to Respondent. The Demand Letter stated that Respondent failed to abide by the terms and conditions of the Final Order. The Demand Letter required Respondent to submit the civil penalty of $500.00 by December 23, 2020, or HDOA would refer the case to the Hawai'i Department of the Attorney General for collection. As of today's date, Respondent has failed to submit the civil penalty of $500.00 to HDOA. A copy of the Demand Letter is attached hereto as "Exhibit C".

AUTHORITY: Pursuant to section 149A-41(b)(4), *Hawai'i Revised Statutes* ("HRS"), in case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the Board may determine, the Board shall refer the matter to the Attorney General, who shall recover the amount by action in the appropriate court.

RECOMMENDATION: The Pesticides Branch of HDOA Plant Industry Division recommends the Board refer the Final Order for In the Matter of SAFEWAY, INC., Docket No. 19-PE-029, to the Attorney General for collection.

It is respectfully requested that the Board follow the recommendation set forth above.

Submitted by:

GREG TAKESHIMA
Acting Pesticides Branch Manager

CONCURRED:

BECKY AZAMA
Acting Administrator, Plant Industry

Attachments – Exhibits "A" through "C"

Request that the Final Order for In the Matter of SAFEWAY, INC., Docket No. 19-PE-029, be Referred to the Attorney General for Collection

APPROVED FOR SUBMISSION:

Phyllis Shimabukuro-Geiser

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

C3

C4

Exhibit A

STATE OF HAWAI'I

DEPARTMENT OF AGRICULTURE

IN THE MATTER OF **SAFEWAY INC.** **Respondent.**	**DOCKET NO. 19-PE-029** **NOTICE OF FINDING OF VIOLATION; AUTHORITY; ALLEGED VIOLATION; CAUSE OF ACTION; ORDER TO CEASE AND DESIST; PROPOSED ORDER TO PAY CIVIL PENALTY; OPPORTUNITY FOR HEARING**

NOTICE OF FINDING OF VIOLATION

NOTICE IS HEREBY GIVEN that inspections conducted by the State of Hawai'i Department of Agriculture yielded evidence of a violation of sections 149A-11(a)(2) and 149A-15(7) of the *Hawai'i Revised Statutes* ("HRS").

AUTHORITY

The State of Hawai'i Department of Agriculture ("HDOA"), pursuant to the authority granted in HRS section 149A-41, and sections 4-66-3, 4-66-43 and 4-66-66.1 of the *Hawai'i Administrative Rules* ("HAR"), does hereby bring this action against Respondent SAFEWAY INC. for violation of HRS sections 149A-11(a)(2) and 149A-15(7). The inspections were conducted pursuant to the authority granted in HRS section 149A-36 and HAR section 4-66-43(a) for purposes of determining compliance with state and federal pesticide laws.

ALLEGED VIOLATION

1. On or about April 30, 2019, Hawai'i Department of Agriculture Environmental Health Specialist Paul De Filippi ("HDOA Inspector") conducted a Marketplace Surveillance Inspection of the retail establishment SAFEWAY INC. ("Respondent"), located at 277 Pikea Avenue, Kihei, Hawai'i 96753 ("Store").

2. The HDOA Inspector met with the Grocery Manager employed by Respondent and issued him a Notice of Inspection pursuant to HRS section 149A-36.

3. The HDOA Inspector reviewed approximately ninety-five (95) purportedly licensed general use pesticide products. The pesticide products were being offered for sale or distribution at the Store and were readily available for purchase by members of the public.

4. The HDOA Inspector recorded the name and Environmental Protection Agency ("EPA") Registration Number of each pesticide product being offered for sale at the Store.

5. The HDOA Inspector documented that individual containers of LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE (EPA Reg. No. 777-128) did not have the EPA registration number and EPA establishment number located in a conspicuous place on the outer container label.

6. The HDOA Inspector collected documentary samples of the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE.

7. On or about May 1, 2019, the HDOA Inspector returned to the Store and met with Respondent's Store Director to continue the inspection. The HDOA Inspector issued Respondent's Store Director a Notice of Inspection.

8. A Receipt for Samples form was issued to Respondent's Store Director for the documentary samples collected on or about April 30, 2019. Respondent's Store Director informed the HDOA Inspector that the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE had been removed from sale.

9. A Dealer's Statement was collected from Respondent's Store Director by the HDOA Inspector. On the Dealer's Statement, Respondent's Store Director claimed that the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE was received from Safeway Distribution, located at 16900 W. Schulte Road, Tracy, California 95377 at an unspecified date.

10. Sales records or shipping invoices for the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE were not provided to the HDOA Inspector.

C6

11. A product is "misbranded" if, among other things, the product "label does not bear the federal registration number assigned to each establishment in which it was produced;" pursuant to HRS section 149A-2.

12. HRS section 149A-11 states: "**Prohibited acts**. (a) Except as other wise exempted in section 149A-12, it shall be unlawful for any person to distribute, solicit, sell, offer for sale, hold for sale, transport, deliver for transportation, or receive and having so received, deliver or offer to deliver to any person in intrastate commerce or between points within this State through any point outside this State any of the following: . . . (2) Any pesticide unless it is in the licensee's or the manufacturer's unbroken immediate container, and there is affixed to the container and to the outside container or wrapper of the retail package, if any, through which the required information on the immediate container cannot be clearly read, a label bearing information pursuant to section 149A-15. . ."

(Emphasis added.)

13. HRS section 149A-15 states: "**Labeling requirements**. Each container of pesticides shall bear or have attached in a conspicuous place, a plainly written or printed label in the English language providing the following information: . . . (7) The EPA registration and establishment numbers. . ."

(Emphasis added.)

14. HDOA has previously initiated enforcement action against Respondent by way of Warning Notices dated June 26, 2017 and October 25, 2018, and a Notice of Violation under Docket No. 16-PE-003, making this a subsequent offense subject to increased penalties.

CAUSE OF ACTION

VIOLATION ONE:

HRS section 149A-11(a)(2) provides in pertinent part as follows:

"**Prohibited acts.** (a) Except as otherwise exempted in section 149A-12, it shall be unlawful for any person to distribute, solicit, sell, offer for sale, hold for sale, transport, deliver for transportation, or receive and having so received, deliver or offer to deliver to any person in intrastate commerce or between points within this State through any point outside this State any of the following:

> (2) Any pesticide unless it is in the licensee's or the manufacturer's unbroken immediate container, and there is affixed to the container and to the outside container or wrapper of the retail package, if any, through which the required information on the immediate container cannot be clearly read, a label bearing information pursuant to section 149A-15. . ."

(Emphasis added.)

HRS section 149A-15 provides in pertinent part as follows:

> **"Labeling requirements.** Each container of pesticides shall bear or have attached in a conspicuous place, a plainly written or printed label in the English language providing the following information: . . . (7) The EPA registration and establishment numbers. . ."

(Emphasis added.)

On or about April 30, 2019, Respondent SAFEWAY INC., located at 277 Pikea Avenue, Kihei, Hawai'i 96753, violated HRS sections 149A-11(a)(2) and 149A-15(7) by distributing, selling, offering for sale, holding for sale, transporting, delivering for transportation or receiving and having so received, deliver or offer to deliver to any person within the State of Hawai'i any pesticide that did not bear the EPA registration and establishments numbers in a conspicuous place as required by HRS sections 149A-11(a)(2) and 149A-15(7), to wit:

> LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE
> (EPA Reg. No. 777-128)

Respondent SAFEWAY INC. has received Warning Notices dated June 26, 2017 and October 25, 2018, and a Notice of Violation under Docket No. 16-PE-003, making this a subsequent violation subject to increased penalties.

Pursuant to HRS section 149A-41(b)(1), a retailer who violates any provision of HRS Chapter 149A may be assessed an administrative penalty of not more than Five Thousand Dollars ($5,000.00) for each offense.

ORDER TO CEASE AND DESIST

The State of Hawai'i Department of Agriculture, pursuant to the provisions of HAR section 4-66-3, **DOES HEREBY ORDER RESPONDENT TO CEASE AND DESIST**

C7

VIOLATION OF HRS CHAPTER 149A. You are hereby notified that any further violation of HRS Chapter 149A will result in increased penalties as provided by law.

YOU ARE SO NOTIFIED.

PROPOSED ORDER TO PAY CIVIL PENALTY

THE STATE OF HAWAI'I DEPARTMENT OF AGRICULTURE finds and concludes that the Respondent's actions, as set forth above, have violated HRS sections 149A-11(a)(2) and 149A-15(7).

HAVING VIOLATED the Hawai'i Pesticides Law as set forth in HRS Chapter 149A, RESPONDENT IS HEREBY ORDERED TO PAY the following civil penalty in accordance with HRS section 149A-41(b)(1) and HAR section 4-66-66.1:

Violation One: Five Hundred Dollars ($500.00) per misbranded product

TOTAL CIVIL PENALTY: Five Hundred Dollars ($500.00).

The civil penalty shall be paid within twenty (20) business days from the date of this Notice of Finding of Violation by delivering payment to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814

The Department of Agriculture may use all reasonable means to collect the full amount of the penalty, if not paid within the specified time period, as authorized by law.

OPPORTUNITY FOR HEARING

Pursuant to HRS section 149A-41(b)(3), **Respondent is entitled to a hearing** to contest this Proposed Order or any portion of this Notice of Finding of Violation. If you wish to contest this Proposed Order or any portion of this Notice of Finding of Violation, you must submit a **written request for hearing** to the Office of the Chairperson **within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation. Upon receipt of the written request for hearing, a notice will be issued setting forth the date, time, and place where such hearing will

be conducted. The hearing will be conducted pursuant to HRS Chapter 91, the Hawai'i Administrative Procedure Act.

In lieu of a hearing, you may request a meeting with representatives of the State of Hawai'i Department of Agriculture concerning an informal disposition pursuant to HRS section 91-9(d). The request to meet with representatives of the State of Hawai'i Department of Agriculture must be made **in writing within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation. If a settlement can be reached, a Consent Agreement and Order will be signed by all parties. A Consent Agreement and Order shall constitute a waiver of your right to a hearing on any matter to which you have agreed.

The **civil penalty and any proposed corrective action** contained in the Notice of Finding of Violation **shall become a Final Order**, as set forth below, **unless the Respondent files a written request for hearing or meeting within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation.

ISSUED THIS 13th DAY OF July, 2020.

Pesticides Program Manager

THIS ORDER IS HEREBY DECLARED FINAL PURSUANT TO HRS Section 149A-41(b)(3).

Dated: _____
Honolulu, Hawai'i

PHYLLIS SHIMABUKURO-GEISER,
Chairperson
Board of Agriculture

cc: file

C9

C10

Exhibit B

STATE OF HAWAI'I

DEPARTMENT OF AGRICULTURE

IN THE MATTER OF **SAFEWAY INC.,** **Respondent.**	**DOCKET NO. 19-PE-029** **FINDING OF VIOLATION; AUTHORITY; VIOLATIONS; CAUSE OF ACTION; FINAL ORDER TO PAY CIVIL PENALTY**

FINDING OF VIOLATION

Inspections conducted by the State of Hawai'i Department of Agriculture yielded evidence of a violation of sections 149A-11(a)(2) and 149A-15(7) of the *Hawai'i Revised Statutes* ("HRS").

AUTHORITY

The State of Hawai'i Department of Agriculture ("HDOA"), pursuant to the authority granted in HRS section 149A-41, and sections 4-66-3, 4-66-43 and 4-66-66.1 of the *Hawai'i Administrative Rules* ("HAR"), does hereby bring this action against Respondent SAFEWAY INC. for violation of HRS sections 149A-11(a)(2) and 149A-15(7). The inspections were conducted pursuant to the authority granted in HRS section 149A-36 and HAR section 4-66-43(a) for purposes of determining compliance with state and federal pesticide laws.

VIOLATION

1. On or about April 30, 2019, Hawai'i Department of Agriculture Environmental Health Specialist Paul De Filippi ("HDOA Inspector") conducted a Marketplace Surveillance Inspection of the retail establishment SAFEWAY INC. ("Respondent"), located at 277 Pikea Avenue, Kihei, Hawai'i 96753 ("Store").

2. The HDOA Inspector met with the Grocery Manager employed by Respondent and issued him a Notice of Inspection pursuant to HRS section 149A-36.

3. The HDOA Inspector reviewed approximately ninety-five (95) purportedly licensed general use pesticide products. The pesticide products were being offered for sale or distribution at the Store and were readily available for purchase by members of the public.

4. The HDOA Inspector recorded the name and Environmental Protection Agency ("EPA") Registration Number of each pesticide product being offered for sale at the Store.

5. The HDOA Inspector documented that individual containers of LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE (EPA Reg. No. 777-128) did not have the EPA registration number and EPA establishment number located in a conspicuous place on the outer container label.

6. The HDOA Inspector collected documentary samples of the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE.

7. On or about May 1, 2019, the HDOA Inspector returned to the Store and met with Respondent's Store Director to continue the inspection. The HDOA Inspector issued Respondent's Store Director a Notice of Inspection.

8. A Receipt for Samples form was issued to Respondent's Store Director for the documentary samples collected on or about April 30, 2019. Respondent's Store Director informed the HDOA Inspector that the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE had been removed from sale.

9. A Dealer's Statement was collected from Respondent's Store Director by the HDOA Inspector. On the Dealer's Statement, Respondent's Store Director claimed that the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE was received from Safeway Distribution, located at 16900 W. Schulte Road, Tracy, California 95377 at an unspecified date.

10. Sales records or shipping invoices for the LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE were not provided to the HDOA Inspector.

C11

11. A product is "misbranded" if, among other things, the product "label does not bear the federal registration number assigned to each establishment in which it was produced;" pursuant to HRS section 149A-2.

12. HRS section 149A-11 states: "**Prohibited acts**. (a) Except as other wise exempted in section 149A-12, it shall be unlawful for any person to distribute, solicit, sell, offer for sale, hold for sale, transport, deliver for transportation, or receive and having so received, deliver or offer to deliver to any person in intrastate commerce or between points within this State through any point outside this State any of the following: . . . (2) Any pesticide unless it is in the licensee's or the manufacturer's unbroken immediate container, and there is affixed to the container and to the outside container or wrapper of the retail package, if any, through which the required information on the immediate container cannot be clearly read, a label bearing information pursuant to section 149A-15. . ."

(Emphasis added.)

13. HRS section 149A-15 states: "**Labeling requirements.** Each container of pesticides shall bear or have attached in a conspicuous place, a plainly written or printed label in the English language providing the following information: . . . (7) The EPA registration and establishment numbers. . ."

(Emphasis added.)

14. HDOA has previously initiated enforcement action against Respondent by way of Warning Notices dated June 26, 2017 and October 25, 2018, and a Notice of Violation under Docket No. 16-PE-003, making this a subsequent offense subject to increased penalties.

CAUSE OF ACTION

VIOLATION ONE:

HRS section 149A-11(a)(2) provides in pertinent part as follows:

"**Prohibited acts.** (a) Except as otherwise exempted in section 149A-12, it shall be unlawful for any person to distribute, solicit, sell, offer for sale, hold for sale, transport, deliver for transportation, or receive and having so received, deliver or offer to deliver to any person in intrastate commerce or between points within this State through any point outside this State any of the following:

> (2) Any pesticide unless it is in the licensee's or the manufacturer's unbroken immediate container, and there is affixed to the container and to the outside container or wrapper of the retail package, if any, through which the required information on the immediate container cannot be clearly read, a label bearing information pursuant to section 149A-15. . ."

(Emphasis added.)

HRS section 149A-15 provides in pertinent part as follows:

"**Labeling requirements.** Each container of pesticides shall bear or have attached in a conspicuous place, a plainly written or printed label in the English language providing the following information: . . . (7) The EPA registration and establishment numbers. . ."

(Emphasis added.)

On or about April 30, 2019, Respondent SAFEWAY INC., located at 277 Pikea Avenue, Kihei, Hawai'i 96753, violated HRS sections 149A-11(a)(2) and 149A-15(7) by distributing, selling, offering for sale, holding for sale, transporting, delivering for transportation or receiving and having so received, deliver or offer to deliver to any person within the State of Hawai'i any pesticide that did not bear the EPA registration and establishments numbers in a conspicuous place as required by HRS sections 149A-11(a)(2) and 149A-15(7), to wit:

LYSOL BRAND KILLS 99% OF BACTERIA LAUNDRY SANITIZER 0% BLEACH USE AS AN ADDITIVE
(EPA Reg. No. 777-128)

Respondent SAFEWAY INC. has received Warning Notices dated June 26, 2017 and October 25, 2018, and a Notice of Violation under Docket No. 16-PE-003, making this a subsequent violation subject to increased penalties.

Pursuant to HRS section 149A-41(b)(1), a retailer who violates any provision of HRS Chapter 149A may be assessed an administrative penalty of not more than Five Thousand Dollars ($5,000.00) for each offense.

FINAL ORDER TO PAY CIVIL PENALTY

THE STATE OF HAWAI'I DEPARTMENT OF AGRICULTURE finds and concludes that the Respondent's actions, as set forth above, have violated HRS sections 149A-11(a)(2) and 149A-15(7).

C13

Pursuant to HRS section 149A-41(b)(3), Respondent was entitled to a hearing to contest the Notice of Finding of Violation if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the Notice of Finding of Violation. Respondent was served with the Notice of Finding of Violation on July 20, 2020. No request for a hearing was received by the Office of the Chairperson of the Department of Agriculture by August 9, 2020. Such inaction constitutes a waiver of Respondent's right to a hearing on this matter.

HAVING VIOLATED the Hawai'i Pesticides Law as set forth in HRS Chapter 149A, RESPONDENT IS HEREBY ORDERED TO PAY the following civil penalty in accordance with HRS section 149A-41(b)(1) and HAR section 4-66-66.1:

Violation One: Five Hundred Dollars ($500.00) per misbranded product

TOTAL CIVIL PENALTY: Five Hundred Dollars ($500.00).

The civil penalty shall be paid within twenty (20) business days from the date of this Notice of Finding of Violation by delivering payment to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814.

Pursuant to HRS section 149A-41(b)(4), "[i]n case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the board may determine, the board shall refer the matter to the attorney general, who shall recover the amount by action in the appropriate court. For any judicial proceeding to recover the administrative penalty imposed, the attorney general need only show that notice was given, a hearing was held or the time granted for requesting a hearing has expired without such a request, the administrative penalty was imposed, and that the penalty remains unpaid."

THIS ORDER IS HEREBY DECLARED FINAL PURSUANT TO HRS SECTION 149A-41(b)(3)

Dated: 10-7-2020
Honolulu, Hawai'i

Phyllis Shimabukuro-Geiser
PHYLLIS SHIMABUKURO-GEISER,
Chairperson
Board of Agriculture

cc: file

C15

C16

DAVID Y. IGE
Governor

JOSH GREEN
Lt. Governor

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

MORRIS M. ATTA
Deputy to the Chairperson

State of Hawaii
DEPARTMENT OF AGRICULTURE
1428 South King Street
Honolulu, Hawaii 96814-2512
Phone: (808) 973-9600 FAX: (808) 973-9613

Exhibit C

December 8, 2020

Certified Mail No. 7020 1810 0000 9420 9296
Return Receipt Requested

The Corporation Company, Inc.
Agent for Safeway, Inc.
1136 Union Mall, Suite 301
Honolulu, Hawai'i 96813

Re: In the Matter of SAFEWAY, INC.
Docket No. 19-PE-029

Dear Agent:

On July 13, 2020, a Notice of Finding of Violation and Proposed Order to Pay Civil Penalty ("NOV") was issued under Docket No. 19-PE-029 to SAFEWAY, INC. ("Respondent"). The NOV was based upon inspections conducted on April 30, 2019 and May 1, 2019 pursuant to the authority granted in Hawai'i Revised Statutes ("HRS") section 149A-36 for purposes of determining compliance with state and federal pesticide laws. On July 20, 2020, the NOV was served on the agent for Respondent.

Pursuant to HRS section 149A-41(b)(3), Respondent was entitled to a hearing to contest the NOV if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the NOV. The NOV clearly advised Respondent that the NOV would become a FINAL ORDER unless Respondent filed a written request for hearing within twenty (20) calendar days.

The twenty (20) day time period expired August 9, 2020; no request for a hearing was received by the Office of the Chairperson of the Department of Agriculture by that time. Accordingly, Respondent waived the opportunity to challenge the finding of violation and the NOV became a FINAL ORDER.

On October 7, 2020, HDOA issued a Final Order to Respondent. The Final Order required the Respondent to submit the civil penalty of five hundred dollars ($500.00) within twenty (20) calendar days from the receipt of the Final Order, or HDOA would refer the matter to the Hawai'i Department of the Attorney General for collection. The agent for Respondent was served with the Final Order on October 14, 2020. As of today's date, Respondent has failed to submit the civil penalty to HDOA and **is now in DEFAULT**.

HRS section 149A-41(b)(4) provides as follows:

> In case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the board may determine, **the board shall refer the matter to the attorney general, who shall recover the amount by action in the appropriate court**. For any judicial proceeding to recover the administrative penalty imposed, **the attorney general need only show that notice was given**, a hearing was held or **the time granted for requesting a hearing has expired without such a request**, the **administrative penalty was imposed**, and that **the penalty remains unpaid**. (Emphasis added.)

Please note that this letter and enclosures will be tendered to the State of Hawai'i Attorney General as evidence of RESPONDENT'S FAILURE TO SUBMIT THE CIVIL PENALTY TO HDOA AS AGREED. Respondent SAFEWAY, INC. may be deemed liable for court costs, attorney fees, and interest should legal action be required to secure payment of the five hundred dollar ($500.00) civil penalty.

Please remit the five hundred dollar ($500.00) civil penalty by December 23, 2020 to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814

Copies of the NOV, Final Order, and signed certified mail return receipts are enclosed. **If no payment is received by December 23, 2020**, pursuant to HRS section 149A-41(b)(4), **the matter will be referred to the Hawai'i Department of the Attorney General for collection.**

Should you have any questions or concerns please contact the undersigned at (808) 973-9404 or via email at greg.y.takeshima@hawaii.gov

Sincerely yours,

GREG TAKESHIMA
Acting Pesticides Program Manager

GT:sn
[K:SAFEWAY]

Enclosures (17 pages)

cc: File
EPA Region IX

C17

C18

State of Hawai'i
Department of Agriculture
Plant Industry Division
Pesticide Branch

March 15, 2021

Board of Agriculture
Honolulu, Hawai'i

Subject: Request that the Final Order for In the Matter of DENBY ERECE, Docket No. 19-PE-004, be Referred to the Attorney General for Collection.

PROCEDURAL BACKGROUND:
On November 13, 2019, a Notice of Finding of Violation and Proposed Order to Pay Civil Penalty ("NOV") was issued under Docket No. 19-PE-004 to DENBY ERECE ("Respondent"). The NOV was based upon inspections conducted on June 27, 2018 and June 28, 2018 pursuant to the authority granted in Hawai'i Revised Statutes ("HRS") section 149A-36 for purposes of determining compliance with state and federal pesticide laws. The NOV stipulated a civil penalty amount of four thousand dollars ($4,000.00). On November 29, 2019, the NOV was served on Respondent. A copy of the NOV is attached hereto as "Exhibit A".

On December 16, 2019, Respondent requested to meet with HDOA for purposes of resolving the NOV by agreement. On January 27, 2020 and March 3, 2020, Respondent met with HDOA to discuss settlement of all matters raised in the NOV. Following the March 3, 2020 meeting, HDOA and Respondent were unable to reach a settlement.

Respondent was issued a letter, dated June 4, 2020, stating that pursuant to HRS section 149A-41(b)(3), Respondent was entitled to a hearing to contest the NOV if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the June 4, 2020 letter. The June 4, 2020 letter clearly advised Respondent that the NOV would become a FINAL ORDER unless Respondent filed a written request for hearing within twenty (20) calendar days. A copy of the June 4, 2020 letter is attached hereto as "Exhibit B".

The twenty (20) day time period expired June 28, 2020. Accordingly, Respondent waived the opportunity to challenge the finding of violation and the NOV became a FINAL ORDER.

Request that the Final Order for In the Matter of DENBY ERECE, Docket No. 19-PE-004, be Referred to the Attorney General for Collection

On July 17, 2020, HDOA issued a Final Order to Respondent. The Final Order required the Respondent to submit the civil penalty of four thousand dollars ($4,000.00) within twenty (20) calendar days from the date of the Final Order, or HDOA would refer the matter to the Hawai'i Department of the Attorney General for collection. Respondent was served with the Final Order on July 28, 2020. A copy of the Final Order is attached hereto as "Exhibit C".

The twenty (20) day time period expired August 6, 2020. HDOA did not receive the civil penalty of four thousand dollars ($4,000.00) from Respondent.

On September 24, 2020, HDOA issued a Demand Letter to Respondent. The Demand Letter stated that Respondent failed to abide by the terms and conditions of the Final Order. The Demand Letter required Respondent to submit the civil penalty of $4,000.00 by October 9, 2020, or HDOA would refer the case to the Hawai'i Department of the Attorney General for collection. As of today's date, Respondent has failed to submit the civil penalty of four thousand dollars ($4,000.00) to HDOA. A copy of the Demand Letter is attached hereto as "Exhibit D".

AUTHORITY: Pursuant to section 149A-41(b)(4), *Hawai'i Revised Statutes* ("HRS"), in case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the Board may determine, the Board shall refer the matter to the Attorney General, who shall recover the amount by action in the appropriate court.

RECOMMENDATION: The Pesticides Branch of HDOA Plant Industry Division recommends the Board refer the Final Order for In the Matter of DENBY ERECE, Docket No. 19-PE-004, to the Attorney General for collection.

It is respectfully requested that the Board follow the recommendation set forth above.

Submitted by:

GREG TAKESHIMA
Acting Pesticides Branch Manager

CP

C20

Request that the Final Order for In the Matter of DENBY ERECE, Docket No. 19-PE-004, be Referred to the Attorney General for Collection

CONCURRED:

BECKY AZAMA
Acting Administrator, Plant Industry

Attachments – Exhibits "A" through "D"

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

STATE OF HAWAI'I

DEPARTMENT OF AGRICULTURE

IN THE MATTER OF DENBY ERECE Respondent.	DOCKET NO. 19-PE-004 NOTICE OF FINDING OF VIOLATION; AUTHORITY; ALLEGED VIOLATION; CAUSE OF ACTION; ORDER TO CEASE AND DESIST; PROPOSED ORDER TO PAY CIVIL PENALTY; OPPORTUNITY FOR HEARING

NOTICE OF FINDING OF VIOLATION

NOTICE IS HEREBY GIVEN that an inspection conducted by the State of Hawai'i Department of Agriculture yielded evidence of violation of sections 149A-31(1) and 149A-33(3), (4) and (5) of the *Hawai'i Revised Statutes* ("HRS"), and sections 4-66-61 and 4-66-62 of the *Hawai'i Administrative Rules* ("HAR").

AUTHORITY

The State of Hawai'i Department of Agriculture ("HDOA"), pursuant to the authority granted in HRS sections 149A-31 and 149A-41(b)(1)-(2), and sections 4-66-3 and 4-66-66.1 of the *Hawai'i Administrative Rules* ("HAR"), does hereby bring this action against the Respondent DENBY ERECE for violations of HRS sections 149A-31(1) and 149A-33, and HAR sections 4-66-61 and 4-66-62. The inspection was conducted pursuant to the authority granted in HRS section 149A-36 for purposes of determining compliance with state and federal pesticide laws.

C22

ALLEGED VIOLATIONS

1. On May 4, 2018, Hawaii Department of Agriculture ("HDOA") Pesticides Branch staff reviewed Restricted Use Pesticide ("RUP") sales records provided by RUP Dealers to HDOA on a monthly basis. Sales reported by J. R. Simplot Company dba Simplot ("Simplot") reported sales to Green Produce II, LLC, which does not employ any certified applicators. The sales report listed the certification number H72426, which belonged to DENBY ERECE ("Respondent"). These sales records generated a Certified Applicator's Inspection with the Respondent, who is employed by Crown Pacific International, LLC, and an Agricultural Use Inspection with Green Produce II, LLC.

2. On or about June 27, 2018, HDOA Inspector Adam Williams ("HDOA Inspector") met with non-certified applicator Mr. Yun Min He, ("Min He") who applies RUPs under the direct supervision of the Respondent at his farm, Green Produce II, LLC, located at 86-446 Kuwale Road, Waianae, HI 96792.

3. The HDOA inspector interviewed Min He about his purchases of the RUPs AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) and DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729).

4. The HDOA Inspector issued Min He a Notice of Pesticide Use/Misuse Inspection pursuant to HRS section 149A-36. Min He voluntarily consented to the HDOA Inspector's request to conduct an inspection.

5. Min He explained that he purchases the RUPs from Simplot under Respondent's commercial certification number H72426.

6. The HDOA Inspector reviewed Green Produce II, LLC's pesticide application records for AGRI-MEK SC MITICIDE/INSECTICIDE and DUPONT CORAGEN INSECT CONTROL kept by Min He. The records show Green Produce II, LLC's most recent application of DUPONT CORAGEN INSECT CONTROL occurred on August 18, 2017 and its most recent application of AGRI-MEK SC MITICIDE/INSECTICIDE occurred on July 22, 2017.

7. With regards to Green Produce II, LLC's July 22, 2017 application of AGRI-MEK SC MITICIDE/INSECTICIDE, Min He mixed 10.5 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE into 250 gallons of water and applied this dilution to 3 acres of basil. This equates to 3.5 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE applied per acre of basil.

8. Green Produce II, LLC's application records further showed that previous applications of 3.5 ounces per acre were made to the same field fourteen (14) times in between February 22, 2017 and July 22, 2017. This equates to 49 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE applied per acre within 5 months.

9. The AGRI-MEK SC MITICIDE/INSECTICIDE label states, in part:

> **"RESTRICTED USE PESTICIDE**
> For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **HERB CROP SUBGROUP 19A**
> * * *
> **Basil**
> * * *
> **Maximum Amount per Year**: Do not apply more than 10.25 fl oz/A (or 0.056 lb ai/A) of Agri- Mek SC or any other foliar-applied abamectin-containing product per year."

10. HRS section 149A-31 provides: "No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

11. Min He, acting under the direct supervision of the Respondent, exceeded the annual rate of application of AGRI-MEK SC MITICIDE/INSECTICIDE.

12. The HDOA inspector asked Min He if he added any wetting, spreaders, penetrating agent, or other adjuvants to his mixtures of AGRI-MEK SC MITICIDE/INSECTICIDE, to which Min He responded he did not always use adjuvants.

13. The AGRI-MEK SC MITICIDE/INSECTICIDE label states, in part:

> **"RESTRICTED USE PESTICIDE**
> * * *
> FOR RETAIL SALE TO AND USE ONLY BY CERTIFIED APPLICATOR OR PERSONS UNDER THEIR DIRECT SUPERVISION, AND ONLY FOR THOSE USES COVERED BY THE CERTIFIED APPLICATOR'S CERTIFICATION.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **USE INFORMATION**
> * * *
> **Adjuvant Requirement:** To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the Directions for Use for each crop on this label.
> * * *

C23

HERB CROP SUBGROUP 19A
* * *
Basil
* * *
Instructions To avoid illegal residues, Agri-Mek SC must be mixed with a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant."

(Emphasis added.)

14. Min He, acting under the direct supervision of the Respondent, did not use a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant as required by the label.

15. During the inspection with Min He, the HDOA inspector documented that no written instructions for applying AGRI-MEK SC MITICIDE/INSECTICIDE and no written instructions for applying DUPONT CORAGEN INSECT CONTROL were provided by the Respondent to Min He.

16. **HRS 149A-33** provides the following: "Rules: The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following . . . (5) To establish, as necessary, procedures for the issuance of guidelines to specify those conditions that constitute use of a pesticide in a manner inconsistent with its label."

17. **HAR 4-66-61** further provides that: A person may apply a restricted use pesticide, provided that: (1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; (2) The person shall be given specific written instructions by the certified applicator for applying the pesticide; (3) The certified applicator shall be responsible for all violations of the Act and this rule ..."

18. Min He stated that he has not used AGRI-MEK SC MITICIDE/INSECTICIDE since the application that occurred on July 22, 2017, and has not used DUPONT CORAGEN INSECT CONTROL since the application that occurred on August 18, 2017.

19. During the inspection, the HDOA inspector found no containers of AGRI-MEK SC MITICIDE/INSECTICIDE or DUPONT CORAGEN INSECT CONTROL in the storage area or elsewhere at Green Produce II, LLC. Min He was not able to show any container of AGRI-MEK SC MITICIDE/INSECTICIDE or DUPONT CORAGEN INSECT CONTROL to the HDOA inspector; Min He was only able to provide the used AGRI-MEK SC MITICIDE/INSECTICIDE and DUPONT CORAGEN INSECT CONTROL labels printed from the HDOA's Currently Licensed Pesticide Listing database.

20. RUP sales records submitted to HDOA from by J. R. SIMPLOT COMPANY dba SIMPLOT (the Dealer) show that between August 1, 2017 and May 23, 2018, the Dealer sold Green Produce II, LLC 26 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE and between September 5, 2017 and May 23, 2018, the Dealer sold Green Produce II, LLC 17

gallons of DUPONT CORAGEN INSECT CONTROL under Respondent's commercial certification number, H72426..

21. On June 13, 2018, the HDOA Inspector collected invoices and delivery tags from The Dealer which showed Green Produce II, LLC had purchased 28 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE and 21 gallons of DUPONT CORAGEN INSECT CONTROL under Respondent's commercial certification number H72426 between July 6, 2017 and May 23, 2018. These invoices and delivery tags show that Green Produce II, LLC had received 27 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE between July 22, 2017 and May 23, 2018 and 19 gallons of DUPONT CORAGEN INSECT between August 18, 2017 and May 23, 2018.

22. RUP application records maintained at Green Produce II, LLC, and statements from Min He document that no applications of AGRI-MEK SC MITICIDE/INSECTICIDE occurred after July 22, 2017, and no applications of DUPONT CORAGEN INSECT CONTROL occurred since August 18, 2017.

23. On or about June 28, 2018, The HDOA Inspector met with the Respondent at Crown Pacific International, LLC's office on Kualalakai Parkway in Kapolei, Hawaii.

24. The HDOA Inspector issued the Respondent a Notice of Pesticide Use/Misuse Inspection pursuant to HRS section 149A-36. The Respondent voluntarily consented to the HDOA Inspector's request to conduct an inspection.

25. The Respondent attested that she provides supervision and oversite for the RUPs purchased by and used at Green Produce II, LLC.

26. The HDOA Inspector inventoried the contents on Crown Pacific International's pesticide storage and documented one and one-quarter (1 ¼) gallon of AGRI-MEK SC MITICIDE/INSECTICIDE and one (1) gallon of DUPONT CORAGEN INSECT CONTROL in the storage area. These items were purchases under Erece's private certification number for Crown Pacific, F78356, not the commercial certification number, H72426.

27. The HDOA Inspector reviewed RUP records produced and maintained by the Respondent. The Respondent provided the HDOA inspector with her records for applications of RUPs. However, these RUP records were clearly labeled for Crown Pacific, and listed the site of application as Crown Pacific Produce, LLC on Luakaha Street in Hilo, Hawaii, not for Green Produce.

28. As the Respondent, under her commercial certification number H7246, (1) purchased at least 27 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE for Green Produce II, LLC after Respondent's last application of AGRI-MEK SC MITICIDE/INSECTICIDE for Green Produce II, LLC, (2) purchased at least 19 gallons of DUPONT CORAGEN INSECT CONTROL for Green Produce II, LLC after Respondent's last application of DUPONT CORAGEN INSECT CONTROL, and (3) does not have any AGRI-MEK SC

MITICIDE/INSECTICIDE or DUPONT CORAGEN INSECT CONTROL in storage for Green Produce II, LLC, the Respondent has failed to keep adequate records of at least 37 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE and 19 gallons of DUPONT CORAGEN INSECT CONTROL purchased under her commercial certification number H7246 for Green Produce II, LLC. [1]

29. On or about July 19, 2018, Respondent e-mailed the HDOA Inspector a copy of written instructions she was providing to Green Produce for the use of AGRI-MEK SC MITICIDE/INSECTICIDE and DUPONT CORAGEN INSECT CONTROL.

30. HRS 149A-33 provides the following: "Rules: The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following . . . (4) To establish, as necessary, record keeping requirements for pesticide use by applicators."

31. HAR 4-66-62 further provides the following: Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators shall keep records of all applications of restricted use pesticides applied, at their principal place of business. (b) These records must be kept for a period of two years and shall be made available for inspection by the head during reasonable working hours. Recordkeeping information shall include: (1) Brand or common name of pesticide product applied; (2) EPA registration number; (3) Type of formulation; (4) Per cent active ingredient; (5) Scientific or common name of target pest; (6) Dilution rate; (7) Total amount of pesticide used; (8) Total area covered; (9) Time and date of application; (10) Address or location of treated site; (11) Name of certified applicator and his or her certification number; (12) Crop, commodity, stored product or other site; (13) Restricted entry interval and whether posting and oral notification are required; and (14) Any other information that the head deems to be necessary.

CAUSE OF ACTION

VIOLATION ONE:

HAR section 4-66-61(1) and (3) states:

"A person may apply a restricted use pesticide, provided that:

(1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; ...
(3) The certified applicator shall be responsible for all violations of the Act and this rule."

HRS section 149A-31 states: "Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

[1] We note that Respondent could not have legally distributed the unaccounted for RUPs as Respondent was not licensed to do so under HRS Section 149A-17.

Between February 22, 2017 and July 22, 2017, Respondent DENBY ERECE violated HRS section 149A-31 by using the pesticide AGRI-MEK SC MITICIDE/INSECTICIDE in a manner inconsistent with its label, to wit.

The AGRI-MEK SC MITICIDE/INSECTICIDE label clearly states:

> **"RESTRICTED USE PESTICIDE**
> For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **HERB CROP SUBGROUP 19A**
> * * *
> **Basil**
> * * *
> **Maximum Amount per Year**: Do not apply more than 10.25 fl oz/A (or 0.056 lb ai/A) of Agri- Mek SC or any other foliar-applied abamectin-containing product per year."

Between February 22, 2017 and July 22, 2017, non-certified applicator Min He, under the direct supervision of the Respondent, applied 49 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE per acre of basil within 5 months.

VIOLATION TWO:

HAR section 4-66-61(1) and (3) states:

"A person may apply a restricted use pesticide, provided that:

(1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; ...
(3) The certified applicator shall be responsible for all violations of the Act and this rule."

HRS section 149A-31 states: "Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

C27

Between February 22, 2017 and July 22, 2017, Respondent DENBY ERECE violated HRS section 149A-31 by using the pesticide AGRI-MEK SC MITICIDE/INSECTICIDE in a manner inconsistent with its label, to wit.

The AGRI-MEK SC MITICIDE/INSECTICIDE label clearly states:

> "**RESTRICTED USE PESTICIDE**
> For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **USE INFORMATION**
> * * *
> **Adjuvant Requirement:** To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the Directions for Use for each crop on this label.
> * * *
> **HERB CROP SUBGROUP 19A**
> * * *
>
> **Basil**
> * * *
> **Instructions** To avoid illegal residues, Agri-Mek SC must be mixed with a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant."

On applications occurring from February 22, 2017 up to and including July 22, 2017, non-certified applicator Min He, under the direct supervision of the Respondent, mixed and applied 14 dilutions of AGRI-MEK SC MITICIDE/INSECTICIDE without adding any wetting, spreaders, penetrating agent, or other adjuvants to his mixtures of AGRI-MEK SC MITICIDE/INSECTICIDE.

VIOLATION THREE

HRS section 149A-33 provides:

The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (3) To establish, as necessary, specific standards and guidelines which specify those conditions which constitute unreasonable adverse effects on the environment[.]

. . . (4) To establish, as necessary, record keeping requirements for pesticide use by applicators[.]

HAR section 4-66-62 provides:

(a) Certified pesticide applicators shall keep records of all applications of restricted use pesticides applied, at their principal place of business.

(b) These records must be kept for a period of two years and shall be made available for inspection by the head during reasonable working hours. Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Per cent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site;
(13) Restricted entry interval and whether posting and oral notification are required; and
(14) Any other information that the head deems to be necessary.

From February 22, 2017 to July 22, 2017, Respondent DENBY ERECE violated HAR Section 4-66-62 by failing to keep adequate records of all applications of AGRI-MEK SC MITICIDE/INSECTICIDE, a restricted use pesticide, at Respondent's principal place of business, to wit.

From February 22, 2017 to July 22, 2017, Respondent failed to keep any of the required recordkeeping information for the applications of at least 27 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE. Additionally, records of AGRI-MEK SC MITICIDE/INSECTICIDE applications that were maintained by Respondent lacked multiple required items, including type of formulation, per cent active ingredient, dilution rate, whether posting and oral notification was required, and at time, a specific date.

VIOLATION FOUR

HRS section 149A-33 provides:

> The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (3) To establish, as necessary, specific standards and guidelines which specify those conditions which constitute unreasona
> ble adverse effects on the environment[.]
>
> . . . (4) To establish, as necessary, record keeping requirements for pesticide use by applicators[.]

HAR section 4-66-62 provides:

> (a) Certified pesticide applicators shall keep records of all applications of restricted use pesticides applied, at their principal place of business.
> (b) These records must be kept for a period of two years and shall be made available for inspection by the head during reasonable working hours. Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Per cent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site;

(13) Restricted entry interval and whether posting and oral notification are required; and
(14) Any other information that the head deems to be necessary.

From February 22, 2017 to July 22, 2017, Respondent DENBY ERECE violated HAR Section 4-66-62 by failing to keep adequate records of all applications of DUPONT CORAGEN INSECT CONTROL, a restricted use pesticide, at Respondent's principal place of business, to wit.

From February 22, 2017 to July 22, 2017, Respondent failed to keep any of the required recordkeeping information for the applications of at least 19 gallons of DUPONT CORAGEN INSECT CONTROL. Additionally, records of DUPONT CORAGEN INSECT CONTROL applications that were maintained by Respondent lacked multiple required items, including type of formulation, per cent active ingredient, dilution rate, whether posting and oral notification was required, and at time, a specific date.

VIOLATION FIVE

HRS section 149A-33 provides:

> The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (5) To establish, as necessary, procedures for the issuance of guidelines to specify those conditions that constitute use of a pesticide in a manner inconsistent with its label.'

HAR section 4-66-61 provides:

> Conditions on the use of restricted use pesticides. A person may apply a restricted use pesticide, provided that: (1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; (2) The person shall be given specific written instructions by the certified applicator for applying the pesticide; (3) The certified applicator shall be responsible for all violations of the Act and this rule; . . ."

From February 22, 2017 to July 22, 2017, Respondent DENBY ERECE violated HAR Section 4-66-61 by failing to provide specific written directions for applying AGRI-MEK SC MITICIDE/INSECTICIDE or for applying DUPONT CORAGEN INSECT CONTROL to Min He, a non-certified applicator under the Respondent's direct supervision.

ORDER TO CEASE AND DESIST

The State of Hawai'i Department of Agriculture, pursuant to the provisions of HAR section 4-66-3, **DOES HEREBY ORDER RESPONDENT TO CEASE AND DESIST VIOLATION OF HRS CHAPTER 149A.** You are hereby notified that any further violation of HRS Chapter 149A will result in increased penalties as provided by law.

YOU ARE SO NOTIFIED.

PROPOSED ORDER TO PAY CIVIL PENALTY

THE STATE OF HAWAI'I DEPARTMENT OF AGRICULTURE finds and concludes that Respondent's actions, as set forth above, have violated HRS sections 149A-31(1) and 149A-33(3), (4) and (5), and HAR sections 4-66-61 and 4-66-62.

HAVING VIOLATED the Hawai'i Pesticide Law as set forth in HRS Chapter 149A, RESPONDENT DENBY ERECE IS HEREBY ORDERED TO PAY the following civil penalty in accordance with HRS section 149A-41(b)(2) and HAR section 4-66-66.1:

Violation One:	Two Thousand Dollars ($2,000.00)
Violation Two:	Seven Hundred Fifty Dollars ($750.00)
Violation Three:	Five Hundred Dollars ($500.00)
Violation Two:	Five Hundred Dollars ($500.00)
Violation Five:	Two Hundred Fifty Dollars ($250.00)

TOTAL CIVIL PENALTY: Four Thousand Dollars ($4,000.00) and SIX (6) MONTH SUSPENSION OF RESPONDENT DENBY ERECE'S COMMERCIAL APPLICATOR CERTIFICATION.

The civil penalty shall be paid within twenty (20) business days from the date of receipt of this Notice of Finding of Violation by delivering payment to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814

The Department of Agriculture may use all reasonable means to collect the full amount of the penalty, if not paid within the specified time period, as authorized by law.

OPPORTUNITY FOR HEARING

Pursuant to HRS section 149A-41(b)(3), **Respondent is entitled to a hearing** to contest this Proposed Order or any portion of this Notice of Finding of Violation. If you wish to contest this Proposed Order or any portion of this Notice of Finding of Violation, you must submit a **written request for hearing** to the Office of the Chairperson **within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation. Upon receipt of the written request for hearing, a notice will be issued setting forth the date, time, and place where such hearing will be conducted. The hearing will be conducted pursuant to HRS Chapter 91, the Hawai'i Administrative Procedure Act.

In lieu of a hearing, you may request a meeting with representatives of the State of Hawai'i Department of Agriculture concerning an informal disposition pursuant to HRS section 91-9(d). The request to meet with representatives of the State of Hawai'i Department of Agriculture must be made **in writing within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation. If a settlement can be reached, a Consent Agreement and Order will be signed by all parties. A Consent Agreement and Order shall constitute a waiver of your right to a hearing on any matter to which you have agreed.

The **civil penalty and any proposed corrective action** contained in the Notice of Finding of Violation **shall become a Final Order**, as set forth below, **unless Respondent files a written request for hearing or meeting within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation.

ISSUED THIS 13th DAY OF November, 2019.

Pesticides Program Manager

C33

THIS ORDER IS HEREBY DECLARED FINAL PURSUANT TO HRS section 149A-41(b)(3).

Dated: _____
Honolulu, Hawai'i

_____
PHYLLIS SHIMABUKURO-GEISER
Chairperson
Board of Agriculture

cc: file

DAVID Y. IGE
Governor

JOSH GREEN
Lt. Governor

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

MORRIS M. ATTA
Deputy to the Chairperson

State of Hawaii
DEPARTMENT OF AGRICULTURE
1428 South King Street
Honolulu, Hawaii 96814-2512
Phone: (808) 973-9600 FAX: (808) 973-9613

Exhibit B

June 4, 2020

Certified Mail No. 7019 1640 0002 1812 5119
Return Receipt Requested

Ms. Denby Erece
25-221 Ua Nahele Street
Hilo, Hawai'i 96720

Re: In the Matter of DENBY ERECE
Docket No. 19-PE-004

Dear Ms. Erece:

On November 13, 2019, a Notice of Finding of Violation and Proposed Order to Pay Civil Penalty ("NOV") was issued under Docket No. 19-PE-004 to DENBY ERECE ("Respondent"). The NOV was based upon inspections conducted on June 27, 2018 and June 28, 2018 pursuant to the authority granted in Hawai'i Revised Statutes ("HRS") section 149A-36 for purposes of determining compliance with state and federal pesticide laws. The NOV documented the following violations:

- Violation One: Between February 22, 2017 and July 22, 2017, a non-certified applicator, under the direct supervision of Respondent, exceeded the maximum application rate of the restricted use pesticide AGRI-MEK SC MITICIDE/INSECTICIDE, in violation of HRS 149A-31(1).
- Violation Two: On applications occurring from February 22, 2017, up to and including July 22, 2017, a non-certified applicator, under the direct supervision of Respondent, mixed and applied the restricted use pesticide AGRI-MEK SC MITICIDE/INSECTICIDE without adding any wetting, spreaders, penetrating agent, or other adjuvants to his mixture of AGRI-MEK SC MITICIDE/INSECTICIDE, in violation of HRS 149A-31(1).
- Violation Three: From February 22, 2017 to July 22, 2017, Respondent failed to keep any of the required recordkeeping information for the applications of at least 27 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE. In addition, records of AGRI-MEK SC MITICIDE/INSECTICIDE that were maintained by Respondent lacked the following information: Type of formulation, per cent active ingredient, dilution rate, whether posting and oral notification were required, and at time, a specific date, in violation of Hawai'i Administrative Rules ("HAR") 4-66-62.
- Violation Four: From February 22, 2017 to July 22, 2017, Respondent failed to keep any of the required recordkeeping information for the applications of at least 19 gallons of the

restricted use pesticide DUPONT CORAGEN INSECT CONTROL. In addition, records of DUPONT CORAGEN INSECT CONTROL that were maintained by Respondent lacked the following information: Type of formulation, per cent active ingredient, dilution rate, whether posting and oral notification were required, and at time, a specific date, in violation of HAR 4-66-62.

- Violation Five: From February 22, 2017 to July 22, 2017, Respondent failed to provide specific written directions for applying AGRI-MEK SC MITICIDE/INSECTICIDE and DUPONT CORAGEN INSECT CONTROL to a non-certified application under Respondent's direct supervision, in violation of HAR 4-66-61.

The NOV stipulated a civil penalty in the amount of four thousand dollars ($4,000) and a six (6) month suspension of Respondent's commercial applicator certification.

Respondent was served with the NOV on November 29, 2019. On December 16, 2019, Respondent requested to meet with HDOA for purposes of resolving the NOV by agreement. On January 27, 2020 and March 3, 2020, Respondent met with HDOA to discuss settlement of all matters raised in the NOV. Following the March 3, 2020 meeting, HDOA and Respondent were unable to reach a settlement. In spite of HDOA's good faith efforts, HDOA and Respondent are not able to reach a settlement in this matter and the NOV is still in effect.

Copies of the NOV and signed certified mail return receipts are enclosed. As of today's date, Respondent has failed to submit the civil penalty to HDOA.

Pursuant to HRS section 149A-41(b)(3), **Respondent is entitled to a hearing** to contest the NOV's Proposed Order or any portion of the NOV. If you wish to contest the Proposed Order or any portion of the NOV, you must submit a **written request for hearing** to the Office of the Chairperson **within twenty (20) calendar days from the date of receipt** of this letter. Upon receipt of the written request for hearing, a notice will be issued setting forth the date, time, and place where such hearing will be conducted. The hearing will be conducted pursuant to HRS Chapter 91, the Hawai'i Administrative Procedure Act.

The **civil penalty and any proposed corrective action** contained in the NOV **shall become a Final Order unless Respondent files a written request for hearing or meeting within twenty (20) calendar days from the date of receipt** of this letter.

DENBY ERECE
June 4, 2020

Should you have any questions or concerns please contact the undersigned at (808) 973-9404 or via email at greg.y.takeshima@hawaii.gov

Sincerely yours,

GREG TAKESHIMA
Acting Pesticides Program Manager

JM:vm

[K:DENBYERECE]

Enclosures (16 pages)

cc: File
EPA Region IX

C37

C38

Exhibit C

STATE OF HAWAI'I

DEPARTMENT OF AGRICULTURE

IN THE MATTER OF	**DOCKET NO. 19-PE-004**
DENBY ERECE	**FINDING OF VIOLATION; AUTHORITY; VIOLATIONS; CAUSES OF ACTION; FINAL ORDER TO PAY CIVIL PENALTY**
Respondent.	

FINDING OF VIOLATION

Inspections conducted by the State of Hawai'i Department of Agriculture yielded evidence of violation of sections 149A-31(1) and 149A-33(3), (4) and (5) of the *Hawai'i Revised Statutes* ("HRS"), and sections 4-66-61 and 4-66-62 of the *Hawai'i Administrative Rules* ("HAR").

AUTHORITY

The State of Hawai'i Department of Agriculture ("HDOA"), pursuant to the authority granted in HRS sections 149A-31, 149A-34, and 149A-41(b)(1)-(2), and sections 4-66-3 and 4-66-66.1 of the *Hawai'i Administrative Rules* ("HAR"), does hereby bring this action against the Respondent DENBY ERECE for violations of HRS sections 149A-31(1) and 149A-33, and HAR sections 4-66-61 and 4-66-62. The inspection was conducted pursuant to the authority granted in

HRS section 149A-36 for purposes of determining compliance with state and federal pesticide laws.

VIOLATIONS

1. On May 4, 2018, Hawaii Department of Agriculture ("HDOA") Pesticides Branch staff reviewed Restricted Use Pesticide ("RUP") sales records provided by RUP Dealers to HDOA on a monthly basis. Sales reported by J. R. Simplot Company dba Simplot ("Simplot") reported sales to Green Produce II, LLC, which does not employ any certified applicators. The sales report listed the certification number H72426, which belonged to DENBY ERECE ("Respondent"). These sales records generated a Certified Applicator's Inspection with the Respondent, who is employed by Crown Pacific International, LLC, and an Agricultural Use Inspection with Green Produce II, LLC.

2. On or about June 27, 2018, HDOA Inspector Adam Williams ("HDOA Inspector") met with non-certified applicator Mr. Yun Min He, ("Min He") who applies RUPs under the direct supervision of the Respondent at his farm, Green Produce II, LLC, located at 86-446 Kuwale Road, Waianae, HI 96792.

3. The HDOA inspector interviewed Min He about his purchases of the RUPs AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) and DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729).

4. The HDOA Inspector issued Min He a Notice of Pesticide Use/Misuse Inspection pursuant to HRS section 149A-36. Min He voluntarily consented to the HDOA Inspector's request to conduct an inspection.

5. Min He explained that he purchases the RUPs from Simplot under Respondent's commercial certification number H72426.

6. The HDOA Inspector reviewed Green Produce II, LLC's pesticide application records for AGRI-MEK SC MITICIDE/INSECTICIDE and DUPONT CORAGEN INSECT CONTROL kept by Min He. The records show Green Produce II, LLC's most recent application of DUPONT CORAGEN INSECT CONTROL occurred on August 18, 2017 and its most recent application of AGRI-MEK SC MITICIDE/INSECTICIDE occurred on July 22, 2017.

7. With regards to Green Produce II, LLC's July 22, 2017 application of AGRI-MEK SC MITICIDE/INSECTICIDE, Min He mixed 10.5 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE into 250 gallons of water and applied this dilution to 3 acres of basil. This equates to 3.5 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE applied per acre of basil.

8. Green Produce II, LLC's application records further showed that previous applications of 3.5 ounces per acre were made to the same field fourteen (14) times in between February

CBJ

C40

22, 2017 and July 22, 2017. This equates to 49 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE applied per acre within 5 months.

9. The AGRI-MEK SC MITICIDE/INSECTICIDE label states, in part:

> **"RESTRICTED USE PESTICIDE**
> For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **HERB CROP SUBGROUP 19A**
> * * *
> **Basil**
> * * *
> **Maximum Amount per Year**: Do not apply more than 10.25 fl oz/A (or 0.056 lb ai/A) of Agri- Mek SC or any other foliar-applied abamectin-containing product per year."

10. HRS section 149A-31 provides: "No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

11. Min He, acting under the direct supervision of the Respondent, exceeded the annual rate of application of AGRI-MEK SC MITICIDE/INSECTICIDE.

12. The HDOA inspector asked Min He if he added any wetting, spreaders, penetrating agent, or other adjuvants to his mixtures of AGRI-MEK SC MITICIDE/INSECTICIDE, to which Min He responded he did not always use adjuvants.

13. The AGRI-MEK SC MITICIDE/INSECTICIDE label states, in part:

> **"RESTRICTED USE PESTICIDE**
> * * *
> FOR RETAIL SALE TO AND USE ONLY BY CERTIFIED APPLICATOR OR PERSONS UNDER THEIR DIRECT SUPERVISION, AND ONLY FOR THOSE USES COVERED BY THE CERTIFIED APPLICATOR'S CERTIFICATION.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **USE INFORMATION**
> * * *
> **Adjuvant Requirement:** To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type

wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the Directions for Use for each crop on this label.

* * *

HERB CROP SUBGROUP 19A

* * *

Basil

* * *

Instructions To avoid illegal residues, Agri-Mek SC must be mixed with a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant."

(Emphasis added.)

14. Min He, acting under the direct supervision of the Respondent, did not use a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant as required by the label.

15. During the inspection with Min He, the HDOA inspector documented that no written instructions for applying AGRI-MEK SC MITICIDE/INSECTICIDE and no written instructions for applying DUPONT CORAGEN INSECT CONTROL were provided by the Respondent to Min He.

16. **HRS 149A-33** provides the following: "Rules: The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following . . . (5) To establish, as necessary, procedures for the issuance of guidelines to specify those conditions that constitute use of a pesticide in a manner inconsistent with its label."

17. **HAR 4-66-61** further provides that: A person may apply a restricted use pesticide, provided that: (1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; (2) The person shall be given specific written instructions by the certified applicator for applying the pesticide; (3) The certified applicator shall be responsible for all violations of the Act and this rule ..."

18. Min He stated that he has not used AGRI-MEK SC MITICIDE/INSECTICIDE since the application that occurred on July 22, 2017, and has not used DUPONT CORAGEN INSECT CONTROL since the application that occurred on August 18, 2017.

19. During the inspection, the HDOA inspector found no containers of AGRI-MEK SC MITICIDE/INSECTICIDE or DUPONT CORAGEN INSECT CONTROL in the storage area or elsewhere at Green Produce II, LLC. Min He was not able to show any container of AGRI-MEK SC MITICIDE/INSECTICIDE or DUPONT CORAGEN INSECT CONTROL to the HDOA inspector; Min He was only able to provide the used AGRI-MEK SC MITICIDE/INSECTICIDE and DUPONT CORAGEN INSECT CONTROL labels printed from the HDOA's Currently Licensed Pesticide Listing database.

CH

20. RUP sales records submitted to HDOA from by J. R. SIMPLOT COMPANY dba SIMPLOT (the Dealer) show that between August 1, 2017 and May 23, 2018, the Dealer sold Green Produce II, LLC 26 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE and between September 5, 2017 and May 23, 2018, the Dealer sold Green Produce II, LLC 17 gallons of DUPONT CORAGEN INSECT CONTROL under Respondent's commercial certification number, H72426..

21. On June 13, 2018, the HDOA Inspector collected invoices and delivery tags from The Dealer which showed Green Produce II, LLC had purchased 28 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE and 21 gallons of DUPONT CORAGEN INSECT CONTROL under Respondent's commercial certification number H72426 between July 6, 2017 and May 23, 2018. These invoices and delivery tags show that Green Produce II, LLC had received 27 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE between July 22, 2017 and May 23, 2018 and 19 gallons of DUPONT CORAGEN INSECT between August 18, 2017 and May 23, 2018.

22. RUP application records maintained at Green Produce II, LLC, and statements from Min He document that no applications of AGRI-MEK SC MITICIDE/INSECTICIDE occurred after July 22, 2017, and no applications of DUPONT CORAGEN INSECT CONTROL occurred since August 18, 2017.

23. On or about June 28, 2018, The HDOA Inspector met with the Respondent at Crown Pacific International, LLC's office on Kualalakai Parkway in Kapolei, Hawaii.

24. The HDOA Inspector issued the Respondent a Notice of Pesticide Use/Misuse Inspection pursuant to HRS section 149A-36. The Respondent voluntarily consented to the HDOA Inspector's request to conduct an inspection.

25. The Respondent attested that she provides supervision and oversite for the RUPs purchased by and used at Green Produce II, LLC.

26. The HDOA Inspector inventoried the contents on Crown Pacific International's pesticide storage and documented one and one-quarter (1 ¼) gallon of AGRI-MEK SC MITICIDE/INSECTICIDE and one (1) gallon of DUPONT CORAGEN INSECT CONTROL in the storage area. These items were purchases under Erece's private certification number for Crown Pacific, F78356, not the commercial certification number, H72426.

27. The HDOA Inspector reviewed RUP records produced and maintained by the Respondent. The Respondent provided the HDOA inspector with her records for applications of RUPs. However, these RUP records were clearly labeled for Crown Pacific, and listed the site of application as Crown Pacific Produce, LLC on Luakaha Street in Hilo, Hawaii, not for Green Produce.

28. As the Respondent, under her commercial certification number H7246, (1) purchased at least 27 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE for Green Produce II, LLC after Respondent's last application of AGRI-MEK SC MITICIDE/INSECTICIDE for

Green Produce II, LLC, (2) purchased at least 19 gallons of DUPONT CORAGEN INSECT CONTROL for Green Produce II, LLC after Respondent's last application of DUPONT CORAGEN INSECT CONTROL, and (3) does not have any AGRI-MEK SC MITICIDE/INSECTICIDE or DUPONT CORAGEN INSECT CONTROL in storage for Green Produce II, LLC, the Respondent has failed to keep adequate records of at least 37 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE and 19 gallons of DUPONT CORAGEN INSECT CONTROL purchased under her commercial certification number H7246 for Green Produce II, LLC. [1]

29. On or about July 19, 2018, Respondent e-mailed the HDOA Inspector a copy of written instructions she was providing to Green Produce for the use of AGRI-MEK SC MITICIDE/INSECTICIDE and DUPONT CORAGEN INSECT CONTROL.

30. HRS 149A-33 provides the following: "Rules: The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following . . . (4) To establish, as necessary, record keeping requirements for pesticide use by applicators."

31. HAR 4-66-62 further provides the following: Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators shall keep records of all applications of restricted use pesticides applied, at their principal place of business. (b) These records must be kept for a period of two years and shall be made available for inspection by the head during reasonable working hours. Recordkeeping information shall include: (1) Brand or common name of pesticide product applied; (2) EPA registration number; (3) Type of formulation; (4) Per cent active ingredient; (5) Scientific or common name of target pest; (6) Dilution rate; (7) Total amount of pesticide used; (8) Total area covered; (9) Time and date of application; (10) Address or location of treated site; (11) Name of certified applicator and his or her certification number; (12) Crop, commodity, stored product or other site; (13) Restricted entry interval and whether posting and oral notification are required; and (14) Any other information that the head deems to be necessary.

CAUSE OF ACTION

VIOLATION ONE:

HAR section 4-66-61(1) and (3) states:

"A person may apply a restricted use pesticide, provided that:

(1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; ...
(3) The certified applicator shall be responsible for all violations of the Act and this rule."

[1] We note that Respondent could not have legally distributed the unaccounted for RUPs as Respondent was not licensed to do so under HRS Section 149A-17.

C43

CHH

HRS section 149A-31 states: "Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

Between February 22, 2017 and July 22, 2017, Respondent DENBY ERECE violated HRS section 149A-31 by using the pesticide AGRI-MEK SC MITICIDE/INSECTICIDE in a manner inconsistent with its label, to wit.

The AGRI-MEK SC MITICIDE/INSECTICIDE label clearly states:

> "**RESTRICTED USE PESTICIDE**
> For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **HERB CROP SUBGROUP 19A**
> * * *
> **Basil**
> * * *
> **Maximum Amount per Year**: Do not apply more than 10.25 fl oz/A (or 0.056 lb ai/A) of Agri- Mek SC or any other foliar-applied abamectin-containing product per year."

Between February 22, 2017 and July 22, 2017, non-certified applicator Min He, under the direct supervision of the Respondent, applied 49 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE per acre of basil within 5 months.

VIOLATION TWO:

HAR section 4-66-61(1) and (3) states:

"A person may apply a restricted use pesticide, provided that:

(1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; ...
(3) The certified applicator shall be responsible for all violations of the Act and this rule."

HRS section 149A-31 states: "Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

Between February 22, 2017 and July 22, 2017, Respondent DENBY ERECE violated HRS section 149A-31 by using the pesticide AGRI-MEK SC MITICIDE/INSECTICIDE in a manner inconsistent with its label, to wit.

The AGRI-MEK SC MITICIDE/INSECTICIDE label clearly states:

> "**RESTRICTED USE PESTICIDE**
> For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification.
> * * *
> **DIRECTIONS FOR USE**
> * * *
> **USE INFORMATION**
> * * *
> **Adjuvant Requirement:** To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the Directions for Use for each crop on this label.
> * * *
> **HERB CROP SUBGROUP 19A**
> * * *
>
> **Basil**
> * * *
> **Instructions** To avoid illegal residues, Agri-Mek SC must be mixed with a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant."

On applications occurring from February 22, 2017 up to and including July 22, 2017, non-certified applicator Min He, under the direct supervision of the Respondent, mixed and applied 14 dilutions of AGRI-MEK SC MITICIDE/INSECTICIDE without adding any wetting, spreaders, penetrating agent, or other adjuvants to his mixtures of AGRI-MEK SC MITICIDE/INSECTICIDE.

VIOLATION THREE

HRS section 149A-33 provides:

CH5

CH6

> The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (3) To establish, as necessary, specific standards and guidelines which specify those conditions which constitute unreasonable adverse effects on the environment[.]
>
> . . . (4) To establish, as necessary, record keeping requirements for pesticide use by applicators[.]

HAR section 4-66-62 provides:

> (a) Certified pesticide applicators shall keep records of all applications of restricted use pesticides applied, at their principal place of business.
>
> (b) These records must be kept for a period of two years and shall be made available for inspection by the head during reasonable working hours. Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Per cent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site;
> (13) Restricted entry interval and whether posting and oral notification are required; and
> (14) Any other information that the head deems to be necessary.

From February 22, 2017 to July 22, 2017, Respondent DENBY ERECE violated HAR Section 4-66-62 by failing to keep adequate records of all applications of AGRI-MEK SC MITICIDE/INSECTICIDE, a restricted use pesticide, at Respondent's principal place of business, to wit.

From February 22, 2017 to July 22, 2017, Respondent failed to keep any of the required recordkeeping information for the applications of at least 27 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE. Additionally, records of AGRI-MEK SC MITICIDE/INSECTICIDE applications that were maintained by Respondent lacked multiple required items, including type of formulation, per cent active ingredient, dilution rate, whether posting and oral notification was required, and at time, a specific date.

VIOLATION FOUR

HRS section 149A-33 provides:

> The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (3) To establish, as necessary, specific standards and guidelines which specify those conditions which constitute unreasona
> ble adverse effects on the environment[.]
>
> . . . (4) To establish, as necessary, record keeping requirements for pesticide use by applicators[.]

HAR section 4-66-62 provides:

> (a) Certified pesticide applicators shall keep records of all applications of restricted use pesticides applied, at their principal place of business.
> (b) These records must be kept for a period of two years and shall be made available for inspection by the head during reasonable working hours. Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Per cent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site;

C47

> (13) Restricted entry interval and whether posting and oral notification are required; and
> (14) Any other information that the head deems to be necessary.

From February 22, 2017 to July 22, 2017, Respondent DENBY ERECE violated HAR Section 4-66-62 by failing to keep adequate records of all applications of DUPONT CORAGEN INSECT CONTROL, a restricted use pesticide, at Respondent's principal place of business, to wit.

From February 22, 2017 to July 22, 2017, Respondent failed to keep any of the required recordkeeping information for the applications of at least 19 gallons of DUPONT CORAGEN INSECT CONTROL. Additionally, records of DUPONT CORAGEN INSECT CONTROL applications that were maintained by Respondent lacked multiple required items, including type of formulation, per cent active ingredient, dilution rate, whether posting and oral notification was required, and at time, a specific date.

VIOLATION FIVE

HRS section 149A-33 provides:

> The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (5) To establish, as necessary, procedures for the issuance of guidelines to specify those conditions that constitute use of a pesticide in a manner inconsistent with its label.'

HAR section 4-66-61 provides:

> Conditions on the use of restricted use pesticides. A person may apply a restricted use pesticide, provided that: (1) The person is under the direct supervision of an applicator certified in a category appropriate to the restricted use pesticide being used; (2) The person shall be given specific written instructions by the certified applicator for applying the pesticide; (3) The certified applicator shall be responsible for all violations of the Act and this rule; . . ."

From February 22, 2017 to July 22, 2017, Respondent DENBY ERECE violated HAR Section 4-66-61 by failing to provide specific written directions for applying AGRI-MEK SC MITICIDE/INSECTICIDE or for applying DUPONT CORAGEN INSECT CONTROL to Min He, a non-certified applicator under the Respondent's direct supervision.

FINAL ORDER TO PAY CIVIL PENALTY

THE STATE OF HAWAI'I DEPARTMENT OF AGRICULTURE finds and concludes that Respondent's actions, as set forth above, have violated HRS sections 149A-31(1) and 149A-33(3), (4) and (5), and HAR sections 4-66-61 and 4-66-62.

Respondent was served with the Notice of Finding of Violation and Order on November 29, 2019. On December 16, 2019, Respondent requested to meet with the Hawai'i Department of Agriculture for purposes of resolving the Notice of Finding of Violation and Order by agreement. On January 27, 2020 and March 3, 2020, Respondent met with the Hawai'i Department of Agriculture to discuss settlement of all matters raised in the Notice of Finding of Violation and Order. Following the March 3, 2020 meeting, the Hawai'i Department of Agriculture and Respondent were unable to reach a settlement.

Respondent was issued a letter, dated June 4, 2020, stating that pursuant to HRS section 149A-41(b)(3), Respondent was entitled to a hearing to contest the Notice of Finding of Violation if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the June 4, 2020 letter. Respondent was served with the June 4, 2020 letter on June 8, 2020. No request for a hearing was received by the Office of the Chairperson of the Hawai'i Department of Agriculture by June 28, 2020. Such inaction constitutes a waiver of Respondent's right to a hearing on this matter.

CM

HAVING VIOLATED the Hawai'i Pesticide Law as set forth in HRS Chapter 149A, RESPONDENT DENBY ERECE IS HEREBY ORDERED TO PAY the following civil penalty in accordance with HRS section 149A-41(b)(2) and HAR section 4-66-66.1:

Violation One:	Two Thousand Dollars ($2,000.00)
Violation Two:	Seven Hundred Fifty Dollars ($750.00)
Violation Three:	Five Hundred Dollars ($500.00)
Violation Four:	Five Hundred Dollars ($500.00)
Violation Five:	Two Hundred Fifty Dollars ($250.00)

TOTAL CIVIL PENALTY: Four Thousand Dollars ($4,000.00) and SIX (6) MONTH SUSPENSION OF RESPONDENT DENBY ERECE'S COMMERCIAL APPLICATOR CERTIFICATION.

The civil penalty shall be paid within twenty (20) calendar days from the date of this Final Order by delivering payment to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814

THIS ORDER IS HEREBY DECLARED FINAL PURSUANT TO HRS SECTION 149A-41(b)(3)

Dated: 7-17-2020
Honolulu, Hawai'i

Phyllis Shimabukuro-Geiser
PHYLLIS SHIMABUKURO-GEISER,
Chairperson
Board of Agriculture

cc: file

DAVID Y. IGE
Governor

JOSH GREEN
Lt. Governor

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

MORRIS M. ATTA
Deputy to the Chairperson

State of Hawaii
DEPARTMENT OF AGRICULTURE
1428 South King Street
Honolulu, Hawaii 96814-2512
Phone: (808) 973-9600 FAX: (808) 973-9613

Exhibit D

September 24, 2020

Certified Mail No. 7019 1640 0000 2239 8303
Return Receipt Requested

Ms. Denby Erece
25-221 Ua Nahele Street
Hilo, Hawai'i 96720

Re: In the Matter of DENBY ERECE
Docket No. 19-PE-004

Dear Ms. Erece:

On November 13, 2019, a Notice of Finding of Violation and Proposed Order to Pay Civil Penalty ("NOV") was issued under Docket No. 19-PE-004 to DENBY ERECE ("Respondent"). The NOV was based upon inspections conducted on June 27, 2018 and June 28, 2018 pursuant to the authority granted in Hawai'i Revised Statutes ("HRS") section 149A-36 for purposes of determining compliance with state and federal pesticide laws. On November 29, 2019, the NOV was served on Respondent. On December 16, 2019, Respondent requested to meet with HDOA for purposes of resolving the NOV by agreement. On January 27, 2020 and March 3, 2020, Respondent met with HDOA to discuss settlement of all matters raised in the NOV. Following the March 3, 2020 meeting, HDOA and Respondent were unable to reach a settlement.

Respondent was issued a letter, dated June 4, 2020, stating that pursuant to HRS section 149A-41(b)(3), Respondent was entitled to a hearing to contest the NOV if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the June 4, 2020 letter. The June 4, 2020 letter clearly advised Respondent that the NOV would become a FINAL ORDER unless Respondent filed a written request for hearing within twenty (20) calendar days.

The twenty (20) day time period expired June 28, 2020. Accordingly, Respondent waived the opportunity to challenge the finding of violation and the NOV became a FINAL ORDER.

On July 17, 2020, HDOA issued a Final Order to Respondent. The Final Order required the Respondent to submit the civil penalty of four thousand dollars ($4,000.00) within twenty (20) calendar days from the date of the Final Order, or HDOA would refer the matter to the Hawai'i Department of the Attorney General for collection. Respondent was served with the Final Order on July 28, 2020. As of today's date, Respondent has failed to submit the civil penalty to HDOA and **is now in DEFAULT**.

DENBY ERECE
September 24, 2020

HRS section 149A-41(b)(4) provides as follows:

> In case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the board may determine, **the board shall refer the matter to the attorney general, who shall recover the amount by action in the appropriate court**. For any judicial proceeding to recover the administrative penalty imposed, **the attorney general need only show that notice was given**, a hearing was held or **the time granted for requesting a hearing has expired without such a request**, the **administrative penalty was imposed**, and that **the penalty remains unpaid**. (Emphasis added.)

Please note that this letter and enclosures will be tendered to the State of Hawai'i Attorney General as evidence of RESPONDENT'S FAILURE TO SUBMIT THE CIVIL PENALTY TO HDOA AS AGREED. Respondent DENBY ERECE may be deemed liable for court costs, attorney fees, and interest should legal action be required to secure payment of the four thousand dollar ($4,000) civil penalty.

Please remit the four thousand dollar ($4,000) civil penalty by October 9, 2020 to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814

Copies of the NOV, June 4, 2020 letter, Final Order, and signed certified mail return receipts are enclosed. **If no payment is received by October 9, 2020**, pursuant to HRS section 149A-41(b)(4), **the matter will be referred to the Hawai'i Department of the Attorney General for collection.**

Should you have any questions or concerns please contact the undersigned at (808) 973-9404 or via email at greg.y.takeshima@hawaii.gov

Sincerely yours,

GREG TAKESHIMA
Acting Pesticides Program Manager

GT:sn
[K:DENBYERECE]

Enclosures (36 pages)
cc: File
EPA Region IX

State of Hawai'i
Department of Agriculture
Plant Industry Division
Pesticide Branch

March 15, 2021

Board of Agriculture
Honolulu, Hawai'i

Subject: Request that the Final Order for In the Matter of HAWAI'I MEDICINAL BOTANICAL GARDEN and PAU CUN CHI, Docket No. 19-PE-036, be Referred to the Attorney General for Collection.

PROCEDURAL BACKGROUND:

On July 13, 2020, a Notice of Finding of Violation and Proposed Order to Pay Civil Penalty ("NOV") was issued under Docket No. 19-PE-036 to HAWAI'I MEDICINAL BOTANICAL GARDEN, INC. and PAU CUN CHI (collectively, "Respondents"). The NOV was based upon an inspection conducted on May 16, 2019 pursuant to the authority granted in Hawai'i Revised Statutes ("HRS") section 149A-36 for purposes of determining compliance with state and federal pesticide laws. On July 30, 2020, the NOV was served on the agent for Respondents. A copy of the NOV is attached hereto as "Exhibit A".

Pursuant to HRS section 149A-41(b)(3), Respondents were entitled to a hearing to contest the NOV if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the NOV. The NOV clearly advised Respondents that the NOV would become a FINAL ORDER unless Respondents filed a written request for hearing within twenty (20) calendar days.

The twenty (20) day time period expired August 19, 2020; no request for a hearing was received by the Office of the Chairperson of the Department of Agriculture by that time. Accordingly, Respondents waived the opportunity to challenge the finding of violation and the NOV became a FINAL ORDER.

On October 12, 2020, HDOA issued a Final Order to Respondents. The Final Order required the Respondents to submit the civil penalty of three thousand two hundred fifty dollars ($3,250.00) within twenty (20) calendar days from the receipt of the Final Order, or HDOA would refer the matter to the Hawai'i Department of the Attorney General for

Request that the Final Order for In the Matter of HAWAI'I MEDICINAL BOTANICAL GARDEN and PAU CUN CHI, Docket No. 19-PE-036, be Referred to the Attorney General for Collection

collection. The agent for Respondents was served with the Final Order on December 3, 2020. A copy of the Final Order is attached hereto as "Exhibit B".

The twenty (20) day time period expired December 23, 2020. HDOA did not receive the civil penalty of three thousand two hundred fifty dollars ($3,250.00) from Respondents.

On February 9, 2021, HDOA issued a Demand Letter to Respondents. The Demand Letter stated that Respondents failed to abide by the terms and conditions of the Final Order. The Demand Letter required Respondents to submit the civil penalty of three thousand two hundred fifty ($3,250.00) by February 24, 2021, or HDOA would refer the case to the Hawai'i Department of the Attorney General for collection. As of today's date, Respondents have failed to submit the civil penalty of three thousand two hundred fifty ($3,250.00) to HDOA. A copy of the Demand Letter is attached hereto as "Exhibit C".

AUTHORITY: Pursuant to section 149A-41(b)(4), *Hawai'i Revised Statutes* ("HRS"), in case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the Board may determine, the Board shall refer the matter to the Attorney General, who shall recover the amount by action in the appropriate court.

RECOMMENDATION: The Pesticides Branch of HDOA Plant Industry Division recommends the Board refer the Final Order for In the Matter of HAWAI'I MEDICINAL BOTANICAL GARDEN and PAU CUN CHI, Docket No. 19-PE-036, to the Attorney General for collection.

It is respectfully requested that the Board follow the recommendation set forth above.

Submitted by:

GREG TAKESHIMA
Acting Pesticides Branch Manager

Request that the Final Order for In the Matter of HAWAI'I MEDICINAL BOTANICAL GARDEN and PAU CUN CHI, Docket No. 19-PE-036, be Referred to the Attorney General for Collection

CONCURRED:

BECKY AZAMA
Acting Administrator, Plant Industry

Attachments – Exhibits "A" through "C"

APPROVED FOR SUBMISSION:

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

CS5

C56
Exhibit A

STATE OF HAWAI'I

DEPARTMENT OF AGRICULTURE

IN THE MATTER OF **HAWAI'I MEDICINAL BOTANICAL GARDEN INC. and PAU CUN CHI,** **Respondents.**	**DOCKET NO. 19-PE-036** **NOTICE OF FINDING OF VIOLATION; AUTHORITY; ALLEGED VIOLATIONS; CAUSES OF ACTION; ORDER TO CEASE AND DESIST; PROPOSED ORDER TO PAY CIVIL PENALTY; OPPORTUNITY FOR HEARING**

NOTICE OF FINDING OF VIOLATION

NOTICE IS HEREBY GIVEN that an inspection conducted by the State of Hawai'i Department of Agriculture yielded evidence of violation(s) of sections 149A-31(1) of the *Hawai'i Revised Statutes* ("HRS") and section 4-66-62 of the *Hawai'i Administrative Rules* ("HAR").

AUTHORITY

The State of Hawai'i Department of Agriculture ("HDOA"), pursuant to the authority granted in HRS sections 149A-31 and 149A-41, and HAR sections 4-66-3, and 4-66-66.1, does hereby bring this action against Respondents HAWAI'I MEDICINAL BOTANICAL GARDEN INC. and PAU CUN CHI for violation of HRS sections 149A-31(1) and HAR section 4-66-62. The inspection was conducted pursuant to the authority granted in HRS section 149A-36 for purposes of determining compliance with state and federal pesticide laws.

ALLEGED VIOLATIONS

1. On or about May 16, 2019, State of Hawai'i Environmental Health Specialist Russell Nishii ("HDOA Inspector") conducted a Certified Applicator Recordkeeping and an after-the-fact Agricultural Use inspection at HAWAI'I MEDICINAL BOTANICAL GARDEN INC. ("Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN"), located at 54-230 Kamehameha Highway, Hauula, Hawai'i 96717 ("Farm").

2. The HDOA Inspector met with PAU CUN CHI ("Respondent CHI"), the owner of Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN. Respondent CHI possesses Certified Private Applicator license number B13076.

3. Pursuant to HRS section 149A-36, the HDOA Inspector issued a Notice of Pesticide Use/Misuse Inspection to Respondent CHI. Respondent CHI voluntarily consented to the HDOA Inspectors' request to conduct an inspection.

4. The HDOA Inspector reviewed the most recent pesticide application made at the Farm with Respondent CHI.

5. Respondent CHI stated that on or about April 16, 2019, he mixed approximately nine (9) fluid ounces of the restricted use pesticide ("RUP") AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) into approximately 300 gallons of water, and applied the dilution to approximately 3.2 acres of basil to treat for leaf miner.

6. Respondent CHI informed the HDOA Inspector that a adjuvant/surfactant was not used during the application of AGRI-MEK SC MITICIDE/INSECTICIDE on or about April 16, 2019.

7. The AGRI-MEK SC MITICIDE/INSECTICIDE label states: "**USE INFORMATION** . . . **Adjuvant Requirement**: To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the **Directions for Use** for each crop on this label. Non-ionic activator type wetting, spreading and/or penetrating spray adjuvants include non-ionic surfactants (NIS) with at least 75% surface active agent and crop oil concentrates (COC), vegetable oil concentrates (VOC), methylated seed/vegetable oils (MSO) and organosilicones (OS) with at least 15% emulsifiers/surfactants and include blends of these non-ionic activator type spray adjuvants. Spray adjuvants must be compatible with Agri-Mek SC and must be used at concentrations specified on the **spray adjuvant product label** directions for use for the targeted crop unless more specific directions are provided in the **Directions for Use** for individual crops on this label. **Do not use binder or sticker type adjuvants because these type adjuvants may reduce translaminar movement of the active ingredient into the plant**. SYNGENTA recommends the use of a Chemical Producers and Distributors Association-certified spray adjuvant. . . **CROP USE DIRECTIONS** . . . **HERB CROP SUBGROUP 19A Crops in this subgroup are:** . . . **Basil** . . . **Adjuvant Requirement**: To avoid illegal residues Agri-Mek SC **must** be mixed with a non-ionic

CST

activator type wetting, spreading and/or penetrating spray adjuvant as instructed in the Use Information section found at the beginning of this Agri-Mek SC label. The spray adjuvant must be approved for use on the intended target crop in the Herb Crop Subgroup 19A. . ."

8. Pursuant to HRS section 149A-31: "Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

(Emphasis added.)

9. During the inspection on or about May 16, 2019, the HDOA Inspector conducted a baseline inventory of the following RUPs in Respondent CHI's storage:

- LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) – 0 gallons
- WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) – 0 gallons
- AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) – 40 fluid ounces
- GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) – 2.6 gallons
- DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) – 0 gallons
- DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) – 5.0 gallons
- DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) – 1 gallon
- ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) – 0 gallons

10. RUP sales records submitted to HDOA from June 27, 2017 to April 30, 2019 indicated that Respondent CHI purchased the following amounts of RUPs:

- LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) – 7.5 gallons
- WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) – 2.0 gallons
- AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) – 7.5 gallons
- GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) – 50.0 gallons
- DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) – 3.0 gallons
- DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) – 80.0 gallons
- DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) – 17.0 gallons
- ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) – 5.0 gallons

11. During the inspection on or about May 16, 2019, the HDOA Inspector asked to see Respondent CHI's records of applications of RUPs under Respondent CHI's certification number.

12. Respondent CHI informed the HDOA Inspector that the RUP application records for the years 2017 and 2018 were located at a farm on Hawai'i island.

13. Respondent CHI's records for the year 2019 indicated that the following RUPs (with respective amounts) were applied: 28 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE; 20 pints of GRAMOXONE SL 2.0 HERBICIDE; 20 pints of DUPONT LANNATE LV INSECTICIDE; 167 fluid ounces of DUPONT CORAGEN INSECT CONTROL.

14. Respondent CHI's RUP application records for the year 2019 omitted the following information required by HAR section 4-66-62: **Dilution rate and whether posting and oral notification are required**.

15. Based on HDOA's RUP sales records from June 27, 2017 to April 30, 2019 and the RUP inventory check conducted on or about May 16, 2019, Respondent CHI was unable to account for the use of the following amounts of RUPs:

- LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) – 7.5 gallons
- WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) – 2.0 gallons
- AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) – 7.0 gallons
- GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) – 44.9 gallons
- DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) – 3.0 gallons
- DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) – 72.5 gallons
- DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) – 14.7 gallons
- ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) – 5.0 gallons

16. Pursuant to HRS section 149A-33(4): "The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (4) To **establish**, as necessary, **record keeping requirements** for pesticide use **by applicators**[.]"

(Emphasis added.)

17. Pursuant to HAR section 4-66-62: "(a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business**. (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours**. Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;

CS9

(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required; and
(14) Any other information that the head deems to be necessary. ..."

(Emphasis added.)

18. At the time of the inspection on or about May 16, 2019, Respondent CHI was unable to provide any RUP application records for the years 2017 and 2018.

19. On or about May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, the HDOA Inspector requested, via email, the RUP application records for the years 2017 and 2018 from Respondent CHI. The requested application records were not submitted to the HDOA Inspector.

20. HRS section 149A-41(d) states: "Liabilities. When construing and enforcing the provisions of this chapter, the act, omission, or failure of any officer, agent, or other person acting for or employed by any person shall in every case be also deemed to be the act, omission, or failure of such person as well as that of the person employed."

21. HRS section 149A-34 states: "The department may deny issuance of a certificate for reasonable cause. Any certificate issued pursuant to rules adopted under section 149A-33(1) may be suspended or revoked by the department, after hearing, for violation of any condition of the certificate or of any law or rule pertaining to the use of any restricted use pesticide. Any order made by the department for the suspension or revocation of a certificate shall be in writing and shall set forth the reasons for the suspension or revocation. ..."

22. Respondent CHI has been issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, which notified him that any further violations of the Hawai'i Pesticides Law may result in administrative action.

CAUSES OF ACTION

VIOLATION ONE:

HRS section 149A-31 provides in pertinent part as follows:

> **"Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"**

(Emphasis added.)

On or about April 16, 2019, Respondents HAWAI'I MEDICINAL BOTANICAL INC. and PAU CUN CHI, violated HRS section 149A-31(1) by using the restricted use pesticide AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) in a manner inconsistent with its label, to wit:

The AGRI-MEK SC MITICIDE/INSECTICIDE label clearly stated:

> "**USE INFORMATION** . . . **Adjuvant Requirement**: To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the **Directions for Use** for each crop on this label. Non-ionic activator type wetting, spreading and/or penetrating spray adjuvants include non-ionic surfactants (NIS) with at least 75% surface active agent and crop oil concentrates (COC), vegetable oil concentrates (VOC), methylated seed/vegetable oils (MSO) and organosilicones (OS) with at least 15% emulsifiers/surfactants and include blends of these non-ionic activator type spray adjuvants. Spray adjuvants must be compatible with Agri-Mek SC and must be used at concentrations specified on the **spray adjuvant product label** directions for use for the targeted crop unless more specific directions are provided in the **Directions for Use** for individual crops on this label. **Do not use binder or sticker type adjuvants because these type adjuvants may reduce translaminar movement of the active ingredient into the plant.** SYNGENTA recommends the use of a Chemical Producers and Distributors Association-certified spray adjuvant. . . **CROP USE DIRECTIONS** . . . **HERB CROP SUBGROUP 19A Crops in this subgroup are:** . . . **Basil** . . . **Adjuvant Requirement**: To avoid illegal residues Agri-Mek SC **must** be mixed with a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant as instructed in the Use Information section found at the beginning of this Agri-Mek SC label. The spray adjuvant must be approved for use on the intended target crop in the Herb Crop Subgroup 19A. . ."

(Emphasis added.)

Respondent CHI, as the certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., did not mix AGRI-MEK SC

C61

MITICIDE/INSECTICIDE with an adjuvant/surfactant during his application on or about April 16, 2019.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION TWO:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years **and shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site:
> (13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 7.5 gallons of LORSBAN ADVANCED INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION THREE:

HRS section 149A-33(4) provides:

C63

Rules. The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:

* * *

(4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL

BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 2.0 gallons of WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION FOUR:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for

CLS

a period of two years and **shall be made available for inspection by the head during reasonable working hours**. Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) for the years 2017

and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 7.0 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE that were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION FIVE:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business**. (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours**. Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;

C67

(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 44.9 gallons of GRAMOXONE SL 2.0 HERBICIDE that were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION SIX:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site:
> (13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified

CM

restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 3.0 gallons of DUPONT LANNATE SP INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION SEVEN:

HRS section 149A-33(4) provides:

Rules. The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:

* * *

(4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours**. Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application

records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 72.5 gallons of DUPONT LANNATE LV INSECTICIDE that were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION EIGHT:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;

(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 14.7 gallons of DUPONT CORAGEN INSECT CONTROL that

were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION NINE:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site:
> (13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

> Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 5.0 gallons of ASANA XL INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION TEN:

HRS section 149A-33(4) provides:

Rules. The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:

* * *

(4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) **Dilution rate**;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and **whether posting and oral notification are required**[.]

(Emphasis added.)

On or about May 16, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a

certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent CHI was only able to provide his application records for the year 2019. The 2019 restricted use pesticide application records provided by Respondent CHI were missing the following information: **Dilution rate; whether posting and oral notification are required.**

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

ORDER TO CEASE AND DESIST

The State of Hawai'i, Department of Agriculture, pursuant to the provisions of HAR section 4-66-3, **DOES HEREBY ORDER RESPONDENTS TO CEASE AND DESIST VIOLATION OF CHAPTER 149A.** You are hereby notified that any further violation of HRS Chapter 149A will result in increased penalties as provided by law.

YOU ARE SO NOTIFIED.

PROPOSED ORDER TO PAY CIVIL PENALTY

THE STATE OF HAWAI'I DEPARTMENT OF AGRICULTURE finds and concludes that Respondents' actions, as set forth above, have violated HRS sections 149A-31(1) and HAR section 4-66-62.

HAVING VIOLATED the Hawai'i Pesticides Law as set forth in HRS Chapter 149A, RESPONDENTS ARE HEREBY ORDERED TO PAY the following civil penalty in accordance with HRS section 149A-41(b)(1)-(2) and HAR section 4-66-66.1:

Violation One:	One Thousand Dollars ($1,000.00)
Violation Two:	Two Hundred Fifty Dollars ($250.00)
Violation Three:	Two Hundred Fifty Dollars ($250.00)
Violation Four:	Two Hundred Fifty Dollars ($250.00)
Violation Five:	Two Hundred Fifty Dollars ($250.00)
Violation Six:	Two Hundred Fifty Dollars ($250.00)
Violation Seven:	Two Hundred Fifty Dollars ($250.00)
Violation Eight:	Two Hundred Fifty Dollars ($250.00)
Violation Nine:	Two Hundred Fifty Dollars ($250.00)
Violation Ten:	Two Hundred Fifty Dollars ($250.00)

TOTAL CIVIL PENALTY: Three Thousand Two Hundred Fifty Dollars ($3,250.00) and three (3) month suspension of PAU CUN CHI's Restricted Use Pesticide Applicator Certification.

The civil penalty shall be paid within twenty (20) business days from the date of this Notice of Finding of Violation by delivering payment to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814

The Department of Agriculture may use all reasonable means to collect the full amount of the penalty, if not paid within the specified time period, as authorized by law.

Pursuant to HAR section 4-66-60, Respondent PAU CUN CHI is also required to participate in a remedial education program. The remedial education program shall be conducted

by the HDOA, Education Section, or by a person deemed qualified by the HDOA, Education Section. Completion of the remedial education program must be made prior to the reinstatement of Respondent PAU CUN CHI's restricted use pesticide certification.

OPPORTUNITY FOR HEARING

Pursuant to HRS section 149A-41(b)(3), **Respondents are entitled to a hearing** to contest this Proposed Order or any portion of this Notice of Finding of Violation. If you wish to contest this Proposed Order or any portion of this Notice of Finding of Violation, you must submit a **written request for hearing** to the Office of the Chairperson **within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation. Upon receipt of the written request for hearing, a notice will be issued setting forth the date, time, and place where such hearing will be conducted. The hearing will be conducted pursuant to HRS Chapter 91, the Hawai'i Administrative Procedure Act.

In lieu of a hearing, you may request a meeting with representatives of the State of Hawai'i Department of Agriculture concerning an informal disposition pursuant to HRS section 91-9(d). The request to meet with representatives of the State of Hawai'i Department of Agriculture must be made **in writing within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation. If a settlement can be reached, a Consent Agreement and Order will be signed by all parties. A Consent Agreement and Order shall constitute a waiver of your right to a hearing on any matter to which you have agreed.

The **civil penalty and any proposed corrective action** contained in the Notice of Finding of Violation **shall become a Final Order**, as set forth below, **unless the Respondents file a written request for hearing or meeting within twenty (20) calendar days from the date of receipt** of this Notice of Finding of Violation.

ISSUED THIS 13th DAY OF July, 2020.

Pesticides Program Manager

THIS ORDER IS HEREBY DECLARED FINAL PURSUANT TO HRS Section 149A-41(b)(3).

Dated: _____
Honolulu, Hawai'i

PHYLLIS SHIMABUKURO-GEISER,
Chairperson
Board of Agriculture

cc: file

C79

C80

Exhibit B

STATE OF HAWAI'I

DEPARTMENT OF AGRICULTURE

IN THE MATTER OF **HAWAI'I MEDICINAL BOTANICAL GARDEN INC., and PAU CUN CHI,** **Respondents.**	**DOCKET NO. 19-PE-036** **FINDING OF VIOLATION; AUTHORITY; VIOLATIONS; CAUSE OF ACTION; FINAL ORDER TO PAY CIVIL PENALTY**

FINDING OF VIOLATION

An inspection conducted by the State of Hawai'i Department of Agriculture yielded evidence of violation(s) of section 149A-31(1) of the *Hawai'i Revised Statutes* ("HRS") and section 4-66-62 of the *Hawai'i Administrative Rules* ("HAR").

AUTHORITY

The State of Hawai'i Department of Agriculture ("HDOA"), pursuant to the authority granted in HRS sections 149A-31 and 149A-41, and HAR sections 4-66-3, and 4-66-66.1, does hereby bring this action against Respondents HAWAI'I MEDICINAL BOTANICAL GARDEN INC. and PAU CUN CHI for violation of HRS section 149A-31(1) and HAR section 4-66-62. The inspection was conducted pursuant to the authority granted in HRS section 149A-36 for purposes of determining compliance with state and federal pesticide laws.

VIOLATIONS

1. On or about May 16, 2019, State of Hawai'i Environmental Health Specialist Russell Nishii ("HDOA Inspector") conducted a Certified Applicator Recordkeeping and an after-the-fact Agricultural Use inspection at HAWAI'I MEDICINAL BOTANICAL GARDEN INC. ("Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN"), located at 54-230 Kamehameha Highway, Hauula, Hawai'i 96717 ("Farm").

2. The HDOA Inspector met with PAU CUN CHI ("Respondent CHI"), the owner of Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN. Respondent CHI possesses Certified Private Applicator license number B13076.

3. Pursuant to HRS section 149A-36, the HDOA Inspector issued a Notice of Pesticide Use/Misuse Inspection to Respondent CHI. Respondent CHI voluntarily consented to the HDOA Inspectors' request to conduct an inspection.

4. The HDOA Inspector reviewed the most recent pesticide application made at the Farm with Respondent CHI.

5. Respondent CHI stated that on or about April 16, 2019, he mixed approximately nine (9) fluid ounces of the restricted use pesticide ("RUP") AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) into approximately 300 gallons of water, and applied the dilution to approximately 3.2 acres of basil to treat for leaf miner.

6. Respondent CHI informed the HDOA Inspector that a adjuvant/surfactant was not used during the application of AGRI-MEK SC MITICIDE/INSECTICIDE on or about April 16, 2019.

7. The AGRI-MEK SC MITICIDE/INSECTICIDE label states: "**USE INFORMATION** . . . **Adjuvant Requirement**: To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the **Directions for Use** for each crop on this label. Non-ionic activator type wetting, spreading and/or penetrating spray adjuvants include non-ionic surfactants (NIS) with at least 75% surface active agent and crop oil concentrates (COC), vegetable oil concentrates (VOC), methylated seed/vegetable oils (MSO) and organosilicones (OS) with at least 15% emulsifiers/surfactants and include blends of these non-ionic activator type spray adjuvants. Spray adjuvants must be compatible with Agri-Mek SC and must be used at concentrations specified on the **spray adjuvant product label** directions for use for the targeted crop unless more specific directions are provided in the **Directions for Use** for individual crops on this label. **Do not use binder or sticker type adjuvants because these type adjuvants may reduce translaminar movement of the active ingredient into the plant**. SYNGENTA recommends the use of a Chemical Producers and Distributors Association-certified spray adjuvant. . . **CROP USE DIRECTIONS** . . . **HERB CROP SUBGROUP 19A Crops in this subgroup are:** . . . **Basil** . . . **Adjuvant Requirement**: To avoid illegal residues Agri-Mek SC **must** be mixed with a non-ionic

C81

activator type wetting, spreading and/or penetrating spray adjuvant as instructed in the Use Information section found at the beginning of this Agri-Mek SC label. The spray adjuvant must be approved for use on the intended target crop in the Herb Crop Subgroup 19A. . ."

8. Pursuant to HRS section 149A-31: "Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"

(Emphasis added.)

9. During the inspection on or about May 16, 2019, the HDOA Inspector conducted a baseline inventory of the following RUPs in Respondent CHI's storage:

- LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) – 0 gallons
- WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) – 0 gallons
- AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) – 40 fluid ounces
- GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) – 2.6 gallons
- DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) – 0 gallons
- DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) – 5.0 gallons
- DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) – 1 gallon
- ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) – 0 gallons

10. RUP sales records submitted to HDOA from June 27, 2017 to April 30, 2019 indicated that Respondent CHI purchased the following amounts of RUPs:

- LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) – 7.5 gallons
- WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) – 2.0 gallons
- AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) – 7.5 gallons
- GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) – 50.0 gallons
- DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) – 3.0 gallons
- DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) – 80.0 gallons
- DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) – 17.0 gallons
- ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) – 5.0 gallons

11. During the inspection on or about May 16, 2019, the HDOA Inspector asked to see Respondent CHI's records of applications of RUPs under Respondent CHI's certification number.

12. Respondent CHI informed the HDOA Inspector that the RUP application records for the years 2017 and 2018 were located at a farm on Hawai'i island.

13. Respondent CHI's records for the year 2019 indicated that the following RUPs (with respective amounts) were applied: 28 fluid ounces of AGRI-MEK SC MITICIDE/INSECTICIDE; 20 pints of GRAMOXONE SL 2.0 HERBICIDE; 20 pints of DUPONT LANNATE LV INSECTICIDE; 167 fluid ounces of DUPONT CORAGEN INSECT CONTROL.

14. Respondent CHI's RUP application records for the year 2019 omitted the following information required by HAR section 4-66-62: **Dilution rate and whether posting and oral notification are required.**

15. Based on HDOA's RUP sales records from June 27, 2017 to April 30, 2019 and the RUP inventory check conducted on or about May 16, 2019, Respondent CHI was unable to account for the use of the following amounts of RUPs:

- LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) – 7.5 gallons
- WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) – 2.0 gallons
- AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) – 7.0 gallons
- GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) – 44.9 gallons
- DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) – 3.0 gallons
- DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) – 72.5 gallons
- DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) – 14.7 gallons
- ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) – 5.0 gallons

16. Pursuant to HRS section 149A-33(4): "The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following: . . . (4) To **establish**, as necessary, **record keeping requirements** for pesticide use **by applicators**[.]"

(Emphasis added.)

17. Pursuant to HAR section 4-66-62: "(a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;

C83

(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required; and
(14) Any other information that the head deems to be necessary. ..."

(Emphasis added.)

18. At the time of the inspection on or about May 16, 2019, Respondent CHI was unable to provide any RUP application records for the years 2017 and 2018.

19. On or about May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, the HDOA Inspector requested, via email, the RUP application records for the years 2017 and 2018 from Respondent CHI. The requested application records were not submitted to the HDOA Inspector.

20. HRS section 149A-41(d) states: "Liabilities. When construing and enforcing the provisions of this chapter, the act, omission, or failure of any officer, agent, or other person acting for or employed by any person shall in every case be also deemed to be the act, omission, or failure of such person as well as that of the person employed."

21. HRS section 149A-34 states: "The department may deny issuance of a certificate for reasonable cause. Any certificate issued pursuant to rules adopted under section 149A-33(1) may be suspended or revoked by the department, after hearing, for violation of any condition of the certificate or of any law or rule pertaining to the use of any restricted use pesticide. Any order made by the department for the suspension or revocation of a certificate shall be in writing and shall set forth the reasons for the suspension or revocation. ..."

22. Respondent CHI has been issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, which notified him that any further violations of the Hawai'i Pesticides Law may result in administrative action.

CAUSE OF ACTION

VIOLATION ONE:

HRS section 149A-31 provides in pertinent part as follows:

> **"Prohibited Acts. No person shall: (1) Use any pesticide in a manner inconsistent with its label[.]"**

(Emphasis added.)

On or about April 16, 2019, Respondents HAWAI'I MEDICINAL BOTANICAL INC. and PAU CUN CHI, violated HRS section 149A-31(1) by using the restricted use pesticide AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) in a manner inconsistent with its label, to wit:

The AGRI-MEK SC MITICIDE/INSECTICIDE label clearly stated:

> "**USE INFORMATION . . . Adjuvant Requirement**: To avoid illegal crop residues, Agri-Mek SC **must always** be mixed with a non-phytotoxic, non-ionic activator type wetting, spreading and/or penetrating spray adjuvant or horticultural oil (not a dormant oil) as specified in the **Directions for Use** for each crop on this label. Non-ionic activator type wetting, spreading and/or penetrating spray adjuvants include non-ionic surfactants (NIS) with at least 75% surface active agent and crop oil concentrates (COC), vegetable oil concentrates (VOC), methylated seed/vegetable oils (MSO) and organosilicones (OS) with at least 15% emulsifiers/surfactants and include blends of these non-ionic activator type spray adjuvants. Spray adjuvants must be compatible with Agri-Mek SC and must be used at concentrations specified on the **spray adjuvant product label** directions for use for the targeted crop unless more specific directions are provided in the **Directions for Use** for individual crops on this label. **Do not use binder or sticker type adjuvants because these type adjuvants may reduce translaminar movement of the active ingredient into the plant**. SYNGENTA recommends the use of a Chemical Producers and Distributors Association-certified spray adjuvant. . . **CROP USE DIRECTIONS . . . HERB CROP SUBGROUP 19A Crops in this subgroup are: . . . Basil . . . Adjuvant Requirement**: To avoid illegal residues Agri-Mek SC **must** be mixed with a non-ionic activator type wetting, spreading and/or penetrating spray adjuvant as instructed in the Use Information section found at the beginning of this Agri-Mek SC label. The spray adjuvant must be approved for use on the intended target crop in the Herb Crop Subgroup 19A. . ."

(Emphasis added.)

Respondent CHI, as the certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., did not mix AGRI-MEK SC MITICIDE/INSECTICIDE with an adjuvant/surfactant during his application on or about April 16, 2019.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION TWO:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years **and shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site:

(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for LORSBAN ADVANCED INSECTICIDE (EPA Reg. No. 62719-591) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 7.5 gallons of LORSBAN ADVANCED INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION THREE:

HRS section 149A-33(4) provides:

Rules. The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:

* * *

(4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE (EPA Reg. No. 100-1295) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 2.0 gallons of WARRIOR II WITH ZEON TECHNOLOGY INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION FOUR:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

C81

HAR section 4-66-62 provides:

Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent

PAU CUN CHI was unable to produce any restricted use pesticide application records for AGRI-MEK SC MITICIDE/INSECTICIDE (EPA Reg. No. 100-1351) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 7.0 gallons of AGRI-MEK SC MITICIDE/INSECTICIDE that were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION FIVE:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;

C91

(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for GRAMOXONE SL 2.0 HERBICIDE (EPA Reg. No. 100-1431) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 44.9 gallons of GRAMOXONE SL 2.0 HERBICIDE that were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's

restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION SIX:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site:
> (13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

> Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for DUPONT LANNATE SP INSECTICIDE (EPA Reg. No. 352-342) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 3.0 gallons of DUPONT LANNATE SP INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION SEVEN:

HRS section 149A-33(4) provides:

Rules. The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:

* * *

(4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business**. (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours**. Recordkeeping information shall include:

(1) Brand or common name of pesticide product applied;
(2) EPA registration number;
(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for DUPONT LANNATE LV INSECTICIDE (EPA Reg. No. 352-384) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 72.5 gallons of DUPONT LANNATE LV INSECTICIDE that were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION EIGHT:

HRS section 149A-33(4) provides:

Rules. The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:

* * *

(4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) Dilution rate;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site:
> (13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application

C97

records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for DUPONT CORAGEN INSECT CONTROL (EPA Reg. No. 352-729) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to account for 14.7 gallons of DUPONT CORAGEN INSECT CONTROL that were sold to him under Respondent's private certification number. Respondent PAU CUN CHI's restricted use pesticide application records for the year 2019 omitted the dilution rate and whether posting and oral notification were required.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION NINE:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business**. (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours**. Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;

(3) Type of formulation;
(4) Percent active ingredient;
(5) Scientific or common name of target pest;
(6) Dilution rate;
(7) Total amount of pesticide used;
(8) Total area covered;
(9) Time and date of application;
(10) Address or location of treated site;
(11) Name of certified applicator and his or her certification number;
(12) Crop, commodity, stored product or other site:
(13) Restricted entry interval and whether posting and oral notification are required[.]

(Emphasis added.)

On or about May 16, 2019, to and including May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent PAU CUN CHI was unable to produce any restricted use pesticide application records for ASANA XL INSECTICIDE (EPA Reg. No. 59639-209) for the years 2017 and 2018 per requests from the HDOA Inspector on or about May 16, 2019, May 20, 2019, May 23, 2019, May 28, 2019, and June 5, 2019. Respondent PAU CUN CHI was unable to

CM

account for 5.0 gallons of ASANA XL INSECTICIDE that were sold to him under Respondent's private certification number.

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

VIOLATION TEN:

HRS section 149A-33(4) provides:

> **Rules.** The department shall have the authority to carry out and effectuate the purpose of this chapter by rules, including but not limited to the following:
> * * *
> (4) To establish, as necessary, **record keeping requirements** for pesticide use by applicators[.]

(Emphasis added.)

HAR section 4-66-62 provides:

> Certified pesticide applicator recordkeeping. (a) Certified pesticide applicators **shall keep records of all applications of restricted use pesticides applied, at their principal place of business.** (b) These records must be kept for a period of two years and **shall be made available for inspection by the head during reasonable working hours.** Recordkeeping information shall include:
>
> (1) Brand or common name of pesticide product applied;
> (2) EPA registration number;
> (3) Type of formulation;
> (4) Percent active ingredient;
> (5) Scientific or common name of target pest;
> (6) **Dilution rate**;
> (7) Total amount of pesticide used;
> (8) Total area covered;
> (9) Time and date of application;
> (10) Address or location of treated site;
> (11) Name of certified applicator and his or her certification number;
> (12) Crop, commodity, stored product or other site:
> (13) Restricted entry interval and **whether posting and oral notification are required**[.]

(Emphasis added.)

On or about May 16, 2019, Respondent HAWAI'I MEDICINAL BOTANICAL INC. and its certified restricted use pesticide applicator Respondent PAU CUN CHI **violated HAR section 4-66-62** by failing to maintain restricted use pesticide application records, to wit:

> Respondent PAU CUN CHI was the certified applicator of restricted use pesticides for Respondent HAWAI'I MEDICINAL BOTANICAL GARDEN INC. As a certified restricted use pesticide applicator for Respondent HAWAI'I MEDICINAL BOTANICAL INC., Respondent CHI was required to maintain records of all restricted use pesticide applications. On or about May 16, 2019, during reasonable working hours, Respondent PAU CUN CHI was asked to make his restricted use pesticide application records available for inspection by the Hawai'i Department of Agriculture. Respondent CHI was only able to provide his application records for the year 2019. The 2019 restricted use pesticide application records provided by Respondent CHI were missing the following information: **Dilution rate; whether posting and oral notification are required.**

Respondent PAU CUN CHI was issued a Warning Notice dated August 27, 2018 and a Notice of Violation under Docket No. 19-PE-009, making this a subsequent offense subject to increased penalties.

FINAL ORDER TO PAY CIVIL PENALTY

THE STATE OF HAWAI'I DEPARTMENT OF AGRICULTURE finds and concludes that the Respondents' actions, as set forth above, have violated HRS section 149A-31(1) and HAR section 4-66-62.

Pursuant to HRS section 149A-41(b)(3), Respondents were entitled to a hearing to contest the Notice of Finding of Violation if a written request for a hearing was submitted to the

C101

Office of the Chairperson within twenty (20) calendar days from the date of receipt of the Notice of Finding of Violation. Respondents were served with the Notice of Finding of Violation on July 30, 2020. No request for a hearing was received by the Office of the Chairperson of the Department of Agriculture by August 19, 2020. Such inaction constitutes a waiver of Respondents' right to a hearing on this matter.

HAVING VIOLATED the Hawai'i Pesticides Law as set forth in HRS Chapter 149A, RESPONDENTS ARE HEREBY ORDERED TO PAY the following civil penalty in accordance with HRS section 149A-41(b)(1)-(2) and HAR section 4-66-66.1:

Violation One:	One Thousand Dollars ($1,000.00)
Violation Two:	Two Hundred Fifty Dollars ($250.00)
Violation Three:	Two Hundred Fifty Dollars ($250.00)
Violation Four:	Two Hundred Fifty Dollars ($250.00)
Violation Five:	Two Hundred Fifty Dollars ($250.00)
Violation Six:	Two Hundred Fifty Dollars ($250.00)
Violation Seven:	Two Hundred Fifty Dollars ($250.00)
Violation Eight:	Two Hundred Fifty Dollars ($250.00)
Violation Nine:	Two Hundred Fifty Dollars ($250.00)
Violation Ten:	Two Hundred Fifty Dollars ($250.00)

TOTAL CIVIL PENALTY: Three Thousand Two Hundred Fifty Dollars ($3,250.00) and three (3) month suspension of PAU CUN CHI's Restricted Use Pesticide Applicator Certification.

The civil penalty shall be paid within twenty (20) business days from the date of this Notice of Finding of Violation by delivering payment to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814.

Pursuant to HRS section 149A-41(b)(4), "[i]n case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the board may determine, the board shall refer the matter to the attorney general, who shall recover the amount by action in the appropriate court. For any judicial proceeding to recover the administrative penalty imposed, the attorney general need only show that notice was given, a hearing was held or the time granted for requesting a hearing has expired without such a request, the administrative penalty was imposed, and that the penalty remains unpaid."

Pursuant to HAR section 4-66-60, Respondent PAU CUN CHI is also required to participate in a remedial education program. The remedial education program shall be conducted by the HDOA, Education Section, or by a person deemed qualified by the HDOA, Education Section. Completion of the remedial education program must be made prior to the reinstatement of Respondent PAU CUN CHI's restricted use pesticide certification.

THIS ORDER IS HEREBY DECLARED FINAL PURSUANT TO HRS SECTION 149A-41(b)(3)

Dated: 10-12-20
Honolulu, Hawai'i

Phyllis Shimabukuro-Geiser
PHYLLIS SHIMABUKURO-GEISER,
Chairperson
Board of Agriculture

cc: file

C103

C104

DAVID Y. IGE
Governor

JOSH GREEN
Lt. Governor

PHYLLIS SHIMABUKURO-GEISER
Chairperson, Board of Agriculture

MORRIS M. ATTA
Deputy to the Chairperson

State of Hawaii
DEPARTMENT OF AGRICULTURE
1428 South King Street
Honolulu, Hawaii 96814-2512
Phone: (808) 973-9600 FAX: (808) 973-9613

Exhibit C

February 9, 2021

Certified Mail No. 7020 2450 0001 2279 4083
Return Receipt Requested

Mr. Pau Cun Chi
Agent for Hawai'i Medicinal Botanical Garden, Inc.
P.O. Box 561
Mountain View, Hawai'i 96771

Re: In the Matter of HAWAI'I MEDICINAL BOTANICAL GARDEN, INC.
Docket No. 19-PE-036

Dear Agent:

On July 13, 2020, a Notice of Finding of Violation and Proposed Order to Pay Civil Penalty ("NOV") was issued under Docket No. 19-PE-036 to HAWAI'I MEDICINAL BOTANICAL GARDEN, INC. and PAU CUN CHI (collectively, "Respondents"). The NOV was based upon an inspection conducted on May 16, 2019 pursuant to the authority granted in Hawai'i Revised Statutes ("HRS") section 149A-36 for purposes of determining compliance with state and federal pesticide laws. On July 30, 2020, the NOV was served on the agent for Respondents.

Pursuant to HRS section 149A-41(b)(3), Respondents were entitled to a hearing to contest the NOV if a written request for a hearing was submitted to the Office of the Chairperson within twenty (20) calendar days from the date of receipt of the NOV. The NOV clearly advised Respondents that the NOV would become a FINAL ORDER unless Respondents filed a written request for hearing within twenty (20) calendar days.

The twenty (20) day time period expired August 19, 2020; no request for a hearing was received by the Office of the Chairperson of the Department of Agriculture by that time. Accordingly, Respondents waived the opportunity to challenge the finding of violation and the NOV became a FINAL ORDER.

On October 12, 2020, HDOA issued a Final Order to Respondents. The Final Order required the Respondents to submit the civil penalty of three thousand two hundred fifty dollars ($3,250.00) within twenty (20) calendar days from the receipt of the Final Order, or HDOA would refer the matter to the Hawai'i Department of the Attorney General for collection. The agent for Respondents was served with the Final Order on December 3, 2020. As of today's date, Respondents have failed to submit the civil penalty to HDOA and **is now in DEFAULT.**

HRS section 149A-41(b)(4) provides as follows:

> In case of inability to collect the administrative penalty or failure of any person to pay all or such portion of the administrative penalty as the board may determine, **the board shall refer the matter to the attorney general, who shall recover the amount by action in the appropriate court.** For any judicial proceeding to recover the administrative penalty imposed, **the attorney general need only show that notice was given,** a hearing was held or **the time granted for requesting a hearing has expired without such a request,** the **administrative penalty was imposed,** and that **the penalty remains unpaid.** (Emphasis added.)

Please note that this letter and enclosures will be tendered to the State of Hawai'i Attorney General as evidence of RESPONDENTS' FAILURE TO SUBMIT THE CIVIL PENALTY TO HDOA AS AGREED. Respondents may be deemed liable for court costs, attorney fees, and interest should legal action be required to secure payment of the three thousand two hundred fifty dollar ($3,250.00) civil penalty.

Please remit the three thousand two hundred fifty dollar ($3,250.00) civil penalty by February 24, 2021 to:

State of Hawai'i Department of Agriculture
Pesticides Branch
1428 S. King Street
Honolulu, Hawai'i 96814

Copies of the NOV, Final Order, and signed certified mail return receipts are enclosed. **If no payment is received by February 24, 2021,** pursuant to HRS section 149A-41(b)(4), **the matter will be referred to the Hawai'i Department of the Attorney General for collection.**

Should you have any questions or concerns please contact the undersigned at (808) 973-9404 or via email at greg.y.takeshima@hawaii.gov

Sincerely yours,

GREG TAKESHIMA
Acting Pesticides Program Manager

GT:sn
[K:HAWAIIMEDICINALBOTANICALGARDEN]

Enclosures (52 pages)
cc: File
EPA Region IX

C105