MORPHOLOGICAL CHARACTERIZATION, VIRULENCE, AND FUNGICIDE SENSITIVITY EVALUATION OF *PHYTOPHTHORA PALMIVORA*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology - Doctor of Philosophy

2016

ABSTRACT

MORPHOLOGICAL CHARACTERIZATION, AND VIRULENCE AND FUNGICIDE SENSITIVITY EVALUATION OF *PHYTOPHTHORA PALMIVORA*

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Phytophthora palmivora is restricting tropical plant pathogen for tropical and subtropical crops. Management of the pathogen has relied mostly on cultural practices and plant breeding; however, absence of these strategies in crops such as oil palm have resulted in catastrophic epidemics. During the present study a total of 150 isolates of *P. palmivora* obtained from 17 countries and 16 different hosts were studied. Differences in morphology, cultural growth, virulence and in-vitro and in-vivo response to nine fungicides, including the broadly used active ingredient mefenoxam, were elucidated.

Differences in sporangia, chlamydospore and oospore measured parameters were observed among isolates; however, they ranged within those described for the species. Differences in sporangia length, sporangia breadth, and chlamydospore diameter seems to be influenced by the host family. This influence was not observed for the oospore characteristics. Virulence of isolates from *Citrus* spp. were also distinguishable from the rest of families when they were tested on apples; the lesion diameter of this isolates (31.9 mm) was about 10 millimeters shorter than the average of the rest of the families (41.7 mm). No significant differences were observed within the other families. Isolates from the Americas caused larger lesions than those from Asia and Oceania. Only one isolate of *P. palmivora* (13720) was intermediately sensitive to mefenoxam at 100 ppm.

In order to identify possible active ingredients that could be used to manage diseases caused by *P. palmivora,* efficacy of the fungicides captan, cyazofamid, dimethomorph, fluopicolide, mandipropamid, mefenoxam, oxathiapiprolin, zoxamide, and potassium phosphite were tested on petunia bedding plants. Total control of the disease was observed at 21 days after inoculation with mefenoxam, captan and oxathiapiprolin. Success in disease control was followed by manidporpamid (83%), and fluopicolide and dimethomorph and zoxamide which presented an intermediate level of control (50%). The rest of the fungicides were not different from the inoculated control.

With the exception of potassium phosphite, the in-vitro response to the selected fungicides was evaluated using the spiral plating technique. The effective concentration (EC_{50}) for each isolate was elucidated by measuring the distance (mm) between the center of the plate and the point where 50% of lateral growth was observed. The data generated during the study were analyzed using the R-package ECX, developed during the present study. All products were effective in controlling most of the isolates at the selected ranges. Four isolates were not controlled with mefenoxam; however, the maximum dose of mefenoxam evaluated (0.289 mg/L) with the spiral plating technique does not implies resistance. The present study demonstrates the variability of the *P. palmivora* population, and provides some insight in its control using fungicides.

To Maritza for her immeasurable love and support,
To Emilia for coming to my life and inspiring my way to happiness
To my parents Gabriel and María Teresa for their support
To my brothers, sisters nephews and niece for be there for me
To my friend Gerardo Martinez for encouraging me to take this journey

ACKNOWLEDGMENTS

I want to express my gratitude to Dr. Mary K. Hausbeck for accepting me into her program and supporting me to conduct the research on *Phytophthora palmivora*. I also want to thank my graduate committee members Drs. Ray Hammerschmidt, Annemiek Schilder, and Christina DiFonzo for their feedback and contribution to accomplish my doctoral studies. I also want to thank Dr. William Kirk for his feedback and support for the fungicide testing chapter and Dr. Mathieu Ngouajio as former committee member. I want to specially thank Dr. Jianjun Hao for supporting me to combine two different sciences (computer and plant pathology) into a useful tool for fungicide testing, and also for his critical review of this thesis. I also want to express my gratitude to my Colombian friends in the department and lab, to my lab friends and members, especially to Dr. Rachel Naegele, and Sheila Linderman for her critical review of parts of this thesis. Finally, but not least, I want to express my gratitude to my wife Maritza, for her unconditional support during this grateful experience.

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CHAPTER I: LITERATURE REVIEW

The genus *Phytophthora* belongs to the Family Pythiaceae, Order Peronosporales, Class Oomycetes, Phylum Oomycota, of the kingdom Straminipila (Brasier, 1992). Members of Straminipila kingdom were classified into the Fungi kingdom for many years because they are morphologically similar, and have similar ecological habits (Brasier, 1992). However, individuals of Straminipila kingdom were distinguished from fungi by their inability to produce sterols, the presence of ß-glucan as the primary constituent of their cell walls, and the ay bsence of a haploid phase during most of their life cycle (Brasier, 1992). Genetic recombination of oomycetes is similar to organisms with diploid or polyploid chromosomes (higher organisms) with the production of gametangia prior to gamete formation (Brasier, 1992). The Kingdom Straminipila contains multiple genera of plant pathogens including *Phytophthora, Pythium,* the downy mildews, and white rust as well as diatoms and brown algae (Tyler, 2002).

The genus *Phytophthora* was described for first time in 1876, and by 2012 had about 117 described species (Martin et al., 2012). The host specificity of *Phytophthora* varies among species. *Phytophthora infestans* has a limited host range while others like *P. cinnamomi* have a wider host range (Brasier, 1992; Hong et al., 2008b). Damage caused by *Phytophthora* spp. and their management was calculated at $10 billion in 2002 and is higher in countries where management practices are limited (Tyler, 2002).

General life cycle of plant pathogenic *Phytophthora* species:

During asexual reproduction, *Phytophthora* produces sporangia, chlamydospores and zoospores (Tyler, 2002). Sporangia can germinate directly by forming a hyphae or by

differentiating into zoospores (Tyler, 2002). Zoospores lack a cell wall but have two flagella that allow them to swim. When a zoospore locates the appropriate host tissue, they release their flagella, encyst, and germinate (Tyler, 2002). Zoospores effectively infect roots (Tyler, 2002). Sexual reproduction occurs in most species of *Phytophthora* when haploid structures, the antheridium (male structure) and the oogonium (female structure), come together to form the oospore (Schumann & D'Arcy, 2007). Development of these organs can occur in the same (homothallic) or different (heterotallic) isolates (Brasier, 1992). Complementary heterothallic species are commonly described as A1 or A2 mating types (Brasier, 1992).

Dispersal of *Phytophthora*

Phytophthora species may be dispersed root to root, or by water or splash movement (Sujkowski et al., 1999); *Phytophthora infestans* can be dispersed via air currents (Granke et al., 2009). Dispersal via surface water was demonstrated for different species of *Phytophthora* in several crops (Bowers et al., 1990; Larkin et al., 1995; Oudemans, 1999; Ristaino et al., 1994; Sujkowski et al., 1999). In Michigan, Gevens et al. (2007) confirmed the role of surface water used for irrigation in the dispersal of *P. capsici* on susceptible vegetable crops. Similarly, Oudemnas (1999) in New Jersey trapped *P. cinnamomi* and *P. megasperma* in water sources used in cranberry production. Hong et al. (2008a), described nine *Phytophthora* spp. found in irrigation water used in ornamental nurseries in Virginia. Six of those species were classified as new, resembling *P. citrophtora, P. citricola* and *P. drechsleri*, but differing in specific morphological and ecological characteristics (Hong et al., 2008a).

Bowers et al. (1990) evaluated the effect of rainfall on the epidemiology of *P. capsici* on peppers and found that cumulative rainfall aided in pathogen dispersal (Bowers et al., 1990). A geostatistical epidemiological analysis of this pathogen confirmed a predominance of movement

within a row rather than across rows (Larkin et al., 1995). Madden and Ellis (1990) reported that the use of plastic mulches in strawberry promoted the movement of *Phytophthora cactorum* between plants when a rain simulator was used. Ristaino and Gumpertz (2000) concluded that rainfall is the main dispersal factor for several *Phytophthora* species. Granke et al. (2009) found that *P. capsici* sporangia were readily detached by water and that airborne dispersal of *P. capsici* sporangia was closely related with rain.

Phytophthora palmivora

Phytophthora palmivora (Butler) is a destructive tropical plant pathogen distributed around the world (Chase & Broschat, 1991; Erwin & Ribeiro, 1996). *Phytophthora palmivora* was first isolated by Hart in 1899 in Trinidad from cacao fruits (*Theobroma cacao L*). In the same year Massee classified the pathogen as *Phytophthora omnivora* (Ashby, 1929). Other names for the pathogen include *Pythium palmivora* (Shaw, 1914; Waterhouse, 1974), *P. faberi* (Ashby, 1929) and *P. theobromae* (Ashby, 1929). In 1996, Erwin and Ribeiro reported the existence of at least 168 host species of *P. palmivora.* This pathogen can affect different organs of its hosts , including, roots, trunks, leaves and fruits (Erwin & Ribeiro, 1996).

Phytophthora. palmivora produces sporangia and chlamydospores during its asexual stage (Ribeiro, 1978). Abundant sporangia are observed 5 days after inoculation and are typically papillate with short pedicels and a length-breadth ratio that exceeds 1.4 (length range= 35-60µm; width range= 20-40µm) (Waterhouse, 1974). Sporangia germinate directly or produce zoospores (Waterhouse, 1974). Zoospore production requires water and is stimulated by cholesterol, root exudates, light and aeration (Waterhouse, 1974). Zoospore discharge begins at room temperature 15 minutes after placing them in water at 20 to 25°C (Gadd, 1924).

Chlamydospores are spherical to ellipsoid structures with a thick cell wall (4µm) and a diameter of 35 to 45µm; They serve as survival structures remaining viable up to 36 months (Waterhouse, 1974) (Fig 1.1).

Oospores, the sexual reproductive structure of *Phytophthora palmivora*, was described initially in 1922 by Ashby, who report for first time the existence of heterotallic species within the genus (Brasier, 1992). Ashby described two types of colonies capable of mating, one with vigorous growth (+) and the other with weak growth (-) (Ashby, 1922). Positive colonies were identified from cotton and coconut palm isolates while negative colonies were obtained from cacao isolates(Ashby, 1922). The sexual stage of *P. palmivora* was reported once in nature. Ashby (1929) reported amphigynous oospores at the base of a leaf rachis from a coconut palm tree in Jamaica in 1920.

In 1924, Gadd found that *P. palmivora* sporangia and chlamydospores from cacao and papaw isolates were wider and larger than structures from isolates obtained from rubber, bread fruit, orchids, and *Odontadenia* sp. Based on this observation, Gadd divided the mating types into ‘cacao’ and ‘rubber’ groups. He found that oospores were produced exclusively when individuals from those groups were mated (Gadd, 1924). In 1968, Savage *et al*. arbitrarily assigned the conventional A1 label to the ‘rubber’ group and A2 to the ‘cacao’ group (Waterhouse, 1974).

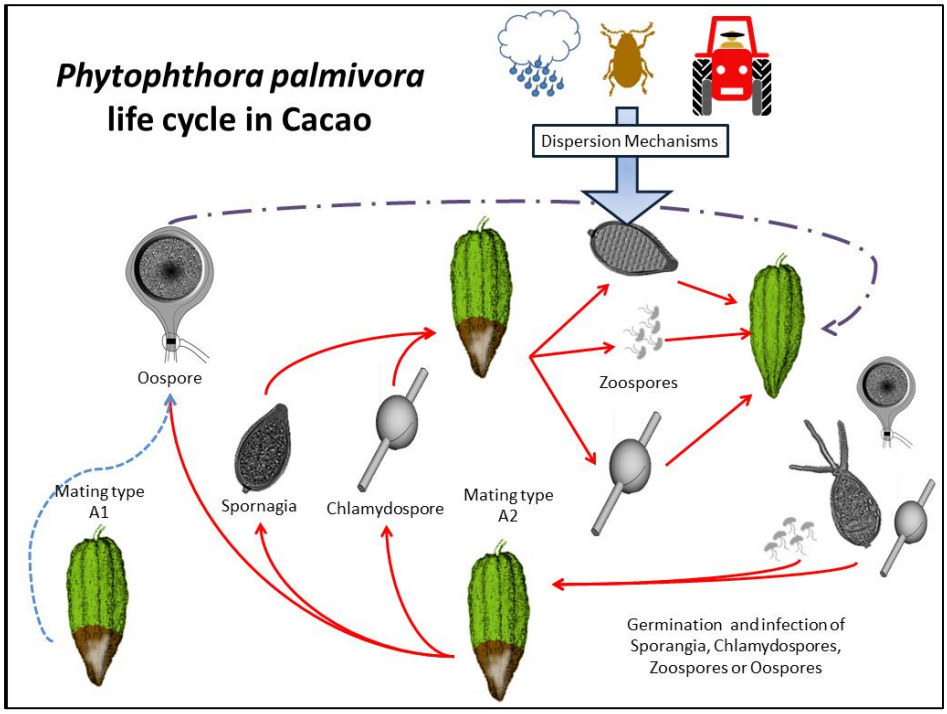

Figure 1.1 General life cycle of *Phytophthora palmivora* on cacao (*Theobroma cacao*)

Some species of *Phytophthora* produce A1(+) and A2(-) in similar proportion, but in *P. palmivora*, the ratio is higher for the A2 mating type (Brasier, 1992). Studies to identify the heritability of *P. palmivora* virulence concluded that heritability of virulence seems to be polygenic and is favored by directional selection (Brasier, 1992). Ashby (1929) classified *P. palmivora* isolates depending on their growth patterns. Isolates with sparse aerial mycelia and abundant sporangia were classified as 'typical' while isolates producing copious aerial mycelia with few sporangia were catalogued 'atypical' (Ashby, 1929). Most of the isolates identified as 'atypical' were later renamed *Phytophthora botryose* (Waterhouse, 1974). Ashby (1929) suggested that *P. palmivora* was a polygamous species based on his observations when *P. palmivora* was paired with *P. parasitica* (microspora).

Development of *P. palmivora* is influenced by environmental conditions; among them temperature have an important role. The optimal growing temperature established for *P. palmivora* is 27.5 to 30 °C with a minimum of 11 °C and a maximum of 35 °C (Waterhouse, 1974). In 1931, Tucker found that 16 of 20 isolates of *P. palmivora* survived when he kept them at -9 °C to -5 °C (Waterhouse, 1974).

Phytophthora palmivora is traditionally identified using morphological characteristics; molecular characterization is achieved using internal transcriber spacers (ITS) of ribosomal DNA. Appiah et al. (2004) used ITS to evaluated 88 isolates of *P. palmivora* isolated from cocoa from different regions of the world. Torres et al. (2010) reported the use of this region to characterize *P. palmivora* affecting oil palm in Colombia. Cacciola et al. (2011) also reported the identification of *P. palmivora* affecting windmill palm using ITS.

A recent outbreak of bus rot, caused by *P. palmivora*, in the oil palm industry in Colombia demonstrated the destructive potential of this pathogen (Torres et al., 2016). Improper management of oil palm bud rot caused the augmentation of pathogen inoculum in some areas and the destruction of more than 70,000 ha of oil palm in Colombia within a period of seven years (Torres et al., 2016). Measures to control the disease were developed, but are restricted to low incidence areas. Management requires at least the improvement of agronomic practices, including drainage and balanced fertilization, removal of affected tissue and the use of pesticides to control *P. palmivora* and other secondary microorganisms (Torres et al., 2016).

Fungicide control of Oomycetes

One of the most common and effective strategies for controlling diseases caused by oomycete pathogens is the use of fungicides (Brent & Hollomon, 2007). Fungicides can affect the initial disease intensity (y_0), the rate of disease increase (r), or both (Madden et al., 2007). Schwinn and Urech (1986) grouped fungicides effective against oomycetes into six classes: butyrolactones, carbamates, isoxazoles, cyanoacetamide oximes, ethyl phosphonates, and acylalanines. Cohen and Coffey (1986) classified acylalanines as a subclass of acylanilides, with butyrolactones, thiobutyrilactones and oxazplidiones. A recent classification of oomycete fungicides by Gisi and Sierotzki (2008) included multi-site compounds (dithiocarbamates, phthalimides, chloronitriles and copper formulations); quinone outside inhibitors (QoIs), phenylamides (Pas), carboxylic acid amides (CAAs), cyano-acetemid-oximes, phosphonates, carbamates and plant defense inducers.

One of the most widely used fungicides for treating diseases caused by *Phytophthora* is the protectant active ingredient captan (National Pesticide Information Center, 2000). interacts with the sulphydryl group of the microorganism (Canadian Council of Ministers of the Environment, 1999), affecting spore germination, mycelial growth and respiration (Dugger Jr. et al., 1959). In 1990, the United States Environmental Protection Agency (US EPA) revoked the registration of captan for citrus crops, cranberries, pineapples and other food crops because of carcinogenic concerns (EPA, 1999)

Cyazofamid belongs to the phenylimidazole fungicide class and has a wide spectrum for controlling oomycetes at low rates (80-115 g a.i./ha) (Ohshima et al., 2004). This fungicide affects all stages of pathogen development including sporulation, zoospore production, zoospore motility and mycelial growth (Ohshima et al., 2004). Cyazofamid blocks electron transfer in the

mitochondrial cytochrome complex bc1 at the Qi region (Ohshima et al., 2004). Cyazofamid is used as protectant fungicide (Enviromental Protection Agency, 2004).

Dimethomorph is a cinnamic acid derivate specific to controlling disease caused by *Phytophthora* and *Peronospora* (Kuhn et al., 1991; Stein & Kirk, 2004; Wicks & Hall, 1990). This fungicide affects mycelial growth, sporulation, sporangia and zoospore cyst germination (Keinath, 2007; Kuhn et al., 1991) and affects the cell wall biosynthesis by targeting the cellulose synthase (Fungicide Resistance Action Committee, 2011). Dimethomorph belongs to the carboxyl acid amide (CAA) group (Cohen & Gisi, 2007).

Mandipropamid is a mandelic acid amide fungicide grouped also within in the CAA group (Cohen & Gisi, 2007). This fungicide inhibits lipids and membrane synthesis (Jackson et al., 2012) and is easily attached to the leaf surface (Cohen & Gisi, 2007). Mandipropamid affects zoospore and sporangial germination, mycelial growth, haustoria formation and sporulation, but not zoospore development, discharge, motility and encystment (Cohen & Gisi, 2007). Blum *et al.*(2010) reported that mandipropamid targets the synthase-like protein PiCesA3 affecting cellulose synthesis.

Ethyl phosphates offer good control *in-vivo* of *Phytophthora* spp. However, *in-vitro* tests with Fosetil-AL are less effective than *in-vivo* and its efficacy is due to a systemic response of the plant (Cohen & Coffey, 1986). Phosphonates target sporulation (Cohen & Coffey, 1986). Fosetyl-AL was the first commercial fungicide with a basipetal movement (Cohen & Coffey, 1986). It targets sporulation and mycelium production (Schwinn & Staub, 1995).

Fluopicolide belongs to the benzamide and the pyridine classes (Jackson et al., 2010). It is active against a wide spectrum of oomycetes with translaminar action (locally systemic) (Toquin et al., 2007). This fungicide affects zoospore release and motility, cyst germination,

sporulation and growth of mycelium, by translocating the spectrin-like protein from the plasma membrane to the cytoplasm (Toquin et al., 2007). An *in-vitro* zoospores screening of *P. capsici* conducted by Lu *et al.* (2011) demonstrated that *P. capsici* populations had moderate to high risk of developing resistance against this fungicide.

The fungicide mefenoxam demonstrates good disease control of *Phytophthora* and it is commonly used among growers (Ware & Withacre, 2004). This fungicide is a purified active isomer of metalaxyl (Ware & Withacre, 2004). Mefenoxam is a protectant and curative fungicide used to control diseases caused by oomycetes in vegetable, ornamental, forestry, and fruit production (Ware & Withacre, 2004). Mefenoxam affects mycelium growth and sporulation (Schwinn & Staub, 1995). The extensive and prolonged use of this fungicide has resulted in resistant *Phytophthora* isolates.

Zoxamide belongs to the benzamide fungicide class (Young & Slawecki, 2001). This fungicide disrupts microtubules during nuclear division by covalently binding to the ß-subunit of tubulin (Young et al., 2001). Young et al.(2001) determined that the risk of oomycetes developing resistance to zoxamide was lower than mefenoxam. Zoxamide is a protectant fungicide that inhibits germ tube elongation, mycelial growth and zoospore development; however, it does not affect zoospore motility, encystment or germination.

Fungicide resistance

Modern fungicides are effective, but have reduced toxicity against humans and non-target organisms within the environment (Lyr, 1995). After site-specific fungicides were introduced in the 1960s, resistance to fungicides has become a major problem for farmers (Brent & Hollomon, 2007; Dekker, 1995). Fungicide resistance is attributed to many factors. Gisi *et al*. (2000) defined the resistance of pathogens to fungicides as the combination of three aspects: 1) the

presence of a resistance gene (r) in the population, originating through mutation; 2) the formation of sub-groups with the (r) gene due to selection and migration processes; and 3) the incapacity to control those populations. Lyr (1995) described five reasons for differences in sensitivity against fungicides: 1) differences in the accumulation of a fungicide in the cell; 2) different structures of the receptor or target systems; 3) difference in ability to toxify (activate) a compound; 4) difference in ability to detoxify a compound; and 5) different degrees of importance of a receptor or target system for survival of the fungus. Similar mechanisms were also proposed by Ma and Michailides (2005). An analysis of the Fungicide Resistance Action Committee (FRAC) code by Leadbeater and Gisi (2010) showed that 19% of fungicides were catalogued as high risk for developing resistance, while 35% were a moderate risk.

The most common methods for detecting fungicide resistance require isolation of the pathogen in a pure culture and subsequent plating on an amended medium containing the fungicide. Inoculating a plant or plant tissue treated with the fungicide is another method (Ma & Michailides, 2005). To evaluate mefenoxam sensitivity for *P. capsici*, Lamour and Hausbeck (2000) placed an agar plug of an actively expanding single-zoospore colony in the center of a V8 agar plate mefenoxam-amended (100 ppm), then compared the diameter after 3 days of incubation with colonies developing on plates lacking the fungicide. They hypothesized that oospores played an important role in the long-term buildup of resistant isolates in agriculture (Lamour & Hausbeck, 2001).

Fungicide resistance has not been reported for *P. palmivora;* however, there is complete or moderate resistance of other species of *Phytophthora*. For instance, in a study of floriculture crops in North Carolina Hwang and Benson (2005) found 122 insensitive, 57 intermediate-sensitive, and 5 sensitive isolates of *P. cryptogea;* 56 insensitive and 217 sensitive isolates of *P.*

nicotinae; and 26 sensitive isolates of *P. palmivora.* Parra and Ristaino (Parra & Ristaino, 2001) reported 59% of 150 *P. capsici* isolates were insensitive to mefenoxam in North Carolina and New Jersey. Keinath (2007) found eight insensitive (7%) and 43 intermediate-sensitive isolates in a sample of 120 isolates of *P. capsici* from S. Carolina. Jackson et al. (2010) reported 74.5% of resistance and 19.6% of intermediate resistance in a study of 51 isolates of *P. capsici* in Georgia. Dunn et al. (2010) reported insensitive isolates of *P. capsici* in Long Island and the Capital District in New York affecting zucchini, peppers, and pumpkins.

In summary, bud rot is a very destructive disease caused by *Phytophthora palmivora*. Despite different approaches exist to manage the disease, there is not a single practice that can control it individually. Fungicides are a shot term solution in other crops affected by *Phytophthora* spp. The use of fungicides requires precise control to limit emergence of resistant individuals. The research objective of this dissertation was to evaluate the effectiveness of different fungicides for controlling *P. palmivora* on a worldwide collection.

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CHAPTER II: CHARACTERIZATION OF MORPHOLOGY, MATING TYPE AND VIRULENCE OF *PHYTOPHTHORA PALMIVORA* ISOLATES

ABSTRACT

Phytophthora palmivora is a destructive plant pathogen in tropical and sub-tropical regions of the world. In this study, a collection of 150 *Phytophthora palmivora* isolates from 17 countries and 16 host crops were characterized. Morphological characteristics measured included: dimensions of sporangia, pedicel, chlamydospores, oospore, oogonia, and antheridia; production of sporangia, chlamydospores; and mycelial growth. Mating type, mefenoxam sensitivity and virulence of the isolates were evaluated. Sporangia were ovoid and ellipsoid, with the dimensions ranging from 13 to 47 µm in width and 20.4 to 75 µm in length. Significant differences of sporangial length were observed for isolates grouped by host family and geographical location. Length:breadth ratio (0.7 to 3.0) and pedicel length (0.8 to 7.5 µm) varied among continent and host family. Chlamydospore diameter ranged from 20.6 to 50.1 µm; significant differences were observed when isolates were grouped by host family. Oogonium (15.2 to 45.3 µm) and oospore (12.0 to 38.0 µm) diameter, and antheridium width (7.0 to 24.8 µm) also varied among isolates, but differences were not observed when isolates were grouped for continent or host family. Colony growth differences were noted among host family, but not among continents. A1 and A2 mating types were distributed in a 2:7 ratio. All isolates, with one exception were sensitive to mefenoxam. Differences in virulence, determined by lesion diameter of inoculated apple fruit after five days of incubation, varied from 9.0 to 51.4 mm. Virulence of isolates obtained from the Rutaceae family was significantly different from the rest.

INTRODUCTION

The oomycete plant pathogen *Phytophthora palmivora* Butler is mainly distributed in tropical regions of the world (Chee, 1974; Erwin & Ribeiro, 1996; Waterhouse, 1974), and in subtropical areas including China (Guo et al., 2012; Li et al., 2011), Italy (Aiello et al., 2011), Japan (Gappa-Adachi et al., 2011; Tashiro et al., 2012), Poland (Orlikowski & Szkuta, 2006), Spain (Moralejo et al., 2009), and Turkey (Dervis et al., 2011). In the continental U.S., *P. palmivora* is found in California (Keim et al., 1976), Florida (Garofalo & McMillan, 1999; Timmer et al., 1991) North Carolina (Hwang & Benson, 2005), South Carolina (Robayo-Camacho, 2009), and Tennessee (Donahoo & Lamour, 2008).

Phytophthora palmivora infects roots, stems, branches, leaves, flowers and fruits of its more than 180hosts (Erwin & Ribeiro, 1996), including cocoa (Zentmyer, 1974), rubber (Zentmyer, 1974), and oil palm (Butler, 1924; Ghesquiere, 1935; Kovachich, 1957; Richardson, 1995; Sarria et al., 2008; Torres et al., 2010). Some commercial crops are seriously affected by the pathogen. On cocoa, *P. palmivora* causes trunk rot, chupon (suckers) wilt and black pod diseases and account for 20 to 30% losses in this crop worldwide (Erwin & Ribeiro, 1996). In rubber, *P. palmivora* causes black stripe, patch canker, pod rot, abnormal defoliation and green twig blight (Chee, 1969; Erwin & Ribeiro, 1996). In 2010, Torres et al. (2010) reported *P. palmivora* as the causal agent of oil palm bud rot in Colombia. Bud rot disease destroyed at least 70,000 hectares of oil palm in Colombia between 2007 to 2013 (Avendaño & Garzon, 2013), accounting for an estimated loss of $250 USD million (Grogan & Mosquera-Montoya, 2014).

In nature, *Phytophthora palmivora* is a heterothallic species with predominance of the A2 mating type (MT) over the A1 mating type (Brasier, 1992). Zentmeyer (1973) found both *P.*

palmivora MTs existed in Asia, Africa, Oceania, Central and South America. However, natural occurrence of *P. palmivora* oospores is extremely limited (Navin, 1990). Chee (1971) suggested predominance of the A2 MT was due to a host adaptability of the species. Mchau and Coffey (1994) found low genetic diversity among *P. palmivora* A2 mating type isolates, while A1 mating type isolates recovered from coconut, durian and other non-cacao hosts originating in south-Asia represented higher diversity. In addition to genetic variability, Mchau and Coffey (1994) reported morphological variation between the mating types.

Pathogenicity and virulence tests of *P. palmivora* commonly used the host from which the isolate was obtained (Chee, 1969; Graham et al., 1998). However, Ribeiro (1978) and Tucker (1967) successfully infected apples with *P. palmivora*. Identification of virulent strains is is important to develop an effective oil palm breeding program or or understand pathogen epidemiology. Virulent *P. palmivora* isolates were used in cocoa (Chowdappa & Chandramohanan, 1997) and rubber (Dantanarayana et al., 1984) breeding programs.

The objective of this study was to characterize *P. palmivora* isolates collected from various hosts and regions using phenotypic and virulence characteristics. The overall goal was to have a wider perspective of its population and the interaction of this pathogen with different crops.

MATERIALS AND METHODS

***Phytophthora palmivora* isolates.** A total of 150 *P. palmivora* isolates were obtained from 17 countries and 16 different host crops (Appendix A). An agar plug (7-mm-diameter, same size throughout the study) of each isolate was transferred to a Petri plate containing unclarified V8 (UCV8)(160 ml unclarified V8 juice, 30 mM calcium carbonate, 16 g of agar), and incubated for seven days at room temperature (23 ± 2°C) under continuous fluorescent light at the. Hausbeck's Laboratory at Michigan State University, East Lansing, MI. An agar plug from the active growing edge of each culture was used to inoculate individual pear (*Pyrus communis*) fruit. Each isolate was recovered from the pear tissue onto BARP (50 mg benomyl, 75 mg ampicillin, 15 mg rifampicin, 100 mg PCNB)-amended filtered-V8 (40 ml clarified V8 juice, 960 ml distilled water, 6 mM $CaCO_3$, 1.2 % agar) for reactivation (Lamour & Hausbeck, 2003; Quesada-Ocampo et al., 2009). A plug of actively growing mycelia from the margin of the recovered isolates was transferred onto fresh UCV8 and maintained under continuous fluorescent light at room temperature (23 ± 2°C). One-week-old plates of each isolate of *P. palmivora* were used for morphological, mating type and virulence characterization.

Morphological characterization. Sporangial density (sporangia/cm^2) and morphology were evaluated using seven-day-old cultures growing on UCV8 plates. One millimeter of distilled water was added to each plate and sporangia were dislodged using a glass spreader bar; the resulting suspension was recovered into a 1.7-ml micro centrifuge tube (Granke & Hausbeck, 2011). The dislodged sporangia were fixed using 50 µl of acid fuchsin (10 mg acid fuchsin, 100 ml double distilled water [ddH_2O], 100 ml 85% lactic acid), and the volume was brought to 1.0 ml with ddH_2O. The total number of sporangia was estimated using a hemocytometer, and the

value was divided by the internal plate area to calculate sporangial density (π * 2.6 cm^2 = 21.2 cm^2). Preliminary sporangia measurements were made visually at 200X magnification (20X objective and 10X eyepiece). Ten sporangia were photographed at100X magnification using a Leica DFC420 camera (Leica Microsystems, Switzerland); the images were imported to ImageJ (National Institutes of Health, USA) and analyzed by setting the scale at 3.6 pixels/µm. Length, breadth, and pedicel length were recorded using a personalized macro. Length/breadth ratio (LB ratio) for each isolate was calculated by dividing the length by the breadth for each sporangium. The characterization of each isolate was conducted twice.

Chlamydospore production was evaluated using 2-week-old cultures. The plates were evaluated at 100X magnification; pictures and diameter measurements were taken using the protocol and software described previously. Chlamydospore density (chlamydospore/cm^2) was calculated by taking pictures of three randomly chosen fields of view per isolate (720 x 540 µm per picture), counting the number of structures present using ImageJ, and estimating the density of chlamydospores/cm^2. The characterization was conducted twice.

The mating type (MT) was determined using the reference isolates 13749 (WPC-11012) for A1 and 13732 (WPC-0500) for A2 obtained from the World *Phytophthora* Collection (WPC) at the University of California in Riverside. The references were originally obtained from Areca palm (*Areca* sp.) in Guam and from cocoa (*Thobroma cacao*) in Colombia, respectively. Clarified V8 (CV8)(120 ml of clarified V8 juice, 880 ml distilled water, 6 mM $CaCO_3$, 1.6 % agar) plates were used for oospore production. All plates for oospore production were incubated under dark conditions for three weeks at room temperature (23 ± 2°C). The mating type evaluation was conducted three times. The first replication of the experiment was conducted by placing one plug of the test isolate in one side of a petri dish and a plug of the reference mating

type on the other side of the plate.(Erwin & Ribeiro, 1996). The second and third replications were conducted by placing both reference isolates in a single 60-mm-diameter, forming a triangle with the test isolate (Fig 2.1). The MT for each test isolate was identified based on oospore production. Evaluation of MT and oospore dimension was conducted at 100x magnification using an Olympus CK2 inverted microscope (Olympus, Melville, N.Y.). An Olympus OM system to Nikon-J1 lens adapter was used to attach a digital Nikon J1 camera (Nikon Inc., Melville, N.Y.) to the microspore OM imaging system (Olympus). Pictures at 100x magnification were taken of ten oospores per isolate, and measurements were done using ImageJ, with an estimated scale value of 3.87 pixels/µm. The antheridium width and oogonium and oospore diameter were measured.

Colony growth diameter was calculated by placing a mycelial plug on fresh CV8-media, and incubating for three days at room temperature under continuous fluorescent light. Three plates per isolates were prepared. The plates were scanned using an EPSON Perfection V30 scanner (Epson America Inc., Long Beach, CA); contrast was adjusted to improve the image quality. Colony diameter was measured with ImageJ, using an scale transformation of 11.72 pixels/ mm. Colony pattern was characterized according to morphology for 7-day-old colonies (Erwin & Ribeiro, 1996); shape was classified as rosette, chrysanthemum, radial, stellate or uniform (Fig 2.2). Mycelial growth was classified as reduced or appressed, scanty-fluffy or densely fluffy following the descriptions of Erwin and Ribeiro (Erwin & Ribeiro, 1996) (Fig 2.3).

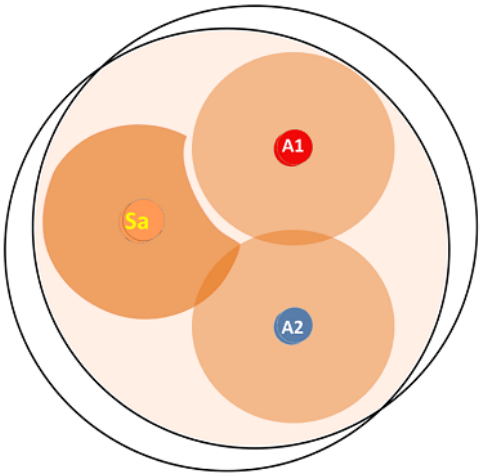

Figure 2. 1 Diagram of mating type distribution on plate: compatibility is represented as the overlapping shadowed area. Sa= test isolate.

Mefenoxam sensitivity. One mycelial plug from the edge of a 7-day-old culture of each isolate were transferred into the center of a UCV8 plate, and two plates of UCV8 amended with 100 mg/l of mefenoxam (Syngenta Crop Protection Inc., Greensboro, NC). The plates were incubated at room temperature (23 ± 2°C) under continuous fluorescent light for three days. Mycelial growth was determined by measuring the diameter of colonies. Fungicide sensitivity was calculated by comparing mycelial growth on the amended plates with growth of the same isolate on the control plates. Fungicides exposed isolates were concidered as sensitive if the growth was less than 30%, intermediately sensitive between 30 to 90%, and resistant for 90% of growth, when they were compared with the control. The experiment was conducted twice.

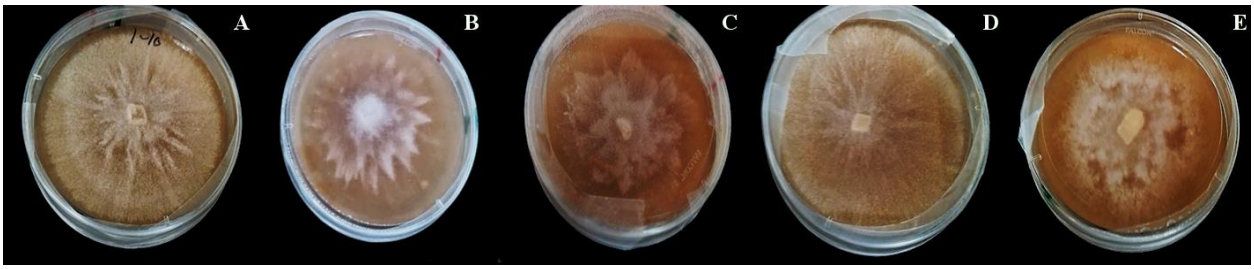

Figure 2. 2 Colony morphologies of *Phytophthora palmivora* on UCV8 plates. A: stellate, B: chrysanthemum, C: rosette, D: radial, and E: uniform.

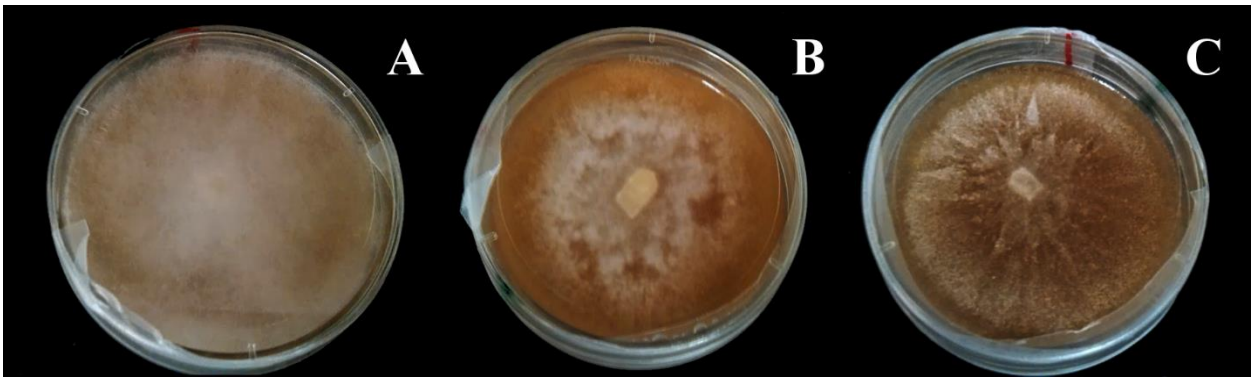

Figure 2. 3 Mycelial fluffiness aspect of *Phytophthora palmivora*. A: densely fluffy, B: scanty fluffy, and C: reduced or appressed.

Pathogenicity tests. Apple fruit of cultivar Gala were selected based on the findings of Ribeiro (1978) and Tucker (1967) for *Phytophthora* spp. virulence assessment. All apples were washed with detergent (Joy ® Ultra dishwashing liquid, Procter and Gamble, Cincinnati, OH) to remove wax that could interfere with the inoculation or rating process followed by air drying. The diameter and height of each apple were recorded prior to inoculation. A wound (1-cm deep) was created in the equatorial portion of the apple using a pushpin dipped in 70% ethanol. A plug obtained from a 7-day-old culture was placed over the wound after the ethanol evaporated. To prevent dehydration, the mycelial plug was covered with a micro-centrifuge screw cap tube

(Axygen Scientific, Union City, CA) with petroleum jelly on its base (Hill, 2005). Control fruit were inoculated with sterile UCV8 plugs using the same method.

A completely randomized block design was used, inoculating one apple per isolate. The inoculated apples were randomly distributed into nine 100-liter plastic boxes (85.7 cm L x 49.2 cm W x 34.0 cm H, Sterlite Corporation, Townsend, MA). Each individual apple was placed on a plastic saucer to reduce movement when the boxes were manipulated. Wet paper towels were added to each box to maintain high relative humidity. A sterile UCV8 plug, and the reference isolates 13749 (A1) and 13732 (A2) were used as negative and positive controls. Controls were included in each box. Five days after inoculation, the virulence of each isolate was calculated by measuring the lesion diameter and height using a digital caliper. BARP-V8 was used to recover the pathogen from symptomatic tissue of all samples. The experiment was conducted five times.

Data analysis. All statistical analyses were performed using R statistical software (version 3.0.2, The R foundation for Statistical Computing, Vienna, Austria). Analysis of variance was performed by grouping isolates based on region and host. Interactions among regions, hosts and morphology, pathogenicity and virulence were also evaluated. When significant statistical differences were observed, Tukey's HSD was used for separation of means to account for differences of sample size among regions or host subgroups. In each analysis, isolate was considered as a fixed factor; sporangial length, sporangial breadth, pedicel length, LB ratios, sporangial density, chlamydospore diameter, chlamydospore density, oospore diameter, oogonium diameter, antheridium width, lesion size, and colony diameter were considered the response variables. All parameters were analyzed to satisfy the assumption of normality; only chlamydospore density was logarithmically transformed to satisfy this assumption.

RESULTS

Morphology. Isolates 13718 and 13720 did not produce asexual structures (sporangia or chlamydospores) but produced sterile coenocytic mycelia similar to that produced by other *Phytophthora* spp. Sporangia were produced by all isolates except 13719, 13722, 13742, 13756 and 14053 (Appendix B). Ovoid and ellipsoid forms were the most common sporangial morphology, with exceptions. *Phytophthora palmivora* sporangia were caducous and papillate with pedicel length ranging from 0.8 to 7.5 µm with a mean of 3.6 ± 0.9 µm (Table 2.1). The longest pedicel was observed on isolate 13716 (5.2 µm) from host *Citrus* sp., Florida, and the shortest on isolate 13738 (1.8 µm) from host *Theobroma cacao,* Colombia. The sporangial length and breadth ranged from 20.4 to 75.0 µm and 12.7 to 47.1 µm, respectively. Isolate 13721 had the longest sporangial length (55.3 µm) on host *Pittosporium undulatum* from California, while isolate 14057 presented the shortest (30.6 µm) on host *T. cacao* from Papua, New Guinea. Isolate 14041 presented the broadest sporangial width (32.4 µm) on host *Theobroma cacao* from Indonesia, and isolate 14021 presented the narrowest (19.1 µm) host *Citrus* sp. from Florida. Length for all *P. palmivora* isolates was 43.2 ± 7.2 µm, and the overall breadth was 25.4 ± 3.9 µm (Table 2.1).

The LB ratio for the evaluated *P. palmivora* isolates ranged from 0.7 to 3.0 (Table 2.1) the LB overall mean was 1.71 ± 0.3, and the sporangial density was $2.33 \times 10^5 \pm 1.62 \times 10^5$ spores/ml (Table 2.2). Maximum and minimum sporangial density was observed for isolate 13732 (8.02×10^5 spores/ml) and isolate 14031 (1.50×10^3 spores/ml), respectively. When isolates were grouped by family or geographical region, sporangia parameters were significantly different **(**Table 2.2). However, when families or geographical areas with a limited number of

isolates (other = fewer than 5 isolates per family or group) were removed from the analysis, significant differences in sporangia breadth were not observed (Table 2.2)

Table 2. 1 Morphological, cultural and pathogenic characteristics of *Phytophthora palmivora*.

Parameter measured	Mean	Min	Max	Standard Error
Number of isolates	150.00	--	--	--
Mating type A1	34.00	--	--	--
Mating type A2	116.00	--	--	--
Sporangia length (µm)	43.24	20.43	75.00	0.14
Sporangia breadth (µm)	25.40	12.72	47.09	0.07
Pedicel length (µm)	3.61	0.83	7.50	0.02
LB ratio	1.71	0.64	3.03	0.01
Sporangial density (x 10^3 spores/ml)	233.00	15.00	1,020.00	0.53
Chlamydospore diameter (µm)	32.72	15.82	58.19	0.15
Chlamydospore/^{cm2}	2,302.00	768.00	13,312.00	0.86
Oogonium diameter (µm)	28.72	15.17	45.27	0.10
Oospore diameter (µm)	22.6	11.99	38.05	0.08
Antheridium width (µm)	13.15	6.97	24.76	0.07
Oospore cell in wall thickness (µm)	1.77	0.55	4.60	0.02
Colony diameter day 2 (mm)	25.44	9.00	46.00	0.28
Colony diameter day 3 (mm)	32.57	9.00	51.40	0.32
Lesion diameter on apple (mm)	41.55	8.00	67.00	0.46

Individual isolates from the Rutaceae family had the longest pedicel (4.13 ± 1.28 µm) followed by isolates from the Malvaceae family (3.83 ± 0.81 µm.). Significant differences of pedicel length were not observed among the remaining families (Table 2.2). When isolates were grouped by family and region (Table 2.3.), significant differences in sporangia length and breadth, pedicel length, and LB ratio were not observed among individuals of the Rutaceae family. The pedicel breadth of isolates from the Malvaceae and Palmaceae families were similar among geographical regions (Table 2.3.)

Chlamydospore production was absent in 14 isolates (Appendix C). For chlamydospore-producing isolates, density averaged 2,302 ± 2,002/cm^2. The common form of chlamydospores was globose intercalary and terminal production. The average chlamydospores diameter was 32.72 ± 5.57 µm and range was 15.85 to 58.19 µm (Table 2.4). Isolate 13722 had the largest chlamydospore diameter (50.1 µm, on host *Coleonema* sp. from California) and isolate 13726, the shortest (20.6 µm). Significant differences in chlamydospores diameter and density were observed among isolates. When grouped by host family, significant differences in chlamydospore density were not observed ($P > 0.05$, Table 2.4). The greatest chlamydospore density was observed for isolate 13754 (6,750 chlamydospore/cm^2, on host *Elaeis guineensis* from Colombia). No significant differences were observed in chlamydospore diameter among isolates from Lauraceae, Malvaceae, Palmaceae, and Rutaceae families when other families with fewer than five individuals per family were grouped as "others" and included in the analysis ($P \leq 0.0001$). However, the diameter of chlamydospores from isolates of Rutaceae and Lauraceae were significantly different from Malvaceae and Palmaceae when the other-group was removed from the analysis ($P = 0.0004$).

Significant differences in chlamydospore diameter were observed among isolates when they were grouped by geographical region ($P \leq 0.0001$) (Table 2.4) (Appendix C). The diameter of chlamydospores from isolates obtained from Asia and Africa (29.2 ± 4.9 µm and 26.62 ± 1.65 µm respectively) were smaller than chlamydospores from the remaining regions ($P \leq 0.0001$). No significant differences in chlamydospore diameter were observed when the African and C. American isolates were removed from the analysis. When isolates were grouped by family and geographic region (Table 2.3), significant differences in chlamydospores density were observed

for Malvaceae, Palmaceae and Rutaceae families. However, significant differences in chlamydospore diameter by region were limited to the Malvaceae family ($P \leq 0.0001$).

Oogonium diameter was significantly different among isolates (mean = 28.72 ± 3.07 µm) (Table 2.5, Appendix D). However, when isolates were grouped by host family, or geographical region, no significant differences were observed ($P = 0.17$ and $P = 0.077$ respectively) (Table 2.5). The largest oogonium diameter was observed in isolate 13950 (34.96 µm, host: *Theobroma cacao* from Colombia, MT: A2), and the smallest in isolate 13727 (22.1 µm, host: NA, Florida from MT: A1). When the analysis was done by families separated by geographical region, significant differences in oospores parameters was observed among the *Citrus* spp. isolates using Tukey's HSD test ($P = 0.0128$).

Oospore diameter ranged from 11.99 to 38.08 µm, with a mean of 22.6 ± 2.55 µm. Similar to oogonium, the largest and shortest diameter was observed in isolates 13950 (28.5 µm) and 13727 (17.5 µm) respectively. Unlike oogonia, no significant differences in oospore diameter were observed among isolates when those were evaluated by family ($P = 0.17$) or geographical region ($P = 0.78$) (Table 2.5).

Antheridium width was 13.15 ± 1.95 µm and ranged from 6.97 to 24.76 µm; Cocoa isolates from the Caribbean, Hawaii (13741) and Oceania were significantly smaller than cocoa isolates from Asia and South America ($P < 0.05$). No significant differences in antheridium width were observed when isolates were grouped by region or host family. Oospores cell wall was thicker for isolates from South America (1.92 ± 0.03 µm) compared to isolates from other regions, except for North America (1.8 ± 0.09 µm). Isolates from the Lauraceae family had a thicker oospore cell wall (2.21 ± 0.51 µm) compared with the rest. When isolates were grouped

by host family and region, only significant difference among region was observed on isolates from the Malvaceae family ($P \leq 0.0001$). Isolates from North and South America had a thicker oospore wall. A correlation analysis (Table 2.7) showed a strong positive correlation between oospore and oogonia diameters (r = 0.92); an intermediate correlation was observed between those parameters (r = 0.58) and the antheridium width (r = 0.59).

Table 2. 2 Measurement (mean ± standard error) of *Phytophthora palmivora* sporangia, grouped by continent and host family of isolate collection

Continent	Number of isolates	Sporangial length (µm)[y]	Sporangia breadth (µm)[y]	Pedicel length (µm)[y]	LB rate[y]	Sporangia density (x 10^3 spores/ml)[y]
Continent						
S. America	64	43.82 ± 0.18 a	24.73 ± 0.09 b	3.57 ± 0.03 b	1.78 ± 0.25 ab	248 ± 166 b
Asia	24	40.13 ± 0.42 b	24.53 ± 0.24 b	3.63 ± 0.08 b	1.66 ± 0.33 c	179 ± 144 b
Caribbean	22	43.67 ± 0.29 a	25.26 ± 0.16 b	3.18 ± 0.04 c	1.75 ± 0.27 b	302 ± 165 a
N. America	22	44.8 ± 0.52 a	25.34 ± 0.31 b	3.97 ± 0.08 a	1.81 ± 0.34 a	191 ± 150 b
Oceania	15	39.72 ± 0.59 b	24.52 ± 0.26 b	3.54 ± 0.10 b	1.62 ± 0.25 c	182 ± 134 b
C. America	2	39.32 ± 1.37 b	29.61 ± 0.97 a	3.65 ± 0.26 ab	1.33 ± 0.12 d	84 ± 47 c
Africa	1	38.81 ± 1.58 b	23.9 ± 0.74 b	3.8 ± 0.26 ab	1.62 ± 0.18 c	262 ± 97 ab
Family						
Malvaceae	103	42.54 ± 0.47 b	25.08 ± 0.23 ab	3.43 ± 0.05 b	1.71 ± 0.02 b	227 ± 3 b
Palmaceae	22	44.11 ± 0.62 ab	24.25 ± 0.21 b	3.81 ± 0.10 a	1.83 ± 0.02 a	291 ± 8 a
Other families combined[z]	7	48.07 ± 2.60 a	27.19 ± 1.3 a	3.38 ± 0.56 b	1.79 ± 0.12 ab	169 ± 19 b
Rutaceae	13	44.29 ± 1.99 ab	24.62 ± 1.18 b	4.13 ± 0.30 a	1.84 ± 0.07 ab	177 ± 10 b
Lauraceae	5	43.57 ± 0.64 ab	25.24 ± 0.59 b	3.24 ± 0.20 b	1.73 ± 0.01 ab	227 ± 15 ab

[y.] Mean ± standard error. Means that share letters within a column are not significantly different (Tukey's HSD, $P < 0.05$).

[z.] Individuals of families with fewer than five isolates were pooled into the "other families" category.

Table 2. 3 Morphological characteristics and pathogenicity of *Phytophthora palmivora* on apple fruit, grouped by geographical region and host family of isolate collection

Family and Region	Sporangia					Chlamydospore		Oospore				
	Length	Breadth	Pedicel	LB ratio	Density	Diameter	Density	Oogonia	Oospore	Anth.	WT	AL
Malvaceae												
Asia	40.39 b	24.59 a	3.64 ab	1.67 b	180 b	28.99 c	1724 b	29.42 a	23.02 a	13.31 ab	1.566 b	39.37 bc
Caribbean	43.67 a	25.26 a	3.18 c	1.75 a	302 a	32.23 b	2548 a	28.33 ab	22.47 a	13.14 b	1.587 b	42.75 ab
N. America	43.63 ab	26.15 a	4.05 a	1.67 b	342 a	30.55 bc	1963 ab	29.82 a	23.84 a	11.66 b	2.288 a	37.8 bc
Oceania	39.64 b	24.5 a	3.53 ab	1.62 b	182 b	33.1 ab	1422 b	29.3 ab	23.12 a	13.91 a	1.643 b	36.06 c
S. America	43.29 ab	24.99 a	3.46 b	1.75 a	224 ab	34.02 a	2671 a	28.56 ab	22.46 a	13.2 b	1.939 a	45.04 a
***P.* value**	<0.0001	0.17	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	0.002	0.024	0.0026	<0.0001	<0.0001
Palmaceae												
Asia	35.14 b	23.16 a	3.04c	1.52 c	164 b	34.74 a	870 a	25.28 b	20.87 a	12.11 a	2.03 a	41.40 a
N. America	42.91 a	25.16 a	4.30 a	1.72 b	138 b	30.79 a	1210 b	26.16 b	21.6 a	12.08 a	1.52 a	37.87 a
S. America	44.46 a	24.22 a	3.78 b	1.85 c	314 a	32.44 a	3109 a	28.6 a	22.0 a	12.83 a	1.80 a	44.53 a
***P.* Value**	<0.0001	0.07	0.0008	<0.0001	<0.0001	0.33	0.0026	0.0003	0.253	0.214	0.124	0.08
Rutaceae												
N. America	44.44 a	24.59 a	4.12 a	1.85 a	194 a	34.10 a	1866 b	27.93 a	22.02	12.40 a	1.55 a	31.95 a
S. America	41.87 a	25.14 a	4.64 a	1.67 a	76.5 b	36.4 a	6451 a	29.88 a	24.91	10.65 a	1.57 a	31.33 a
***P.* Value**	0.38	0.75	0.43	0.13	0.0062	0.265	<0.0001	0.128	0.008	0.06	0.88	0.88

Sporangia: length, breadth and pedicel lentgth in µm; LB = Pedicel Length/Breadth Ratio; Density= spore number *1000/ml; Chlamydospores diameter in µm; Chlamydospores density; Oogonia and oospore diameter in µm; Anth.= Antheridium width in µm; WT = Oospore cell wall thickness in µm; and AL = Apple Lesion in mm.

Table 2. 4 Diameter chlamydospores of *Phytophthora palmivora* ordered by continents and host families.

Continent	Number of isolates	Chlamydospore diameter (µm)[y]	Chlamydospores /cm^2
Continent			
S. America	64	33.60 ± 0.42 ab	2893 ± 153 b
Asia	24	29.21 ± 0.68 c	1683 ± 139 c
Caribbean	22	32.23 ± 0.53 b	2548 ± 216 b
N. America	22	33.92 ± 1.47 b	1657 ± 133 c
Oceania	15	33.10 ± 1.17 ab	1422 ± 132 c
C. America	2	37.26 ± 2.72 a	4595 ± 854 a
Africa	1	26.62 ± NA	2458 ± 1088 bc
Family			
Malvaceae	103	32.60 ± 0.39 a	2332 ± 105 a
Palmaceae	22	32.41 ± 0.78 a	2764 ± 225 a
Other families combined[z]	7	35.56 ± 2.73 a	1459 ± 276 b
Rutaceae	13	34.72 ± 2.27 a	2399 ± 317 a
Lauraceae	5	35.05 ± 1.00 a	2395 ± 662 a

[y.] Mean ± standard error. Means that share letters within a column are not significantly different (Tukey's HSD, $P < 0.05$).

[z.] Individuals of families with less than five isolates per where pooled into the "other families" category.

Mating type was identified for 142 isolates (Appendix D). Mating type A1 was predominant (>50%) among isolates from Rutaceae family (77%) and among North America isolates (72%) (Table 2.6). Isolate 13718 did not mate with the references isolates, while isolate 13748 mated with both reference isolates. Colony shape and mycelia growth on agar varied among isolates (Figs. 2.2 and 2.3, Table 2.1, Appendix D).

The largest colony size was observed on isolate 13730 (41.8 mm) and the smallest was observed on isolate 14053 (12.7 mm) at day 3 (Appendix E). Chrysanthemum and stellate patterns were most common (36% and 25%, respectively) when evaluated by host family and

geographical region (Figs 2.4 and 2.5). Significant interactions were not observed between colony size and day measured (2 or 3 days) ($P = 0.202$) (Table 2.8). Significant differences were observed in colony size when grouped by geographical region ($P = 0.0003$) or host family ($P \leq 0.0001$) (Table 2.8)/ Significant differences on colony size were observed between isolates from the Malvaceae family hosts from South America and isolates from Asia or Oceania ($P \leq 0.0001$).

Colony size of isolates from North America and the Caribbean were not significantly different from those originating from other geographical areas. Significant differences in colony size were not observed among geographical regions for the Rutaceae ($P > 0.05$) and Palmaceae families ($P > 0.05$) (Table 2.8).

During the mefenoxam test, almost all *P. palmivora* isolates were sensitive to mefenoxam. However, Isolate 13720 (host: *Magnolia grandiflora*, California) was intermediately sensitive to the fungicide.

Pathogenicity and virulence. Lesion diameter on apples ranged from 9 to 67 mm (Table 2.1; Appendix E). The largest lesion was caused by isolate 13993 (59.2 mm) from host *Theobroma cacao* from Costa Rica, and the smallest by isolate 14063 (12.5 mm) from host *Theobroma cacao* from Papua New Guinea. Significant differences in apple lesion size were observed when isolates were grouped by region (Table 2.8) When analyzed by family, isolates from the Rutaceae produced a significantly smaller lesion (31.86 mm) than the rest of the families (Table 2.8)

Table 2. 5 Measurement (mean ± standard error) of the sexual reproductive structures of *Phytophthora palmivora* grouped by continents and host families.

	Number of isolates	Oogonia diameter (µm)[y]	Oospore diameter (µm)[y]	Antheridium width (µm)[y]	Oospore cell wall thickness (µm)[y]
Continent					
S. America	64	28.58 ± 0.16 a [y]	22.42 ± 0.14 a	13.1 ± 0.12 ab	1.92 ± 0.03 a
Asia	24	29.12 ± 0.24 a	22.87 ± 0.17 a	13.22 ± 0.15 ab	1.6 ± 0.04 b
Caribbean	22	28.33 ± 0.17 a	22.47 ± 0.17 a	13.14 ± 0.13 ab	1.59 ± 0.04 b
N. America	22	28.95 ± 0.42 a	22.83 ± 0.35 a	12.41 ± 0.18 ab	1.8 ± 0.09 ab
Oceania	15	29.25 ± 0.25 a	22.99 ± 0.21 a	13.84 ± 0.19 a	1.67 ± 0.04 b
C. America	2	29.02 ± 0.51 a	23.35 ± 0.48 a	12.90 ± 0.75 ab	1.64 ± 0.12 b
Africa	1	30.01 ± 0.67 a	23.26 ± 0.68 a	14.01 ± 0.63 a	1.5 ± 0.25 b
Family					
Malvaceae	103	28.81 ± 0.18 ab	22.72 ± 0.16ab	13.27 ± 0.13 a	1.74 ± 0.03 b
Palmaceae	22	28.30 ± 0.47 ab	21.93 ± 0.32 b	12.75 ± 0.19 ab	1.80 ± 0.05 b
Other families combined[z]	7	29.71 ± 0.95 a	23.60 ± 1.12 a	13.01 ± 0.47 ab	1.77 ± 0.34 b
Rutaceae	13	28.70 ± 0.65 b	23.25 ± 0.56 b	11.86 ± 0.33 ab	1.56 ± 0.05 b
Lauraceae	5	28.77 ± 1.98 ab	23.03 ± 1.64 ab	13.32 ± 1.57 a	2.21 ± 0.21 a

[y.] Means that share letters within a column are not significantly different (Tukey's HSD, $P < 0.05$).
[z.] Individuals of families with less than five isolates per where pooled into the "other families" category.

Table 2. 6 Mating type distribution of *Phytophthora palmivora* isolates grouped by continent and host family.

Group	Number of isolates	Mating Type A1	Mating Type A2	Mating Type A1+A2
Continent				
S. America	58	1	56	1
Asia	22	11	11	0
Caribbean	23	1	22	0
N. America	22	15	7	0
Oceania	13	4	9	0
C. America	2	0	2	0
Africa	1	0	1	0
Family				
Malvaceae	96	19	77	0
Palmaceae	22	3	19	0
Other families combined[z]	7	4	3	0
Rutaceae	13	10	2	1
Lauraceae	4	0	4	0

[z.] Individuals of families with less than five isolates per where pooled into the "other families" category.

Table 2. 7 Correlation analysis of all quantitative parameters measured on *Phytophthora palmivora*.

	L	B	P	WT	AW	Oo	Og	Ch	SN	CN	CLD2	CLD3	AL
Sporangia length (µm) (L)	**1.00**	0.12	0.13	0.13	0.04	0.11	0.10	0.07	0.13	0.07	0.08	0.17	0.26
Sporangia width (µm) (B)		**1.00**	-0.06	0.03	0.13	0.15	0.11	0.09	-0.28	-0.04	0.09	0.07	0.34
Pedicel length (µm) (P)			**1.00**	0.11	-0.02	0.17	0.19	-0.08	-0.23	0.05	0.08	-0.06	-0.21
Oospore cell wall thickness (µm) (WT)				**1.00**	0.26	0.33	0.31	0.29	-0.26	-0.01	-0.08	0.05	0.05
Antheridium width (µm) (AW)					**1.00**	**0.58***	**0.59***	-0.02	0.05	-0.16	-0.09	0.00	0.12
Oospore diameter (µm) (Oo)						**1.00**	**0.92***	-0.11	0.06	-0.13	0.06	0.17	-0.10
Oogonia diameter (µm) (Og)							**1.00**	-0.19	0.18	-0.12	0.01	0.11	-0.11
Chlamydospores diameter (µm) (Ch)								**1.00**	-0.10	0.08	-0.06	0.00	0.12
Sporangia/cm^2 (SN)									**1.00**	0.02	-0.20	-0.05	-0.09
Chlamydospores/cm^2 (CN)										**1.00**	-0.10	-0.01	0.09
Colony diameter (mm) on day 2 (CLD2)											**1.00**	**0.64***	0.10
Colony diameter (mm) on day 3 (CLD3)												**1.00**	0.22
Apple lesion diameter (mm) (AL)													**1.00**

* Correlation value > 50%

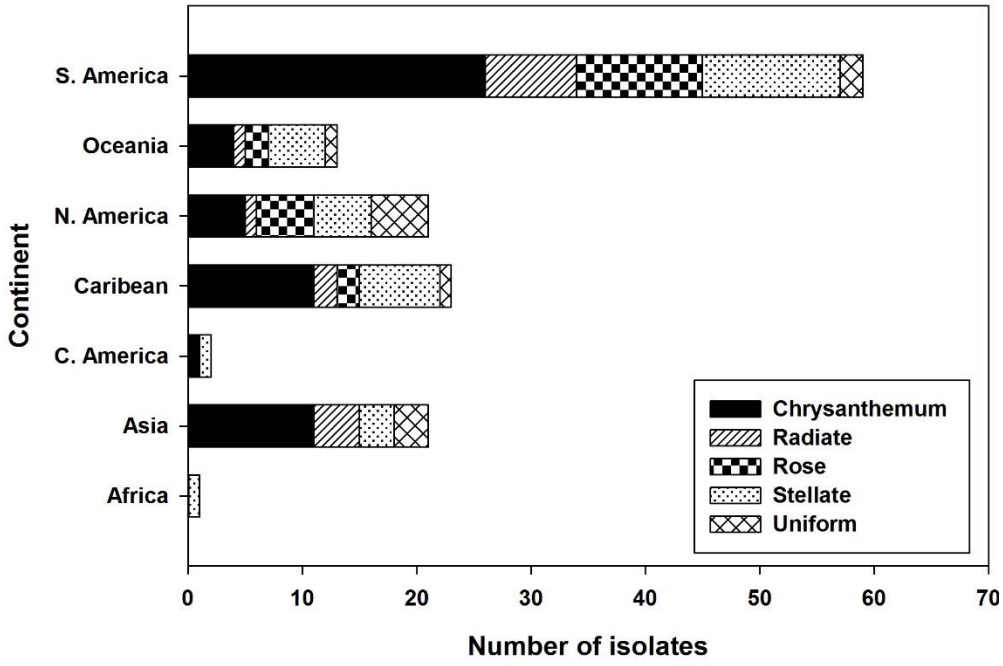

Figure 2. 4 Morphological patterns of *Phytophthora palmivora* colonies determined by geographical location.

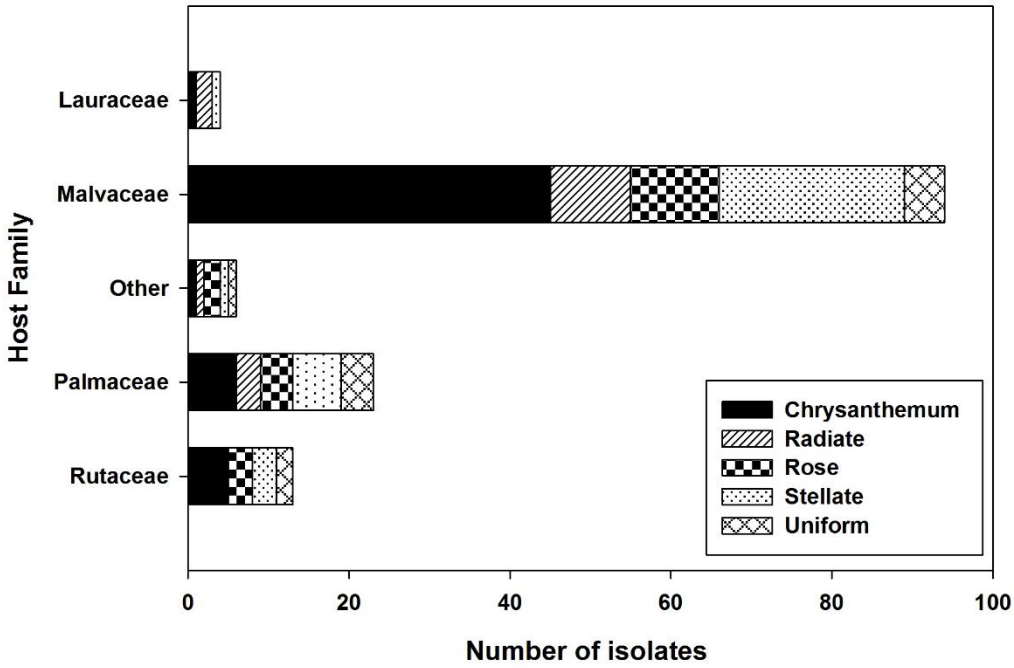

Figure 2. 5 Morphological patterns of *Phytophthora palmivora* colonies determined by host family.

Table 2. 8 Mycelial growth on clarified V8 and host ('Gala' apple) infection by *Phytophthora palmivora*.

Group	Number of isolates	Colony diameter (mm) on day 2[y]	Colony diameter (mm) on day 3[y]	Lesion diameter (mm)[y]
Continent				
South America	64	25.77 ± 0.4 ab	33.64 ± 0.4 ab	44.37 ± 0.6 b
Asia	24	24.41 ± 0.8 b	31.58 ± 0.8 bc	39.47 ± 1.4 cd
Caribbean	22	25.02 ± 0.5 b	33.17 ± 0.6 abc	42.75 ± 1.2 bc
North America	22	25.47 ± 1.2 b	31.86 ± 1.2 bc	35.15 ± 1.9 d
Oceania	15	23.67 ± 1.0 b	29.16 ± 1.2 c	36.06 ± 1.5 d
Central America	2	31.59 ± 0.6 a	38.76 ± 0.6 a	53.87 ± 1.4 a
Africa	1	24.92 ± 1.5 b	33.29 ± 1.7 abc	39.6 ± 3.1 bcd
Family				
Malvaceae	103	25.22 ± 0.3 b	32.71 ± 0.4 bc	42.29 ± 0.6 a
Palmaceae	22	25.72 ± 0.5 ab	33.57 ± 0.5 b	43.65 ± 1.0 a
Other families combined[z]	7	30.01 ± 2.8 a	38.25 ± 2.4 a	39.75 ± 2.3 a
Rutaceae	13	23.73 ± 1.3 b	28.29 ± 1.3 c	31.86 ± 1.4 b
Lauraceae	5	24.39 ± 1.4 b	33.55 ± 1.7 bc	43.12 ± 2.5 a

y. Mean ± standard error. Means that share the same letters within a column are not significantly different (Tukey's HSD, $P < 0.05$).

z. Individuals of families with less than five isolates per where pooled into the "other families" category.

DISCUSSION

This study demonstrated the variability in morphology and virulence of *P. palmivora* from different regions and hosts by examining a large number of isolates, which strengthens published data (Erwin & Ribeiro, 1996; Kroon et al., 2012; Martin et al., 2012). This variability may be the result of evolution. Morphological characteristics were previously used to distinguish subgroups of *P. palmivora* by geographical region (Chee, 1971; Mchau & Coffey, 1994) and host (Brasier & Griffin, 1979; Chee, 1969). The present research included isolates from various hosts and geographical regions showed that the isolates differed significantly in the morphology of sporangia, chlamydospore and oospore.

The sporangia characterized in this study weremainly ovoid and ellipsoid. Spornagia length (mean 43.24 µm) was in agreement with values reported previously for this species (Ashby, 1929; Brasier & Griffin, 1979; Chee, 1969; Mchau & Coffey, 1994; Ocfemia & Roldan, 1927; Tucker, 1967; Waterhouse, 1974). Observed sporangial length followed a normal distribution for the 150 studied isolates, including values designated by Waterhouse (Waterhouse, 1974) to belong to *P. arecae* (sporangial length shorter than 35 µm). Isozyme analysis concluded that *P. palmivora* and *P. areace* are co-specific (Mchau & Coffey, 1994; Oudemans & Coffey, 1991). In this study, sporangia with length shorter than 35 µm, were restricted exclusively to isolates collected from Malvaceae from Asia and Oceania. The results from Waterhouse (1963) may be biased as a result of limited number of isolates or sampling from a limited geographical area or hosts.

In the present study, sporangia breadth ranged from 19.1 to 31.5 µm, with a mean of 24.9 µm when measured with ImageJ; however, visual measurements had a mean of 29.1 µm. During the visual assessment, most *P. palmivora* sporangia breadth, laid between lines 5 and 6 of the micrometer (25 -30 µm), and were rounded mainly to 5.5 or 6 (27.5 or 30 µm respectively). This round up can explain why the visual records were larger than the values recorded using ImageJ. Similar situation could happen in other sporangia breadth reports, including Mchau and Coffey (1994). Sporangia breadth from this study was in agreement with values reported by Brasier and Griffin (1979). When breadth of *P. palmivora* isolates were grouped by family, significant differences were not observed except for the cluster of families with fewer than five isolates per family. Variability of the limited group (“others”) could be related with the narrow sample size.

The ratio of sporangial length and breadth (LB) was used to distinguish among some *Phytophthora* species (Aragaki & Uchida, 2001; Waterhouse, 1963). However, LB ratio is no

longer used to differentiate *P. palmivora* from other species, as is affected by culture media (Brasier & Griffin, 1979). Ovoid and ellipsoid sporangia were most frequently observed and is defined by a LB ratio larger than 1:1. As expected, the LB ratio followed a normal distribution, similar to the distribution of *P. capsici* (Granke & Hausbeck, 2011). In this study, significant differences in LB ratio were observed for *P. palmivora* isolates when they were grouped by family or geographical region, having the Palmaceae and Rutaceae families a ratio 0.11 larger than the Lauraceae and the Malvaceae families.

Pedicel length was used to discriminate among the four *P. palmivora* morphotypes, and to separate *P. capsici* (*P. palmivora* MF4) and *P. megakarya* (*P. palmivora* MF3) from the *P. palmivora* complex (Al-Hedaithy & Tsao, 1979; Brasier & Griffin, 1979; Kaosiri et al., 1978; Zentmyer et al., 1977). The pedicel length observed in this study was 3.5 µm ± 2.6 µm, which is short in comparision with other species of *Phytophthora*. No significant difference in pedicel length was observed when isolates were grouped by region (> 5 isolates/region), but significant differences were observed when isolates were grouped by host.

Chlamydospores of *Phytophthora* species serve as a long term survival structure (Chee, 1973; Hwang & Ko, 1978). Survival of *P. palmivora* chlamydospores was reported up to 24 weeks on soil (Chee, 1973). The isolates in this study produced different density of chlamydospores, ranging from none to many. This variation is believed normal in the *P. palmivora* community (Mchau & Coffey, 1994). The size of chlamydospores was in the range that has previously been reported (Brasier & Griffin, 1979; Mchau & Coffey, 1994; Tucker, 1967), Differences in chlamydospore density were observed when isolates were grouped by geographical location, but not by host.

The A2 and A1 mating types of *P. palmivora* isolates in the present study were distributed in a 7:2 ratio, similar to what Chowdappa and Chandramohanan (1997) have found in India, but slightly different with that by Chee (1971) (5:1 ratio), and significant different from Zentmeyer (1973) who found a 3:2 ratio. Brasier (1992) indicated that despite a 1:1 heritability could be expected, deviation from that ratio is commonly observed in some species, and is favored by asexual reproduction of an specific MT that is better adapted to it host than the opposite MT. Graham et al. (1998) found that *P. palmivora* isolated from cacao and milkweed did not infect Valencia orange seedlings; Ashby (1922) demonstrated isolate from coconut palm did not infect cacao; Zitko et al. (1991) failed to infect *Citrus* spp. roots with an isolate from areca palm. Host selectivity of *P. palmivora* was proven by Chee (1971) for the MT A2 on cacao, and may explain the failure to recover A1 isolates in some countries, including Colombia. Cacao pods were used frequently as *P. palmivora* traps in the tropics (Brasier & Griffin, 1979; Waterhouse, 1974).

Colony morphology of *P. palmivora* was variable on agar plates, but scanty-fluffy with stellate and chrysanthemum patterns were most common, as Brasier and Griffin (1979) reported. Colony size of the isolates seemed to be associated with host or geographical region. Isolates from the Rutaceae and from Oceania had the smallest colonies.

The virulence of the test isolates varied, as others indicated (Ashby, 1929). Virulence was correlated with geographical region, and isolates from the Central America, South America, and the Caribbean caused larger lesions than other isolates. In addition, Rutaceae-obtained isolates caused lesions slightly smaller than those caused by isolates from Palmaceae and Malvaceae families. This result was in agreement with Tucker (Tucker, 1967) on cacao and citrus.

ACKNOWLEDGMENTS

The author want to thanks Dr. Gerardo Martínez, who passed away in 2015 (Cenipalma-Colombia), Surendra Surujdeo-Maharaj (Cocoa Research Centre, Trinidad and Tobago), Dr. André Drenth (The University of Queensland- Australia), Drs. Ute Albrecht, Kim Bowman, Rachel Melnick, and Frank Martin (USDA), Dr. Cheryl L. Blomquist (California Department of Food and Agriculture), Ms. Gail E. Ruhl (Purdue University), Drs. Jim Graham and Aaron J. Palmateer (University of Florida), and Dr. Felipe D. Arredondo (Virginia Polytechnic Institute and State University) for providing the isolates used in the present study. My gratitude is also expressed to Drs Rachel Naegele, Lina Rodriguez, Leah Granke for their advice; Blair Harlan, Charles Krasnow, Ryan Gustafson, Paula Samohan, Matt Bour and the rest of the Dr. Haubeck lab members for their help on conducting the present study.

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CHAPTER III: SENSITIVITY OF *PHYTOPHTHORA PALMIVORA* TO NINE FUNGICIDES

ABSTRACT

Phytophthora palmivora is a tropical plant pathogen affecting more than 160 species of plants, including subtropical species. As cultural practices and resistant cultivars are not adequate to limit the detrimental effects of the pathogen, fungicides may be the first option in an integrated pest management program. The commercial fungicides captan, cyazofamid, dimethomorph, fluopicolide, mandipropamid, mefenoxam, oxathiapiprolin, zoxamide, and potassium phosphite were tested for efficacy against *P. palmivora* using petunia bedding plants. Identification of the half maximal effective concentration (EC_{50}) was estimated for each active ingredient of the fungicide (excluding potassium phosphite) using a *P. palmivora* population of 150 isolates obtained from 16 hosts representing 17 geographical locations. For the in-vivo trial, mefenoxam, oxathiapiprolin, and captan provided complete control of the disease. Mandipropamid reduced disease incidence by 83% 21 days after inoculation. Fluopicolide, dimethomorph, and cyazofamid provided an intermediate level of control, reducing plant death incidence by 50%. Zoxamide and potassium phosphite did not significantly reduce the disease and incidence was similar to the inoculated control. An in-vitro test showed that captan had the highest EC_{50} value (38.082 mg/L), and oxathiapiprolin had the lowest (0.004 mg/L). The EC_{50} for zoospore inhibition with dimethomorph was 0.170 mg/L and was approximately one half of the concentration needed for mycelial inhibition (0.32 mg/L). The EC_{50} values were 0.34 mg/L for fluopicolide, 0.12 mg/L for dimethomorph and zoxamide, and 0.017 mg/L for both mefenoxam and mandipropamid. Four isolates of *P. palmivora* were not sensitive to mefenoxam

at the studied rates. In the in-vitro studies all fungicides demonstrated good control of *P. palmivora*; the effectiveness was often corroborated in-vivo.

INTRODUCTION

Phytophthora palmivora is a globally distributed plant pathogen (Chee, 1974; Erwin & Ribeiro, 1996; Waterhouse, 1974) belonging to the class Oomycota within the kingdom Straminipila (Brasier, 1992). This pathogen infects more than 160 species of plants (Erwin & Ribeiro, 1996). It is a major problem in cacao (*Theobroma cacao*) (Ashby, 1929; Chee, 1974; Tucker, 1967; Waterhouse, 1974), rubber (*Hevea brasilensis*) (Chee, 1973, 1969; Churngchow & Rattarasarn, 2000; Dantanarayana et al., 1984) coconut (*Cocos nucifera*) (Ashby, 1929; Butler, 1924), areca palm (*Areca cachetu*) (Butler, 1924; Chee, 1974), palmyra palm (*Borassus flabellifer*) (Butler, 1924; Chee, 1974) and oil palm (*Elaeis guineensis* Jacq.) (Sarria et al., 2008; Torres et al., 2010). *Phytophthora palmivora* has caused losses exceeding $1 billon for the cocoa industry, and has destroyed of thousands of hectares of crops causing great social impact. The recent epidemics of bud rot in two Colombian regions destroyed more than 70.000 ha of oil palm in eight years (Torres et al., 2016). In Colombia, more than 30,000 ha were destroyed in Tumaco (Pulido, 2011), impacting approximately 73,200 people, 46% of Tumaco's population (Martínez & Silva, 2008) by 2011.

Diseases caused by *P. palmivora* are limited by using integrated pest management (IPM) strategies (Bajwa & Kogan, 2002) that include the combination of cultural, biological, and chemical practices to minimize, delay or avoid the pathogen's detrimental impacts (Kogan, 1998). Fungicides may be required within an IPM approach (Schwinn & Staub, 1995); disease

progress of *P. palmivora* on some crops is limited by the use of copper-based fungicides (Gorenz, 1974; Tey & Wood, 1983). However, the number of fungicides registered to control *P. palmivora* in some developing countries is limited. In Nigeria for example, copper fungicides are the only products registered to control black pod of cacao (Agbeniyi & Oni, 2014). In Colombia, only Inifnito® (propamocarb HCl0 62.5% + fluopicolide 6.25 %, Bayer CropScience AG, Rheim, Germany) is registered for use on oil palm

To screen fungicides for efficacy against pathogens, in-vitro tests can be performed, and then be correlated with efficacy in field tests (Toquin et al., 2010). Fungicide efficacy is measured in-vitro by calculating the minimum inhibitory concentration (MIC) or the concentration that inhibits 50% of the pathogen growth or germination (EC_{50}) (Förster et al., 2004; Sebaugh, 2011). In serial dilution or spiral gradient endpoint (SGE) tests (Förster et al., 2004; Gachango et al., 2012). These techniques were used to assess fungicide efficacy by Gachango *et al.* (2012) and Miles *et al.* (2013) when evaluating products for control of *Fusarium* spp. and *Alternaria solani* on potatoes, respectively. The SGE technique was also used to calculate EC_{50} in *Penicillium* spp. (Förster et al., 2004; Kanetis et al., 2008; McKay et al., 2012), *Monilinia* spp. (Förster et al., 2007, 2004), *Botrytis cinerea* (Amiri et al., 2014, 2013; Förster et al., 2007, 2004), and *Galactomyces citri-aurantii* (McKay et al., 2012).

In-vivo assessment of fungicide efficacy can be accomplished using a susceptible host. Petunia plants are susceptible to many species of *Phytophthora*, including *P. parasitica* (Hagan & Akridge, 1999; Miller & Noegel, 1967), *P. palmivora* (Ann, 2000; Barsam et al., 2011; Erwin & Ribeiro, 1996), *P. nicotianae* (Gerberich & Beckerman, 2012; Warfield & Sugar, 2006; Yang et al., 2012), and *P. citrophthora* (Olson & Benson, 2011). Different fungicides have been tested for their ability to control *P. nicotianae* (Gerberich & Beckerman, 2012; Warfield & Sugar,

2006; Yang et al., 2012) on petunia, with variable results. The susceptibility of petunia to a wide range of *Phytophthora* spp., makes this plant a suitable model for testing fungicide efficacy. The overall objective of this study was to identify fungicides effective against *P. palmivora* and included *i*) evaluations using in-vitro tests and in-vivo petunias assay and *ii)* characterizing isolates for fungicide sensitivity.

MATERIALS AND METHODS

Isolates. A total of 150 isolates of *P. palmivora* were collected from 16 hosts and 17 geographical locations worldwide (Appendix A) with the aid of several research groups. In addition to isolates of *P. palmivora*, several *Phytophthora* spp. from Dr. M.K. Hausbeck's laboratory collection at Michigan State University were used, including three isolates of *P. capsici* (12889-insensitive to mefenoxam, OP97 and OP98), two isolates of *P. asparagi* (SP318 and SP326), two isolates of *P. nicotianae* (1005 and 13597), one isolate of *P. cactorum* (4001), and one isolate of *P. tropicalis* (13715).

The *P. palmivora* isolates were reactivated by inoculating pears and recovering the pathogen from infected tissue by isolating onto BARP (50 mg benomyl, 75 mg ampicillin, 15 mg rifampicin, and 100 mg PCNB)-amended and filtered-V8 (40 ml clarified V8 juice, 960 ml distilled water, 6 mM $CaCO_3$, and 12 g agar) (Lamour & Hausbeck, 2003; Quesada-Ocampo et al., 2009). The isolates were transferred and maintained on unclarified V8 (UCV8) (160 ml unclarified V8 juice, 30 mM calcium carbonate, 16 g of agar) plates until use.

In-vitro assays on the sensitivity of *P. palmivora* to fungicides. In the first of two trials, all isolates were tested for sensitivity to the following fungicides: mefenoxam (Ridomil Gold

EC®, 49 % a.i., Syngenta Crop Protection, Greensboro, NC), fluopicolide (Presidio®, 39.5% a.i., Valent U.S.A. Corporation, Walnut Creek, CA), mandipropamid (Micora®, 23.3 % a.i., Syngenta Crop Protection), zoxamide + mancozeb. (Gavel 75 DF®, (zoxamide) 8.3 % a.i and (mancozeb) 66.7 % a.i., Gowan Company, Yuma, AZ), captan (Captan 80 WDG, 80% a.i., Arysta LifeScience North America, Cary, NC), cyazofamid (Ranman®, 34.5 % a.i., FMC corporation, Philadelphia, PA), ametoctradin + dimethomorph (Zampro®, (ametoctradin) 26.9 % a.i., and (dimethomorph) 20.2 % a.i., BASF Corporation, Research Triangle Park, NC), potassium phosphite (Vital, 54.5 % a.i, Phoenix Environmental Care, Valdosta, GA) and oxathiapiprolin (Orondis, 10% a.i. Syngenta Crop Protection). In the second trial, Zoxium® (Zoxamide, 80 % a.i. Dow AgroSciences, Indianapolis, IN) and Forum® (Dimethomorph 80% a.i., BASF Corporation) were used as source of zoxamide and dimethomorph respectively.The selected fungicides included different modes of action (Table 3.1).

A modified spiral gradient endpoint method and analysis system was used (Förster et al., 2004). A volume of 40 ml of clarified V8 agar (CV8 agar: 120 ml clarified V8 juice, 30 mM $CaCO_3$, 1.6% agar, 880 ml distilled water) was poured into each 150-mm-diameter Petri dish and allowed to solidify and dry for two days. Each fungicide for the in-vitro assay was prepared in a stock concentration, and then deposited in a spiral gradient pattern using a Jet 2 spiral plater (IUL, S.A., Barcelona, Spain). Stock concentrations of test fungicides were determined based on results of a preliminary study (Table 3.2). After the fungicide was applied, the plates were maintained for 24 to 48 hours before a 20-mm-diamter agar disc was removed from the center.

Table 3. 1 Characteristics of active ingredients in selected fungicides tested for efficacy against *Phytophthora palmivora*.

Active ingredient	Mode of Action	Target site	FRAC code[x]	Risk of developing resistance
Captan	Multisite	Multisite	M4	Low
Cyazofamid	Respiration	Complex III: Cytochrome bc1	21	Medium-high
Dimethomorph	Cell wall biosynthesis	Cellulose synthase	40	Low-Medium
Fluopicolide	Mitosis and cell division	Delocalization of spectrin-like proteins	43	Medium-high
Mandipropamid	Cell wall biosynthesis	Cellulose synthase	40	Low-Medium
Mefenoxam	Nucleic acid synthesis	RNA polymerase I	4	High
Oxathiapiprolin	Unknown	Oxysterol binding protein (OSBP) inhibition (proposed)	U15	Medium-high
Potassium phosphite	Unknown	Unknown	33	Low
Zoxamide	Mitosis and cell division	ß-tubulin assembly in mitosis	22	Low-Medium

[z] Fungicide Resistance Action Committee (FRAC)

Mycelial growth inhibition was evaluated for the selected fungicides with the exception of cyazofamid. Zoosporic germination was evaluated only for cyazofamid and dimethomorph. After incubation, mycelial growth and zoosporic germination were measured. The 50% of growth inhibition (EC_{50}) was calculated for mycelium using a wooden stick method (Appendix F) and by spreading an individual drop of released zoospores from each isolate from the outer to the inner part of the cyazofamid and dimethomorph plates at the position designated for each isolate. Briefly, the wooden stick method consisted of one milliliter of double distilled water (ddH_2O) added to a 6-mm-diamter V8 agar plate with a seven-day old culture of each isolate and then mycelia were scraped with a glass spreader bar. Three-hundred microliters of the suspension was recovered using a micropipette and were applied to the top of ten 50x6x1-mm wood sticks

that had been placed on top of the CV8 plate. Each plate was wrapped with Parafilm® and maintained under continuous fluorescent light at room temperature (23 ± 2 °C) until mycelia completely covered the sticks. The sticks were then removed from the plates and placed mycelial side down on the agar within the plates prepared with the various fungicides. Eight different isolates were spaced on each plate such that a 45° angle formed between neighboring sticks.

The spiral plates were scanned two days after inoculation using an Epson Perfection V30 scanner (Epson America Inc., Long Beach, CA). To improve the imaging acquisition, a black background was used on top of the plates with a tone curve adjustment of the multichannel option of the software provided by the scanner manufacturer; the input and output values were set to 132 and 43, respectively. Pictures were saved as JPG files. The Tail Ending Radius (TER - point where maximum inhibition is obtained) and Ending Radius (ER- point where minimal inhibition is obtained) and growth width was obtained using ImageJ (National Institutes of Health, USA). The EC_{50} concentrations for the first repetition were calculated using the EC50 R package (Chapter IV) and a custom ImageJ macro. The EC_{50} values for the second repetition were calculated using a custom "2-Spiral-plater" macro for ImageJ.

This experiment was conducted as an incomplete balanced block design (IBBD), using time as a blocking factor. A total of nine blocks, that included all fungicide treatments, were used and included 64 isolates for each block. Each of the 150 isolates were evaluated three times. Isolates 13732 (A2) and 13749 (A1) were used as positive controls and were included in all blocks. Sterile wood sticks were used as a negative control. Isolates for each individual block were identified, independently randomized, and assigned to each fungicide. The experiment was conducted twice.

Table 3. 2 Characteristics and concentrations of fungicides used for in-vitro and in-vivo assays.

Fungicide	Molecular weight (gr/mole)	Suggested application rate (g ai/ha)	Actual rate (g/plot)	Stock concen-tration (mg/L)	Range evaluated (mg/L)	
					Max.	Min.
Captan	**300.6**	**4500**	**0.18**	**10000**	**96.232**	**0.549**
Cyazofamid	324.8	80	0.0032	10000	95.918	0.518
Dimethomorph	387.9	209	0.0084	250	2.368	0.011
Fluopicolide	383.6	140	0.0056	1000	9.482	0.046
Mandipropamid	411.9	237	0.0095	31	0.353	0.002
Mefenoxam	279.3	30	0.0012	56	0.289	0.002
Oxathiapiprolin	345	142	0.0057	10	0.096	0.0005
Zoxamide	336.7	113	0.0045	100	0.957	0.005
Potassium phosphite	120	70000	2.8	-	-	-

In-vivo assays on the sensitivity *of P. palmivora* to fungicides. All fungicides used for the in-vivo tests, except for potassium phosphite, were evaluated for in-vitro assays. Petunia cv. Yellow Madness® seed (*Petunia* x hybrid) were obtained from Ball Horticultural Company (West Chicago, IL). Studies were conducted in the greenhouses of Michigan State University greenhouse. Seeds were planted in 18 x 30.5 cm plastic trays containing 288 plant cells (12 x 24 cells). One seed was planted per cell into Suremix potting media (Michigan Grower Products, Galesburg, MI). One tray was prepared for each fungicide and for each of the negative and the positive controls (A1 and A2 isolate). Four weeks after germination, 900 ml of the assigned fungicide, representing the registrant's highest labeled rate (Table 3.2) was applied as a drench to the seedlings. The trays containing the treated seedlings were left overnight in the greenhouse and the following day eight individual plants were treated and transferred as a subplot within a 288-cell seedling tray (Fig 3.1) based on the assigned treatment. A total of six trays (288 wells) were prepared and placed individually within a plastic box on top of a 33 x 53 x 10 cm aluminum pan. Wet paper towel was placed along the edges within the plastic box to increase

relative humidity. All subplots, except the negative control, were inoculated with 300 µl of 4 x 10^5 zoospores/ml of A1 (13721 originally collected from *Citrus* sp., Florida) or A2 isolate (13752 originally collected from *Elaeis guineensis*, Colombia).

The experiment was conducted as an incomplete block design using each incubation box as a blocking factor. Each block consisted of twelve subplots including one negative (ddH_2O) and two positive controls (A1 or A2 each treated with ddH_2O). The remaining nine subplots were assigned randomly for each fungicide and isolate. Each treatment combination (isolate + fungicide) was conducted three times. Disease were evaluated using a 1 to 5 disease symptom scale, where 1 = healthy with green tissue, 2 = slight chlorosis, 3 = advanced chlorosis, 4 ≤ 50% necrotic tissue, and 5 ≥ 50% necrotic tissue. Plants were evaluated every 3 to 4 days during a three-week period. Pathogenicity of *P. palmivora* was confirmed by re-isolation of the pathogen to V8 media.

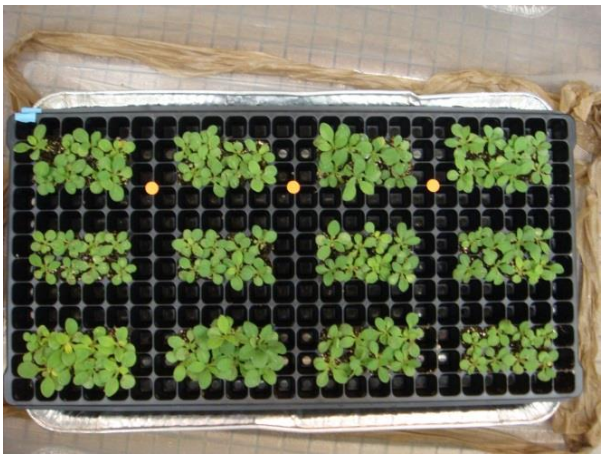

Figure 3. 1 Arrangement of seedling blocks prior to inoculation with *Phytophthora palmivora*.

Data analysis. All statistical analyses were performed using R statistical software version 3.0.2 (The R foundation for Statistical Computing, Vienna, Austria). For in-vitro tests, log

transformation of EC50 was performed for fluopicolide, mandipropamid, mefenoxam, and oxathiapiprolin. Results were back transformed for analysis. The in-vivo tests were evaluated following the Shah and Madden (Shah & Madden, 2004) recommendation for qualitative data. Multinomial analysis and probabilities were performed and calculated using the MULTINORM function of the NNET R package (Venables & Ripley, 2002).

RESULTS

In-vitro assay. The EC_{50} values of 150 isolates of *P. palmivora* and the outgroup isolates were calculated using the spiral plating methodology. Since the concentration selected for the study varied among fungicides, comparisons of in-vitro performance at the same concentrations among treatments were not performed.

Significant differences in EC_{50} values among isolates were observed when mycelial inhibition was measured for captan ($P = 0.003$), dimethomorph ($P \leq 0.001$), fluopicolide ($P = 0.003$), mandipropamid ($P = 0.013$), and oxathiapiprolin ($P = 0.014$). No significant differences were observed for mefenoxam ($P = 0.453$) and zoxamide ($P = 0.865$).

The isolates of *P. palmivora* and other *Phytophthora* spp. included in the study exhibited an EC_{50} value for captan of 38.1 mg/L (±1.5 SE); all isolates were sensitive at the evaluated range of concentrations (Table 3.3). Fluopicolide (0.34 mg/L ± 1.06 SE) did not limit the growth of isolates 13597 (*P. nicotianae*), 13739, 13743 and OP97 (*P. capsici*) at the maximum evaluated concentration (9.48 mg/L, about 15 times lower than the highest recommended commercial dose). EC_{50} values for mandipropamid and mefenoxam were similar (0.014 and 0.017 mg/L, respectively), however, the growth of isolate 13721 was not inhibited by mandipropamid, but was inhibited by mefenoxam at the selected concentrations. Isolates 12889 (insensitive *P. capsici* control), 13720, 13740, 13753, 14010 SP97 (*P. asparagi*) and SP98 (*P. asparagi*) were not controlled by mefenoxam. Differences were not observed between the minimum and maximum EC_{50} mean for mefenoxam and mandipropamid (Table 3.3). Oxathiapiprolin had an EC_{50} mean of 0.004 mg/L (SE ± 1.03), with the lowest being 0.0012 mg/L (on isolate 14033) and the highest being 0.020 mg/L (on isolate 13722). Zoxamide completely controlled the growth of all *P.*

palmivora isolates in-vitro; however, isolates SP98 (*P. asparagi*), 13945 (*P. nicotianae*) and 12889 (*P. capsici*) from the outgroup were not limited when the maximum concentration of 0.96 mg/L were not controlled.

Table 3. 3 Effective concentrations of fungicides for 50% growth inhibition (EC50 in mg/L) of *Phytophthora palmivora*.

Product	Mean EC_{50}	Standard error	Minimum	Maximum
Captan	38.082	1.464	3.740	94.118
Dimethomorph (for mycelial growth)	0.320	1.492	0.020	0.750
Dimethomorph (for zoosporic germination)	0.170	1.312	0.020	0.670
Fluopicolide	0.340	1.033	0.084	0.975
Mandipropamid	0.014	1.039	0.003	0.137
Mefenoxam	0.017	1.052	0.003	0.105
Oxathiapiprolin	0.004	1.035	0.001	0.020
Zoxamide	0.120	1.033	0.011	0.440

Zoosporic germination was measured for cyazofamid and dimethomorph. The effective concentration for cyazofamid was established for six isolates of *P. palmivora* (13732, 13949, 13954, 13957, 13987, 14031 and 14033; EC_{50} mean = 70 mg/L). Due the reduced number of representatives of the population, cyazofamid was removed from the analysis for the in-vitro tests. Unlike cyazofamid, spore germination (EC_{50} = 0.17 mg/L) was observed for dimethomorph-amended plates. Isolates 4001 (*P. cactorum*), 13729, 13731, 13954, 14039, 14053 14056, 14060, and the two *P. asparagi* isolates were completely inhibited, but 13722 and 13756 were not inhibited by dimethomorph at the evaluated dose (0.011 – 2.368 mg/L).

In-vivo assay. Significant differences in disease severity were observed among treatments. Disease incidence (percentage of plants with 50% of necrosis or more) at 0, 7, 14 and 21 days post inoculation was 0, 10, 49 and 83% respectively ($P < 0.05$) for the non-treated control plants(Table 3.4, Fig 3.2).Zoxamide and potassium phosphite were not effective in

limiting plant death caused by *P. palmivora* infection; the probability of a plant becoming necrotic within three weeks after inoculation did not differ from the positive control (inoculated, but not treated) (Table 3.4). Treatment with cyazofamid, fluopicolide and dimethomorph delayed disease symptoms compared to untreated inoculated plants, but otherwise did not limit disease. The probability of having a diseased petunia plant in blocks treated with captan, mandipropamid, mefenoxam or oxathiapiprolin was less than 1.2% at 21 days post inoculation, similar to non-inoculated control plants. No detrimental effect was observed on non-inoculated plants treated with the fungicides. Diseases incidence under non-inoculated scenario remained at 6% or lower values for all treatments (Fig 3.2). Significant differences were not observed among the three replicates of this experiment, thus data were pooled for statistical analysis.

Table 3. 4 Incidence of petunia plants inoculated with *Phytophthora palmivora* succumbing to disease and resulting in plant death on day 21 when treated with different fungicides.

Treatment	Disease incidence[1]
Non-inoculated	0 [a]
Mefenoxam	0 [a]
Oxathiapiprolin	0 [a]
Captan	4 [a]
Mandipropamid	17 [a]
Fluopicolide	42 [ab]
Dimethomorph	50 [b]
Cyazofamid	54 [bc]
Zoxamide	67 [cd]
Potassium phosphite	75 [cd]
Untreated	83 [c]

[1] Values followed by the same letter are not significantly different ($P<0.005$).

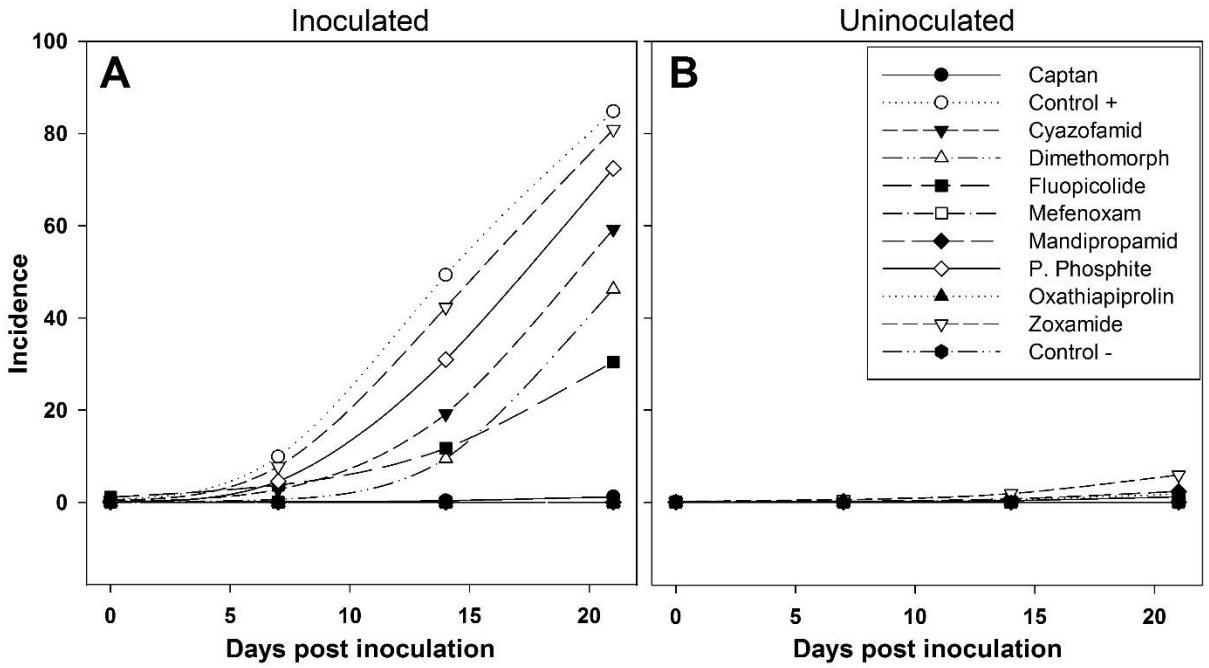

Figure 3. 2 Disease progress of plant death over time that were treated with fungicides, then either inoculated with *P. palmivora* (A) or non-inoculated (B).

DISCUSSION

A total of 150 *P. palmivora* isolates were evaluated. The in-vitro efficacies for eight fungicides with different modes of action exceeded the optimal (>50) number of isolates suggested by Russell to identify a fungicide's baseline (Russell, 2002) and assure an unbiased estimate of baseline sensitivity (Kanetis et al., 2008). The isolates selected for the in-vivo trial were highly pathogenic to petunias. The majority of *P. palmivora* isolates evaluated were sensitive to the fungicides tested, and could be considered valuable tools within an IPM framework.

The ECX program provides free, accurate, and high quality data analysis similar to other commercial software. As Pong et al. (2010) highlighted, the SGE software is significantly

important to calculation the MIC for antimicrobial compounds and provides a powerful tool for its determination; the low-cost-effectiveness of the spiral plating technique made it specially suitable for antimicrobial studies in developing countries (Pong et al., 2010). However, some limitations of the commercial software, such as single user license, single product and plate analysis, and the necessity of manually input of the data were observed at the initial stage of the present study for effective concentrations. These flaws were satisfactorily overcome by developing a more efficient system for calculating the final concentration (Chapter IV). In addition to the software, the development of a wooden stick method to support the *Phytophthora* growth, improved the methodology proposed by Förster et al. (2004) and validates the spiral technique as an efficient method for effective concentration calculation.

In-vitro testing does not assure in-vivo success of a fungicide, but may provide an indicator of its response (Russell, 2002). In the present study, most of the fungicides provided good control both in-vitro and in-vivo; only zoxamide did not control the pathogen at the evaluated ranges.

Captan, mandipropamid, mefenoxam, and oxathiapiprolin reducedd petunia plant infection by day 21, close to levels found in the non-inoculated control. Cyazofamid, fluopicolide and dimethomorph demonstrated some control of disease development with a single application, but did not restrict disease. Good control with these fungicides was obtained by Meyer and Hausbeck (2013) on summer squash when applied every week over a 5- week period. The present study did not test multiple sprays, and could explain why disease protection was not maintained for a longer period of time as observed with the drench application for *P. capsici* (Meyer & Hausbeck, 2013). Further research is needed to determine the response of *P. palmivora* to multiple fungicide applications.

Physiological differences among plant species can affect how a fungicide is transported internally and processed metabolically (Edgington, 1981). Control with potassium phosphite was not observed for *P. palmivora* in the present study with petunia plants; however, it has been satisfactory when tested on cocoa and other ornamental species (Holderness, 1992; McMahon et al., 2010; Palmer & Vea, 2010). The baseline for eight fungicides to control *P. palmivora* was established in this study. This constitutes an important information for tracing fungicide resistance changes for *P. palmivora* as stated by Russell (2002) and may help to manage fungicides in disease management of oil palm production.

ACKNOWLEDGMENTS

The author want to thanks Dr. Gerardo Martínez, who passed away in 2015 (Cenipalma-Colombia), Surendra Surujdeo-Maharaj (Cocoa Research Centre- Trinidad and Tobago), Dr. André Drenth (The University of Queensland- Australia), Drs. Ute Albrecht, Kim Bowman, Rachel Melnick, and Frank Martin (USDA), Dr. Cheryl L. Blomquist (California Department of Food and Agriculture), Ms. Gail E. Ruhl (Purdue University), Drs. Jim Graham and Aaron J. Palmateer (University of Florida), and Dr. Felipe D. Arredondo (Virginia Polytechnic Institute and State University) for providing the isolates used in the present study. My gratitude is also expressed to Drs Rachel Naegele, Lina Rodriguez, Leah Granke for their advice; Blair Harlan, Ryan Gustafson, Paula Samohan, Matt Bour and the rest of the Dr. Haubeck lab members for their help on conducting the present study

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CHAPTER IV: ECX- AN R PACKAGE FOR STUDYING SENSITIVITY OF ANTIMICROBIAL SUBSTANCES USING SPIRAL PLATING TECHNOLOGY

A paper submitted to the Plant Health Progress Journal

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ABSTRACT

Spiral plating technique (SPT) is reliable, repeatable and more efficient than dilution plating methods in studying the efficacy of antimicrobial products. In the SPT method, the concentration of chemicals is calculated using an associated software. To establish a user-friendly and cost-free platform, the R- package, ECX was developed to calculate effective concentrations of different antimicrobial compounds. Mathematical models were established for calculating dispensed volume on agar plates, using variables that affect diffusion (molecular weight and agar height). In addition to the R packages, the web-based Shiny extensions ECX, multi and ppm were developed to provide a graphical interface for calculating individual concentrations, multiple concentrations, and stock concentration. By comparing ECX with a commercial software, no significant differences were observed between them ($P > 0.05$). However, the ability to import and process large datasets made the ECX package a better option for SPT studies. Furthermore, the multiplatform nature of the ECX package overcomes limitations presented in other software. Therefore, the ECX characteristics can enhance the use of the spiral plating technique for sensitivity studies.

INTRODUCTION

Chemicals like fungicides and other antimicrobial substances, are evaluated for their efficacy in inhibiting the growth of microorganisms by using effective concentration (EC) values and the minimal inhibitory concentration (MIC) (Förster et al., 2004; Sebaugh, 2011b). Depending on the purpose of the study, the effective concentration is typically defined as 50% or 90% of growth or development inhibition (EC_{50} or EC_{90}), or the minimum inhibitory concentration where no inhibition occurs (Gachango et al., 2012; Hill & Schalkowsky, 1990; Paton et al., 1990; Pong et al., 2010; Russell, 2002; Wexler et al., 1996, 1991). Traditionally, bioassays or plate assays using pesticide-amended media have been used to determine the EC, which involves a series of chemical concentrations (minimum of 4) and replicated plates (Förster et al., 2004). These techniques are relatively expensive and time consuming (Wexler et al., 1996). Förster et al. (2004) found that the use of the spiral gradient dilution method using the Spiral Autoplate was an efficient substitute for traditional methods in fungicide sensitivity assays; those observations were corroborated by Gachango et al. (2012), Miles et al. (2013), Fairchild et al. (2013) Amiri et al. (2013), and others (Driever et al., 2012; Kanetis et al., 2008; Martini, 2012). Several studies using this technique on human bacterial pathogens have also shown success (Hill & Schalkowsky, 1990; Paton et al., 1990; Pong et al., 2010; Wexler et al., 1996, 1991);.This technique has also been successfully used to evaluate the EC_{50} and baseline sensitivities for agricultural pesticides on different plant pathogens (Adaskaveg et al., 2011; Amiri et al., 2013; Förster et al., 2004; Gachango et al., 2012; Miles et al., 2013).

The first spiral plating equipment was initially developed by Gilchrist et al. in 1973, for counting bacterial colonies (Gilchrist et al., 1973; Paton et al., 1990), but Spiral System

Instruments Inc. developed the spiral gradient endpoint test to determine antimicrobial susceptibility of bacteria using this continuous method in 1985 (Paton et al., 1990). The machine functions by dispensing a volume of ~50 µl in a gradient concentration. This deposition forms an Archimedean spiral, beginning at 12 to 13 mm from the center of the plate, towards the edge of the plate (Fig 4.1) (Förster et al., 2004; Paton et al., 1990). The deposited volume can be precisely tracked and calculated, resulting in a radially-decreasing concentration gradient (Gilchrist et al., 1973; Hill & Schalkowsky, 1990; Wexler et al., 1991). The innermost loops of the spiral are omitted for evaluation, since diffusion in this area is highly affected by being next to a zero concentration area (center to dispensing starting point) (Paton et al., 1990). Förster *et al* (Förster et al., 2004) recommended removing this fungicide-free zone with a cork borer to avoid growing of insensitive pathogens from one side of the plate to the other. Since high concentrations occur in the first laps, researchers recommend to analyze plates from radius 20 mm to the edge (Förster et al., 2004; Paton et al., 1990). Data collected from the spiral plate technique usually requires a licensed software for analysis, such as the Spiral Gradient Endpoint by Spiral Biotech Inc. (Wexler et al., 1996). The EC, minimum inhibition concentration (MIC) and total inhibition concentration (no growth of the pathogen - TIC) can be elucidated by calculating the volume dispensed at the point where the desired effect is observed; Values are calculated using the distance from the center of the plate to the point where these parameters are observed (Förster et al., 2004).

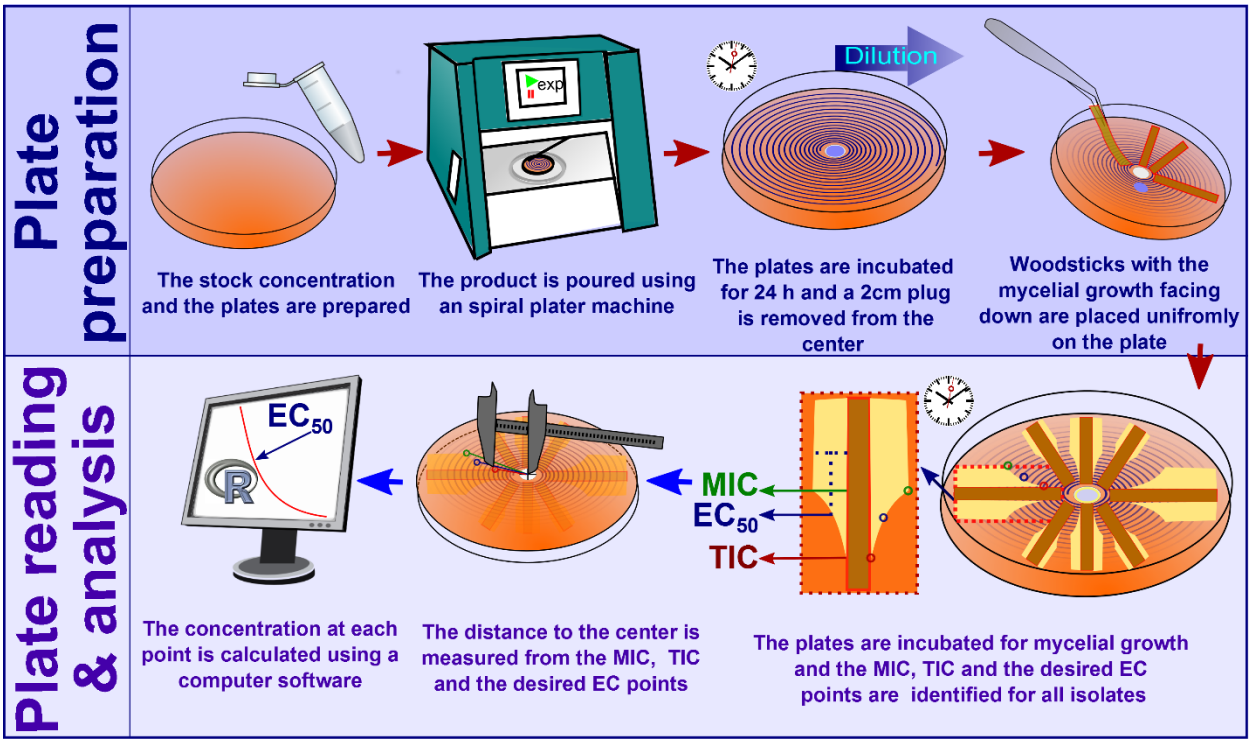


Figure 4. 1 Schematic procedure for calculating concentrations using spiral plate technique.

In addition to the distance to the MIC, the TIC or the EC, the calculation of the effective concentration by the commercial software includes the molecular weight (MW) of the chemical, the concentration (µg/ml) of the stock solution, incubation time (1 or 2 days), and agar medium height (AH). The concentrations of the compound corresponding to both the MIC and TIC are generated by the software SGE (Förster et al., 2004). Although the commercial software provides satisfactory calculations, its use is limited by the high cost of license and single computer installation policy. To provide an alternative platform for users, our objective was to establish mathematical models based on the mechanisms of SPT, using an open source R project package and Rstudio Shiny web applications that expanded the use of the spiral plate technique for effective concentration assessment.

MATERIALS AND METHODS

Mathematical models for calculating dispensing volume. The radial length of each section of the template provided by the Spiral Autoplate 4000® manufacturer for counting colonies was scanned and measured with ImageJ (National Institute of Health – NIH, Maryland), and compared with the volume dispensed for each individual section by the manufacturer. The data were plotted and different exponential models were tested for goodness of fit using JMP® 11.0 (The SAS Institute Inc. Cary, NC. 2013).

The length of each individual loop of the Archimedean spiral created by the spiral plate was calculated for all sections, and the volume dispensed at each loop was calculated. Dispensed volume of 10 different molecular weight compounds was calculated by simulate six different agar height (data not shown) using the SGE software. The volumes were compared with the values reported by a commercial software output using the statistical software R (R Foundation for Statistical Computing, Vienna, Austria) and a quantic polynomial model (Appendix G, equation 3) was developed to adjust the effect of molecular weight to diffusion. The effect of agar height was also extrapolated from the commercial software outputs and incorporated to the model by a linear regression model.

Package building. Once the general model for dispensing was elucidated, a series of R functions were developed in order to incorporate the diffusion affected by the variables of agar height, molecular weight, and concentration. Due to the open source of R (The R Core Team, 2015), all models were packed within an R package that could be freely distributed and adopted by users. The maximum and minimum inhibitory concentrations (MIC and TIC, respectively) in

the output are equivalent to tail ending radius (TER) and ending radius (ER) referred to by some software (5).

To fulfill the minimal structure for an R based package, the function received differential names; the help and example files were created and integrated within the package. Multiplatform integrity was tested on Windows, Ubuntu and Apple OS X.

Results of the developed package and SGE software were compared using the paired t-test, correlation, Chi-square analysis, with the R functions *t.test, cor* and *chisq.test* respectively. Statistically significant differences were determined at $\alpha = 0.05$. Equivalence between the software was evaluated using the two one-sided test (TOST) proposed by Robinson and Froese (2004).

Single product and stock concentration evaluation. To calculate the dilution observed on a single spiral plate with a specific product stock concentration, the function **"spcal ( )"** was developed. This function calculates the dilution observed every 2 mm from the TIC to the MIC points when these values (rad1 and rad2 respectively) are input, in addition to the molecular weight (mw), stock concentration (ppm), and agar height (ah). For example, to calculate the dilution obtained between the radius 20 and 64 mm, with a EC_{90} observed at 30 mm, and the mw, ppm, ah values of 385, 1000 and 3, respectively, the calculation is accomplished using the following command:

```
spcal(rad1 = 20, rad2 = 64, ec = 30, ECx = 90, mw = 385, ppm = 1000, AH = 3)
```

Following the recommendation for minimum optimal value, the function was set to accept values higher or equal to 20 mm. Similarly, the maximum value was set to 64 mm, which is the length of the maximum radius produced by the spiral plater. Any attempt to introduce a lower or higher value than the recommended range will result in an error message.

The output of the "**spcal"** function is presented as a data table in the R console, and it can be saved if the argument "**write = T**" is added to the command. In Windows environment, the table can be copied to the clipboard by introducing the **"copy = T"** argument; the copy argument does not work under Linux nor Apple OS-X operative systems environments. In addition to the table, the "**spcal"** function produce a graphical output, that includes 1) a scheme of the growth observed in the plate with a yellow circle representing the effective concentration; 2) a plot with the exponential dilution observed with the MIC, TIC and EC values; and 3) a linear regression plot with its formula (Fig. 4.2). This formula can be used to find values for the same product under the same conditions by replacing X for the desired radius, and powering ten to the obtained result from the formula (back transformation); this reduces the dependence on R or SGE for routine analysis under specific repeated conditions. The arguments "Product" and "ECx" are merely informative, and are used in the plating diagram.

Individual and multiple concentrations calculator. The function **"ECcal"** was used to calculate individual or multiple concentrations obtained with the spiral gradient technique. Calculations for a single isolate are done by inputting the distance to the center of the plate in millimeters of the TIC, EC and MIC points individually or in a group, in companion with the parameters included for **"spcal"**. Individual concentrations (TIC, EC and MIC) for multiple isolates plated under the same conditions (same mw, ppm and ah), or the concentration for multiple isolates plated with different specifications can be obtained. Since many effective concentration studies require the use of several samples and the comparison of two or more antimicrobial compounds, the **"ECcal"** includes the possibility of import (write = T) or paste (Read = T `# Not tested on Linux`) data tables, when the columns headers are described (Table

1). If the data points order was mistakenly input (e.g., TIC where placed as MIC and vice versa) the function was programed to correct them.

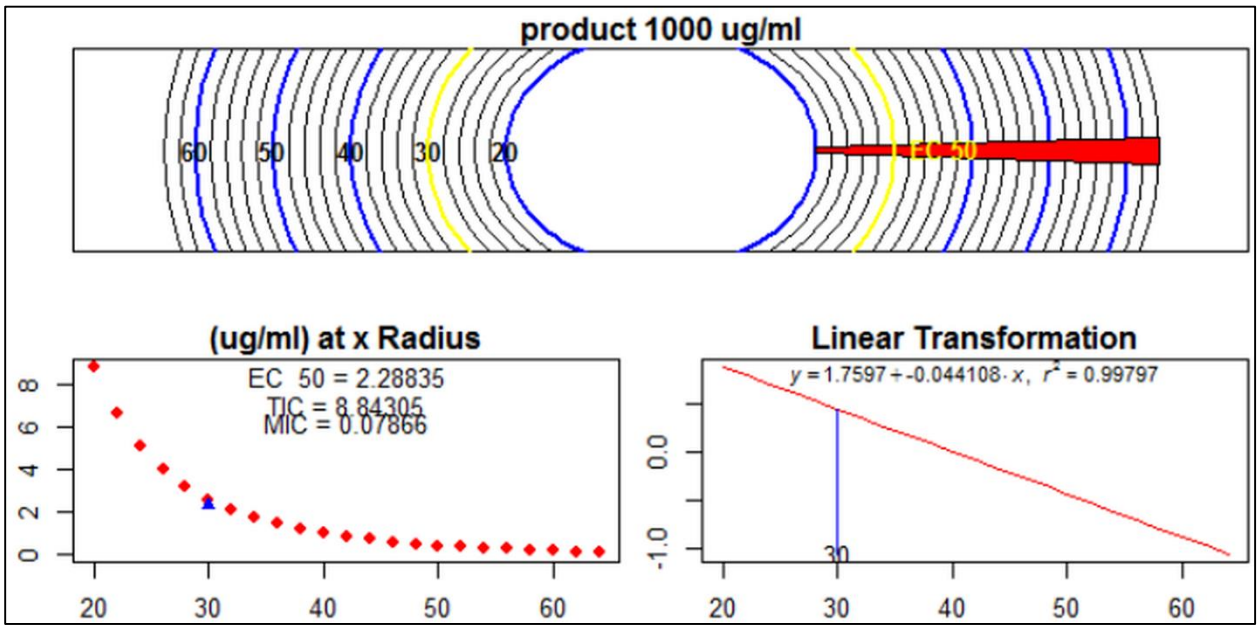


Figure 4. 2 Output generated from the "`spcal`" function, with the parameters of TIC = 20, TER = 64, EC = 30, ppm = 1000, mw = 385 and AH = 3.

Table 4. 1 Explanation of the parameters used within the function "ECcal" of the R ECX package.

Parameter[w]	Represents	Class	Description
Calculation			
rad1	TIC -Total inhibitory concentration	Numeric	Distance in mm from observed total inhibition point to the center of the plate.
rad2	MIC – Minimal inhibitory concentration	Numeric	Distance in mm from observed zero inhibition point to the center of the plate.
EC	EC - Effective Concentration	Numeric	Distance in mm from observed the desired inhibition point to the center of the plate.
Mw	Molecular Weight	Numeric	Molecular weight of the product.
Ppm	mg/liter	Numeric	Stock concentration of the product used to load the spiral plater.
AH	Agar height	Numeric	Agar Height. If the height is unknown, see the parameter **"vol".**
Vol	Volume	Numeric	If AH is unknown the function calculates the height if the volume used per plate is known.
Informative			
Product	Product	Text[2]	Name of the product evaluated.[x]
iso	Isolate	Text[2]	Name of the isolate evaluated. [x]
ECx	EC evaluated	Numeric	Value of the Effective concentration used in the study.
sens	Sensitivity	True/**False**[z]	Calculates based on the EC value if the isolate is sensitive, intermediate sensitive, or insensitive, the sensitive range are introduced in the parameters "**insens**" and "**intsens"**
insens	Insensible Concentration	Numeric	Stablish the lower limit to consider a specific concentration as insensible
intsens	Intermediate sensible concentration	Numeric	Stablish the lower limit to consider a specific concentration as intermediate insensible
Data management			
info	Information	True/**False**[y]	Add the informative cells to the output
Read	Read file	**True**/False[z]	Open a Window for browsing a file to be imported
sep	Separator	Comma "," semicolon ";" or tab **"\t"**	Sep is referred to the parameter used to separate the imported or pasted table. The options must be entered within quotation marks.
Paste	Paste table	True/**False**[z]	Paste a copied table to the function for being analyzed
write	Write table	True/**False**[z]	Crates a *csv file with the output in the working directory
copy	Copy Table	True/**False**[z]	Copy a table in the clipboard

w. The parameters are case sensitive and must be entered as presented on the table.
x. Text must be entered within quotation marks " ", for manual input.
y. Informative value only. It is used exclusively if the **"info"** parameter is set as True.
z. Bold font indicates default value of the parameter.

The "**ECcal**" function enables the importation and analysis of one of the tables provided from the package by inputting the information included below. The output parameters such as saving, coping or having additional information can be used as another option.

```
# Lines with # symbol will not run
# Only importing
ECcal( )

#Importing and saving
ECcal (write=T)

#Importing and pasting into the clipboard
ECcal(Paste=T)

#Importing and setting the intermediate sensitivity and sensitivity

# values to 1 and 10 mg/liter respectively

ECcal(sens = T,intsens = 1,insens = 10)
```

A single calculation can be done by manually inputting the terms. For instance, if the TIC, EC_{90} and MIC values of an isolate where observed at 25, 35 and 43 mm from the center of the plate respectively, on a spiral plate prepared with an antimicrobial compound of MW = 350 g/mol, a stock concentration of 1000 mg/liter and agar height of 3 mm, the code is:

```
ECcal(rad1 = 25, rad2 = 43, EC = 25, mw = 350, ppm =1 000, AH = 3)
```

Similar to a single calculation, the values of multiple concentration with the same parameters can be analyzed manually. Suppose that the observed radius of the effective concentration for 5 isolates with the same plate description of the past example were: 25,32,31,45, and 36. The EC for all of them can be obtained by:

```
ECcal(rad1 = c(25,32,31,45,36),mw = 350,ppm = 1000,AH = 3)
```

Concentration by radius. The "**Radconc"** function calculates the radius at which a specific concentration is observed. For instance, to identify at which radius 3.5mg/liter is observed using the same parameters than the previous examples, the code is:

```
Radconc(Ec = 3.5, ppm = 1000, mw = 350, AH = 3)
```

Stock concentration calculator for specific concentration range. To observe a specific concentration at a determined point on the plate (e.g. 5 mg/liter at radius 35), the **"ConcCAL"** function can be used. The default value for the concentration distance is 42 mm, since it is the midpoint of 150 mm Ø the plate workable area. However, it can be changed with the parameter "rad1 ="; this parameter is only required in the function, if a value different from 42mm is desired. The other information required by the function is the concentration desired (E50 =), molecular weight (mw =), and the agar height (AH). The code can be executed as:

```
# For having 5 mg/liter at radius 42
ConcCAL(E50 = 5, mw= 350, AH = 3)

# For having 5 mg/liter at radius 38
ConcCAL(E50 = 5,rad1 = 38, mw = 350,AH = 3)
```

Web-based applications. In order to reduce the gap between R users and non-R users, a series of local graphical web-based applications were developed using the R package **Shiny** (install it by typing the following command in R: install.packages("shiny")) from Rstudio Inc. (RStudio, 2015).

The "ECX" function produces the same output files than the "spcal" function. However, the function is dynamic and changes the output as the parameters are changed. In addition, the output plots and the dilution table is presented in independent tabs of the results area (Fig 4.3).

The function is called without introducing any parameter. The functions will call the package and then a webpage will open. The code to launch this function is:

```
# Not introduce anything within the parenthesis
ECX()
```

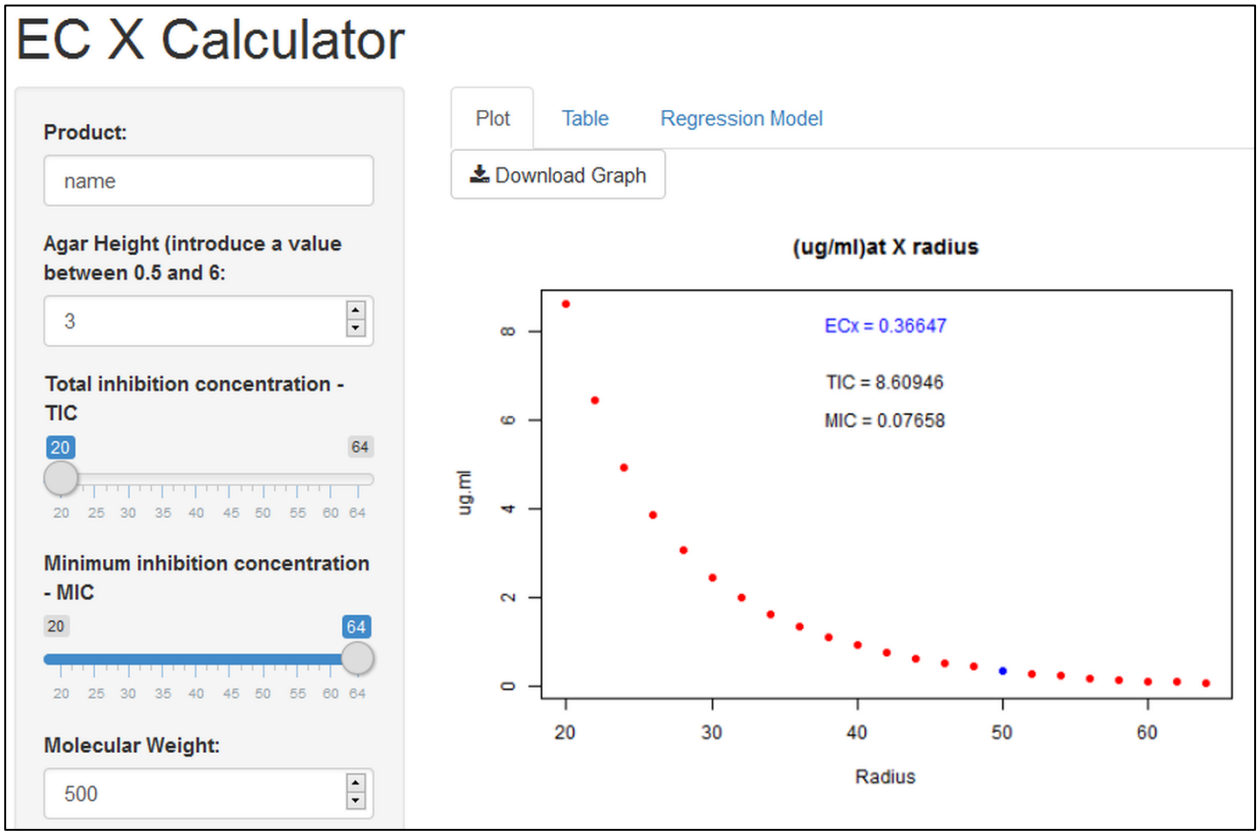
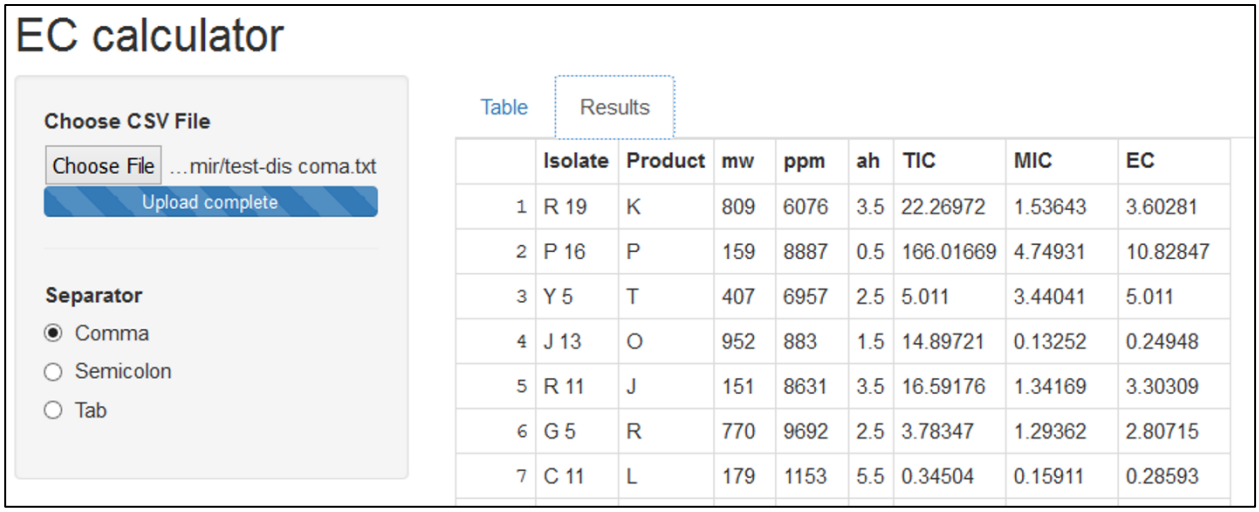


Figure 4. 3 Screenshot of interface of function "ECX".

A graphical version of the "ECcal" importing function is used for previewing the table with the values to be analyzed, and to obtain the concentrations at the same time. The "multi" function runs similar to the "ECX" function but the parameters input is simpler for function "multi". By browsing a file table, the type of separator used can be specified. In the table tab, the original table is loaded; while in the results tab, the TIC, EC, and MIC values are incorporated to the table (Fig 4.4). The table can be selected and copied.

The function runs as follows:

multi()



	Isolate	Product	mw	ppm	ah	TIC	MIC	EC
1	R 19	K	809	6076	3.5	22.26972	1.53643	3.60281
2	P 16	P	159	8887	0.5	166.01669	4.74931	10.82847
3	Y 5	T	407	6957	2.5	5.011	3.44041	5.011
4	J 13	O	952	883	1.5	14.89721	0.13252	0.24948
5	R 11	J	151	8631	3.5	16.59176	1.34169	3.30309
6	G 5	R	770	9692	2.5	3.78347	1.29362	2.80715
7	C 11	L	179	1153	5.5	0.34504	0.15911	0.28593

Figure 4. 4 Screenshot of the output of function "`multi`".

As an additional tool to prepare the stock concentration, the function "`ppm`" can be used to determinate the exact product amount required to obtain a specific volume with a specific concentration of the product (mg/liter or µl/liter). The function requires the name of the product, percentage of active ingredient, product presentation (liquid or solid), desired final concentration (mg/liter) and desired final volume in ml. The output is a text indicating the required amount of product necessary to obtain the final volume at the desired concentration (Fig 4.5). The calculator is launched by the code:

ppm( )

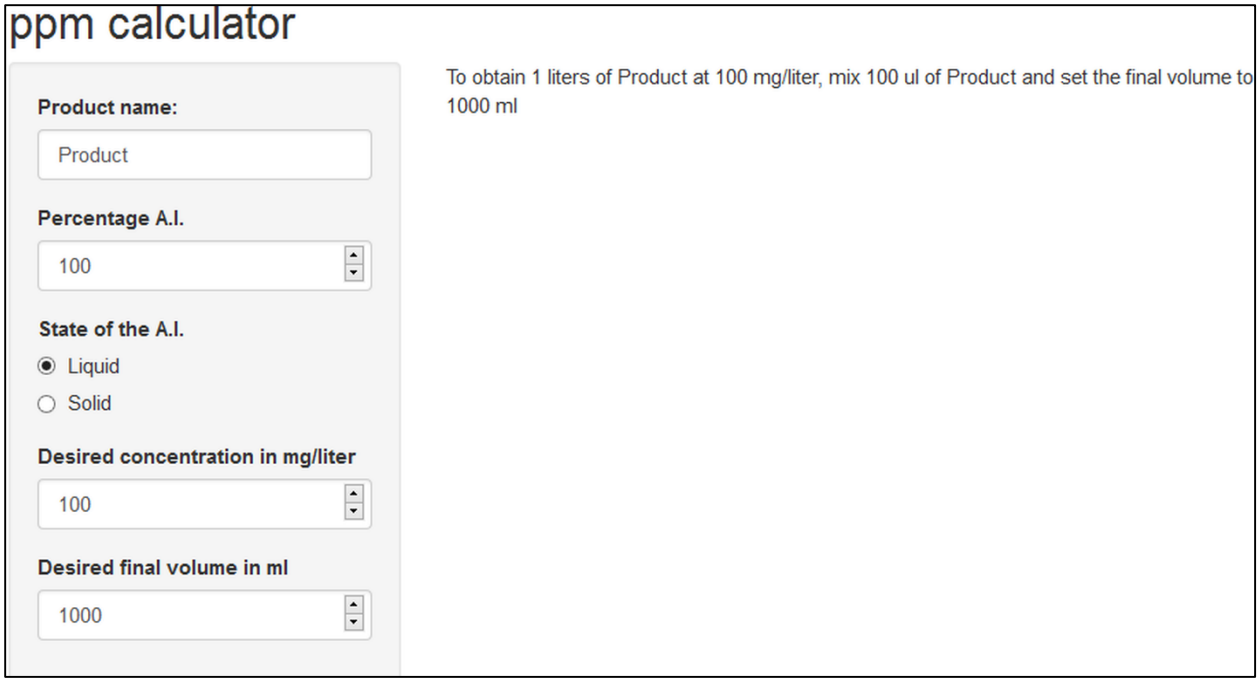


Figure 4. 5 Screenshot of the output of function "`ppm`".

In some R environments when the webpages are closed, R console appears like "`Browse[1]>`", users are required to stop its running by pressing the escape key.

Supporting files. A series of tables with 10.000 randomly created data points were prepared as an example for running the **"ECcal"** function (https://github.com/GabrielTorres/ECX). The columns of all of them, except "test-dis coma.txt", were separated by a tab space (sep = "\t"). The files include a complete table with TIC, MIC and EC values. Tables with only TIC and MIC values, and with only EC Values were also included. The tables with TIC, EC and MIC ordered, (`RC_ord`), TIC, EC and MIC disordered (`RC_disord`), with TIC and MIC (`RC_2v`), and EC only (RC_sv), were incorporated within the package and can be called and tested as follow.

```
Data(EC)
#The ECcal function ca be run as:
ECcal(RC_2v)
```

RESULTS

A group of functions for calculating the concentration deposited by spiral plater machines were developed. All individual functions presented similar results to SGE (Fig 4.6 and Table 4.2). The Shiny ECX application had the same performance as the `spcal` function package. The Shiny ppm extension was also tested and proven to be accurate when the concentrations of commercial diluted products were calculated. The result displays the number of grams, milligrams, milliliters or microliters that the user must add to the final volume.

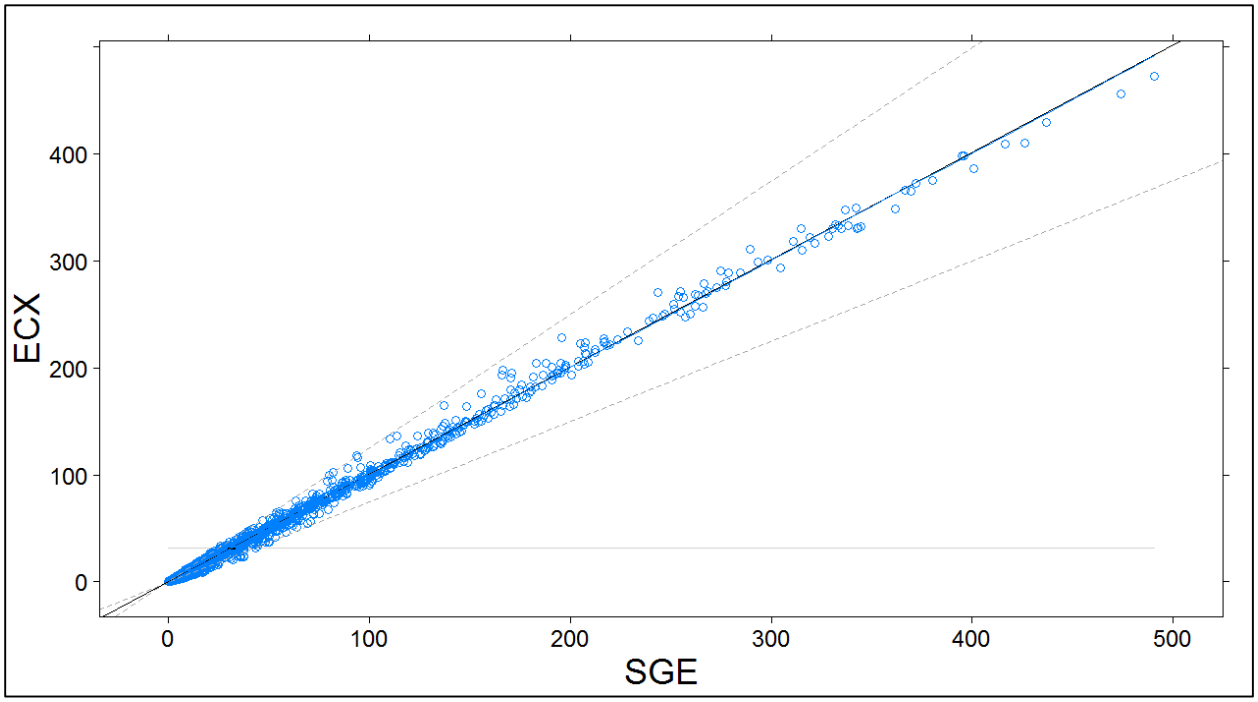


Figure 4. 6 Equivalence test (TOST) between the values obtained with SGE and the values estimated with ECX. A high similarity is observed between the software.

Table 4. 2 Concentration calculated at radius 30 mm for different antimicrobial compounds (ppm= 650 mg/L, AH=3 mm)

Product	Molecular weight	SGE	ECX
Amikacin	586	1.77	1.83
Control	1000	1.74	1.82
Erythromycin	734	1.74	1.81
Fluopicolide	385	1.91	1.91
Mefenoxam	279	2.04	1.94
Metronidazole	171	2.19	1.89
Nalidixic acid	232	2.11	1.93
Prothioconazole	480	1.83	1.86
Propamocarb Hydrochloride	188	2.17	1.91
Rifampicin	823	1.73	1.82
Thiabendazole	201	2.15	1.92
Topsin	342	1.96	1.92
Tobramycin	468	1.84	1.87
Zoxamide	337	1.96	1.93

No significant differences were observed between the SGE software and the r-package when the output of a mock dataset of 3435 data points was evaluated at P= 0.95. The Chi square and the t-test produced *P-values* of 1 and 0.88 respectively at α=0.05. A correlation higher than 99.8% was observed.

DISCUSSION

We have developed an R-based package and web-based extension freeware to calculate effective concentrations of test compounds. Results obtained with this new package were similar to those obtained with the commercial SGE software. The cost-free packages developed during this research were reliable and reproducible. The user-friendly interface, the ability to import datasets from other software and the multi-platform nature of those packages provide extra tools for broad and powerful analyses.

Spiral gradient endpoint techniques are precise and cost effective compared to traditional dilution methods (Förster et al., 2004; Paton et al., 1990; Pong et al., 2010). As Hill and Schalkowsky (1990) indicated, we found MW is the main component to explain diffusion. The effectiveness of the diffusion adjustment done by Hill and Schalkowsky to the SGE formula, was validated by Wexler et al. (1996). For plant pathogens, Föster et al. (2004) found that the 1-day and 2-day parameters designed for aerobic and anaerobic bacteria, respectively, were adequate to calculate the EC_{50} value for conidial germination and mycelial growth, specifically. The present study focused on mycelial growth; results were similar to those obtained in the commercial software when the "day" option is set to 2.

In the R package ECX, concentration was expressed as "ppm" and "ug/ml" for variables and outputs, respectively. Users can define any other units, as long as they are kept constant. SGE exclusively calculates the concentration at the specified TER and ER points. In contrast, the Conc function and the Shiny web application in ECX calculate the concentrations for the range between TER and ER in a two-millimeter increment. The output of those functions also provides the linear regression formula of the linear transformation. This formula can be used to find

values for the same product under the same conditions by replacing X for the desired radius, and powering ten to the obtained result from the formula (back transformation); this reduces the dependence on R or SGE for routine analysis under specific repeated conditions.

The ECX package will be available at: **https://github.com/GabrielTorres/ECX/releases/tag/3.01**. While the objective of these new packages is not to be a substitute of the commercial software, its open source characteristics, wide range of data that can be manipulated under a single analysis process and its relative user friendly interface, can enhance the use of spiral autoplate for antimicrobial compound analysis.

ACKNOWLEDGMENT

The authors express their gratitude to Dr. William Kirk, Qianwei Jiang, Paula Somohan for the package testing and feedback. Also to Dr. Andrew Robinson for his comments on the equivalence test analysis.

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FUTURE WORK

Despite the present study represents a valuable advances in understand *Phytophthora palmivora* and its management, still there are scientific questions that remain unsolved. There is some of the question that I want to answer:

- How fungicides are moving within the palm?

  This is a very interesting question, since there is not clarity how chemicals move within an oil palm tree. This is especially critical for bud rot disease, because it is necessary to find a fungicide that can move into the zone where *P. palmivora* is able to infect (above the meristematic tissues).

- Are the fungicides tested also optimal choices for bud rot management?

  This question is not only linked to the previous question, but also requires different tests to understand if palm physiology interferes with the fungicide and reduces its efficacy.

- Is the *P. palmivora* mating type A1 present in Colombia?

  My research in Colombia showed the presence of oospores within affected tissue; however, isolation of the A1 mating type has not been done yet. This will help to identify possible sources of variation among *P. palmivora* populations.

APPENDICES

APPENDIX A

Phytophthora palmivora isolates used for morphological, virulence and fungicide studies.

Isolate[1]	Continent	Country	State	Host	Family
13716	N. America	USA	Florida	*Citrus* spp.	Rutaceae
13717	N. America	USA	Florida	*Citrus* spp.	Rutaceae
13718	S. America	Ecuador	NA	*Theobroma cacao*	Malvaceae
13719	N. America	USA	California	*Ceanothus* sp.	Rhamnaceae
13720	N. America	USA	California	*Magnolia grandiflora*	Magnoliaceae
13721	N. America	USA	California	*Pittosporium undulatum*	Pittosporaceae
13722	N. America	USA	California	*Coleonema* sp.	Rutaceae
13723	N. America	USA	Ohio	*Verbena* sp.	Verbenaceae
13724	N. America	USA	Florida	*Citrus* spp.	Rutaceae
13725	N. America	USA	Florida	*Citrus* spp.	Rutaceae
13726	N. America	USA	Florida	*Citrus* spp.	Rutaceae
13727	N. America	USA	Florida	NA	NA
13729	N. America	USA	Florida	NA	NA
13730	N. America	USA	Virginia	NA	NA
13731	S. America	Colombia	NA	*Theobroma cacao*	Malvaceae
13732	S. America	Colombia	NA	*Theobroma cacao*	Malvaceae
13733	S. America	Trinidad	NA	*Theobroma cacao*	Malvaceae
13734	S. America	Brazil	NA	*Theobroma cacao*	Malvaceae
13735	N. America	USA	Hawaii	*Chamaedorea elegans*	Palmaceae
13736	Caribbean	Jamaica	NA	*Theobroma cacao*	Malvaceae
13737	S. America	Uruguay	NA	*Citrus* spp.	Rutaceae
13738	S. America	Colombia	Chigorodo	*Theobroma cacao*	Malvaceae
13739	S. America	Colombia	Palmira	*Theobroma cacao*	Malvaceae
13740	N. America	USA	Hawaii	*Carica papaya*	Caricaceae
13741	N. America	USA	Hawaii	*Theobroma cacao*	Malvaceae
13742	S. America	Venezuela	NA	*Theobroma cacao*	Malvaceae
13743	N. America	USA	Hawaii	Orchid	Orchideaceae
13744	N. America	USA	Florida	*Chamaedorea seifrizii*	Palmaceae
13745	S. America	Peru	Tingo Maria	*Theobroma cacao*	Malvaceae
13746	S. America	Colombia	NA	*Theobroma cacao*	Malvaceae

Appendix A (*Cont´d)*

Isolate[1]	Continent	Country	State	Host	Family
13747	Asia	America Samoa	NA	*Theobroma cacao*	Malvaceae
13748	S. America	Argentina	NA	Soil	NA
13749	Asia	Guam	NA	*Areca catechu*	Palmaceae
13750	N. America	USA	California	*Howea forestiana*	Palmaceae
13751	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae
13752	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae
13753	S. America	Colombia	Nariño	*Elaeis guineensis*	Palmaceae
13754	S. America	Colombia	Nariño	*Elaeis guineensis*	Palmaceae
13755	S. America	Colombia	Nariño	*Elaeis guineensis*	Palmaceae
13756	S. America	Colombia	Meta	*Elaeis guineensis*	Palmaceae
13757	N. America	USA	Florida	*Liriope* sp.	Asparagaceae
13940	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13941	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13943	S. America	Colombia	Santander	*Theobroma cacao*	Malvaceae
13945	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae
13946	S. America	Colombia	Caldas	*Persea americana*	Lauraceae
13949	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13950	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13952	S. America	Colombia	Caldas	*Persea americana*	Lauraceae
13954	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13957	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13958	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13960	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13961	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13962	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13963	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13964	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13965	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13966	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13967	S. America	Colombia	Caldas	*Persea americana*	Lauraceae
13968	S. America	Colombia	Magdalena	*Elaeis guineensis*	Palmaceae
13972	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13973	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13974	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13975	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13976	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13978	S. America	Colombia	Nariño	*Elaeis guineensis*	Palmaceae
13979	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae
13980	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae

Appendix A (*Cont´d)*

Isolate[1]	Continent	Country	State	Host	Family
13982	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13983	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13984	S. America	Colombia	Valle del Cauca	*Persea americana*	Lauraceae
13985	S. America	Colombia	Caldas	*Persea americana*	Lauraceae
13987	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13988	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13989	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13990	S. America	Colombia	Caldas	*Theobroma cacao*	Malvaceae
13991	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae
13993	C. America	Costa Rica	NA	*Theobroma cacao*	Malvaceae
13995	N. America	USA	Florida	*Citrus* spp.	Rutaceae
13996	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
13997	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
13998	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
13999	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14000	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14001	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14002	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14003	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14004	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14005	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14006	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14007	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14008	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14009	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14010	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14011	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14012	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14013	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14014	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14015	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14016	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14017	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14018	Caribbean	Trinidad	NA	*Theobroma cacao*	Malvaceae
14019	C. America	Costa Rica	NA	*Theobroma cacao*	Malvaceae
14021	N. America	USA	Florida	*Citrus* spp.	Rutaceae
14022	Oceania	P.N.G. [2]	NA	*Theobroma cacao*	Malvaceae
14024	Oceania	P.N.G. [2]	NA	*Theobroma cacao*	Malvaceae
14025	Africa	Ivory Coast	NA	*Theobroma cacao*	Malvaceae
14026	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae

Appendix A (*Cont´d)*

Isolate[1]	Continent	Country	State	Host	Family
14027	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae
14028	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14029	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14031	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14032	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14033	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14034	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14036	Asia	Indonesia	South East Sulawesi	*Theobroma cacao*	Malvaceae
14037	Asia	Indonesia	South East Sulawesi	*Theobroma cacao*	Malvaceae
14038	Asia	Indonesia	South East Sulawesi	*Theobroma cacao*	Malvaceae
14039	Asia	Indonesia	South East Sulawesi	*Theobroma cacao*	Malvaceae
14040	Asia	Indonesia	South East Sulawesi	*Theobroma cacao*	Malvaceae
14041	Asia	Indonesia	South East Sulawesi	*Theobroma cacao*	Malvaceae
14042	Asia	Indonesia	South West Sulawesi	*Theobroma cacao*	Malvaceae
14043	Asia	Indonesia	South West Sulawesi	*Theobroma cacao*	Malvaceae
14046	Asia	Indonesia	South West Sulawesi	*Theobroma cacao*	Malvaceae
14047	Asia	Indonesia	Java	*Theobroma cacao*	Malvaceae
14048	Asia	Indonesia	Java	*Theobroma cacao*	Malvaceae
14049	Asia	Indonesia	Java	*Theobroma cacao*	Malvaceae
14050	Asia	Indonesia	South West Sulawesi	*Theobroma cacao*	Malvaceae
14051	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae
14053	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae
14054	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae
14055	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae
14056	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae
14057	Asia	Malaysia	Sabah	*Theobroma cacao*	Malvaceae
14059	Asia	Malaysia	Sarawak	*Theobroma cacao*	Malvaceae
14060	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14061	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14062	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14063	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14064	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae
14065	Oceania	P.N.G.[2]	NA	*Theobroma cacao*	Malvaceae

Appendix A (*Cont´d)*

Isolate[1]	**Continent**	**Country**	**State**	**Host**	**Family**
14066	Oceania	P.N.G. [2]	NA	*Theobroma cacao*	Malvaceae
14078	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae
14081	S. America	Colombia	Tumaco	*Elaeis guineensis*	Palmaceae
14083	S. America	Colombia	Tumaco	*Elaeis guineensis*	Palmaceae
14086	S. America	Colombia	Tumaco	*Elaeis guineensis*	Palmaceae
14092	S. America	Colombia	Magdalena	*Elaeis guineensis*	Palmaceae
14095	S. America	Colombia	Tumaco	*Elaeis guineensis*	Palmaceae
14101	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae
14102	S. America	Colombia	Santander	*Elaeis guineensis*	Palmaceae

1. Internal identification code used in the laboratories of M. K. Hausbeck at Michigan State University
2. P.N.G. Papua New Guinea

APPENDIX B

Morphological characterization of sporangia of the *Phytophthora palmivora* collection by isolate

Isolate	Length (µm)	Breadth (µm)	LB ratio[z]	Pedicel length (µm)	Spores/cm^2
13716	49.6	24.3	2.0	5.2	11786
13717	48.8	23.4	2.1	5.0	15143
13718	NA	NA	NA	NA	NA
13719	NA	NA	NA	NA	NA
13720	NA	NA	NA	NA	NA
13721	55.3	28.0	2.0	1.8	7214
13722	NA	NA	NA	NA	NA
13723	48.7	31.4	1.6	4.2	5420
13724	40.1	23.1	1.7	5.0	4571
13725	49.5	26.9	1.8	4.2	2071
13726	44.4	21.0	2.1	4.4	3786
13727	45.5	24.3	1.9	4.5	12643
13729	43.1	22.1	1.9	4.2	2964
13730	46.3	30.1	1.5	4.7	3321
13731	34.9	22.0	1.6	4.1	2536
13732	34.5	24.3	1.4	3.4	3571
13733	42.2	22.3	1.9	4.2	7679
13734	41.5	24.6	1.7	4.2	2714
13735	51.9	27.7	1.9	4.7	643
13736	42.5	24.1	1.8	2.5	NA
13737	41.9	25.1	1.7	4.6	3571
13738	47.6	24.2	2.0	1.3	1883
13739	45.6	24.2	1.9	3.7	5357
13740	46.8	23.5	2.0	3.9	6214
13741	43.6	26.1	1.7	4.1	7929
13742	NA	NA	NA	NA	NA
13743	39.0	28.4	1.4	NA	786
13744	43.0	26.2	1.6	4.4	5429
13745	41.5	24.2	1.7	3.9	6571
13746	46.2	26.2	1.8	3.9	4714
13747	44.6	25.4	1.8	4.0	3714
13748	43.2	25.2	1.7	4.0	612
13749	35.1	23.2	1.5	3.0	2964
13750	42.8	24.2	1.8	4.2	2393

Appendix B (*cont´d)*

Isolate	Length (µm)	Breadth (µm)	LB ratio[z]	Pedicel length (µm)	Spores/cm^2
13751	43.8	23.5	1.9	3.8	2964
13752	44.2	24.0	1.8	4.3	11893
13753	43.8	24.7	1.8	4.0	7286
13754	42.6	23.4	1.8	4.2	6500
13755	47.8	25.1	1.9	4.0	6786
13756	43.6	35.1	1.2	4.4	NA
13757	46.1	29.4	1.6	3.9	5536
13940	46.8	25.4	1.8	3.9	4393
13941	45.8	25.4	1.8	3.7	1429
13943	48.4	26.1	1.9	3.6	4321
13945	42.9	23.6	1.8	3.9	5321
13946	41.3	31.0	1.3	3.0	5223
13949	48.5	25.5	1.9	3.7	3107
13950	47.2	24.8	1.9	4.2	3929
13952	45.3	26.9	1.7	3.9	3036
13954	47.4	25.4	1.9	4.2	7107
13957	40.5	21.1	1.9	3.7	12286
13958	44.0	24.0	1.8	3.4	5642
13959	46.0	24.0	1.9	1.5	NA
13960	42.4	24.0	1.8	3.8	3714
13961	42.3	22.4	1.9	3.4	11179
13962	43.4	25.3	1.7	3.0	11929
13963	44.2	24.8	1.8	3.3	10214
13964	42.9	24.6	1.7	3.2	15179
13965	44.5	23.7	1.9	3.3	5786
13966	47.7	26.6	1.8	3.9	3857
13967	44.2	25.6	1.7	3.4	4357
13968	45.0	25.4	1.8	3.3	5786
13972	44.6	25.8	1.7	3.2	9036
13973	44.7	26.1	1.7	3.3	9107
13974	42.3	24.3	1.7	3.8	4393
13975	42.9	24.5	1.8	3.7	7643
13976	42.1	24.3	1.7	3.5	6500
13978	44.1	24.8	1.8	3.7	3857
13979	42.7	20.7	2.1	3.3	NA
13980	43.3	24.6	1.8	3.1	9821
13982	39.4	28.0	1.4	3.0	5500
13983	46.6	24.7	1.9	3.0	12071
13984	44.2	25.9	1.7	3.2	10179
13985	42.2	24.0	1.8	2.9	12857
13987	43.5	25.3	1.7	3.3	4643
13988	39.2	28.4	1.4	3.0	10679

Appendix B (*cont´d)*

Isolate	Length (µm)	Breadth (µm)	LB ratio[z]	Pedicel length (µm)	Spores/cm^2
13989	43.6	26.4	1.6	3.5	8321
13990	45.1	24.4	1.8	2.8	6821
13991	46.3	23.8	1.9	3.1	9393
13993	41.1	31.0	1.3	4.1	3429
13995	36.1	25.8	1.4	4.6	1571
13996	40.4	23.9	1.7	2.8	15179
13997	42.7	23.9	1.8	2.9	8893
13998	48.8	25.1	1.9	3.8	12357
13999	43.7	24.1	1.8	3.0	14464
14000	40.5	26.6	1.5	3.1	9714
14001	46.7	24.3	1.9	3.4	10649
14002	37.8	27.5	1.4	2.8	5571
14003	46.0	23.7	1.9	3.1	8286
14004	41.8	22.9	1.8	3.0	22571
14005	44.7	23.3	1.9	3.1	19107
14006	44.1	31.1	1.4	3.4	9857
14007	43.5	24.8	1.8	3.3	10036
14008	39.7	27.3	1.5	3.0	14643
14009	43.9	24.2	1.8	3.4	6071
14010	44.3	24.4	1.8	3.2	21071
14011	44.4	24.5	1.8	3.1	15893
14012	49.1	29.6	1.7	2.7	1929
14013	47.3	26.9	1.8	3.6	18321
14014	38.7	26.8	1.4	3.3	4857
14015	44.8	23.9	1.9	3.1	8607
14016	44.8	24.3	1.8	3.1	13750
14017	45.4	25.8	1.8	3.4	14429
14018	44.3	27.4	1.6	3.4	5214
14019	37.1	27.9	1.3	3.2	3929
14021	37.3	19.1	2.0	3.8	15643
14022	37.8	28.0	1.4	3.6	7071
14024	43.6	28.6	1.5	3.7	1143
14025	38.8	23.9	1.6	3.8	10750
14026	40.6	19.6	2.1	4.6	10500
14027	41.7	23.8	1.8	2.9	19536
14028	33.4	20.9	1.6	3.2	14357
14029	43.4	25.2	1.7	3.5	71
14031	38.3	24.2	1.6	3.4	6321
14032	37.8	25.5	1.5	2.6	16357
14033	30.8	21.3	1.4	3.1	4071
14034	34.9	22.8	1.5	3.8	8357
14036	49.4	28.8	1.7	3.9	8571

Appendix B (*cont´d)*

Isolate	Length (µm)	Breadth (µm)	LB ratio[z]	Pedicel length (µm)	Spores/cm^2
14037	45.8	29.0	1.6	4.0	4429
14038	51.8	32.2	1.6	5.1	286
14039	49.2	28.8	1.7	4.1	35
14040	48.9	24.2	2.0	3.8	9179
14041	49.0	32.4	1.5	5.1	1357
14042	41.2	27.3	1.5	4.7	9250
14043	45.9	30.9	1.5	3.6	35
14046	40.0	29.6	1.4	3.5	5286
14047	38.3	27.1	1.4	3.2	13821
14048	40.5	24.7	1.6	3.2	18357
14049	39.6	28.5	1.4	3.3	7357
14050	40.3	23.5	1.7	3.4	15964
14051	37.7	29.6	1.3	NA	286
14053	NA	NA	NA	NA	NA
14054	30.7	24.8	1.2	3.4	4607
14056	31.5	25.0	1.3	3.8	4571
14057	30.6	23.6	1.3	3.1	7893
14059	38.9	23.0	1.7	3.2	11071
14060	48.7	31.1	1.6	4.8	6214
14061	47.1	24.4	1.9	4.3	12857
14062	47.9	27.8	1.8	4.6	NA
14063	42.7	26.5	1.6	3.6	NA
14064	44.0	25.9	1.7	3.7	19286
14065	39.4	26.2	1.5	4.1	11893
14066	44.7	27.2	1.6	3.0	9500
14078	45.1	25.2	1.8	4.1	18000
14081	44.3	23.0	1.9	3.6	23250
14083	43.9	23.9	1.8	3.7	16071
14086	44.2	24.8	1.8	3.1	19464
14092	44.1	23.9	1.8	3.5	75
14095	43.5	25.0	1.7	3.8	16964
14101	44.9	24.7	1.8	3.9	8758
14102	47.3	25.1	1.9	3.7	24786

z. LB ratio= Length breadth ratio.
NA= Information no available.

APPENDIX C

Morphological characteristics of chlamydospores of the *Phytophthora palmivora* collection by isolate.

Isolate	Chlamydospore diameter (µm)	Chlamydospores /cm^2
13716	34.4	1725
13717	27.0	600
13718	NA	NA
13719	33.4	450
13720	NA	NA
13721	39.1	713
13722	50.1	1550
13723	39.8	1575
13724	30.1	450
13725	30.5	1238
13726	20.6	450
13727	28.4	1179
13729	NA	NA
13730	28.3	1550
13731	33.5	1613
13732	35.9	638
13733	37.9	1400
13734	32.4	2850
13735	31.8	638
13736	NA	NA
13737	36.4	2450
13738	31.5	975
13739	36.6	1725
13740	24.6	550
13741	30.6	1150
13742	29.6	2450
13743	33.2	1013
13744	31.1	525
13745	33.4	2300
13746	35.9	4725
13747	33.4	450
13748	NA	NA
13749	34.7	510

Appendix C (*Cont'd*)

Isolate	Chlamydospore diameter (µm)	Chlamydospores /cm^2
13750	26.8	1050
13751	33.8	2700
13752	33.2	6750
13753	33.4	2550
13754	32.3	3450
13755	36.1	1530
13756	36.1	788
13757	31.6	450
13940	31.4	1875
13941	NA	NA
13943	32.0	1500
13945	32.7	4100
13946	NA	NA
13949	35.9	930
13950	37.9	990
13952	37.9	1125
13954	32.8	1088
13957	32.4	1950
13958	33.2	2950
13959	NA	NA
13960	32.3	2475
13961	39.5	1125
13962	36.1	1725
13963	31.6	800
13964	34.1	2850
13965	35.5	720
13966	31.3	1163
13967	34.7	2175
13968	35.3	2550
13972	37.2	1388
13973	35.1	2550
13974	36.1	1150
13975	33.6	1550
13976	35.2	1500
13978	35.8	1950
13979	NA	NA
13980	33.6	1425
13982	43.0	525
13983	36.0	900
13984	33.6	2700
13985	33.8	570
13987	30.3	1725

Appendix C (*Cont'd*)

Isolate	Chlamydospore diameter (µm)	Chlamydospores /cm^2
13988	37.6	1350
13989	29.2	3075
13990	28.7	2550
13991	32.1	3075
13993	39.7	3257
13995	37.3	900
13996	32.4	863
13997	30.1	975
13998	33.9	750
13999	31.8	1200
14000	29.6	1500
14001	34.5	1725
14002	29.9	1250
14003	31.9	1350
14004	33.1	4950
14005	35.5	450
14006	34.9	713
14007	28.3	1650
14008	39.0	2640
14009	34.8	1400
14010	31.6	1710
14011	32.7	2190
14012	33.0	2350
14013	31.1	2600
14014	34.7	1838
14015	28.6	2213
14016	31.4	1050
14017	29.0	800
14018	31.7	1230
14019	31.5	1110
14021	30.9	870
14022	31.2	1575
14024	40.6	1275
14025	26.6	1440
14026	30.7	493
14027	28.5	1440
14028	NA	NA
14029	33.7	510
14031	29.8	570
14032	32.7	450
14033	NA	NA
14034	35.4	600

Appendix C (*Cont'd*)

Isolate	Chlamydospore diameter (µm)	Chlamydospores /cm^2
14036	23.6	550
14037	31.7	600
14038	31.5	1260
14039	33.7	863
14040	33.5	875
14041	NA	NA
14042	25.4	1163
14043	30.3	2050
14046	29.8	825
14047	28.9	975
14048	29.0	720
14049	29.3	725
14050	27.9	1175
14051	25.7	700
14053	33.9	1000
14054	30.2	2213
14055	NA	NA
14056	26.5	2100
14057	26.9	1080
14059	32.3	870
14060	29.4	713
14061	32.1	1425
14062	NA	NA
14063	NA	NA
14064	35.8	810
14065	32.2	1050
14066	31.1	800
14078	29.4	960
14081	31.7	870
14083	30.3	1950
14086	36.2	1170
14092	24.1	1450
14095	28.2	1388
14101	30.4	788
14102	32.9	1200

NA= Information not available

APPENDIX D

Morphological and mating type characterization of the *Phytophthora palmivora* collection by isolate.

Isolate	Antheridium width (µm)	Oospore diameter (um)	Oogonia Diameter (µm)	Mating type
13716	11.5	21.3	26.1	A1
13717	13.9	21.6	28.5	A1
13718	NA	NA	NA	NA
13719	10.4	20.7	26.1	A2
13720	12.6	26.3	30.7	A2
13721	13.1	19.7	26.0	A1
13722	14.9	22.8	29.6	A2
13723	12.7	20.3	26.0	A1
13724	14.0	22.9	27.7	A1
13725	11.0	18.4	23.4	A1
13726	13.3	21.4	28.4	A1
13727	12.8	17.5	22.1	A1
13729	12.4	22.2	27.5	A1
13730	13.5	23.3	29.3	A2
13731	13.9	23.8	30.0	A2
13732	14.4	21.2	27.5	A2
13733	14.0	23.9	26.9	A2
13734	12.7	18.8	25.0	A2
13735	12.8	21.5	27.5	A1
13736	cont	cont	cont	cont
13737	13.2	24.5	30.0	A2
13738	12.6	23.7	30.6	A2
13739	10.8	19.5	24.8	A2
13740	12.1	25.2	30.7	A1
13741	11.7	23.8	29.8	A2
13742	11.6	22.4	26.5	A2
13743	13.8	22.2	28.8	A1
13744	12.5	24.6	27.5	A1
13745	11.4	20.0	27.0	A1
13746	12.2	21.2	27.3	A2
13747	11.8	22.4	28.6	A2
13748	10.6	24.9	29.9	Both
13749	12.1	20.9	25.3	A1
13750	11.7	19.1	24.8	A2

Appendix D (*cont´d*)

Isolate	Antheridium width (µm)	Oospore diameter (um)	Oogonia Diameter (µm)	Mating type
13751	12.4	21.4	26.5	A2
13752	10.7	21.4	27.1	A2
13753	13.2	21.3	26.3	A2
13754	11.9	19.7	25.7	A2
13755	12.3	22.4	27.2	A2
13756	13.4	20.8	26.4	A2
13757	12.7	23.1	30.8	A2
13940	13.4	20.5	26.7	A2
13941	cont	cont	cont	cont
13943	12.0	22.8	29.0	A2
13945	12.6	21.0	27.9	A2
13946	cont	cont	cont	cont
13949	14.0	21.2	26.3	A2
13950	16.3	28.5	35.0	A2
13952	17.8	27.4	34.3	A2
13954	16.9	27.7	33.7	A2
13957	14.0	25.4	33.8	A2
13958	15.3	23.4	30.4	A2
13959	15.3	26.5	34.4	A2
13960	15.5	21.4	30.0	A2
13961	15.0	21.2	27.0	A2
13962	13.8	21.1	27.6	A2
13963	12.5	19.8	24.5	A2
13964	14.0	20.7	27.8	A2
13965	12.5	20.3	26.6	A2
13966	15.2	24.2	29.5	A2
13967	10.7	19.8	25.2	A2
13968	12.1	19.2	25.1	A2
13972	13.7	24.8	31.4	A2
13973	12.1	22.3	28.8	A2
13974	10.7	21.9	27.4	A2
13975	12.0	22.5	28.1	A2
13976	14.3	22.6	28.9	A2
13978	12.0	21.8	27.0	A2
13979	cont	cont	cont	cont
13980	14.5	22.3	29.1	A2
13982	12.2	20.7	26.2	A2
13983	11.7	20.0	25.7	A2
13984	11.6	21.9	27.3	A2
13985	13.8	21.8	27.2	A2
13987	12.1	24.4	30.6	A2
13988	14.2	22.6	27.7	A2

Appendix D (*cont´d*)

Isolate	Antheridium width (µm)	Oospore diameter (um)	Oogonia Diameter (µm)	Mating type
13989	15.1	23.1	28.8	A2
13990	13.2	22.5	28.1	A2
13991	12.8	22.3	28.2	A2
13993	12.5	23.0	28.4	A2
13995	12.3	20.0	25.4	A1
13996	14.5	23.5	28.5	A2
13997	11.4	22.0	26.6	A2
13998	12.6	23.8	28.5	A2
13999	12.8	22.4	28.2	A2
14000	15.5	24.3	29.2	A2
14001	13.9	21.0	27.6	A2
14002	12.3	22.7	28.4	A2
14003	13.1	20.4	26.6	A2
14004	12.0	22.6	28.2	A2
14005	11.5	22.5	28.5	A2
14006	13.0	22.4	28.3	A2
14007	13.6	21.7	27.9	A2
14008	14.2	23.5	29.6	A2
14009	13.4	23.1	28.5	A2
14010	15.2	24.0	30.0	A2
14011	13.8	23.5	29.2	A2
14012	13.4	25.3	29.9	A2
14013	12.4	22.9	29.3	A2
14014	12.4	21.8	28.7	A2
14015	15.1	21.9	29.0	A2
14016	11.5	21.9	27.6	A2
14017	13.4	22.3	28.5	A2
14018	11.7	23.2	27.8	A1
14019	14.0	24.4	30.6	A2
14021	12.5	22.7	28.7	A1
14022	12.8	21.9	29.0	A2
14024	16.1	22.5	30.3	A2
14025	14.0	23.3	30.0	A2
14026	12.5	22.3	28.0	A1
14027	14.8	24.7	32.6	A2
14028	14.5	21.5	27.6	A1
14029	13.7	22.0	28.4	A1
14031	14.2	23.6	29.8	A2
14032	13.0	22.8	29.5	A2
14033	13.1	24.4	28.9	A2
14034	13.1	22.1	27.7	A2
14036	13.2	22.9	29.4	A1

Appendix D (*cont´d*)

Isolate	Antheridium width (µm)	Oospore diameter (um)	Oogonia Diameter (µm)	Mating type
14037	14.6	25.5	32.2	A1
14038	14.0	21.6	28.6	A1
14039	12.5	21.1	26.1	A1
14040	14.2	23.8	32.4	A1
14041	12.7	23.5	30.0	A2
14042	14.1	25.1	32.8	A1
14043	12.4	25.7	27.6	A1
14046	13.4	22.5	28.7	A2
14047	13.8	22.4	29.0	A2
14048	13.6	21.9	28.5	A2
14049	12.8	21.1	26.9	A2
14050	12.9	21.9	27.5	A2
14051	14.0	22.9	31.0	A2
14053	cont	cont	cont	cont
14054	12.5	23.3	28.7	A1
14056	13.6	23.9	30.7	A2
14057	13.3	23.4	29.8	A1
14059	14.0	23.6	30.4	A2
14060	14.1	23.5	29.2	A1
14061	13.2	24.7	30.4	A2
14062	cont	cont	cont	cont
14063	cont	cont	cont	cont
14064	12.8	23.7	30.3	A2
14065	16.2	24.4	29.2	A1
14066	13.3	21.5	28.3	A2
14078	13.0	22.3	31.7	A2
14081	12.0	23.7	30.1	A2
14083	13.1	22.8	30.4	A2
14086	12.1	21.9	28.8	A2
14092	12.9	21.5	29.4	A2
14095	13.3	24.7	33.3	A2
14101	14.6	23.3	30.1	A2
14102	14.8	23.8	30.1	A2

NA= Information not available
Both= Mating type A1 and A2 presents
Cont= Contaminated.

APPENDIX E

Cultural characteristics of the *Phytophthora palmivora* collection by isolate.

Isolate	Colony growth at day 2 (mm)	Colony growth at day 3 (mm)	Apple Lesion Diameter (mm)	Mycelial growth[z]	Growth Pattern
13716	12	12	25	HF	Radiate
13717	17	23	33	HF	Radiate
13718	20	29	48	HF	Rose
13719	22	31	27	HF	Uniform
13720	10	12	22	HF	Uniform
13721	23	31	47	SF	Chrysanthemum
13722	19	28	49	MF	Rose
13723	21	34	46	SF	Uniform
13724	14	21	26	MF	Chrysanthemum
13725	7	19	29	SF	Chrysanthemum
13726	16	28	25	SF	Chrysanthemum
13727	7	17	21	MF	Rose
13729	13	14	36	SF	Uniform
13730	31	42	39	SF	Rose
13731	22	32	45	SF	Chrysanthemum
13732	22	29	26	SF	Chrysanthemum
13733	15	21	26	SF	Stellate
13734	18	24	31	MF	Uniform
13735	20	30	32	MF	Uniform
13736	NA	NA	36	NA	NA
13737	7	19	21	SF	Rose
13738	19	28	51	SF	Chrysanthemum
13739	25	35	46	MF	Stellate
13740	31	37	39	SF	Chrysanthemum
13741	19	26	35	SF	Uniform
13742	18	25	46	SF	Stellate
13743	30	42	46	HF	Stellate
13744	20	30	43	SF	Uniform
13745	26	32	45	SF	Stellate
13746	17	24	48	SF	Rose
13747	18	29	50	SF	Stellate
13748	24	34	36	MF	Chrysanthemum
13749	26	31	37	SF	Stellate

Appendix E (*cont´d*)

Isolate	Colony growth at day 2 (mm)	Colony growth at day 3 (mm)	Apple Lesion Diameter (mm)	Mycelial growth[z]	Growth Pattern
13750	20	27	33	MF	Uniform
13751	17	25	45	SF	Stellate
13752	17	25	46	SF	Chrysanthemum
13753	16	23	38	MF	Chrysanthemum
13754	14	25	46	SF	Rose
13755	17	25	52	SF	Uniform
13756	20	29	50	MF	Uniform
13757	21	32	25	SF	Stellate
13940	18	27	48	MF	Chrysanthemum
13941	35	41	25	NF	Radiate
13943	14	23	42	SF	Rose
13945	18	25	43	SF	Rose
13946	36	37	26	NF	Radiate
13949	13	26	35	SF	Chrysanthemum
13950	19	24	42	SF	Chrysanthemum
13952	14	28	50	MF	Radiate
13954	11	28	46	MF	Radiate
13957	12	26	39	SF	Rose
13958	14	24	43	SF	Rose
13959	13	28	28	SF	Rose
13960	16	25	42	SF	Chrysanthemum
13961	18	25	40	SF	Chrysanthemum
13962	13	26	36	MF	Uniform
13963	16	24	55	MF	Uniform
13964	16	24	49	SF	Radiate
13965	20	27	51	SF	Stellate
13966	17	29	44	SF	Rose
13967	19	26	43	MF	Stellate
13968	22	32	49	MF	Radiate
13972	21	33	42	SF	Chrysanthemum
13973	19	28	39	SF	Rose
13974	19	30	45	SF	Uniform
13975	21	30	44	SF	Chrysanthemum
13976	22	34	45	SF	Chrysanthemum
13978	22	28	52	SF	Chrysanthemum
13979	38	42	33	HF	Radiate
13980	21	36	47	MF	Chrysanthemum
13982	16	27	45	SF	Chrysanthemum
13983	17	22	42	SF	Stellate
13984	15	26	41	MF	Chrysanthemum
13985	12	26	50	SF	Chrysanthemum

Appendix E (*cont´d*)

Isolate	Colony growth at day 2 (mm)	Colony growth at day 3 (mm)	Apple Lesion Diameter (mm)	Mycelial growth[z]	Growth Pattern
13987	13	26	43	SF	Rose
13988	17	29	46	SF	Radiate
13989	17	28	50	SF	Stellate
13990	19	25	44	SF	Rose
13991	16	26	44	SF	Radiate
13993	27	32	59	HF	Stellate
13995	18	25	30	MF	Chrysanthemum
13996	17	26	50	SF	Chrysanthemum
13997	15	27	46	MF	Chrysanthemum
13998	16	22	35	SF	Chrysanthemum
13999	15	26	55	SF	Rose
14000	19	29	44	SF	Chrysanthemum
14001	17	25	52	HF	Radiate
14002	18	26	49	SF	Stellate
14003	20	25	46	SF	Chrysanthemum
14004	14	31	31	SF	Chrysanthemum
14005	18	35	29	SF	Chrysanthemum
14006	13	22	39	MF	Uniform
14007	13	23	32	MF	Uniform
14008	19	27	39	SF	Rose
14009	17	29	50	SF	Chrysanthemum
14010	16	26	44	SF	Stellate
14011	14	25	40	SF	Stellate
14012	20	29	41	MF	Stellate
14013	19	30	48	HF	Stellate
14014	15	25	44	SF	Stellate
14015	15	23	46	SF	Radiate
14016	19	27	39	SF	Stellate
14017	22	28	46	SF	Chrysanthemum
14018	20	25	15	HF	Uniform
14019	23	32	51	HF	Stellate
14021	14	22	30	SF	Stellate
14022	21	32	22	SF	Stellate
14024	23	33	41	SF	Chrysanthemum
14025	22	33	41	SF	Stellate
14026	18	29	19	SF	Chrysanthemum
14027	19	27	42	SF	Radiate
14028	13	14	22	HF	Uniform
14029	12	17	29	HF	Stellate
14030	22	35	NA	SF	Stellate
14031	24	27	27	SF	Chrysanthemum

Appendix E (*cont´d*)

Isolate	Colony growth at day 2 (mm)	Colony growth at day 3 (mm)	Apple Lesion Diameter (mm)	Mycelial growth[z]	Growth Pattern
14032	18	31	39	SF	Stellate
14033	12	16	34	SF	Radiate
14034	10	14	26	HF	Uniform
14036	26	32	42	MF	Stellate
14037	19	28	36	SF	Stellate
14038	9	23	21	SF	Chrysanthemum
14039	9	17	23	SF	Chrysanthemum
14040	16	23	38	HF	Radiate
14041	17	21	33	HF	Radiate
14042	23	26	42	SF	Radiate
14043	7	11	32	SF	Uniform
14046	12	24	51	SF	Stellate
14047	16	27	53	SF	Chrysanthemum
14048	16	28	52	SF	Stellate
14049	14	24	54	SF	Stellate
14050	15	27	49	SF	Chrysanthemum
14051	9	17	27	MF	Uniform
14053	7	10	NA	MF	Uniform
14054	16	24	33	HF	Uniform
14055	7	7	NA	SF	Stellate
14056	9	15	21	MF	Uniform
14057	21	28	38	HF	Uniform
14059	22	25	39	SF	Chrysanthemum
14060	15	19	42	MF	Stellate
14061	19	27	39	MF	Stellate
14062	7	14	13	HF	Uniform
14063	7	13	13	HF	Uniform
14064	17	21	30	SF	Radiate
14065	20	24	48	SF	Stellate
14066	13	21	38	SF	Chrysanthemum
14078	15	24	45	SF	Radiate
14081	17	26	39	SF	Radiate
14083	18	28	41	SF	Rose
14086	17	22	45	SF	Rose
14092	29	30	36	MF	Uniform
14095	15	28	35	SF	Chrysanthemum
14101	12	22	39	SF	Stellate
14102	18	26	44	SF	Stellate

z. Mycelial fluffiness of the growth: SF: Slightly fluffy; MF: Moderate fluffy; HF: Highly fluffy.
NA. Information not available.

APPENDIX F

Protocol for *Phytophthora palmivora* mycelium growth on wood stirrers.

Wood stick preparation

Materials: cut wood sticks (50x5x2mm), magenta boxes and V8 broth.

1) Put 320 to 350 cut wood sticks on a magenta box (1 box for every 30 plates).

Figure F- 1 Wood sticks cut and placed on mangenta box.

2) Cover the sticks with v8 broth (1 can of V8 juice adjusted to one liter with distilled water), and autoclave for 60 min.

3) Autoclave 500 ml of V8 broth and let it cool down. When cooled add 7.5mg of Rifampicin and 37.5mg of Ampicillin properly diluted (amended V8 broth)

4) After autoclaving the wood sticks should have absorbed the broth. When the sticks have cooled down, add enough amended V8 broth to cover the sticks under aseptic conditions and keep them closed until use.

Figure F- 2 Magenta box filled with the sticks and V8 broth.

Plating the sticks

Materials: autoclaved wood stirrers, tweezers and V8 plates (Ø100 millimeters)

In the laminar flow hood:

1) Flame the tweezers.
2) Pick a small bunch of sticks and place on an V8 agar plate.
3) Place up to 10 sticks on the plate. Do not leave spaces between neighbor sticks.

Figure F- 3 *Phytophthora palmivora* growth on wood sticks

4) Pack the plates on the sleeves and seal properly to reduce drying and avoid contaminations until use.

Inoculating the sticks

Materials: Wood stick plates, isolates, double distilled water, glass spreader, two 1000 µl pipettes, 1000 µl tips, Parafilm®.

1) Label 3 wood stick plates with the selected isolate ID and date.

2) Set one of the pipettes to 1000 µl, and add 2000 µl of ddH_2O into the isolate plate.

3) Embed the glass spreader in 95% ethanol and then flame it.

4) Scrap the mycelia with the spreader trying to promote sporangia releasing.

5) Set the other pipette to 300 µl and collect 300 µl of the spore suspension from the scraped plate.

6) Incline the wood stick plate and distribute the collected volume on the top of the sticks. Distribute the rest of the volume at the mid height of the sticks. Repeat it for the other two plates.

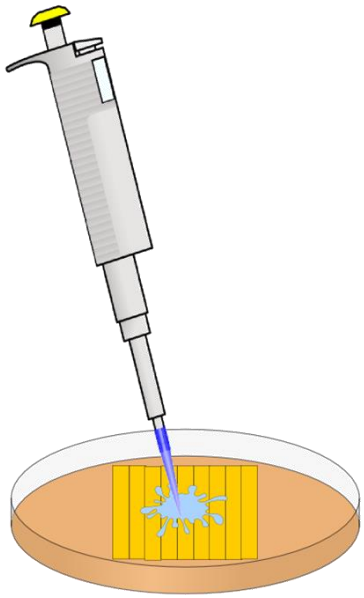

Figure F- 4 A 300 µl of *P. palmivora* suspension is deposited on the sticks.

7) Flame the glass spreader and distribute uniformly the suspension dispensed. Flame the spreader between plates of the same isolate (not alcohol embedding is necessary for the same isolate).

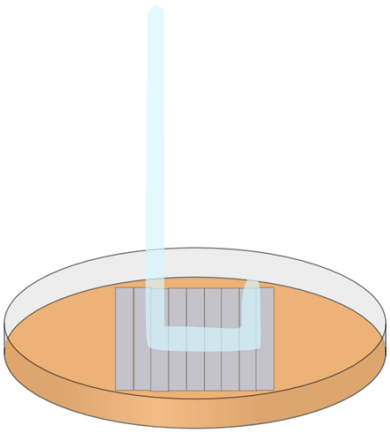

Figure F- 5 The P. palmivora suspension is distributed uniformly

8) Wrap with Parafilm ® and incubate at room temperature until mycelia growth uniformly covers the sticks

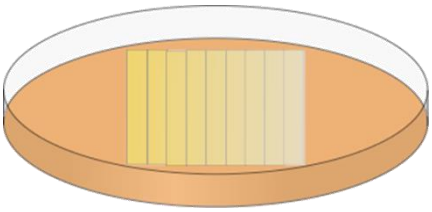

Figure F- 6 The plate is incubated until sticks are covered with mycelia

APPENDIX G

Models developed to build the ECX R Package.

Based on the dispensed volume described by the spiral plate manufacturer, a four points bi-exponential model (1) was initially fitted to identify the dispensed volume at a specific point of the plate. This volume is calculated by replacing in (1), the variable "rad" which means radius, by the distance in millimeter from the desired point to the center of the plate.

$$id_1 = 23.44294 \times e^{(-0.0826534 \times \mathrm{rad})} + \left(325.32176 \times e^{(-0.237215 \times \mathrm{rad})}\right) \quad (1)$$

A quadratic model (2) was developed in order to enhance the model (1) fitting. In this model id_1 is replaced by the result of model (1).

$$id_1 = 23.44294 \times e^{(-0.0826534 \times \mathrm{rad})} + \left(325.32176 \times e^{(-0.237215 \times \mathrm{rad})}\right) \quad (2)$$

The effect of diffusion was calculated by introducing the molecular weight of the evaluated product, into the quantic polynomial model (3), following the values reported with SGE software.

$$dif = id_2 \times \left(0.7433336 + (0.0033412 \times mw) + \left(-1.21E-05 \times mw^2\right) + \left(1.91E-08 \times mw^3\right) + \left(-1.40E-11 \times mw^4\right) + \left(3.92E-15 \times mw^5\right)\right) \quad (3)$$

The linear effect of agar height (AH) and stock concentration (ppm or mg/L) were joined into the model (3) in order to account for variation in these parameters (4).

$$Concentration = dif \times (ppm/1000) \times (3/AH) \quad (4)$$