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STUDIES ON MANGO SOILBORNE DISEASES WITH SPECIAL REFERENCE TO PHYTOPHTHORA ROOT ROT

This thesis is presented for the degree of

Doctor of Philosophy by

YOSRA MAHMOUD MOHAMED AHMED

COORDINATOR:
Prof. Carmelo RAPISARDA

SUPERVISORS:
Prof. Gabriella CIRVILLERI
Dr. Anna Maria D'ONGHIA
Dr. Thaer YASEEN

Dedication

I would like to dedicate my thesis

To all those people who were my sun and my stars

To those who were my lights and my inspiration.

To those who parted the clouds for me

To those who believed that I could fly.

To those who encouraged me along in bad weather.

To all those who believe in the richness of learning

To all those who believe in the value of the science

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SUMMARY

Mango (*Mangifera indica* L.) is an important fruit crop in many tropical and subtropical countries. Despite the importance of the crop worldwide, the literature review highlighted the lack of knowledge about the impact of soilborne diseases on mango expansion and productivity. Recently, mango has been introduced into Italy mainly in some provinces of Sicily. However, its future as approaching commodity in Sicilian agriculture is threatened by diverse biotic and abiotic threats. This study aimed to assess the occurrence of the fungal soilborne diseases and their causal agents in the island. Special reference was provided to *Phytophthora* species, oomycetes -like fungi that cause Phytophthora root and crown rot on mango.

Surveys were conducted over summer and spring (2010- 2011) in different mango orchards located in five provinces (Palermo, Messina, Catania, Agrigento and Ragusa) in Sicily. Several diseases induced by soilborne pathogens were reported in all the investigated orchards. Typical symptoms of damping off, root rot, crown rot, wilt, Armillaria root rot and wood decays diseases were observed. Morphological and molecular identification of the isolated fungi and oomycetes showed that they belong to different genera: *Rhizoctonia*, *Fusarium*, *Pythium* and *Armillaria*. The percentage of disease incidence and fungal frequency were recorded.

Verticillium wilt, a vascular disease caused by *Verticillium dahliae*, was reported for the first time in a new mango grove in Catania province. Typical symptoms of the disease were observed. The pathogen identity was initially made based on colony morphology and formation of microsclerotia and further confirmed by molecular method. Greenhouse inoculation trial, performed on young Kensington Pride cv. mango plants, fulfilled its pathogenicity.

Phytophthora root and crown rot disease was reported in provinces of Messina and Palermo. *P. cryptogea* was consistently isolated from diseased tissues taken from the crown and necrotic roots of mango. The fungus was identified on the basis of colony morphology, characterization of the sexual and asexual reproductive structures, and temperature range. In addition, DNA sequence data of ITS, COI, LSU and 60S loci were

used for phylogenetic inferences. Pathogenicity tests conducted to assess its ability to cause disease revealed that the fungus is a possible pathogen of this crop. This study showed that *P. cryptogea* is the cause of crown and root rot on mango in Italy and represented its first occurrence on mango worldwide.

The diversity of *P. cryptogea*, the causal pathogen of mango root rot, within *Phytophthora* worldwide populations was assessed. In this study, re-evaluation of global collection of 140 isolates assigned to *P. cryptogea*, *P. drechsleri* and *P. erythroseptica* was carried out. Single and multiple gene phylogenetic analyses were performed on DNA sequences of nuclear (Internal Transcribed Spacers, ITS) and mitochondrial (Cytochrome c Oxidase subunit I, COI) genes. Both markers provided an acceptable resolution for these species. High levels of intraspecific variation were found within *P. cryptogea* population in which two different clades were inferred. *P. cryptogea* isolates recovered from mango in Italy were fall all together in *P. cryptogea* group II (GII), along with other 20 isolates collected from different woody trees and diverse origins.

The possibility to set up a molecular approach to provide an accurate detection of *P. cryptogea* was investigated. Species-specific primer pairs for *P. cryptogea*, and two other species (*P. megasperma* and *P. citrophthora*), were designed from the most variable fraction of IGS regions. *P. cryptogea* specific primer (Cry5F/Cry5R) amplified 79 bp short fragment, while the primers Cit3F/Cit3R and Mega10F/Mega10R amplified 144 and 121bp fragments in *P. citrophthora* and *P. megasperma,* respectively. The above three sets of species specific primers pair were chosen to develop specific probes for the detection of the three *Phytophthora* species in the Real-time PCR (TaqMan) assay.

An extensive number of potentially pathogenic fungi including species of *Phytophthora*, *Verticillium* and *Armillaria* were found associated with mango in Italy. As some of these fungal species could serve as sources of inoculum onto economically important crops, the present research provides basis for understanding phytosanitary issues in mango. Hopefully results of this study will serve as valuable tools in mango integrated crop management.

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LIST OF ABBREVIATIONS

List of Abbreviations	
A	Adenine
bp	Bas pair
C°	Celsius degree
CBS	Centraalbureau voor Schimmelcultures
cm	Centimeter
cv	Cultivar
C	Cytosine
DNA	Deoxyribuneoclic acid
dNTPs	Deoxyribonucleotide triphosphate
EtOH	Ethanol
FAO	Food and Agriculture Organization
Fig.	Figure
cm	Centimeter
G	Guanine
g	Gram
h	Hour
H_2O	Water
HCl	Hydrochloridric Acid
Kb	Kilo Base Pair
ITS	Internal transcript spacer region
Kbp	Kilo base pair
M	Molar
mg	Milligram
ml	Milliliter
min	Minute
MgCl2	Magnesium chloride
mM	Millimolar
PCR	Polymerase chain eaction
PDA	Potato dextrose agar
%	Percentage
µl	Microliter
µM	Micrometer

List of Abbreviations	
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Analysis
rpm	Revolutions per minute
sec	Second
spp.	Species
SSCP	Single Strain Conformation Polymorphism
T	Thymine
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
U	Enzymatic Unit
UV	Ultraviolet
V	Volt
V/V	Volume per Volume
WA	Water agar
WPC	World Phytophthora collection
SNP	Single-Nucleotide Polymorphism

CHAPTER 1.

1. GENERAL INTRODUCTION, LITERATURE REVIEW AND THESIS OBJECTIVES

1. General Introduction

Mango (*Mangifera indica* L.) is one of the best-known and most widely cultivated tropical fruit species, with production occurring in most countries in the tropics and subtropics areas (Paull and Duarte, 2010). The fruit is marketed fresh, dried, and is used as a source of flavors and colorants (Litz, 2009).

Botany and Taxonomy: Mango belongs to the family Anacardiaceae, also known as the cashew family, with about 75 genera and 700 species, mostly tropical, with some subtropical and temperate species. The genus *Mangifera* consists of 69 species but not all bear edible fruits (Paull and Duarte, 2010). The mango fruit is large, fleshy and sometimes fibrous. The other edible *Mangifera* species generally have lower quality fruit and are commonly referred to as wild mangos (Litz, 2009).

Common Names: Mangos have been grown throughout the tropical and subtropical world for thousands of years and have become an integral part of many cultures. Many of the names have common derivations, reflecting the origins and spread of the mango tree along with the spread of human communities (Paull and Duarte, 2010). Some of these names are: mango (English), mangue, manguier (French), manja- maggo- manja (Dutch), manga- mango (Spanish), manga (Portuguese), manga (Malaysia), mangga (Indonesia), Mangobaum (German), Al mango (Arabic) and Il mango (Italian) (Bally, 2006).

Origin and History: The mango originated in the Indo-Burma region and has been cultivated in India for more than 4000 years. This fruit is closely associated with the Hindu religion (Singh, 1960). Indian traders and Buddhist priests perhaps introduced the mango into Malaysia and other East Asian countries during the 4th or 5th century BC, and to the Philippines between AD 1400 and 1450. The Portuguese, the first Europeans to establish trade routes with India, transported the mango to East Africa and Brazil. Spanish traders took the mango from the Philippines to the west coast of Mexico before the English arrived in the Hawaiian Islands in 1778. The mango was introduced into Hawaii from the west coast of Mexico between 1800 and 1820. Apparently, the Brazilian introductions were spread to Barbados and to other islands in the Caribbean area. Mango is now found in all

tropical areas, as well as many subtropical regions of the world, attesting to its wide range of adaptability (Litz, 2009).

Climate: Although grown widely, mangos prefer a warm, frost-free climate with a well-defined winter dry season, rain and high humidity during flowering and fruit development reduces fruit yields. The tree generally flowers in mid- to late winter, with fruit maturing in the early to mid-summer months (Bally, 2006). Mango trees are evergreens that grow to 60 feet tall and the tree fruits 4 to 6 years after planting (Paull and Duarte, 2010).

Mango Production: Mango covered an area of 4,946,313 ha with production of 37.12 million tons in the world during the year of 2010 (Table 1.1). India occupies top position among mango growing countries of the world and produces 40.48% of the total world mango production. China and Thailand stand at second and third position among mango producing countries in the world with 4,366 and 2,551 thousand tons respectively. The other major mango producing countries in the world during 2010 were Thailand (2550 thousand tons), Pakistan (1784 thousand tones), Mexico (1633 thousand tones) and Indonesia (1314 thousand tones) respectively (FAOSTAT, 2011). In 2010, Mexico dominated the export trade with shares of 22% followed by Philippines (16%), Pakistan (16%), Brazil (13%) and India (11%). Other major exporters include the Netherlands and Peru (Fig.1.1A). United States imported approximately half of total mango imports (48%), followed by China with shares of (11%) (Fig.1.1B). The Netherlands imports of mangoes are (8%), but most of this is redistributed throughout the European Union. Other major importing redistributors of mangoes are the United Arab Emirates (6%) and Saudi Arabia (4%), with most of these imports being redistributed within the Middle East (FAOSTAT, 2011).

Mango production in Italy: There are no official records available for mango production in Italy since the crop is relatively new.

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Table 1.1 Major producing countries of mango in the world during 2010 (FAOSTAT, 2011).

Country	Area (ha)	Production (Tons)	Productivity (Tons/Ha)	% Share in world Total production
India	2312.3	15026.7	6.5	40.48
China	465.337	4351.29	9.35	11.72
Thailand	311.048	2550.6	8.2	6.87
Pakistan	173.7	1845.5	10.62	4.97
Mexico	174.97	1632.65	9.33	4.4
Indonesia	131.674	1287.29	9.78	3.47
Brazil	75.111	1188.91	15.83	3.2
Bangladesh	170.8	1047.85	6.13	2.82
Philippines	189.437	825.68	4.36	2.22
Nigeria	114.9	790.2	6.88	2.13
Other Countries	827.04	6578.07	7.95	17.72
World	4946.314	37124.74	7.51	

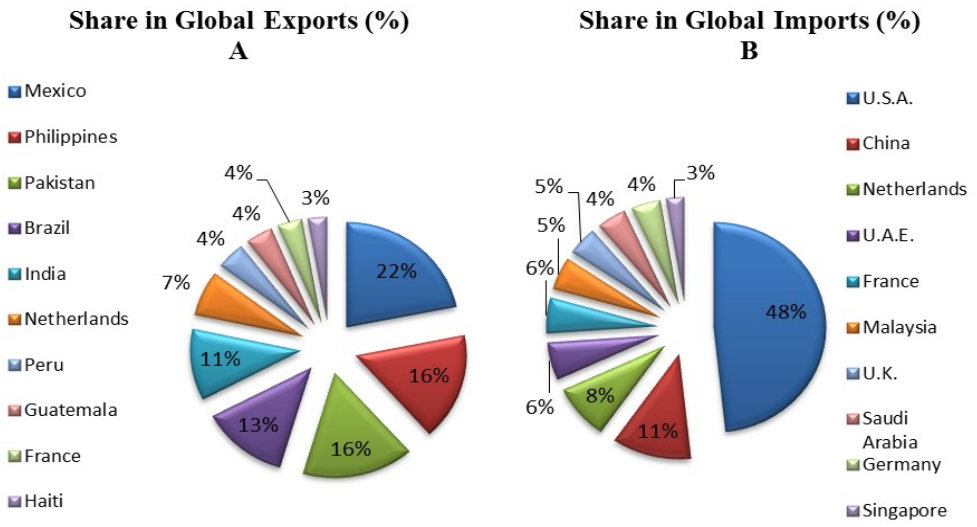

Figure 1.1 The major countries share in global mango exports % (A) and global imports % (B).

Mango Nutritional Value: Ripe mango is delicious fruits with pleasant flavor, the fruits are highly nutritious that contain carbohydrates, proteins, fats, minerals, and vitamins (Litz, 2009; Anonymous, 2010; Chen *et al.*, 2010; Paull and Duarte, 2010).

Mango Varieties: There are over a thousand mango varieties grown around the world. India having the greatest number of varieties almost over 500 named cultivars (Bally, 2006). Florida (USA) has developed a large number of cultivars, using mainly Indian cultivars, which have shown wide geographic adaptability. Most mango varieties planted in the Americas, the Canary Islands and many African countries were developed in Florida The Florida varieties are also dominant in international trade (Paull and Duarte, 2010). The selection of mango varieties is usually based on keeping quality of fruits and tree growing characteristics. Preferences for mango varieties often differ among countries, regions, traditions, and cuisines of markets in which they are consumed. Among the most important mango varieties are:

***Kensington Pride (Australia*):** It is grown widely in the tropical and sub-tropical regions. Kensington Pride was the first cultivar introduced into Sicily (Southern Italy), because it is resistant to low temperatures particularly well represented in Catania and also along the north-eastern coast (Anonymous, 2010). Its fruits are produced early (late August and September) and sells well. The only problem of this cultivar is fruit color that looks a little pale, with little blush, so when it arrives in the markets, the cultivar Glenn, which has the same period of maturation, the Kensington appears to have less success (Litz, 2009).

***Glenn (Florida, USA)**:* The best for the time of maturation (August) for both color and flavor that has far surpassed all other varieties (Litz, 2009).

***Kent (Florida, USA)**:* The fruit color is greenish yellow with red blush. Its weight is around at 600-700 grams. The flesh is of excellent flavor and has a pleasant and a rich aroma (Litz, 2009).

Tommy Atkins (Florida, USA): Is the most important commercial cultivar in the western hemisphere. It is highly resistant to anthracnose disease, handling and shipping stress (Campbell, 1992).

***Zebda (Egypt)*:** Is the most important cultivar in Egypt. The tree is vigorous and regularly productive. It is highly tolerant for anthracnose and resistant to malformation (Litz, 2009).

Mango industry in Italy: In the eighties, mango cultivation was introduced in Italy with limited extensions mainly in Sicily (Southern Italy). Sicily is the largest island in the Mediterranean Sea. It has a typical Mediterranean climate with mild, wet winters and hot, dry summers. Mango industry started as a result of studies conducted by Prof. F. Calabrese and his team of researchers through a project funded by the tropical fruit MIPAAF (Anonymous, 2010). The objectives of these studies were i) trying to cultivate mango under Sicilian mild climate, especially that mango had already been successfully cultivated in countries with comparable climate like Spain, France and Israel, and ii) to provide an alternative crop in areas of Sicily that well suited to tropical and subtropical crops. Therefore, small mango pitches began to arise in different parts of Sicily in order to ascertain what would be the area most suitable for mango cultivation and with which varieties. These researches concluded that the cultivar Kensington Pride could easily be grown even in areas where temperatures down or are close to zero, while for other cultivars was chosen the Tyrrhenian coast that goes from Palermo to Messina. In the near future, it is expected that commercial and backyard plantings of mango trees will increase, being a profitable crop in Sicily (Anonymous, 2010).

Mango Production boundaries: Mangos grow and produce in many tropical and subtropical climates, although fruit production is limited by wet weather during the flowering and fruiting period (Prakash, 2004; Bally, 2006; Paull and Duarte, 2010). Unreliable yields and fruit quality from season to season are also limiting characteristics of many mango varieties. Generally, the low productivity is due to the wide range of climatic conditions, environment situation and the diversity of the associated disease and disorder problems (Ploetz, 2004; Prakash, 2004).

Mango Physiological Disorders: Physiological disorders are any aberrations that have not been caused by infecting organisms. True physiological disorders cannot be transmitted from plant to plant, mechanically or by insect bites (Litz, 2009). They are results of imbalances in metabolism induced by some factors in the pre-harvest or postharvest environment that leads to cell collapse. Pre-harvest factors include growing location, orchard conditions and tree nutrition while post-harvest storage conditions such as temperature, oxygen and carbon dioxide levels, packaging and surface coating treatments are contributing factors to the occurrence of the disorders (Campbell, 1992; Prakash, 2004; Litz, 2009; Paull and Duarte, 2010). Internal necrosis of mango fruit is an emerging physiological disorder due to boron deficiency which may also lead to fruit cracking (Chin *et al.*, 2010; Saran and Kumar, 2011).

Mango Pests: Of the 260 species of insects and mites that have been recorded as pests of mango, 87 are fruit feeders, 127 are foliage feeders, 36 feed on the inflorescence, 33 inhabit buds, and 25 feed on branches and the trunk (Peña *et al.*, 1998). The major key pests (fruit flies, seed weevils, tree borers and mango hoppers) require annual control measures. Secondary pests generally occur at sub-economic levels, but can become serious pests as a result of changes in cultural practices and cultivar or because of indiscriminate use of pesticides against a key pest (Peña *et al.*, 1998; Litz, 2009; Paull and Duarte, 2010).

Mango Diseases: Over 140 pathogens are known to cause damage to mango plants (Prakash, 2004). Mango diseases are caused mainly by some true fungi, fungus-like Oomycetes, nematodes and bacteria. The first step in overcoming the threats from diseases is to accurately identify the problems followed by adequate management and judicious use of fungicides and bactericides (Bally, 2006; Litz, 2009).

2. Literature Review

An Overview of Mango Diseases

Mango as any fruit trees can be affected by different factors, which can influence the healthy status of trees either biotic or abiotic factors. Mango is affected by a number of diseases at all stages of its growth from the seedling and grafted plants in the nursery to the fruits in storage or transit (Ploetz, 2004). Most of mango parts specifically, seeds, root, trunk, branch, twig, leaf, petiole, flower and fruit are attacked by a number of pathogens including fungi and bacteria. They may cause several kinds of rot, die back, wilt, anthracnose, scab, necrosis, blotch, spots, mildew, fruit rot, etc. Diseases in the field result in crop loss, while post-harvest diseases are directly linked with the losses in export and domestic market. In several regions, they are the most important constraint to fruit production as they cause heavy losses in mango yield (Ploetz, 1994a;2004; Bally, 2006; Litz, 2009).

1.1 Fungal Soilborne Diseases of Mango

Soilborne diseases are caused by fungi or pathogenic microbes that persist in soil without plant hosts. These pathogens can induce different kind of symptoms such as damping off, root rot, stem rot and wilt. Plant diseases caused by soilborne pathogens are considered major problems in agricultural production throughout the world. These diseases can reduce yield and quality of many crops. The main fungal and oomycete genera reported as containing species that are pathogenic toward mango, include the fungal genera *Fusarium, Rhizoctonia* and *Verticillium*, and the oomycete genera *Phytophthora* and *Pythium* (Prakash and Singh, 1980; Tsao *et al.*, 1994; Prakash, 2004; Ploetz and Freeman, 2009). Although soilborne diseases of mango are relatively less important than foliar diseases, they can cause significant damage to seedlings, nursery stock and mature trees (Litz, 2009).

a. Pythium Damping Off and Root rot Diseases

Damping-off and root rot diseases occur worldwide in tropical, temperate climates, and in every greenhouse. The disease affects seeds, seedlings, and plant root. The greatest damage arises during germination either before or after emergence. Losses vary considerably with soil moisture, temperature, and other factors (Spies *et al.*, 2011). In many cases, seedlings in seedbed are entirely destroyed by damping-off or they die soon after they are transplanted. Generally, poor germination of seeds or poor emergence of seedlings is the result of damping-off infections in the pre-emergence stage (Spies *et al.*, 2011). Older plants are rarely killed when infected with the damping-off pathogen, but they develop root and stem lesions and root rots. Their growth may be slowed significantly, and their yields may be reduced severely. In mango damping off of seedlings was reported as a serious problem in nurseries in Indonesia, Pakistan and India (Prakash, 2004).

Symptoms: Lim and Khoo (1985) indicated that overcrowding, excessive moisture and the use of polybags favoured this disease. Symptoms include wilting of foliage, which initially becomes pale green, but later develops necrotic patches. Roots develop a wet, blackened necrosis that begins in fine roots and progresses to larger roots and the root collar. Death of seedlings often occurs (Prakash, 2004; Litz, 2009).

The Causal Organism: The genus *Pythium* contains several species that are facultative pathogens. These species causing severe diseases in many crops and acting either individually or in complexes with other organisms (André LéVesque and De Cock, 2004). In Malaysia, *Pythium vexans* de Bary, has been reported as the causal pathogen of mango root rot and wilt of seedlings (Lim and Khoo, 1985) and induced seedling losses of up to 30% in nurseries (Lim and Khoo, 1985; Litz, 2009).

Life Cycle: *Pythium* species occur in surface waters and soils. They live on dead plant and as saprophytes or as parasites of fibrous roots of plants. The pathogen needs free water for its zoospores to swim and infect the host. When a wet soil is infested heavily with *Pythium*, any seeds or young seedlings in such a soil may be attacked by the pathogen (Agrios, 2005). *Pythium* mycelium or spore germ tubes come in contact with mango seeds and seedling tissues then enter by direct penetration (Fig 1.2). The mycelium

grows between and through the plant cells. Pectinolytic enzymes secreted by the fungus dissolve the pectins and the protoplasts of invaded cells, and, in some cases, cellulolytic enzymes cause complete collapse and breakdown of the cell walls. As a result, the invaded tissues cannot support the seedling, which falls over and dies. The disease and losses caused by *Pythium* infections are more severe when the soil is kept wet for long periods

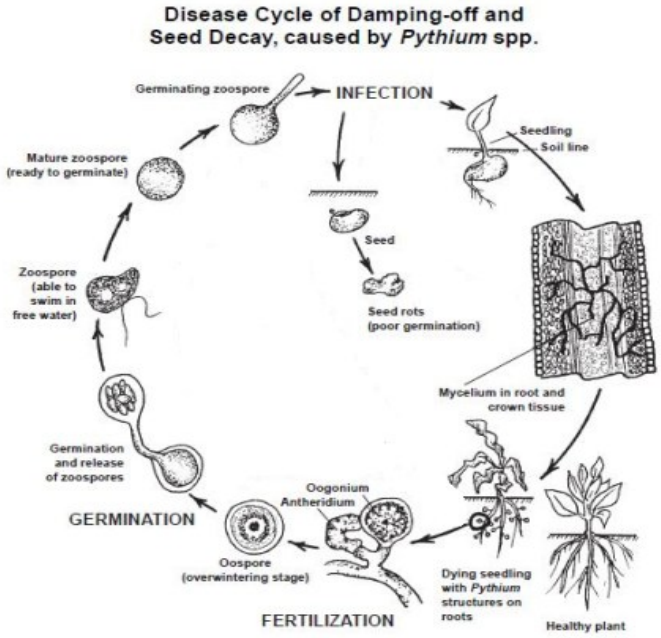

Figure 1.2 Disease cycle of Pythium damping off and root rot (Agrios, 2005).

Disease management: Cultural practices are helpful in reducing the amount of infection. Such practices include providing good soil drainage and good air circulation among plants, planting when temperatures are favourable for fast plant growth, avoiding application of excessive amounts of nitrate forms of nitrogen fertilizers. Seed chemical treatment and spraying of seedlings are important when the soil is infested heavily with *Pythium* or when the soil stays wet for prolonged periods during the early stages of plant growth (Agrios, 2005).

b. Rhizoctonia Damping Off and Root Rot Disease

Rhizoctonia diseases occur throughout the world. They cause losses on almost all vegetables and fruit trees. The most common symptoms on utmost plants are damping-off of seedlings and root rot. It occurs primarily in cold, wet soils. Very young seedlings may be killed before or soon after they emerge from the soil. Damping off of mango seedlings is a serious problem in nurseries in Indonesia and India (Prakash and Singh, 1980).

Symptoms: The disease is characterised by sudden dropping of leaves after the emergence of seedlings from the soil. During prolonged rainy and humid weather, infection occurs below the ground level with circular to irregular water-soaked patches. These patches enlarge and ultimately girdle the entire base of the stem. On account of rotting, the diseased tissues become soft, dark brown or black and the entire seedling collapses and dies (Litz, 2009). Fungal mycelia and sclerotia are densely present on the severely infected parts. Rotting may spread both above and below the stem down up to roots and roots are disintegrated (Prakash and Singh, 1980; Prakash, 2004).

The Causal Organism: *Rhizoctonia* is soil inhabitant basidiomycetes. The fungus was recognized as sterile fungi for many years, because they were thought to produce only sclerotia and to be incapable of producing sexual or asexual spores (Sneh *et al.*, 1991). It is known now that at least some species of *Rhizoctonia*, can produce basidiospores as their sexual spores only under special conditions in the laboratory or are extremely rare in nature. Therefore, fungus basiospores have little value in identifying the fungus (Sneh *et al.*, 1991). The genus *Rhizoctonia* is comprised of a diverse group of fungi that can broadly be divided into multinucleate and binucleate groups based on the number of nuclei per hyphal cell (Sneh *et al.*, 1991). Isolates are classified further into anastomosis groups (AGs), based on their hyphal compatibility with known tester isolates (Tewoldemedhin *et al.*, 2011).

Disease Cycle: The pathogen overwinters usually as mycelium or sclerotia in the soil and in or on infected perennial plants. The fungus spreads with rain, irrigation, and with infected propagative materials. After the seedlings have emerged, the fungus attacks their stem and makes it water soaked, soft, and incapable of supporting the seedling,

which then falls over and dies. In older seedlings, invasion of the fungus is limited to the outer cortical tissues, which develop reddish-brown lesions. The lesions may increase in length and width until they finally girdle the stem, and the plant may die (Agrios, 2005).

Disease Management: Conditions that delay seed germination and slow seedling growth, such as cool, moist, poorly drained soils, favor seedling diseases. Because damping-off is most severe when crops are grown in conditions not favorable to rapid seed germination and seedling emergence, avoid planting into cool, wet, and poorly drained soil. Fields should be prepared so that water does not stand. Seed treatments with appropriate fungicides may provide some protection from seedling diseases (Naqvi, 2004).

c. Fusarium Black Root Rot Disease

Black root rot is reported to be an uncommon problem on young mango trees (Lim and Khoo, 1985). Several species of fungi have been recovered from affected plants, mainly the genera *Fusarium* such as *Fusarium solani*, *F. oxysporum* and in addition to *Lasiodiplodia. theobromae*, but these were thought to be secondary colonizers of roots (Lim and Khoo, 1985).

Symptoms: Canopies of affected plants wilt suddenly and subsequently defoliate. Roots show a water-soaked, darkened decay with association with an unpleasant odour. root discoloration may cover the tap root and the stem below the soil line (Lim and Khoo, 1985; Prakash, 2004).

The Causal Organism: *Fusarium* genus contains over 70 species (Leslie *et al.*, 2006). Many species inhabit soil ecosystems where they are rhizosphere or endophytic colonizers. The interaction of *Fusarium* species with plants can range from highly pathogenic to beneficial plant growth stimulation (Tewoldemedhin *et al.*, 2011). *F. solani* generally produces only asexual spores, although under certain conditions it produces its perithecial stage, *Netria haematococca*. The asexual spores are microconidia, macroconidia and thick-walled chlamydospores. *F. oxysporum* is the most widely dispersed of the *Fusarium* species. The fungus reproduces only asexually (Leslie *et al.*, 2006).

Disease Cycle: The pathogen can live on dead plant tissue and can overwinter as mycelium or spores in infected or dead tissues (Leslie *et al.*, 2006). The fungus is already present in many soils as spores, which are spread easily by air, equipment, water, and contact. Fusarium root rot disease becomes more severe when plants are stressed by low temperature, by irregular drought or excessive soil water (Prakash, 2004).

Disease Management: Treatment of propagative stock with appropriate fungicides or application of fungicide sprays on the plants has helped reduce Fusarium rots (Naqvi, 2004).

d. Phytophthora Diseases of Mango

Phytophthora crown and root rots are common and destructive diseases of fruit trees throughout the world (Erwin and Ribeiro, 1996). *Phytophthora* species cause diseases of mango in several areas. It caused wilt, crown rot, root rot and the death of nursery trees in Arizona USA (Matheron and Matejka, 1988), the Philippines and Thailand (Tsao *et al.*, 1994).

Symptoms: Diseased trees are most likely to be found in heavy, wet soils or sections of the orchard where water collects or is slow to drain. Above-ground symptoms include poor growth with sparse off-color foliage and develop of gumming and visible bark lesions develop above ground on these plants, whereas root and crown rots are evident at or below the ground level (Prakash, 2004; Litz, 2009; Ploetz and Freeman, 2009). Mortality of trees is not observed, but substantial stem cracking and bleeding does occur.

The Causal Organism: The Oomycete pathogens of *Phytophthora* are the most destructive plant pathogens known. There are over 82 species in the genus many with a wide host range. Many *Phytophthora* species have been associated with mango root rot in many countries. *Phytophthora palmivora* was isolated from infected mango trees showing crown rot, root rot and wilt symptoms in the Philippines (Tsao *et al.*, 1994). *P. palmivora* has been reported also as the causal agent of mango root rot and the death of nursery plants in Arizona USA and Thailand (Matheron and Matejka, 1988). *P. parasitica* has been reported in India to cause leaf blight disease on mango (Prakash and Srivastava, 1987; Prakash, 2004). Recently, *P. citricola* was reported in Spain, the fungus was isolated from

mango trees that were wilted, chlorotic and had sparse canopies and cracked bark (Zea-Bonilla *et al.*, 2007)

Disease Cycle: *Phytophthora* species can survive in cold winters or hot, dry summers as oospores, chlamydospores, or mycelium in infected roots, stems and in soil. In the spring, the oospores and chlamydospores germinate by means of zoospores, whereas the mycelium grows further and produces zoosporangia that release zoospores. The zoospores swim around in the soil water and infect roots of susceptible hosts with which they come in contact (Fig. 1.3). More mycelium and zoospores are produced during wet, cool weather and spread the disease to more roots (Erwin and Ribeiro, 1996).

Disease Management: All planting stock should be free of infection. In greenhouses, the soil mixture, seedbeds and planting pots should be sterilized with steam before planting. Sanitary measures must be performed to avoid introduction of pathogen in the nurseries (Agrios, 2005). Overcrowding should be avoided and frequency of irrigation must be adjusted. Seed treatments and transplant dips with suitable systemic fungicides. Some protection of trees can be obtained by injections of selected fungicides into their trunks. Also, application of a solution of some fungicides in the soil around trees seems to inhibit the growth and activity of *Phytophthora* (Naqvi, 2004; and Prakash, 2004).

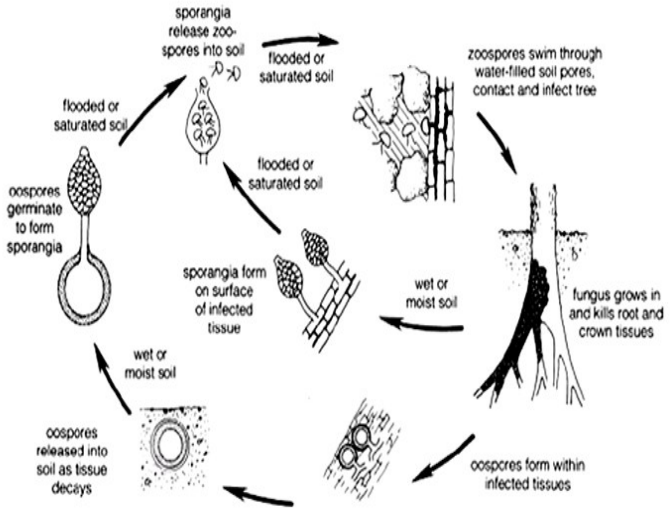

Figure 1.3 Disease cycle of Phytophthora root rot (Agrios, 2005).

e. Verticillium Wilt Disease of Mango

Verticillium species are soil-borne pathogens responsible for Verticillium wilt diseases in temperate and subtropical regions. They affect over 200 hosts, including many economically important fruit tree crops (Klosterman *et al.*, 2009). Verticillium wilt of mango was first reported in Florida USA (Marlatt *et al.*, 1970).

Symptoms: The problem is usually observed in young trees planted in soil previously cropped with vegetables that are also susceptible to Verticillium disease. Disease symptoms comprise wilting, chlorosis, stunting, necrosis and vein clearing. In many of these trees the symptoms expanded, leading to decline and eventual death. Killed leaves usually remain attached to the tree and cross sections of affected branches revealed brown vascular discoloration (Baeza-Montanez *et al.*, 2010).

The Causal Organism:: Verticillium wilt of mango was first reported in Florida USA (Marlatt *et al.*, 1970). The disease was originally attributed to *Verticillium albo-atrum* but this was before *Verticillium dahliae* was recognized as a distinct species. Recently, Baeza-Montanez *et al.* (2010) reported Verticillium wilt caused by *V. dahliae* in Southern Spain.

Disease Cycle: The typical disease-cycle of Verticillium wilt begins with the germination of resting structures (microsclerotia) in soil, and penetration of the root epidermis of host plants to reach the cortex (Fradin and Thomma, 2006). After crossing the endodermis, the hyphae invade the vascular tissues where conidia can be formed (Fig. 1.4). The conidia are drawn up into the plant with the water stream in the xylem, and trapped in pit cavities, where they germinate. The expression of Verticillium wilt symptoms is usually associated with the colonization of adjacent vascular and cortical tissue (Klosterman *et al.*, 2009). The fungus produces microsclerotia, which are returned to the soil with the decomposition of the plant, and the disease cycle starts again (Fradin and Thomma, 2006; Sanei *et al.*, 2008; Klosterman *et al.*, 2009).

Disease Management: Control of Verticillium wilt depends on planting disease-free materials in disease-free soil and using resistant rootstocks. Avoid planting where solanaceous crops (i.e. potato, tomato and eggplant) have been grown repeatedly is recommended. New mango orchards should not be planted on such sites (Ploetz and Freeman, 2009). Sharma *et al.* (1993) found that soil drenching with Carbendazim (0.1%) or Captafol (0.25%) has been found effective in controlling the seedling wilt.

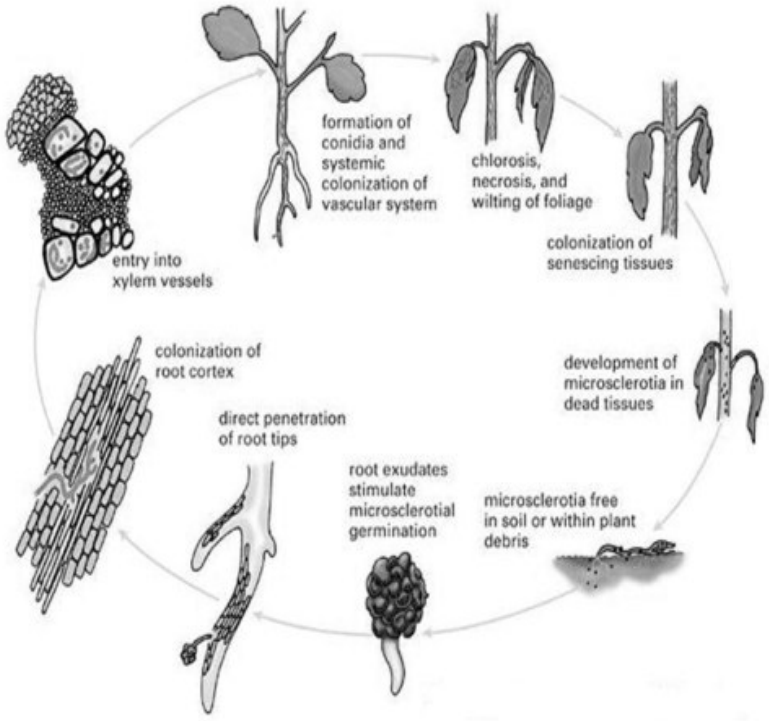

Figure 1.4 Disease cycle of Verticillium wilt (Agrios, 2005).

f. Armillaria Root Rot Disease of Mango

The disease is also known as shoestring root rot, mushroom root rot, or oak root fungus disease. Armillaria root rot occurs throughout temperate and tropical regions. It affects fruit trees, vines, shrubs, and forest trees (Aguín-Casal *et al.*, 2004; Matsushita and Suzuki, 2005). The threat of Armillaria root rot is of serious concern in Europe, Australia, America and other developed nations of the world (Popoola, 2004).

Symptoms: The pathogen grows underneath the bark and destroys the structural root system of plant. Chlorosis and abscission of leaves are the common symptoms when the sufficient amount of large roots is destroyed by the fungus. Diagnostic characteristics of Armillaria root rot appear at decayed areas in the bark. White mycelial mats, their margins often veined and shaped like fans, form between the bark and wood (Popoola, 2004). The mycelium may extend for a few feet upward in the phloem and cambium of the trunk and may cause white rot decay. Another characteristic sign of the disease is the formation of black rhizomorphs. These are consisting of a compact outer layer of black mycelium and a core of white or colorless mycelium (Prakash, 2004).

The Causal Organism: The most common *Armillaria* species that can cause root rot on fruit trees are *A. tabescens* and *A. mellea.* The two species are generally very similar but there are a few features that distinguish them. The basidiocarps of *A. tabescens* lack an annulus or ring just below the cap and can be a darker shade of brown than those of *A. mellea*. In contrast, an annulus is present in the basidiocarps of *A. mellea* (Prakash, 2004).

Disease Cycle: *Armillaria mellea* is a polyphagus pathogen very aggressive on old plants, sometime on young plants. The fungus overwinters as mycelium or rhizomorphs in diseased trees or in decaying roots (Fig.1.5). The main dispersal means are the mycelium and rhizomorphs and not the basidiospores produced by the fungus. Rhizomorphs can grow in the soil 0.5 - 3 m per year, spreading the disease as an oil spot. The fungus can survive for many years in dead or living roots. The pathogen requires cool, moist soil conditions for spreading and disease development. In autumn-winter and a few days after rains, *Armillaria* often forms clusters of mushrooms at the base of infected trees (Prakash, 2004).

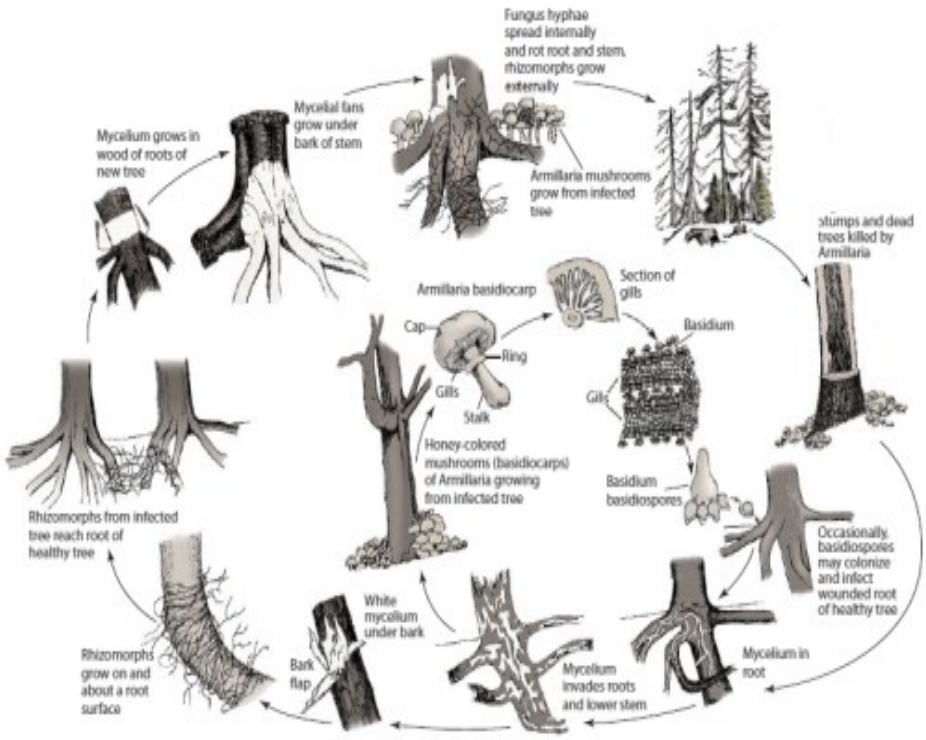

Figure 1.5 Disease cycle of Armillaria rot (Agrios, 2005).

Disease Management: Good sanitary measures are the best practice to reduce the disease and to manage Armillaria root. Provide a good growing environment and proper cultural practices. Good drainage is important, as *Armillaria* fungus is very susceptible to drying (Prakash, 2004). The rhizosphere soil and affected root system should be removed carefully by deep diggings. Removal of stumps and as much as possible root pieces with rhizomorphs from the soil helps in controlling the further spread of the disease (Agrios, 2005).

g. Wood Rots And Decays of Mango

Wood rots and decays diseases are considered to be minor diseases on mango.

Symptoms: Depending on the tree part attacked, wood rots may be called root rots, root and butt rots, or stem rots. The wood decay is usually associated with wounds or cankers, whereas in wood pieces the decay is usually at or near the surface of wood that has high moisture content.

The causal organism: Different species belong to basidiomycetes fungi can induce wood rot and decay such as *Rigidoporus lignosus* and *Ganoderma lucidum*. Some species are saprophytic that grow on dead or declining heartwood of trees, while several are pathogens that cause decay of roots and stems.

Disease Cycle: Fungi that cause tree or wood decays grow inside the wood cells and utilize the cell wall components (Fig.1.6). Some of them attack softwoods and break down and utilize primarily the cell wall polysaccharides like cellulose and hemicellulose giving the rotten wood symptoms.

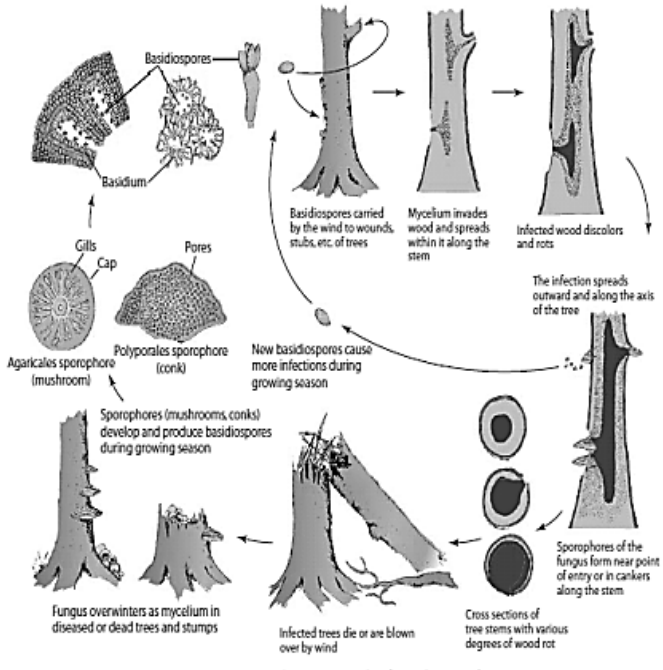

Figure 1.6 Typical disease cycle of wood rot and decay fungi (Agrios, 2005).

Disease Management: Reduce the chance of introducing the fungi into healthy orchards. Avoiding or preventing wounds on the trees. Keep the trees in good vigor through adequate irrigation and proper fertilization. Treatment the large cuts or wounds with a wound dressing or tree paint.

1.2 Fungal Foliar Diseases of Mango

Foliar diseases are any disease above ground and localized in the foliage. They are the most important and visible problems on mango. Since many of the pathogens that caused foliar diseases also affect panicles (Litz, 2009; Ploetz and Freeman, 2009).

a. Grey Leaf Spot Disease

Grey leaf spot or grey blight of mango disease caused by *Pestalotiopsis mangiferae* is prevalent in many mango grown areas.

Symptoms: Initial symptoms are small, yellow-to-brown spots on leaves. Later, the irregularly shaped spots, ranging from a few millimetres to a few centimetres in diameter, turned white to grey and coalesced to form larger grey patches. Lesions had slightly raised dark margins. On mature lesions, numerous black acervuli, may become visible to the naked eye in the central region and more on upper surface of the leaf (Prakash, 2004; Ko *et al*., 2007).

The Causal Organism: *Pestalotiopsis mangiferae* is the main species causes grey leaf spot and stem end rot of mango fruit. Two other species of *Pestalotiopsis* that have been reported on mango are *P. mangifolia* and *P. versicolor* (Litz, 2009).

Disease Cycle: The fungus produces abundant conidia in acervuli that develop in grey leaf spot lesions and necrotic areas on fruit (Lim and Khoo, 1985). *Pestalotiopsis* does not kill the plant wholly but reduces the photosynthesis activity. The fungus is a weak parasite that infects the injured tissues through wounds. Moist conditions are favorable by the fungus and help in increasing infection.

Disease Management: No specific control measures are required. Control methods used to control other foliar disease are usually sufficient and effective to control grey leaf spot disease (Prakash, 2004).

b. **Mango Anthracnose**

Anthracnose is the most important disease on mango. It is affecting plants by killing inflorescences, causing spots on leaves, and, especially, by causing dark brown to black decay spots on fruit when it nears the ripening stage (Agrios, 2005)). Mango anthracnose occurs throughout the tropics and humid production where mangos are grown and is less important than other diseases in dry production area (Litz, 2009; Ploetz and Freeman, 2009). Mango anthracnose is particularly severe and may destroy the total crop as a postharvest disease (Prakash, 2004).

Symptoms: The disease appears as blossom blight, as leaf blight, and, when moisture conditions are favorable, as tree dieback. Blossom blight kills individual flowers or it affects parts of or the complete inflorescence. Infected leaves develop irregular shaped black necrotic spots that often merge and form large necrotic areas. Young twigs may also be invaded and killed, resulting in dieback of twigs. Under wet or very humid conditions, fruit become infected in the field but remain symptomless until the beginning of ripening, which takes place after harvest. Symptoms on fruit are brownish-black lesions on the surface. The lesions form larger dark lesions that cover large areas of the fruit spreading downward from the stem end toward the distal end of the fruit (Campbell, 1992; Litz, 2009).

The Causal Organism: *Colletotrichum gloeosporioides* is a major fungal pathogen of anthracnose on mango and avocado fruits. It causes anthracnose and stem-end rot in these crops but has also been identified as the causal pathogen of pepper spot of avocado and tear stain of mango. *C. gloeosporioides var. minor* and *C. acutatum* have been also reported (Giblin *et al.*, 2010).

Disease Cycle: Mango anthracnose fungi produce abundant conidia on infected leaves, inflorescences, and on mummified aborted fruit. Conidia are spread by splashing rain and cause new infections on leaves, blossoms, and fruits. In the infected fruit, in the field, the fungus remains quiescent until the fruit is harvested and ripening begins. The fungus then becomes activated and the lesions begin to develop and to enlarge. In storage, however, the fungus does not move from one fruit to the next. Conidia of *Colletotrichum* spp. produced on hosts, such as avocado, papaya, banana, and citrus, can also infect and

cause the disease on mango fruit. The fungus could infect mangoes through both pedicel and peel wounds, causing stem end rot and anthracnose in ripe fruit (Dinh *et al.*, 2003). *Colletotrichum gloeosporioides* produces a phytotoxin *in vitro*. The partially purified phytotoxin, likely *colletotrichin*, that caused anthracnose-like symptoms on young mango leaves when they are tested by Jayasankar *et al.* (1999).

Disease Management: The successful management of anthracnose depends on understanding the conditions that promote disease development. Anthracnose is one of several fruit diseases that affect pre- and post-harvest quality (Ploetz, 2003). Pre-harvest control measures of anthracnose include orchard sanitation by removing sources of inoculum altering the time of flowering to ensure that fruit set and development occur during dry conditions; proper irrigation and fertilizer to maintain the tree vigour. Many of the most effective fungicides are systemic and they have selective modes of action but the efficacy might be reduced due to the development of resistance in the targeted pathogens (Lim and Khoo, 1985; Prakash, 2004; Litz, 2009). For post-harvest control measures, regular chemical spray in the field to reduce the latent infection is the most effective method to control the disease (Ploetz, 1994b; Ploetz, 2003; Ploetz and Freeman, 2009). The benzimidazoles are still effective as post-harvest treatments.

c. Mango Malformation Disease (MMD)

Malformation is one of the most economically important diseases of mango (Britz *et al.*, 2002; Kumar *et al.*, 2011). The disease was reported in Asia, Africa, Middle East and America. Although trees are not killed, the disease causes serious losses since malformed inflorescences do not bear fruit (Prakash and Srivastava, 1987).

Symptoms: Mango malformation is characterised by the abnormal development of vegetative shoots and inflorescences. The vegetative form of the disease is observed more frequently on young seedlings, where axillary or apical buds produce misshapen shoots, have shortened internodes and brittle leaves that are significantly smaller than those of healthy plants. Malformed shoots tend to remain compact thus giving rise to a bunchy-top appearance (Britz *et al.*, 2002; Ploetz, 2003; Marasas *et al.*, 2006). Malformed panicles may

also produce dwarfed and distorted leaves. The flowers are unusually enlarged, sterile and do not bear fruit (Kvas *et al*., 2008).

The Causal Organism: Although the disease was first reported over a century ago in India, the aetiology of malformation has been controversial and unwell understood as long as the disease has been recognized. Many factors such as physiological abnormalities, nutritional, hormonal imbalances viral infection, mite (*Aceria mangiferae*) infestation and fungal infections have been suggested as possible causal agents of the disease (Prakash and Srivastava, 1987; Ploetz, 1994a; Britz *et al*., 2002; Ploetz, 2003; Ploetz, 2004; Prakash, 2004; Marasas *et al*., 2006; Litz, 2009; Ploetz and Freeman, 2009; Chakrabarti, 2011). *F. moniliforme* was the first fungus that found to be associated with malformation. The fungus was further identified as *F. moniliforme* var. *subglutinans*. Several *Fusarium* species have been also associated with the disease (Marasas *et al*., 2006). *Fusarium oxysporrum* has been isolated from malformed tissues (Prakash, 2004). Another *Fusarium* species that was described from a small area in South Africa is *F. sterilihyphosum* (Britz *et al*., 2002). *F. mangiferae*, is the only species which has conclusively been shown to cause mango malformation (Britz *et al*., 2002; Marasas *et al*., 2006). *F. mangiferae* has been reported in Egypt, Florida, Israel, Malaysia and South Africa (Britz *et al*., 2002; Marasas *et al*., 2006).

Disease Cycle: The spread of malformation disease relies on the movement of infected nursery stock, but the means by which within-tree and tree-to-tree spread is accomplished is not known. Malformation moves slowly in affected orchards. Conidiospores are probable infective propagules since they form abundantly on dead, malformed tissues. *F. mangiferae* is spread by grafting and in infected nursery stock. *F. mangiferae* has a highly localized distribution within malformed mango trees (Ploetz, 1994a). It predominates in symptomatic tissues, and is found far less frequently, or not all, in asymptomatic organs. This distribution suggests that vegetative and floral buds of mango are the primary sites of infection, and that systemic colonization of older, subtending tissues does not occur (Ploetz, 2004).

Disease Management: Once the disease is found in an orchard, control measures are possible, but more difficult. Cultural practices have been effective to reduce mango malformation. New plantings should be established with pathogen free nursery stock. Scion material should never be taken from an affected orchard (Ploetz, 2004). Nurseries should not be established in orchards that are affected by malformation. All affected terminals and the subtending three nodes are cut from trees, removed from the field and should be burned. Removing symptomatic tissues from trees is usually effective, presumably because it reduces the levels of inoculum in an orchard (Ploetz, 2004). Integrated management program that include the use of tolerant cultivars and treatment with systemic fungicides might be useful for controlling the disease (Ploetz, 1994a;2004; Prakash, 2004).

1.3 Bacterial Diseases of Mango

Some bacterial diseases of mango trees caused by *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Erwinia carotovora* subsp. *carotovora* and *Xanthomonas campestris* pv. *mangiferaeindicae* (Cazorla *et al*., 1998).

a. Mango Bacterial Canker Disease (MBC)

Mango bacterial canker is one of the most important bacterial diseases for mango industries worldwide (Gagnevin and Pruvost, 2001). The disease is also known as bacterial spot, leaf spot, black spot, mango blight, and bacterial black spot. Bacterial black spot is a destructive leaf, stem and fruit disease in many production areas. (Ploetz, 2004; Willis, 2009). It can be the most important disease where fungal-induced diseases are well controlled (Gagnevin and Pruvost, 2001).

Symptoms: The disease was noticed on leaves, leaf stalks, stem, twigs, branches and fruits, initially producing water soaked lesions, later turning into typical canker. Under severe infections, the leaves turn yellow and dropped off, canker on leaf stalks. In some excessive infected fruits crack and become brown in colour, pulp and stones were also infected (Prakash, 2004).

The Causal Organism: The disease is caused by *Xanthomonas campestris* pv. *mangiferae indicae* (Ah-You *et al.*, 2009).

Disease Cycle: Bacterium survives in infected plant parts on the tree. Cankers on mango leaves are reduced by fall of infected leaves but pathogen was found to survive up to 8 months in diseased leaves. Twig canker initiates the infection on fruits (Prakash, 2004).

Diseases Management: Regular inspection of orchards, sanitation and seedling certification are recommended as preventive measures against the disease. In areas with high disease pressure, resistant varieties should be used (Prakash, 2004). Cultural measures, such as the use of wind breaks to reduce wounding and reduce inoculum load from the tree are used effectively to prevent the disease. Treatment with Copper oxychloride (0.3%) was found to be effective in controlling bacterial canker (Prakash, 1994).

b. Apical Necrosis

The apical necrosis is one of the primary factors limiting mango fruit production in many areas. Apical necrosis affects mango crops in subtropical regions such as Spain, Portugal, Italy, Israel and Australia (Kennelly *et al.*, 2007). The disease has produced severe production losses in Southern Europe in some years (Cazorla *et al.*, 2006). Apical necrosis is characterized by a rapid expansion of necrotic lesions on buds and leaves.(Gutiérrez-Barranquero *et al.*, 2012).

Symptoms: Mango buds, leaves, and stems are all susceptible to infection, while fruit lesions have not been observed. Symptoms include necrosis of vegetative and flower buds and bud failure before bud break. Necrotic lesions in buds sometimes extend to the leaf petiole through the stem. Necrotic symptoms on flower panicles are the most severe economic losses because of the decrease in fruit set (Cazorla *et al.*, 1998; Cazorla *et al.*, 2006; Gutiérrez-Barranquero *et al.*, 2012).

Causal Organism: The disease is caused by *Pseudomonas syringae* pv. *syringae* van Hall. The bacterium affects many perennial fruit crops (Kennelly *et al.*, 2007).

Disease Cycle: *P. syringae pv. syringae* causes disease only when the host is stressed. Rain is important for inoculum dissemination to other buds and leaves, and wind facilitates disease development by causing microinjuries (Cazorla *et al.*, 2006).

Diseases Management: The most effective treatment for controlling apical necrosis was the spraying of copper compounds Bordeaux mixture (Cazorla *et al.,* 2006). Treatments with Fosetyl-Al as an alternative to copper compound were also efficient to control the disease (Cazorla *et al.*, 2006).

3. Scope of the thesis

The aim of the research described in this thesis was to gain detailed insight in the impact of fungal soilborne diseases on mango. In the introductory chapter, we gave some basic botanical descriptions and the history of mango industry in Italy. This was followed by short descriptions of various diseases affecting mango by briefly discussing disease symptoms, causal pathogens and control measures.

Chapter 2 deals with survey of soilborne diseases of mango in different production nurseries and orchards located in Sicily. The prevalence of soilborne pathogens and disease incidence were recorded. Morphological characteristics and growth characteristics based methods were used to identify the main soilborne pathogens obtained during the survey.

In **Chapter 3,** the occurrence of Verticillium wilt disease in mango orchards was reported. Fungal characterization and its pathogenicity were further described.

Chapter 4 focuses on characterization and identification of *Phytophthora* species causing the root and crown rot diseases of mango in Sicily. Detailed morphological descriptions of the pathogen are given. This chapter describes also the use of molecular tools based on DNA sequencing to identify the causal agent. The pathogenicity ability of chosen five *Phytophthora* isolates was tested on mango under greenhouse conditions.

In **Chapter 5** and **Chapter 6,** the central theme of the study is the usefulness of molecular tools to assess the diversity of *Phytophthora cryptogea*, the causal pathogen of mango root rot, within worldwide population, and detect its presence with rapid, reliable and selective molecular-based protocols.

Chapter 5 deals with the phylogenetic position and taxonomy of *P. cryptogea*, *P. drechsleri* and *P. erythroseptica* that has long been a matter of controversy. The objectives of this study were to re-assess the status of these taxa using molecular methods based on mitochondrial and nuclear DNA sequences. Two genetic markers were chosen, the DNA

barcoding with cytochrome c oxidase subunit I (COI) and internal transcribed spacer (ITS) region.

In **Chapter 6,** the possibility to set up a molecular approach that can provide an accurate detection of *P. cryptogea* and other close *Phytophthora* species (*P. megasperma* and *P. citrophthora*) was explored. This assay could be used and optimized for detection of the pathogen in plants and soils and finally, a quantitative capability of the assay was evaluated using TaqMan ® real-time PCR systems.

4. Literature Cited

Agrios G. N. (2005). *Plant pathology*. Dana Dreibelbis , Elsevier Academic Press.

Aguín-Casal O., Sáinz-Osés M. and Pedro Mansilla-Vázquez J. (2004). *Armillaria* species infesting vineyards in Northwestern Spain. *European Journal of Plant Pathology*, 110(7): 683-687.

Ah-You N., Gagnevin L. G. P. A., Brisse S., Nesme X., Chiroleu F., Bui Thi Ngoc L., Jouen E., Lefeuvre P., Verniere C. and Pruvost O. (2009). Polyphasic characterization of Xanthomonads pathogenic to members of the Anacardiaceae and their relatedness to species of *Xanthomonas*. *International Journal of Systematic and Evolutionary Microbiology*, 59(2): 306-318.

André LéVesque C. and De Cock A. W. A. M. (2004). Molecular phylogeny and taxonomy of the genus *Pythium*. *Mycological Research*, 108(12): 1363-1383.

Anonymous (2010). La coltivazione del mango in Sicilia. In: *Seminario sullo stato dell'arte e linee guida dell'impianto.* Capo d'Orlando (ME), Villa Piccolo - C.da Forno Alto - Capo d'Orla.Messina.

Baeza-Montanez L., Gomez-Cabrera R. and Garcia-Pedrajas M. D. (2010). First report of Verticillium wilt caused by *Verticillium dahliae* on Mango trees (*Mangifera indica*) in Southern Spain. *Plant Disease*, 94(3): 380-381.

Britz H., Steenkamp E. T., Coutinho T. A., Wingfield B. D., Marasas W. F. and Wingfield M. J. (2002). Two new species of *Fusarium* section Liseola associated with mango malformation. *Mycologia*, 94(4): 722-730.

Cazorla F., Arrebola E., Olea F., Velasco L., Hermoso J., Pérez-García A., Torés J., Farré J. and Vicente A. ((2006). Field evaluation of treatments for the control of the bacterial apical necrosis of mango (*Mangifera indica*) caused by *Pseudomonas syringae* pv. *syringae. European Journal of Plant Pathology,* 116(4): 279-288.

Cazorla F. M. T. J. A., Olalla L., Perez-Garcia A., Farre J. M. and de Vicente A. (1998). Bacterial Apical Necrosis of Mango in Southern Spain: A Disease Caused by *Pseudomonas syringae* pv. *syringae. Phytopathology* 88(7): 614-620.

Chen F. M. N. P. D., M. L., F. G., -B. (2010). *Handbook of Fruit and Vegetable*. FlavorsWiley.

Chin D., Condé B., M N., Hamilto D., Hoult M., Moore C., Thistleton B., Ulyatt L. and L. Z. (2010). *Field Guide to Pests, Beneficials, Diseases and Disorders of Mangoes Northern Territory Government.* Department of Resources. *AUSTRALIA.*

Bally, I.S.E. 2006. *Mangifera indica* (mango), ver. 3.1. In: Elevitch, C.R. (ed.). *Species Profiles for Pacific*

Island Agroforestry. Permanent Agriculture Resources (PAR), Hōlualoa, Hawai'i.

Dinh S.-Q., Chongwungse J., Pongam P. and Sangchote S. (2003). Fruit infection by *Colletotrichum gloeosporioides* and anthracnose resistance of some mango cultivars in Thailand. *Australasian Plant Pathology*, 32(4): 533-538.

Erwin D. C. and Ribeiro O. K. (1996). *Phytophthora diseases worldwide.* APS Press.

FAOSTAT (2011). FAO Statistics Food and Agriculture Organization of the United Nations. Rome.

Fradin E. F. and Thomma B. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology*, 7(2): 71-86.

Gagnevin L. and and Pruvost O. (2001). Epidemiology and control of mango bacterial black spot. *Plant Disease*, 85(9): 928-935.

Giblin F. R., Coates L. M. and Irwin J. A. G. (2010). Pathogenic diversity of avocado and mango isolates of *Colletotrichum gloeosporioides* causing anthracnose and pepper spot in Australia. *Australasian Plant Pathology*, 39(1): 50-62.

Gutiérrez-Barranquero J. A., Arrebola E., Bonilla N., Sarmiento D., Cazorla F. M. and de Vicente A. (2012). Environmentally friendly treatment alternatives to Bordeaux mixture for controlling bacterial apical necrosis (BAN) of mango. *Plant Pathology*, 61(4): 665-676.

Campbell R. J., Campbell C. W.(1992). *A guide to mangos in Florida*, Fairchild Tropical Botanic Garden. Miami, Florida, USA.

Chakrabarti D. K. (2011). Chronological History of Mango Malformation. In: Chakrabarti, D. K.(ed.) *Mango Malformation*. Springer, Netherlands, pp. 1-9.

Jayasankar S., Litz R., Gray D. and Moon P. (1999). Responses of embryogenic mango cultures and seedling bioassays to a partially purified phytotoxin produced by a mango leaf isolate of *Colletotrichum gloeosporioides* penz. *In Vitro Cellular & Developmental Biology*, 35(6): 475-479.

Kennelly M. M., Cazorla F. M., de Vicente A., Ramos C. and Sundin G. W. (2007). *Pseudomonas syringae* diseases of fruit trees: Progress toward understanding and control. *Plant Disease*, 91(1): 4-17.

Klosterman S. J., Atallah Z. K., Vallad G. E. and Subbarao K. V. (2009). Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology*, 47(1): 39-62.

Ko Y., Yao K. S., Chen C. Y. and Lin C. H. (2007). First Report of Gray Leaf Spot of Mango (*Mangifera indica*) caused by *Pestalotiopsis mangiferae* in Taiwan. *Plant Disease*, 91(12):

1684-1684. .

Kumar P. and Modi D. R. (2011). Current status of mango malformation in India. *Asian Journal of Plant Sciences*, 10(1): 1-23.

Kvas M., Steenkamp E. T., Adawi A. O., Deadman M. L., Jahwari A. A., Marasas W. F. O., Wingfield B. D., Ploetz R. C. and Wingfield M. J. (2008). *Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. *European Journal of Plant Pathology*, 121(2): 195-199.

Leslie J. F., Summerell B. A. and Bullock S. (2006). *The Fusarium Laboratory Manual*. John Wiley & Sons.

Lim T. K. and Khoo K. C. (1985). *Diseases and disorders of mango in Malaysia*. Tropical Press.

Litz R. E. (2009). *The Mango: Botany, Production and Uses*. CABI Direct, Wallingford. UK.

Marasas W. F., Ploetz R. C., Wingfield M. J., Wingfield B. D. and Steenkamp E. T. (2006). Mango malformation disease and the associated *Fusarium* species. *Phytopathology* 96(6): 667-672.

Marlatt R. B., Knight R. J., Jr. and Goldweber S. (1970). Verticillium wilt of mango (*Mangifera indica*) in Florida. *Plant Disease Reporter* 54: 569-571.

Matheron M. E. and Matejka J. C. (1988). Phytophthora crown and root rot of nursery grown mango trees delivered to Artizona (Abs.). *Phytopathology*, 78: 1572. .

Matsushita N. and Suzuki K. (2005). Identification of *Armillaria* species in Japan using PCR-RFLP analysis of rDNA intergenic spacer region and comparisons of *Armillaria* species in the world. *Journal of Forest Research*, 10(3): 173-179.

Naqvi S. A. M. H. (2004). Diseases of fruits and vegetables. In: *Diagnosis and management*, Academic K., New York.

Paull R. E. and Duarte O. (2010). Tropical Fruits. *Crop Production Science in Horticulture*, 1(20 CABI.)

Peña J. E., Mohyuddin A. I. and Wysoki M. (1998). A review of the pest management situation in mango agroecosystems. *Phytoparasitica*, 26 (2): 129-148.

Ploetz R. C. (1994a). Distribution and prevalence of *Fusarium subglutinans* in mango trees affected by malformation. *Canadian Journal of Botany*, 72 (1): 7-9.

Ploetz R. C. (2004). Strategies and potential for sustainable management the major diseases of mango. In: Pinto A. C. Q., Pereira M. E. C. and Alves R. E., *Acta Horticulturae* 137-150.

Ploetz R. C. (2003). *Diseases of Mango Diseases of Tropical Fruit Crops*. Wallingford, UK., CABI Publishing.

Ploetz R. C. and Freeman S. (2009). Foliar, floral and soilborne diseases. In: *The Mango: Botany, production and uses*. CABI.

Ploetz R. C., Zentmyer, G. A. N., W. T., , Rohrbach K. G. and Ohr H. D. (1994b). *Compendium of Tropical Fruit Diseases*. The American Phytopathological Society, St. Paul, MN.

Popoola T. O. S. and (2004). Problems of Armillaria root and Butt rot in tropical Africa: The case of Nigerian forests. In: College of Natural Sciences Proceedings F. U. O. A. (eds). *Proceedings of the International Conference on Science & National Development.* Abeokuta, Nigeria.

Prakash O. (2004). Diseases and Disorders of Mango and their Management. In: Naqvi S. A. M. H (ed). *Diseases of Fruits and Vegetables*, Netherlands S., pp. 511-619.

Prakash O., Misra A. K. and Raoof M. A. (1994). Studies on mango bacterial canker disease *Bio. Memoirs*, 20: 95-107.

Prakash O. and Srivastava K. C. (1987). Mango diseases and their management. In: *A world review.* Today & Tomorrow's Printers and Publishers.

Prakash O. m. and Singh U. N. (1980). Root rot and damping off of mango seedling caused by *Rhizoctonia solani*. *Indian Journal of Mycology and Plant Pathology*, 10(1): 69.

Sanei S. J., Waliyar F., Razavi S. I. and Okhovvat S. M. (2008). Vegetative compatibility, host range and pathogenicity of *Verticillium dahliae* isolates in Iran. *International Journal of Plant Production*, 2(1): 37-45.

Saran P. L. and Kumar R. (2011). Boron deficiency disorders in mango (*Mangifera indica*): field screening, nutrient composition and amelioration by boron application. *Indian Journal of Agricultural Sciences*, 81(6): 506-510.

Sharma I. M., Badiyala S. D. and Sharma N. K. (1993). Effect of fungicidal drenching against wilt of mango seedlings caused by *Fusarium solani*. *Indian Journal of Mycology and Plant Pathology*, 23(326-327).

Singh L. B. (1960). *The Mango. Botany, cultivation, and utilization*. World crop series, Martnus Nijhoff, the Hauge, Netherland.

Sneh B., Burpee L. and Ogoshi A. (1991). *Identification of Rhizoctonia species*. APS Press.

Spies C., Mazzola M. and McLeod A. (2011). Characterisation and detection of *Pythium* and

Phytophthora species associated with grapevines in South Africa. *European Journal of Plant Pathology*, 131(1): 103-119.

Tewoldemedhin Y., Mazzola M., Botha W., Spies C. and McLeod A. (2011). Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. *European Journal of Plant Pathology*, 130(2): 215-229.

Tsao P. H., Luzaran P. B., Delossantos A. B., Portales L. A., Gochangco A. M. and Gruber L. C. (1994). Phytophthora crown and root-rot of mango detected in Philippine nurseries. *Plant Disease*, 78(1): 100-100.

Willis A. (2009). Evaluation of alternatives to copper oxychloride for the control of bacterial black spot and post -harvest diseases in mango. In: A. O. S. (ed) *Acta Horticulturae*, pp. 535-540.

Zea-Bonilla T., Martín-Sánchez P. M., Hermoso J. M., Carmona M. P., Segundo E. and Pérez-Jimenéz R. M. (2007). First report of *Phytophthora citricola* on *Mangifera indica* in Spain. . *Plant Pathology*, 56 2): 356-356.

CHAPTER 2

2. SURVEY OF SOILBORNE DISEASES OF MANGO IN SICILY (SOUTHERN ITALY)

1. Abstract

Mango (*Mangifera indica* Linn.) is a promising crop in Sicily thanks to the favorable climatic and soil conditions. Few years ago, mango cultivation was introduced in the island with limited extensions, mainly in the provinces of Palermo, Ragusa, Messina, Agrigento and Catania. Kensington was the main variety obtained by locally produced seeds, probably originated from Australia. In the near future, it is expected that commercial and backyard plantings of mango trees will increase, being a profitable crop. Due to the unknown phytosanitary status of this crop in Sicily, surveys were conducted over Summer and Spring (2010-2011) in different mango orchards located in five provinces (Palermo, Messina, Catania, Agrigento and Ragusa. The aim of the study was to assess the occurrence of the fungal soilborne diseases and their causal agents. Several symptoms induced by soilborne pathogens were reported in all the investigated orchards. Typical symptoms of damping off, root rot, crown rot, Verticllium wilt, Armillaria root rot and wood decays diseases were observed. Samples were collected from symptomatic plant tissues in mango nursery and orchards then isolation was made on different media. Morphological and molecular identification of the isolated fungi and oomycetes showed that they belong to different genera. *Rhizoctonia solani* (50%), *Fusarium* spp. (33%), *Pythium* spp. (10.69%) were found to be the most common pathogens associated with damping off and root diseases of mango in the nursery. In the surveyed mango groves, the percentage of disease incidence of black root rot diseases was the highest in Ragusa. Armillaria rot disease was reported in three provinces (Catania, Palermo and Messina). The current work reports for the first time the occurrence of these fungi in mango groves in Italy.

Keywords: Mango, damping off, root rot, Armillaria rot, wood decay

2. Introduction

Mango is an important fruit crop in many tropical and subtropical countries (Litz, 2009; Ploetz and Freeman, 2009). Mango suffers from several diseases at all stages of its life. Although soilborne diseases of mango are relatively less important than foliar and floral diseases, they can cause significant damage to seedlings, nursery stock and mature trees (Ploetz and Freeman, 2009). Several soilborne pathogens have been shown to adversely affect mango root systems world-wide, including fungal genera such as *Fusarium*, *Rhizoctonia*, *Verticillium* and the oomycetes *Pythium* and *Phytophthora* (Prakash and Singh, 1980; Tsao *et al.,* 1994; Prakash, 2004). In Malaysia, *Pythium vexans* de Bary, has been reported as the causal pathogen of mango root rot and wilt of seedlings (Lim and Khoo, 1985). In Indonesia and India, damping off on mango seedlings has been reported as serious problem in nurseries (Prakash and Singh, 1980).

In order to manage the soilborne pathogens, it is essential to raise or select pathogen free plant material to avoid the introduction of possibly devastating diseases. Good drainage and aeration of soil should be maintained for healthy and adequate growth of root system. Resistant and compatible rootstock should always be used considering the diseases prevalent in the locality.

Correct diagnosis and regular monitoring of the diseases are required for their quick and economically promising management. Predisposing factors for disease development should be minimized or eliminated to control the diseases.

Mango cultivation in Italy is relatively new and no assessment of the pathological problems has been carried before. Because the phytosainatry status of the trees is still unknown, our study aimed to survey and monitor mango plantations in different growing areas in Sicily in order to identify the causal agents associated with soilborne diseases symptoms and signs. This information could help to understand the ecology of those pathogens and the influence of chemical, physical, and biological soil factors upon disease development in nurseries and also they can be useful for proper timing of control measure application.

3. Materials and methods

Survey locations

A survey was conducted in Sicily over summer and spring (2010- 2011) in different mango orchards located in five provinces (Palermo, Messina, Catania, Agrigento and Ragusa), to assess the occurrence of the fungal soilborne diseases and their relative causal agents (Fig. 2.1). The survey covered 2 to 6 orchards per province (except one orchard in Ragusa).

Sampling method

In each orchard several symptomatic trees were investigated for fungal diseases. Samples were collected from different plant organs (feeder roots, roots, bark, stem) and from the soil. Then samples were placed in marked bags and were used for analysis.

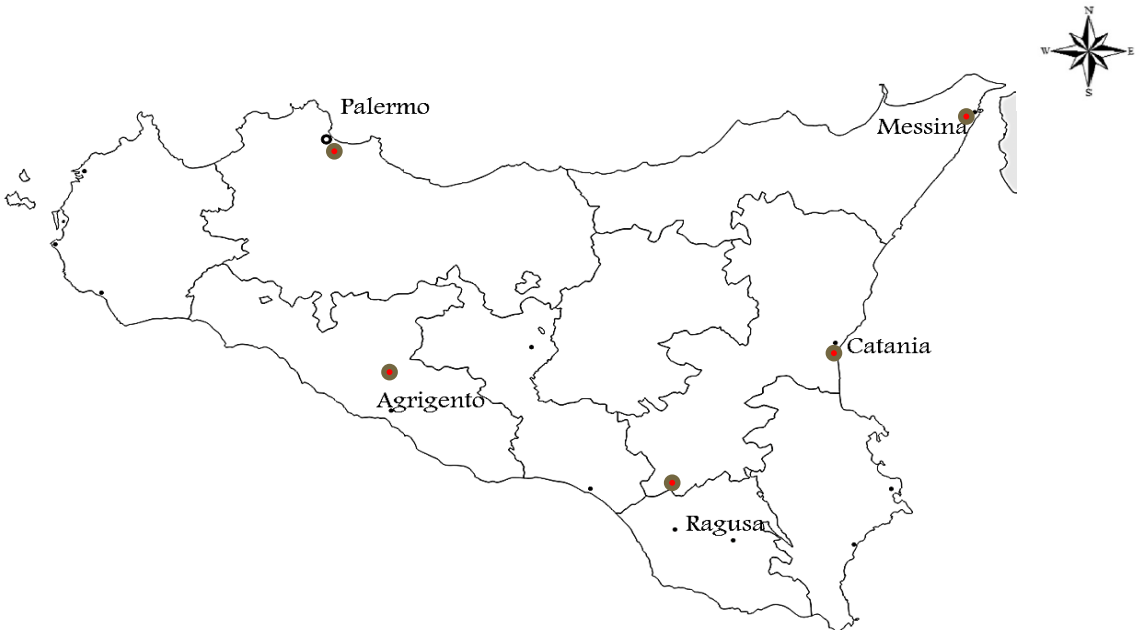

Figure 2.1 Map of Sicily (Southern Italy) showing the geographical locations of the surveyed orchards (Palermo, Messina, Ragusa, Agrigento and Catania).

Fungal isolation from roots

The isolation from infected tissues was carried out using semi selective Potato Dextrose Agar (PDA) amended with 250 mg of ampicillin and 250 mg of streptomycin sulfate per 1 liter. The tissues were surface disinfected with 1% sodium hypochlorite to reduce contamination. Then the tissue was cut into small pieces and placed on the medium. Two Petri-dishes were used for each sample and all isolation plates were incubated at 25°C for 5 -7 days. Fungal and oomycete growth coming out of the root pieces was subcultured into PDA plates.

Fungal isolation from woods

Tissues were removed in the laboratory from portions of mycelial fans and decayed wood that were previously not exposed. Small pieces were placed on PDA and/or Malt Extract Agar (MEA) medium. The plates were incubated at 27°C for 7 days then the colonies were transferred into a new PDA plates.

Fungal purification

To obtain purified culture, conidia were separated by dilution plating and the spores allowed germinating overnight. The mycelium of the germinated spores was excised with a small sterile wire mounted in a standard needle holder, and transferred to the target medium. All single-spored of *Fusarium* and hyphal-tipped of *Pythium* and *Rhizoctonia* cultures were stored for subsequent species identification. Pure cultures of *Fusarium* spp. were stored in 15% glycerol at −85°C while all *Rhizoctonia* isolates were stored on PDA slants at 4°C. Oomycetes were stored on PDA at 15°C and on PDA slants at 15°C.

Fungal frequency and disease incidence

The isolation frequencies of soilborne pathogens in mango tissues and disease incidence in the field were calculated according to Zak and Willig (2004) using the following formula:

$$\text{Relative frequency (\%)} = \frac{\text{Number of isolates for each species}}{\text{Total number of isolates}} \times 100$$

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of plants assessed}} \times 100$$

Morphological characterization of the isolated fungi and oomycetes

Fungal cultures were sub-cultured onto water agar (WA) and identified to the genus level. Isolates were identified as *Fusarium* according to Leslie *et al.* (2006), as *Pythium* according to Dick (1990), and as *Rhizoctonia* according to Sneh *et al.* (1991).

Molecular characterization of the isolated fungi and oomycetes

Representative isolates of soilborne pathogens collected from mango nursery and orchards were submitted for molecular identification.

Growing mycelium for DNA extraction

The purified isolates were grown in Petri dishes containing Malt Extract Agar (MEA) covered with sterile cellophane sheets to facilitate collection of the mycelium. Inoculation of each fungal isolate was carried out using a 5 mm mycelial plug taken from the edge of actively growing colony on PDA.

DNA extraction

DNA was extracted using the method of Hoffman and Winston (1987). Genomic DNA was obtained from fungal mycelium (0.3-0.5 g), suspended in 400 µl of breaking buffer (2% Triton X-100; 1% sodium dodecyl sulfate; 100 mM NaCl; 10 mM Tris-HCl, pH 8; 1mM EDTA), and extracted with 400 µl of phenol/chloroform/ isoamyl alcohol (25:24:1) in presence of acid-washed glass beads (425-600 µm diameter) and 2 iron balls (5 mm diameter). The eppendorf tubes were vortexed at 3000 rpm for 10 min and centrifuged at 13000 rpm for 10 min. The supernatant was extracted twice with an equal volume of chloroform/ isoamyl alcohol (24:1), 200 µl of phenol and 200 µl chloroform/isoamyl alcohol, respectively. Tubes were vortexed again and centrifuged under the same conditions. DNA was precipitated with 2 vol of cold isopropanol (-20°C) and 1/10 vol of sodium acetate (3M, pH 5.2). Then, DNA was washed with 70% ethanol (-20°C), dried (under vacuum) for 20 min, and re-suspended in 50 µl of nuclease free water. DNA was quantified by spectrophotometer, diluted to 50 ng/µl and stored at -20°C.

PCR amplification and sequencing of oomycetes and fungi

Isolates that were morphologically classified as belonging to the genera *Pythium* and other fungi were identified to the species level using sequencing data from the internal transcribed spacer (ITS) 1, ITS2 and the 5.8 S genes. The species identity of *Fusarium* isolates was conducted using partial beta tubulin gene sequence data. The ITS regions and

5.8 S gene were amplified using primers ITS6 and ITS4 (Cooke *et al.*, 2000a). The *β*-tubulin was amplified from *Fusarium* isolates using primers T-1 and T-2 (Lees *et al.*, 2012).

Primers used in this study, along with annealing temperature and references, are listed in Table 2.1. The PCR reaction was done in C-1000 Thermal cycler (Bio Rad, USA). Reactions were performed in a 25-µl mixture final volume. Each reaction contained 1 µl of 50 ng/µl of genomic DNA, 5 µl of 5x PCR buffer, 15.3 ml of sterile distilled water, 1µl of DNTPs buffer, 1.5 µl of 25 mM MgCl2, 0.5 µl each of forward and reverse primers and 0.2 µl of GoTaq® DNA polymerase (5 U/µl Promega, Madison WI-USA). The thermal cycling conditions for ITS region were initial denaturation step at 94°C for 3 min followed by 35 cycles consisting of denaturation step at 94°C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 30 sec. A final extension at 72°C for 10 min was done at the end of the amplification. For *Fusarium* amplification, PCR was performed using reagents described above, and following the program of : 5 min initial denaturing at 94°C, followed by 35 cycles of 30 sec. at 94°C, 45 sec. annealing at 61°C, 1 min at 72°C, and 7 min final extension at 72°C. Negative controls (no template) were used in each experiment to test for the presence of contaminant DNA in the reagents. Amplicons were separated by electrophoresis in 1 % agarose gels in 1X TAE buffer (0.04M Tris-acetate and mM EDTA) at 100V for 1 h, and stained for 10 min with ethidium bromide (2µg ml-1), and analysed under UV light. PCR products were sent to the Primm SR1 sequencing service and fungal isolates were identified by BLAST the sequence in NCBI genBank.

Table 2.1 Primers used for PCR amplification and sequencing

Target DNA	Primer	Primer sequence 5'- 3'	Temp.[a]	Reference
ITSs 1, 2 and 5.8S gene of rDNA	ITS6 forward	5' GAAGGTGAAGTCGTAACAAGG 3'	51	(Cooke *et al.*, 2000a)
	ITS4 reverse	5' TCCTCCGCTTATTGATATGC 3'		
β-tubulin	T1	5′-AACATGCGTGAGATTGTAAGT-3′	61	(Lees *et al.*, 2012)
	T2	5′-TAGTGACCCTTGGCCCAGTTG-3′		

[a]= Annealing temperature used for PCR amplification

4. Results

Survey and monitoring of soilborne pathogens in mango nurseries

Only one mango nursery was present in Sicily during the survey period. The nursery was located in commune Fiumefreddo (Province of Catania) and it was established in 1990, and was the first one to introduce the mango industry in Sicily. It is mainly engaged in production of mango plants and fruits. The main mango variety was Kensington which obtained by locally produced seeds. General symptoms of damping off and root rot were observed in the investigated nursery.

Damping off disease was characterized on the young seedling by sudden dropping of leaves after the emergence of seedlings from the soil. The basal part of the seedling stem was softer and much thinner than the uninvaded parts above it. The infected tissues were dark brown and the entire seedling wilted, collapsed and dead (Fig. 2.2 A,B).

Infection by Pythium damping off disease was characterized with wet odorless rots. The lower portion of the stem became slimy and black. The soft rotted outer portion of the root easily separated from the inner core.

Infected seedlings by Rhizoctonia damping off disease showed slightly sunken lesions and circular to irregular water-soaked patches on the stem. In advanced infection these patches enlarged and ultimately girdled the entire base of the stem.

Root rot diseases were also associated with the seedlings and the symptoms of wilting of foliage, which initially becomes pale green, but later develops necrotic patches. Roots developed blackened necrosis that begins in fine roots and progresses to larger roots and the root collar (Fig. 2.2C).

Figure 2.2 Damping off and root rot symptoms observed in the mango nursery. A and B, general wilt, collapse and death symptoms on young seedlings. C, destruction of young seedlings roots by root rot fungi.

Frequency of fungi associated with soilborne pathogens in mango nursery

The survey revealed that seven fungal genera were isolated from diseased mango plants in the nursery (Fig. 2.3). The isolated fungi were purified and identified as *Rhizopus* sp., *Alternaria* sp., *Aspergillius* sp., *Fusarium* sp., *Rhizoctonia solani* and *Pythium* sp. The highest number of frequency was recorded by *R. solani* while *Pythium* sp. was the highest one collected from both soil followed by the samples. No *Phytophthora* propagules were obtained from the soil or from the roots.

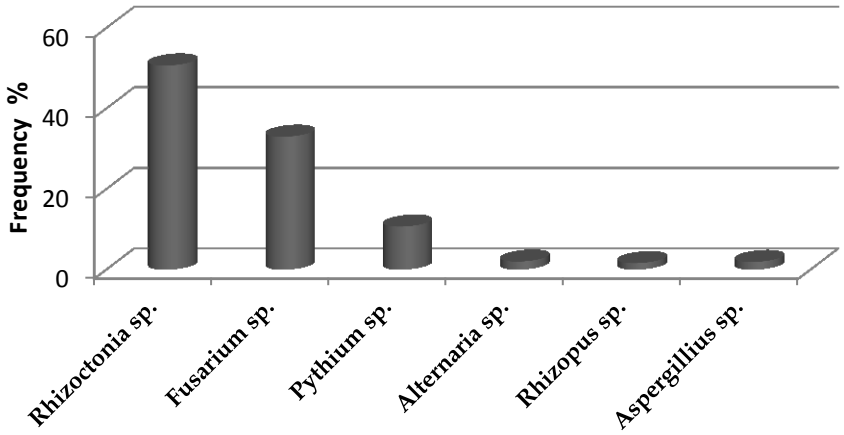

Figure 2.3 The frequency (%) of fungi recovered from mango roots in the nursery

Survey and monitoring of soilborne pathogens in mango orchards

During summer 2010 and spring 2011, survey was carried in Sicily on different mango orchards in five different provinces in Sicily (Palermo, Messina, Catania, Agrigento and Ragusa). A total of 15 orchards and one nursery were examined (Fig. 2.4). Based on our observations, cv. Kensington Pride variety was the main mango varieties in most of monitored orchards. Several symptoms types induced by soilborne pathogens were observed on roots and trunks of mango trees. The main diseases associated with mango trees were: Black root rot, Armillaria rot, wood decay, Verticillium wilt and Phytophthora root rot.

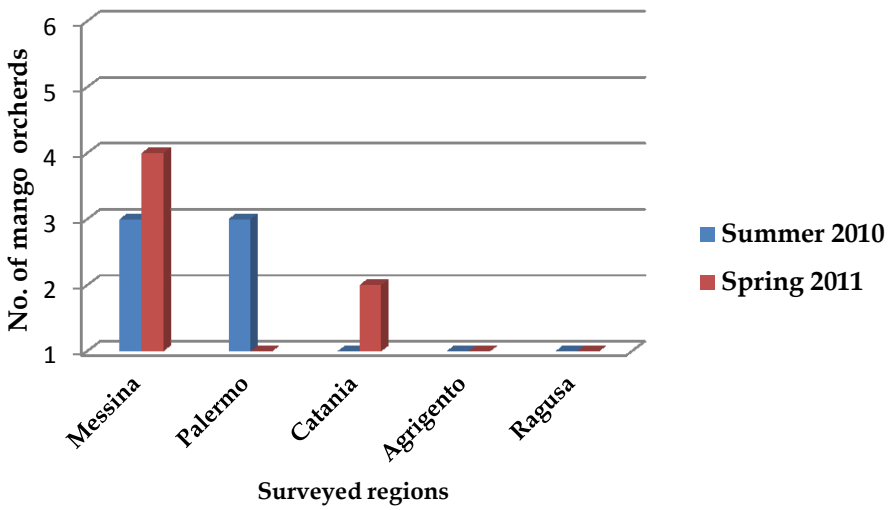

Figure 2.4 Number of mango surveyed orchards surveyed during summer 2010 and spring 2011 in five regions in Sicily.

The occurrence of root rots diseases in mango orchards

Root rot diseases were found in all surveyed orchards. Symptoms observed on the young infected plants were wilt, defoliation and roots exhibit a water-soaked, blackened decay (Fig. 2.5), with an unpleasant rotten odor. Several species of fungi were recovered from the infected plants, including *Fusarium, Rhizoctonia, Phytophthora* and *Pythium* species.

The occurrence of wood decay diseases in mango orchards

Gandoderma fungus can invade trees through wounds and is capable of attacking living trees causing white rot as extensive decay of roots and the trunk. The fungus conks (Fig. 2.6), appeared at the base of the trunk during Summer. The fungus was found in low frequency in mango orchard located in Palermo.

The occurrence of Armillaria root rot diseases in mango orchards

Generally symptoms observed on mango were, the foliage thins and discolor, turning yellow, then brown; branches die back; and reduction of shoot and foliar growth. When Armillaria was present, removing the bark covering infections exposed the characteristic, white mycelial mats or the rhizomorphs that grow between the wood and the bark (Fig. 2.7). The white mycelial mats were marked by irregular fanlike striations of the mycelium. In some areas trees mortality was observed.

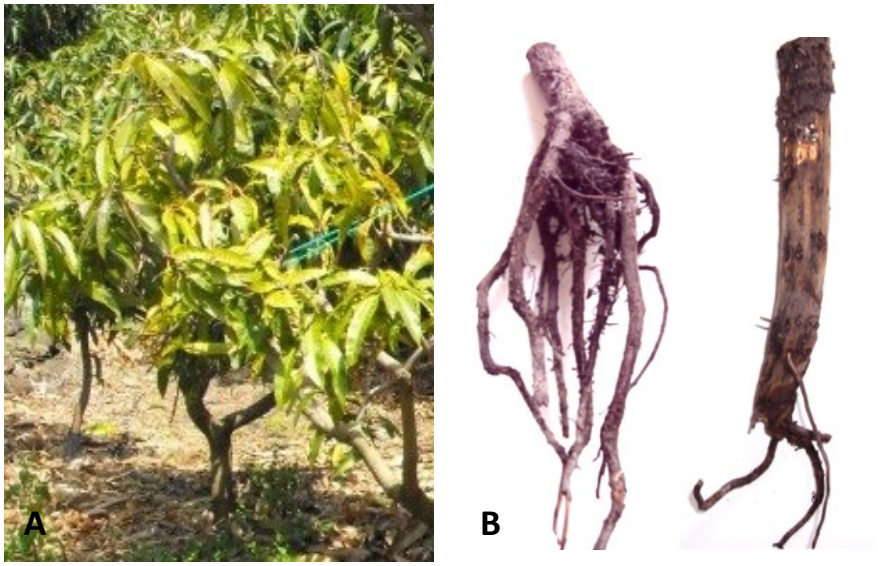

Figure 2.5 Symptoms of black root diseases observed on mango trees in the field. A, symptoms of stunting and foliage yellowing appear on the canopy. B, symptoms of root rot and decay on mango roots

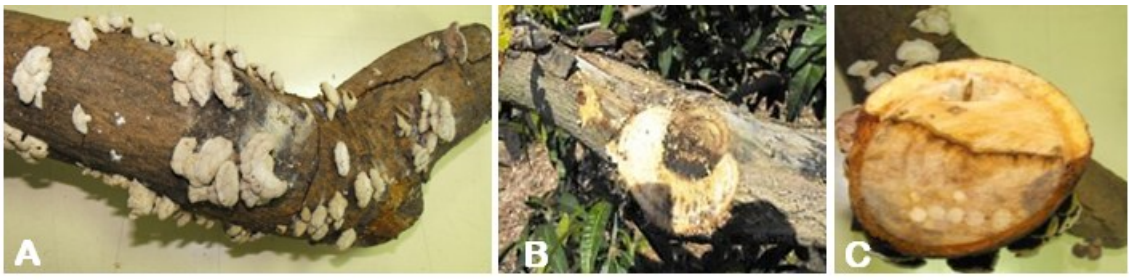

Figure 2.6 Symptoms of wood rot and decay diseases observed on mango trees in the fields. A, a limb extensively invaded with wood rotting fungi. B and C, wood discoloration.

Figure 2.7 Symptoms of Armillaria root diseases observed on mango trees in fields. A, overall appearance of the tree infected with Armillaria rot. B, symptoms of yellowing and wilted foliage associated with rot on the trunk. C, mortality and death of the entire mango tree. D, rot and decay on mango trunk. E, outer bark removed from tissues of an infected mango tree to reveal the presence of mycelial sheets of *Armillaria*. F, severe infection by *Armillaria* with symptoms extended to mango limbs. G, H and I, stumps and roots of mango tree showing mycelial sheets with typical *Armillaria* infection.

Disease incidence

Data presented in Table 2.2 revealed that root rot, Phytophthora rot, Verticillium wilt, Armillaria rot, and fungal wood decay were recorded in mango orchards in Sicily. Root rot diseases were found in all surveyed orchards during the two surveys and the high disease incidence was recorded in Ragusa. Meanwhile Phytophthora rot was only found in province of Messina during the first survey (Summer 2010). Verticillium wilt disease was only recorded in the orchard located in Catania during the two times of survey. Wood decay diseases were prevalent in all the monitored orchards with exception of Ragusa

Fungal frequency and fungal occurrence

The obtained results in Table 2.3 and Fig. 2.8, showed the frequency occurrence of different fungi and oomycetes isolated from assayed mango samples. The frequency (%) varied according to the location and the survey time. The root fungal pathogens (*e.g. Pythium* spp., *Fusarium* sp., *Rhizoctonia solani* and *Phytophthora* sp.), were recorded in high frequency comparing with other fungal genera. The genus *Fusarium* represented the highest records followed by the genus *Rhizoctonia, Phytophthora* and *Verticillium*. Meanwhile, the pathogens referred to genus *Ganoderma* and *Diaporthe* were represented in a lesser frequency.

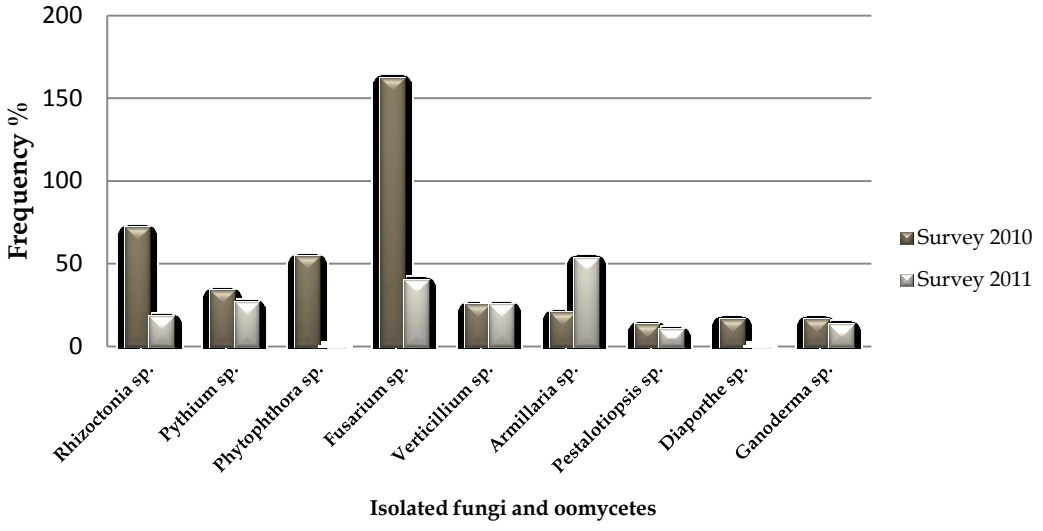

Figure 2.8 The frequency (%) of fungi and oomycetes isolated from mango tissues.

Table 2.2 Disease incidence (%) of soilborne diseases occurred in mango orchards located in five Sicilian provinces during two survey periods

Disease	Diseases incidence (%)[a]						
	Survey 2010					Survey 2011	
	Ragusa	Messina	Palermo	Agrigento	Catania	Catania	Messina
Root rot	80	14.28	58.3	62.5	50	40	42.8
Phytophthora rot	0.0	71.43	25	0.0	0.0	0.0	0.0
Verticillium wilt	0.0	0.0	0.0	0.0	20	30	0.0
Armillaria rot	20	0.0	0.0	0.0	15	10	28.57
Wood decay	0.0	7.41	16.6	25	5	10	18.28
Others	0.0	7.41	8.3	21.5	10	10	10.28

[a] Disease incidence calculated as follows: Number of infected plants/the total number of plants assessed x 100.

Table 2.3 Frequency (%) of fungi and oomycetes occurred in mango orchards located in five provinces in Sicily during two survey periods

Survey	Province	% Frequency and occurrence of fungi and oomycetes [a]									
		Rhizoctonia **sp.**	*Pythium* **sp.**	*Phytophthora* **sp.**	*Fusarium* **spp.**	*Verticillium* **sp.**	*Armillaria* **sp.**	*Pestalotiopsis* **spp.**	*Diaporthe* **sp.**	*Ganoderma* **sp.**	**Others** [b]
Summer 2010	Ragusa	28.57	0.0	0.0	57.1	0.0	14.3	0.0	0.0	0.0	0.0
	Messina	0.0	0.0	41.6	25.0	0.0	0.0	0.0	16.6	16.6	0.0
	Palermo	12.5	24.5	12.5	12.5	0.0	0.0	0.0	0.0	0.0	38.0
	Agrigento	18.18	9.0	0.0	36.3	0.0	0.0	9.0	0.0	0.0	27.2
	Catania	12.5	0.0	0.0	31.25	25.0	6.25	4.25	0.0	0.0	20.75
Spring 2011	Messina	8.0	16.6	0.0	25.0	0.0	33.33	0.0	0.0	8.33	8.33
	Catania	10.0	10.0	0.0	15.0	25.0	20.0	10.0	0.0	5.0	5.0

[a] Frequency % calculated as follows: Number of isolates for each species/the total number of isolates x 100.

[b] Others fungi were recovered from mango tissues in very low frequency such as *Penicillium*, *Aspergillus*, *Alternaria*, *Trichoderma*, *Cylindrocladium* and *Gliocladium*.

Morphological characterization of the isolated fungi and oomycetes

- ***Pythium* sp.**

Isolates produced colonies with indistinct patterns on PDA (Fig. 2.9). The sporangia were wide, globose and sometimes pyriform. The sexual structures were observed on WA media after 3 days of incubation. Oogonia were globose, smooth, terminal or intercalary and oospores were aplerotic.

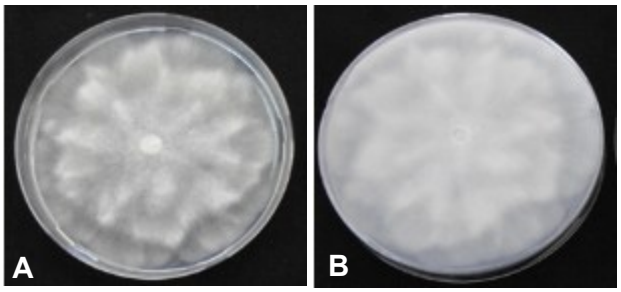

Figure 2.9 Colony morphology of *Pythium* sp. on PDA after 7 days at 25°C. A, colony from the front side; B, reverse colony

- ***Rhizoctonia solani***

R. solani were isolated from mango nursery and orchards and identified based on colony morphology and mycelium characteristics (Fig. 2.10A,B). Mycelium was colorless when young then turned yellowish to light brown with age of the colony.

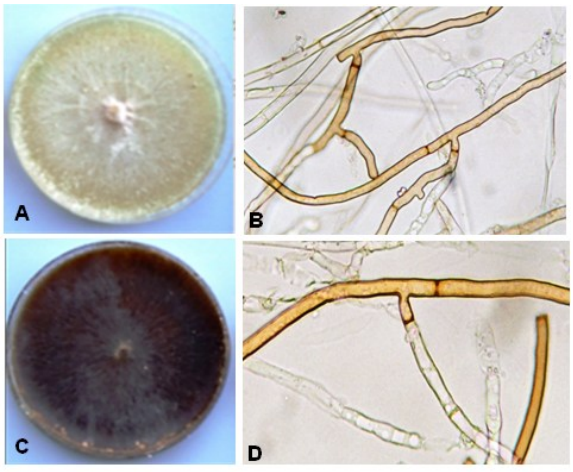

Figure 2.10 Morphological characteristics of *R. solani*. A, C, colony morphology of two *R. solani* isolates on PDA after 7 days at 25°C.; B and D, typical *Rhizoctonia* mycelium showing its branching at a right angle and septa close to the branching point.

- ***Fusarium* spp.**

Morphological characters were observed on PDA and carnation leaf agar (CLA) media after 7 days of incubation at 25°C. Twenty isolates of *Fusarium* were identified morphologically based on conidia and conidiophores characteristics.

F. solani colonies varied on PDA form white to cream with sparse mycelium (Fig. 2.11 A,B). Sporodochia abundantly produced with cream, blue and green color. On CLA media, macroconidia were abundant straight to slightly curved, 3 to 7-septate with rounded ends. Microconidia were oval to ellipsoidal, 0- or 1-septate and formed in round false heads on long monophialides (Fig. 2.11C,E). Chlamydospores were produced abundantly in pairs in hyphae (Fig. 2.11G).

F. oxysporum colonies on PDA varied widely in most of the isolates. Some isolates of *F. oxysporum* produced a pale to dark violet or dark pigment in the agar (Fig. 2.12A,B), while some isolates didn't produce any pigment. Mycellium color ranged from white to light violet. Abundant pale orange or violet macroconidia were produced in a central spore mass. Small pale brown, blue to blue-black or violet sclerotia were formed abundantly by some isolates.

On CLA, macroconidia were formed abundantly in sporodochia in few isolates and were rare in other isolates. Macroconidia were short to medium in length, almost straight, with thin wall and usually 3-septate. The apical cell was short and slightly hooked in some isolates. And the basal cell was notched or foot-shaped (Fig. 2.12D). Microconidia were 0-septate and formed abundantly in false heads on short monophialides (Fig. 2.12E). Chlamydospores formed abundantly in hyphae (Fig. 2.12F).

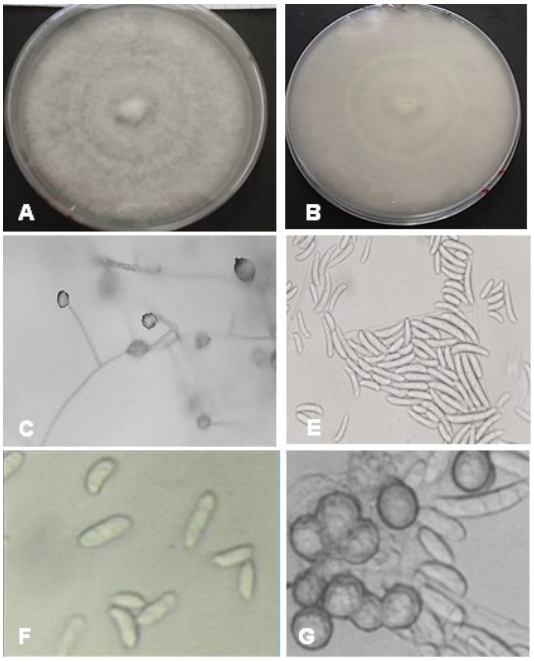

Figure 2.12 Morphological characteristics of *F. solani*. A, colony morphology from the front side; B, reverse side; C, long conidiophore; E, macroconidia; F, microconidia and G, chlamydospores.

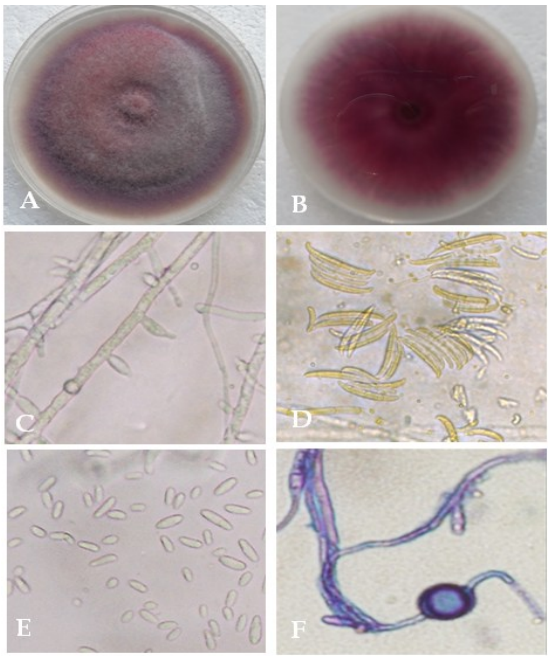

Figure 2.12 Morphological characteristics of *F. oxysporum*. A, colony morphology from the front side; B, reverse side; C, short conidiophore; E, macroconidia; F, microconidia and F, chlamydospores.

- ***Armillaria* sp.**

Several isolates of *Armillaria* sp. were successfully isolated from symptomatic trees and werer grown on MEA media. The colony morphology varied from isolate to another. Generally, in the culture *Armillaria* colonies showed fluffy appearance and produced a sparse flat whitish mycelium with brown, cylindrical rhizomorphs produced in abundance (Fig. 2.13).

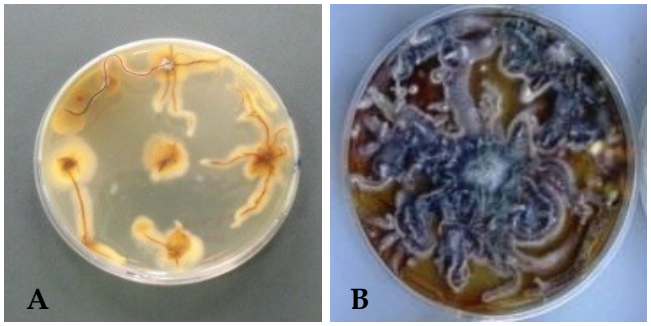

Figure 2.13 Morphological characteristics of *Armillaria mellea*. A. colonies recovered from mango wood tissues. B, colony morphology on MEA media after 21 days at 25°C. The long spidery out growths are the rhizomorphs.

***Ganoderma* sp.**

The mycelium started to grow weakly from the center of the agar (Fig.2.14). Then it increased intensity and showed white pigmentation. The spores had brown color and round shape.

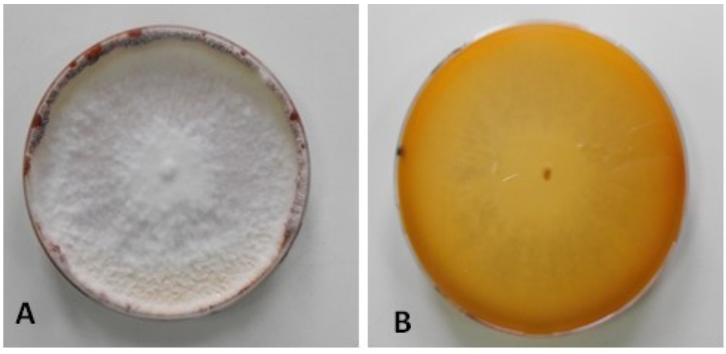

Figure 2.14 Morphological characteristics of *Ganoderma* sp. A, colony morphology from the front side; B, reverse side on MEA media after 15 days at 25°C.

Molecular characterization of the isolated fungi and oomycetes

A subset of 8 isolates was selected for molecular characterization and DNA sequencing. ITS rDNA region amplified a fragment of approximately 900 bp from *Pythium*. For *Fusarium* isolates, a portion of *β*-tubulin gene was amplified with primers T1 and T2 (Fig. 2.15). Based on a comparison of obtained sequences, the isolates showed high similarity with *Pythium vexans, Fusarium solani* and *F. oxysporum*.

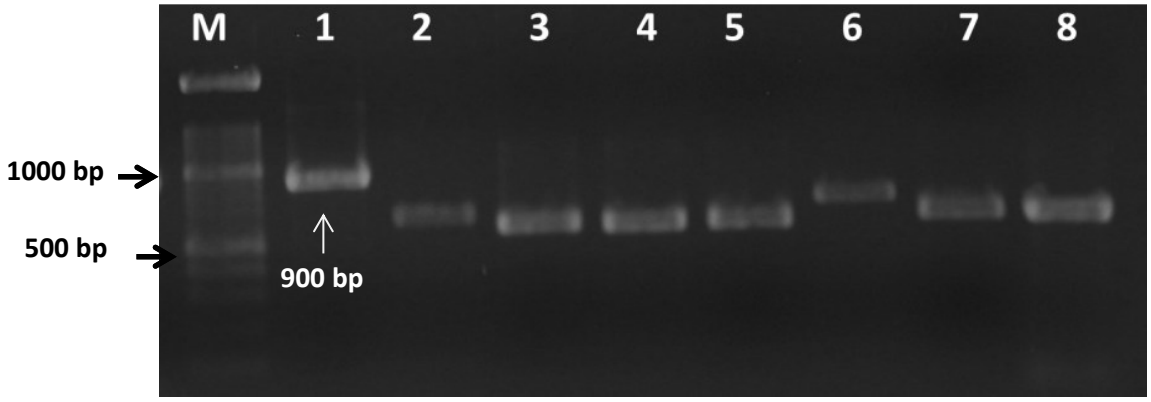

Figure 2.15 Figure 21 Agarose gel electrophoresis of amplified PCR products from genomic DNA of *Pythium* and *Fusarium* isolates collected from mango plants in Sicily. Lane 1, amplified PCR product of ITS using ITS4 and ITS6 primer pairs from *Pythium vexans.* Lanes 2 and 6, amplified PCR products of *β*-tubulin using T1-T2 from *F. oxysporum* isolates. Lanes 3, 4, 5, 7 and 8 amplified PCR products of *β*-tubulin using T1-T2 from *F. solani* isolates.

5. Discussion

The phytosanitary status of mango trees in Italy is still not well studied. Little is known regarding the role played by fungal soilborne agents in the crop productivity. Therefore, our research intended mainly to assess the occurrence of soilborne diseases and their causal agents. During survey conducted on summer 2010 and spring 2011, several symptoms and fungal signs were observed on mango seedlings and trees. Only one nursery was found in province of Catania and investigated for soilborne diseases. A total of 15 orchards located in five Sicilian provinces in Sicily (Palermo, Messina, Catania, Agrigento and Ragusa) were examined.

Damping off symptoms were predominant on high number of seedlings. The disease was characterized by sudden dropping of leaves after the emergence of seedlings from the soil. Infection occurred below the ground level with circular to irregular water-soaked patches.

Seedlings could be attacked by more than one root rot pathogen in the nursery (Agrios, 2005). This was the observation in the present study when more than one of the isolated fungi were found associated with the damping off and root rot of seedlings but the most important, *R. solani*, *Pythium*, *F. oxysporum* and *F. solani*. The isolated fungi have been reported before on mango seedlings by Prakash and Singh (1980); Tsao *et al.* (1994). The severity of damping off disease is proportional to the amount of soil moisture. The increased moisture seems to affect primarily the pathogen, which multiplies and moves (zoospores in the case of *Pythium*) best in wet soils. Increased moisture may also decrease the ability of the host to defend itself through a reduced availability of oxygen in water-logged soil and by lowering the temperature of such soils. For *Rhizoctonia*, usually causes the most severe symptoms on plants when the soil is wet but not flooded. Several other fungi, e.g., *F. solani*, grow fairly well rather in dry environments. Apparently that characteristic enables them to cause more severe diseases in drier soils on plants that are stressed by insufficient water (Leslie *et al.*, 2006).

Root rot diseases were found in all surveyed orchards while Phtophthora crown and root rot diseases were only detected in Messina and Palermo provinces (See chapter. 4). All the fungi isolated from mango roots in this study, are known common soil fungi that cause various diseases in underground parts of trees either in the nursery or in the field. Root rot pathogens attack the fine root hairs that are responsible for water and nutrient uptake as well as the structural roots that stabilize the plants. These pathogens above the ground cause foliar discoloration and thinning of the crown due to destruction of the root and subsequent reduction in nutrients and water supply to the foliage (Agrios, 2005).

Verticillium wilt was detected during the two times of survey in Catania province. The infected trees had one-sided branch dieback. Cross sections of affected branches revealed brown vascular discoloration (See chapter. 3).

Armillaria rot disease was abundantly detected in several orchards during the survey. The most common symptoms observed were the yellowing of the foliage, followed by leaf desiccation. In many cases noticeable mortality of mango trees was observed. Clusters of fruiting bodies were rare. Based on our observations, mango plants rarely showed foliar symptoms for long before they submit to root disease; then symptoms development are immediately preceding the death from Armillaria root disease. Similar findings were observed in vineyards in Spain (Aguín-Casal *et al*., 2004). Generally *Armillaria* acts as a saprophyte and decomposer of woods, breaking down both lignin and cellulose components of wood. However, *Armillaria* can also act as an opportunistic pathogen on stressed or decline trees and as virulent pathogen. As a pathogen, *Armillaria* causes soilborne disease using rhizomorph to spread through the soil from tree to tree (Shaw and Roth, 1978). The rhizomorphes penetrate the roots, the mycelial spread out across the vascular cambium and phloem, forming white mycelial fans which are a diagnostic feature of Armillaria infection. The destruction of phloem and the cambium causes tree death. *Armillaria* species infect a broad range of trees, shrubs, vines, and some herbaceous plants.

Wood decay symptoms were observed on few mango trees. The removal of mango bark from the infected trees revealed small holes on the surface of the woody cylinder made by wood beetles. Fungal mycelia gradually invade the xylem tissues from the top of the branches and spread basipetally ultimately causing death of the infected branches. Similar observations were reported by on mango trees in India by Rajput and Rao (2007). During wet and rain period, the cracks on the surface of bark supported the growth of basidiomycetes fungi like *Pleurotus* and *Polyporous* sp.

Fungi that cause tree or wood product decays grow inside the wood cells and utilize the cell wall components for food and energy. Wood decay in the living trees starts immediately after wounding. Some individual trees have the genetic capacity to recognise injury and infection rapidly and the ability to react effectively to set firm boundaries to restrict the microorganisms to very small sizes in the wood.

6. Conclusion

A large amount of research on mango has focused on the role of fungi contributing to foliar diseases, such as species in the Botryosphaeriaceae (Ramos *et al.*, 1997; Jayasankar *et al.*, 1999; Dinh *et al.*, 2003; Pilotti *et al.*, 2012). Although these pathogens can infect mango and cause disease on their own, severe symptom expression is often only initiated once the host is physiologically stressed. Stress-causing microbial soilborne root pathogens may therefore be an important factor in the predisposition of mango to attack by foliar disease pathogens.

Many citrus and olive orchards sites in Sicily are being converted into mango plantations for several reasons. Mango growers, not knowing the history of the orchard site are often unaware that *Verticillium* and *Armillaria* are common pathogens in olive and citrus and as well as in mango.

The study showed that mango plants are susceptible to damping off and root rot diseases in the nursery and orchards. Seedling damping off, plants wilt, foliar discoloration and eventual death symptoms were results of the occurrence of the root rot condition; more than one fungus was found associated in most of the cases. Root disease causing pathogens of seedlings or trees need proper identification in order to apply appropriate management. Our findings provide baseline data for the management of root rot diseases in mango plants in the nursery and orchards. Prevention of root rot disease in seedlings or trees is essential. An integrated disease management will be the best option, include; seed treatments; avoid wounding seedlings during transplanting in the nursery and adjusting irrigation frequency. In all cases, sanitary measures must be performed to avoid introduction of the pathogen in the nursery.

7. References

Agrios G. N. (2005). *Plant pathology*. Elsevier-Academic Press, Amsterdam-Boston.

Aguín-Casal O., Sáinz-Osés M. and Pedro Mansilla-Vázquez J. (2004). *Armillaria* Species Infesting Vineyards in Northwestern Spain. *European Journal of Plant Pathology*, 110(7): 683-687.

Ah-You N., Gagnevin L., Grimont P. A., Brisse S., Nesme X., Chiroleu F., Bui Thi Ngoc L., Jouen E., Lefeuvre P., Verniere C. and Pruvost O. (2009). Polyphasic characterization of xanthomonads pathogenic to members of the Anacardiaceae and their relatedness to species of *Xanthomonas*. *International Journal of Systematic and Evolutionary Microbiology,* 59(Pt 2): 306-318.

André LéVesque C. and De Cock A. W. A. M. (2004). Molecular phylogeny and taxonomy of the genus *Pythium*. *Mycological Research*, 108(12): 1363-1383.

Anonymous (2010). *La coltivazione del mango in Sicilia. Seminario sullo stato dell'arte e linee guida dell'impianto*. Capo d'Orlando (ME), Villa Piccolo - C.da Forno Alto - Capo d'Orla. Messina.

Baeza-Montanez L., Gomez-Cabrera R. and Garcia-Pedrajas M. D. (2010). First Report of Verticillium Wilt Caused by *Verticillium dahliae* on Mango Trees (*Mangifera indica)* in Southern Spain. *Plant Disease*, 94(3): 380-381.

Bally I. S. E. (2006). *Mangifera indica (mango): Species Profiles for Pacific Island Agroforestry*. Elevitch C. R. e. Hōlualoa - Hawai, Permanent Agriculture Resources (PAR). 3.1.

Britz H., Steenkamp E. T., Coutinho T. A., Wingfield B. D., Marasas W. F. and Wingfield M. J. (2002). Two new species of *Fusarium* section Liseola associated with mango malformation. *Mycologia*, 94(4): 722-730.

Campbell R. J. (1992). *A guide to mangos in Florida*, Fairchild Tropical Garden, Miami, Florida, USA.

Cazorla F., Arrebola E., Olea F., Velasco L., Hermoso J., Pérez-García A., Torés J., Farré J. and Vicente A. (2006). Field evaluation of treatments for the control of the bacterial apical necrosis of mango (*Mangifera indica*) caused by *Pseudomonas syringae* pv. *syringae*. *European Journal of Plant Pathology*, 116(4): 279-288.

Cazorla F. M., Tores J. A., Olalla L., Perez-Garcia A., Farre J. M. and de Vicente A. (1998). Bacterial apical necrosis of mango in Southern Spain: A Disease caused by *Pseudomonas syringae* pv. *syringae*. *Phytopathology*, 88(7): 614-620.

Chakrabarti D. K. (2011). Chronological history of mango malformation. In: *Mango Malformation*. Springer Netherlands, pp. 1-9.

Chen F., M. N. P. D., M. L., F. G., Ph.D. R. P., Olga M., and Ph.D. n.-B. (2010). *Handbook Of Fruit And Vegetable.* Flavors, Wiley.

Chin D., Condé B., M N., Hamilto D., Hoult M., Moore C., Thistleton B., Ulyatt L. and L. Z. (2010). *Field Guide to Pests, Beneficials, Diseases and Disorders of Mangoes.* Northern Territory Government. Department of Resources. AUSTRALIA.

Cooke D., Drenth A., Duncan J., Wagels G. and Brasier C. (2000). A Molecular Phylogeny of *Phytophthora* and Related Oomycetes. *Fungal Genetics and Biology*, 30(1): 17-32.

Dinh S.-Q., Chongwungse J., Pongam P. and Sangchote S. (2003). Fruit infection by *Colletotrichum gloeosporioides* and anthracnose resistance of some mango cultivars in Thailand. *Australasian Plant Pathology*, 32(4): 533-538.

Erwin D. C. and Ribeiro O. K. (1996). *Phytophthora diseases worldwide.* APS Press.

FAOSTAT (2011). FAO Statistics. Rome, Food and Agriculture Organization of the United Nations.

Fradin E. F. and Thomma B. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology*, 7(2): 71-86.

Gagnevin L. and Pruvost O. (2001). Epidemiology and control of mango bacterial black spot. *Plant Disease*, 85(9): 928-935.

Giblin F. R., Coates L. M. and Irwin J. A. G. (2010). Pathogenic diversity of avocado and mango isolates of *Colletotrichum gloeosporioides* causing anthracnose and pepper spot in Australia. *Australasian Plant Pathology*, 39(1): 50-62.

Gutiérrez-Barranquero J. A., Arrebola E., Bonilla N., Sarmiento D., Cazorla F. M. and de Vicente A. (2012). Environmentally friendly treatment alternatives to Bordeaux mixture for controlling bacterial apical necrosis (BAN) of mango. *Plant Pathology*, 61(4): 665-676.

Hoffman C. S. and Winston F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, 57(2-3)

Jayasankar S., Litz R., Gray D. and Moon P. (1999). Responses of embryogenic mango cultures and seedling bioassays to a partially purified phytotoxin produced by a mango leaf isolate of *Colletotrichum gloeosporioides* penz. *In Vitro Cellular & Developmental Biology.*, 35(6): 475-479.

Kennelly M. M., Cazorla F. M., de Vicente A., Ramos C. and Sundin G. W. (2007). *Pseudomonas syringae* diseases of fruit trees: Progress toward understanding and control. *Plant Disease*, 91(1): 4-17.

Klosterman S. J., Atallah Z. K., Vallad G. E. and Subbarao K. V. (2009). Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology*, 47(1): 39-62.

Ko Y., Yao K. S., Chen C. Y. and Lin C. H. (2007). First Report of Gray Leaf Spot of Mango (*Mangifera indica*) Caused by *Pestalotiopsis mangiferae* in Taiwan. *Plant Disease*, 91(12): 1684-1684.

Kumar P., Misra A. K. and Modi D. R. (2011). Current status of mango malformation in India. *Asian Journal of Plant Sciences*, 10(1): 1-23.

Kvas M., Steenkamp E. T., Adawi A. O., Deadman M. L., Jahwari A. A., Marasas W. F. O., Wingfield B. D., Ploetz R. C. and Wingfield M. J. (2008). *Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. *European Journal of Plant Pathology*, 121(2): 195-199.

Leslie J. F., Summerell B. A. and Bullock S. (2006). *The Fusarium Laboratory Manual.* John Wiley & Sons.

Lim T. K. and Khoo K. C. (1985). *Diseases and disorders of mango in Malaysia.* Tropical Press.

Litz R. E. (2009). *The Mango: Botany, Production and Uses.* CABI Direct, Wallingford. UK.

Marasas W. F., Ploetz R. C., Wingfield M. J., Wingfield B. D. and Steenkamp E. T. (2006). Mango malformation disease and the associated *Fusarium* species. *Phytopathology*, 96(6): 667-672.

Marlatt R. B., Knight R. J., Jr. and Goldweber S. (1970). Verticillium wilt of mango (*Mangifera indica*) in Florida. *Plant Disease Reporter*, 54: 569-571.

Matheron M. E. and Matejka J. C. (1988). Phytophthora crown and root rot of nursery grown mango trees delivered to Artizona (Abs.). *Phytopathology*, 78: 1572.

Matsushita N. and Suzuki K. (2005). Identification of *Armillaria* species in Japan using PCR-RFLP analysis of rDNA intergenic spacer region and comparisons of *Armillaria* species in the world. *Journal of Forest Research*, 10(3): 173-179.

Naqvi S. A. M. H. (2004). *Diseases of fruits and vegetables: Diagnosis and management*Kluwer Academic, New York.

O'Donnell K., Cigelnik E. and Nirenberg H. I. (1998). Molecular Systematics and Phylogeography of the *Gibberella fujikuroi* Species Complex. *Mycologia*, 90(3): 465-493.

Paull R. E. and Duarte O. (2010). *Tropical Fruits, Volume 1. Crop Production Science in Horticulture.* CABI.

Peña J. E., Mohyuddin A. I. and Wysoki M. (1998). A review of the pest management situation in mango agroecosystems. *Phytoparasitica*, 26(2): 129-148.

Plan M. R. R., Joyce D. C., Ogle H. J. and Johnson G. I. (2002). Mango stem-end rot (*Botryosphaeria dothidea*) disease control by partial-pressure infiltration of fungicides. *Australian Journal of Experimental Agriculture*, 42(5).

Ploetz R. C. (1994a). Distribution and prevalence of *Fusarium subglutinans* in mango trees affected by malformation. *Canadian Journal of Botany*, 72(1): 7-9.

Ploetz R. C. (ed) (2003). Diseases of Mango. In: *Diseases of Tropical Fruit Crops*. CABI Publishing, Wallingford, UK.

Ploetz R. C. (2004). The major diseases of mango: strategies and potential for sustainable management. In: Pinto A. C. Q., Pereira M. E. C. and Alves R. E. (ed). *Acta Horticulturae*, pp. 137-150.

Ploetz R. C. and Freeman S. (ed) (2009). Foliar, floral and soilborne diseases. In: *The Mango: Botany, production and uses*. 2nd edition. CABI.

Ploetz R. C., Zentmyer, G. A., Nishijima, W. T., Rohrbach, K. G., and Ohr, H. D. (ed) (1994b). *Compendium of Tropical Fruit Diseases*. The American Phytopathological Society, St. Paul, MN.

Popoola T. O. S. (2004). Problems of Armillaria root and butt rot in tropical Africa: The case of Nigerian forests.In: (eds). *Proceedings of the International Conference on Science & National Development.* 25- 28 October, Abeokuta, Nigeria. College of Natural Sciences Proceedings, Federal University of Agriculture.

Prakash O. (2004). Diseases and disorders of mango and their management. In: Naqvi S. A. M. H. (ed). *Diseases of Fruits and Vegetables Volume I*. Springer Netherlands, pp. 511-619.

Prakash O., Misra, A.K. and Raoof, M.A. (1994). Studies on mango bacterial canker disease. *Bio. Memoirs*, 20: 95-107.

Prakash O. and Srivastava K. C. (1987). *Mango diseases and their management: a world review.* Today & Tomorrow's Printers and Publishers.

Prakash O. m. and Singh U. N. (1980). Root rot and damping off of mango seedling caused by *Rhizoctonia solani*. *Indian Journal of Mycology and Plant Pathology*, 10(1): 69.

Rajput K. S. and Rao K. S. (2007). Death and decay in the trees of Mango (*Mangifera indica* L.). *Microbiol Res*, 162(3): 229-237.

Ramos L. J., Davenport T. L., McMillan R. and Lara S. P. (1997). The resistance of mango *(Mangifera indica*) cultivars to tip dieback disease in Florida. *Plant Disease*, 81(5): 509-514.

Sanei S. J., Waliyar F., Razavi S. I. and Okhovvat S. M. (2008). Vegetative compatibility, host range and pathogenicity of *Verticillium dahliae* isolates in Iran. *International Journal of Plant Production*, 2(1): 37-45.

Saran P. L. and Kumar R. (2011). Boron deficiency disorders in mango (*Mangifera indica*): field screening, nutrient composition and amelioration by boron application. *Indian Journal of Agricultural Sciences*, 81(6): 506-510.

Sharma I. M., Badiyala S. D. and Sharma N. K. (1993). Effect of fungicidal drenching agaisnt wilt of mango seedlings caused by *Fusarium solani*. *Indian Journal of Mycology and Plant Pathology*, 23: 326-327.

Shaw C. G. and Roth L. F. (1978). Control of Armillaria root rot in managed coniferous forests. *European Journal of Forest Pathology*, 8(3): 163-174.

Singh L. B. (1960). *The Mango. Botany, cultivation, and utilization.* London, Leonard Hill (Books) Ltd; New York, Interscience Publishers, Inc.

Sneh B., Burpee L. and Ogoshi A. (1991). *Identification of Rhizoctonia species.* APS Press.

Spies C., Mazzola M. and McLeod A. (2011). Characterisation and detection of *Pythium* and *Phytophthora* species associated with grapevines in South Africa. *European Journal of Plant Pathology*, 131(1): 103-119.

Tewoldemedhin Y., Mazzola M., Botha W., Spies C. and McLeod A. (2011). Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. *European Journal of Plant Pathology*, 130(2): 215-229.

Tsao P. H., Luzaran P. B., Delossantos A. B., Portales L. A., Gochangco A. M. and Gruber L. C. (1994). Phytophthora crown and root-rot of mango detected in Philippine nurseries. *Plant Disease*, 78(1): 100-100.

Willis A. (2009). Evaluation of alternatives to copper oxychloride for the control of bacterial black spot and post harvest diseases in mango. In: Oosthuyse S. A. (ed). *Acta Horticulturae*, pp. 535-540.

Zak J. C. and Willig M. R. (2004). Fungal biodiversity patterns. In: (ed). *Biodiversity of Fungi*. Academic Press, Burlington, pp. 59-75.

Zea-Bonilla T., Martín-Sánchez P. M., Hermoso J. M., Carmona M. P., Segundo E. and Pérez-Jimenéz R. M. (2007). First report of *Phytophthora citricola* on *Mangifera indica* in Spain. *Plant Pathology*, 56(2): 356-356.

CHAPTER 3

3. VERTICILLIUM WILT DISEASE OF MANGO IN SICILY (SOUTHERN ITALY)

1. Abstract

Mango (*Mangifera indica* L.) is an important fruit crop in many tropical and subtropical countries. The crop has been introduced into Sicily lately. However, its future as approaching commodity in Sicilian agriculture is threatened by diverse biotic and abiotic threats. One of the main constrains for mango cultivation in many areas is Verticillium wilt, a vascular disease caused by *Verticillium dahliae*. The soilborne fungus challenging agronomic production of over 300 crops worldwide. Vertcillium wilt is an increasing problem in areas where young mango trees are planted on areas previously planted in susceptible crops. In summer 2010 and spring 2011 during a survey for soilborne diseases of mango, typical symptoms of Verticillium wilt were observed. The disease was found in a new mango grove of cv. Kensington Pride, previously planted with olive in Catania province. The trees had one-sided branch dieback. In many of these trees the symptoms extended, leading to decline and eventual death. Cross sections of affected branches revealed brown vascular discoloration. *Verticillium* was isolated from surface-sterilized segments of symptomatic branches placed on semi selective potato dextrose agar (PDA). After incubation, slow-growing colonies were transferred to PDA. Isolates were single spored before storing on potato dextrose agar at 4°C. Identity of the pathogen was initially based on colony morphology and formation of microsclerotia and further confirmed by molecular method. In root-dip inoculation tests in the greenhouse, two *V. dahliae* isolates infected 6 months-old mango plants that wilted within 10 months after inoculation. *V. dahliae* was reisolated from the inoculated plants showed vascular discoloration. To our knowledge, this is the first report of *Verticillium* wilt on mango in Sicily.

Keywords: Mango, wilt, *Verticillium dahliae*, Sicily.

2. Introduction

Verticillium dahliae is a soilborne fungus that causes a vascular wilt disease of plants and losses in a broad range of economically important crops worldwide. The fungus attacks roots through wounding and spreads to the aerial parts of plant by systemic invasion, inducing both external and internal symptoms. The symptoms include, leaf vein browning, chlorosis and necrosis, stunting, wilting, leaf defoliation, vascular discoloration and death in the later stages of the disease (Agrios, 2005). *V. dahliae* produces microsclerotia as survival structures that can remain in the soil for nearly 10-15 years (Xiao and Subbarao, 1998). Germination of microsclerotia is stimulated by plant root exudates released in the soil. The germinated hyphae penetrate host roots, colonize the cortex and enter the xylem. Then, the fungus reproduces asexually by forming conidiospores, which move upward, germinate and penetrate new vessels. Hyphae and plant reactions to the infection eventually plug the vessels and prevent water from reaching the plant's aerial parts, resulting in characteristic wilt symptoms (Agrios, 2005).

Verticillium wilt of mango was first reported in Florida USA (Marlatt *et al.*, 1970). The disease was originally attributed to *Verticillium albo-atrum* that before *V. dahliae* was recognized as distinct species. Marlatt *et al.* (1970) observed 100% of Verticillium wilt infection on a new mango grove planted on land previously cropped with tomatoes. Young mango trees very quickly developed moderate to severe symptoms of die back, leaf necrosis and discoloration in the cambial area of the stems accompanied by stem bleeding. No symptoms of this disease were evident in the other part of the grove planted on virgin land. Recently the disease was reported in Southern Spain. Baeza-Montanez *et al.* (2010) observed on 20 % of the trees typical Verticillium wilt symptoms in mango orchards that previously cultivated with potatoes and tomatoes.

The objectives of this study were i) to survey Verticillium wilt disease in mango in Sicily; ii) to determine the prevalent species of *Verticillium* across the sampled mango fields and iii) to evaluate the pathogenicity toward mango.

3. Materials and methods

Field surveys and fungal isolation

Commercial mango orchards in Sicily were surveyed to assess the prevalence of *Verticillium* species and the incidence of Verticillium wilt. Inspections were carried out during Summer 2010 and Spring 2011. A total of 15 orchards in 5 provinces were surveyed (See chapter 2). For isolation of *Verticillium* sp., infected tissues were surface disinfested by immersion in sodium hypochlorite 1 % for 30 sec. then, rinsed in sterile distilled water and left to dry on sterilized filter paper. Subsequently, small pieces of discolored wood were seeded directly on semi selective PDA media amended for 1 liter with 250 mg of ampicillin and 250 mg of streptomycin sulphate; plates were incubated for 7 days at 25°C.

Morphological characterization

All the isolates were single spored and maintained on potato dextrose agar (PDA) or as a spore suspension. The cultures were grown for 4 weeks at 25°C prior to morphological characterization. The presence and characteristics of conidia, microsclerotia, and chlamydospores were recorded.

Molecular characterization

DNA was extracted following the method described by Hoffman and Winston (1987). For PCR, the Intergenic Spacer region (IGS) was amplified using two primer pairs (Ver2-Ver3 and Vd7b-Vd10). The primer pairs Ver2-Ver3 is a genus specific for *Verticillium* while Vd7b-Vd10 is primer pairs specific to *V. dahliae* (Table 3.1). Reactions were performed in a 25 µl mixture containing 100 ng of genomic DNA, 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 100 µM each dNTPs, 1 mM MgCl2, 1 unit of Taq polymerase, and 1 µM of forward and reverse primers (Ver2-Ver3) for the first PCR amplification and (Vd7b-Vd10) for the second PCR amplification. The thermal cycling parameters were initial denaturation step at 94°C for 5 min followed by 40 cycles, each consisting of 45 sec at 94°C and annealing at 55°C for 45 sec, and extension at 72°C for 30 sec. A final extension at 72°C for 10 min was done at the end of the amplification. Negative controls (no template) were used in each experiment to test for the presence of contaminant DNA in the reagents (Nigro *et. al.,* 2002).

Table 3.1 Primers used in the molecular characterization of *Verticillium* isolates.

Fungus	Target DNA	Orientation	Primer sequence 5'- 3'
***Verticillium* sp.**	Intragenic spacer region (IGS)	Ver2	5' TCGCCGATTCCTCCACCA3'
		Ver3	5' CCAATTCCCGGGTAGCTC 3'
V. dahliae	Intragenic spacer region (IGS)	Vd7	5' TTCAAGAGTCTGCTTGCGG3'
		Vd10	5' GACCGGCCATAGGGTAATCT 3'

Pathogenicity test

Two representative isolates of *Verticillium* (Vd-1 and Vd-2) were tested for their pathogenicity using the root dip inoculation method. Ten months old mango plants cv. Kensington Pride with intact roots were inoculated with a condial suspension of approximately 4×10^6 condia/ml. Three plants for each isolate were dipped in the conidial suspension treatment (100 ml) for at least 10 min. The plants were kept in the greenhouse at 25°C and 90–95% relative humidity Plants dipped in sterile water were treated as controls. All plants were gently uprooted 10 months after transplanting, washed free of soil, and examined for disease infection. Plant height was measured. The fresh foliar and root weight were determined using a portable electric scale. The stem and root portion of each plant was longitudinally split to estimate the percentage of vascular tissue discolored (disease severity) on a scale of 0 to 5, in which 0 = no discoloration, 1 = 1 to 10% discoloration, 2 = 11 to 30% discoloration, 3 = 31 to 50% discoloration, 4 = 51 to 75% discoloration, and 5 = 76 to 100% discoloration (Xiao and Subbarao, 1998). For reisolation, 1- to 2-cm sections of excised stem tissue were placed on semi modified PDA medium and incubated in the dark at 18 to 25°C. After 10 days of incubation, morphological characteristics were examined under both stereo and compound microscopes. The experiment was arranged in completely randomized factorial design and was repeated once. Data were subjected to the analysis of variance one way ANOVA and mean values of the lesion were compared using the Least Significant Difference (LSD) test.

4. Results

Field surveys and fungal isolation

Among 15 mango orchards that were surveyed in Sicily, Verticillium wilt was found only in one orchard in province of Catania. The main mango variety found in the orchards was cv. Kensington Pride.

Symptoms observed in the field were yellow foliage, leaf scorch (marginal browning), slow growth and dieback of shoots and branches. The foliage on one or more branches wilts suddenly, trees decline and die back slowly often was the key diagnostic feature for the disease. The dead leaves often remain attached to infected branches, giving the tree a "fired" appearance. Discoloration streaking of the vascular wood tissues was associated with the external symptoms (Fig. 3.1). Isolations were made from wood mango tissues. Typical cultures of *Verticillium* colonies were obtained and further identified.

Morphological characterization

Based on morphological characteristics, six *Verticillium* isolates recovered from mango infected trees were identified as *V. dahliae*. On PDA, colonies of *Verticillium* isolates changed from white to black as they melanised during the period of incubation. *Verticillium* colonies in general showed white and dense mycelium in the center and black on the margins (Figs. 3.2).

Microscopic examination showed the presence of conidiophores, which were more or less erect, hyaline, with verticillate branches (Fig. 3.3A), and with 2-3 phialides at each nod (Fig. 3.3B). Conidia were also produced at the phialide tips (Fig. 3.3C) and were hyaline, ovoid to elongate (Fig. 3.3D). Microsclerotia were produced on hyaline hyphae (Fig. 3.3). They were very irregular in shape, subglobose to elongate and had dark brown to black color (Fig. 3.3F).

Figure 3.1 Symptoms of Verticillium wilt on mango trees in the field. A, yellowing, wilting and dieback on one side of mango tree. B, dead leaves attached to infected branches giving the tree fired appearance. C and D, cross sections of mango trunk showing vascular discoloration. E and F, characteristic vascular streaking caused by Verticillium wilt. Arrows in A and B, showing mango trees closely planted with olive trees.

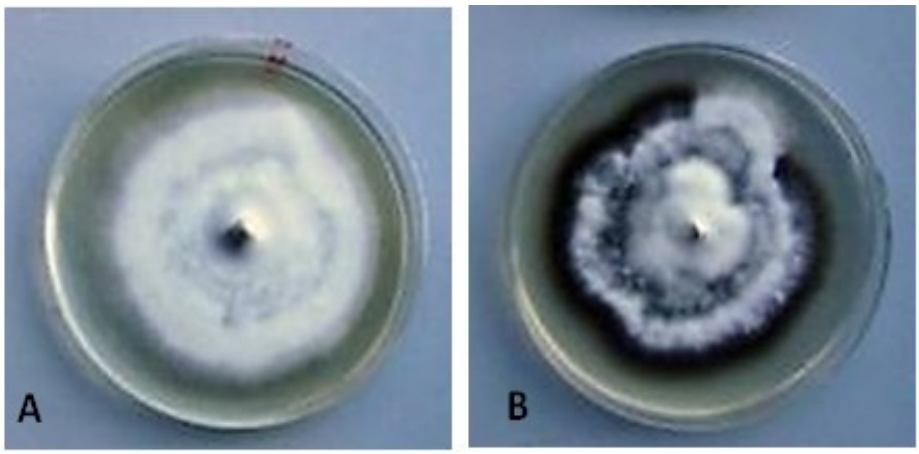

Figure 3.2 Colony morphology of two different isolates of *V. dahliae* on PDA after 30 days at 25°C. (Vd-1 on the left side and Vd-2 on the right one).

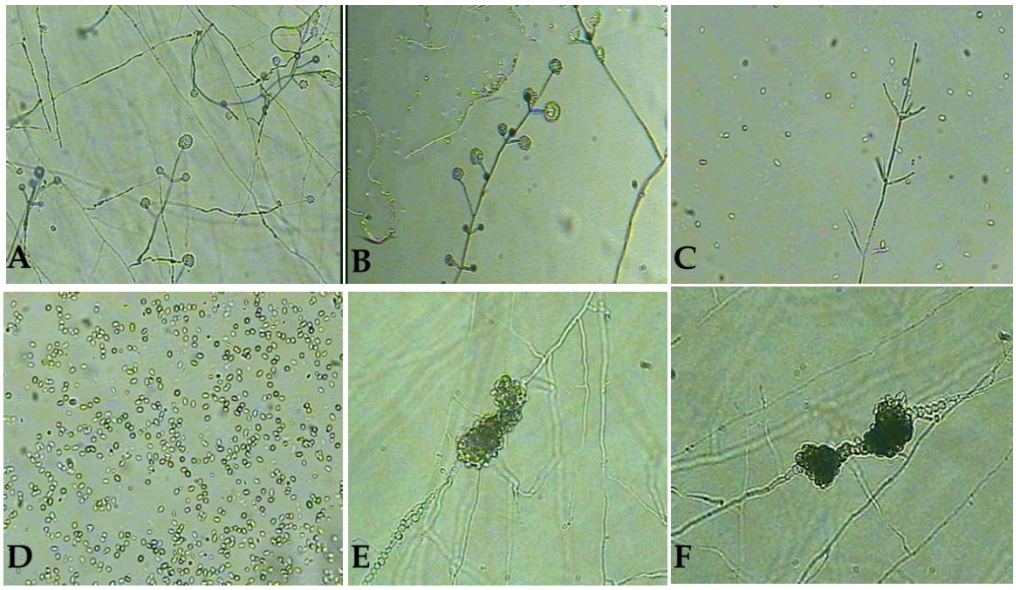

Figure 3.3 Morphological characteristics of *V. dahliae* recovered from mango trees. A and B, erect, hyaline conidiophores; C, typical verticillate branches of *Verticillium*; D, hyaline, ovoid conidia; E, microsclerotia formed on hyphae; F, typical melanised microsclerotia.

Molecular identification of *Verticillium* isolates

Different *Verticillium* isolates were morphologically identified based on morphological features. Six isolates were molecularly characterized using two primer pairs (Ver2-Ver3 and Vd7b-Vd10) of the IGS region. A fragment of 339 bp was amplified by Ver2-Ver3 primer pair which is genus specific primer pairs (Fig. 3.4, lanes 1- 6), while fragment of 139 bp was amplified by Vd7b-Vd10 primer pair specific for *V. dahliae* (Fig 3.4, lanes 8- 13), and the same fragment of the expected size was obtained from the positive controls (Fig. 3.4, lanes 7,14). No amplification was achieved with the negative DNA control (lane H_2O).

The results of PCR amplification indicate that all *Verticillium* isolates obtained from mango trees are *V. dahlia*; these results confirmed the morphological identification.

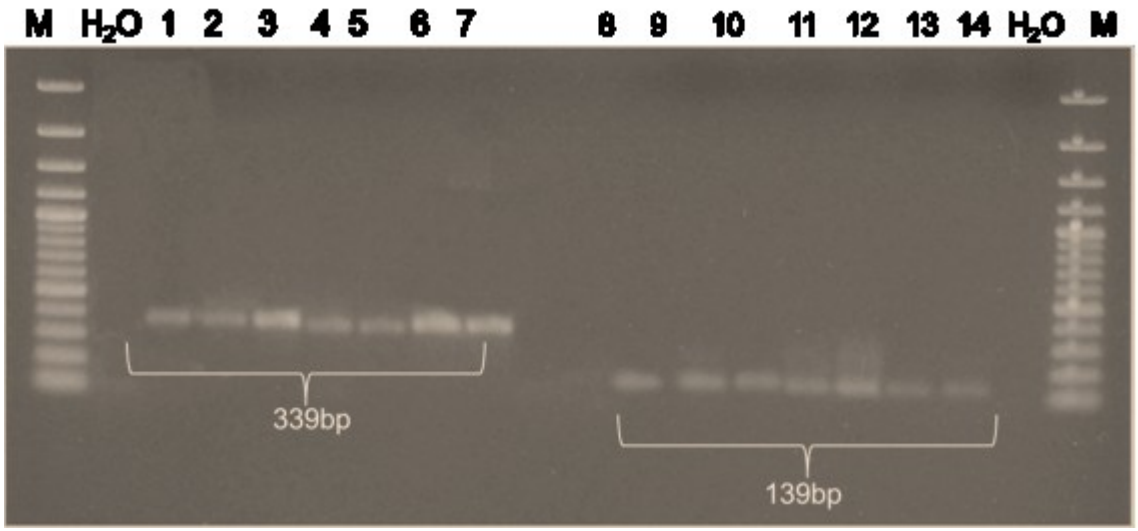

Figure 3.4 Agarose gel electrophoresis of amplified products from genomic DNA of *Verticillium* isolates. Lanes 1 to 7 are PCR products obtained by the genus universal primer (Ver2-Ver3), with a fragment of 339 bp. Lanes 8 to 14 are PCR products obtained by the specific primer (Vd7b-Vd10) with a fragment of 139 bp. with Lanes 7 and 14 positive control of *Verticillium* isolates from IAMB collection, and H_2O contains negative DNA control. Lane M contains 100-bp DNA ladder.

Pathogenicity test

Two isolates of *V. dahliae* were evaluated with respect to pathogenicity and aggressiveness on mango plants. Vd-1 and Vd-2 isolates were capable of causing disease symptoms in the tested plants. Slight variation in the virulence between the two isolates was observed in term of plant height. The prevalent symptoms were leaf chlorosis and necrosis, yellowing, wilts and stunting. All analyzed sections in mango tissues displayed vascular discoloration (Figs. 3.5, 3.6).

Significant differences ($P<0.05$) were recorded among data sets. For disease severity data (Fig. 3.7), no significant differences ($P<0.05$) were observed among the tested isolates. Similar results were obtained with the mean of plant height, fresh weight of foliar and root tissus (Fig. 3.8).

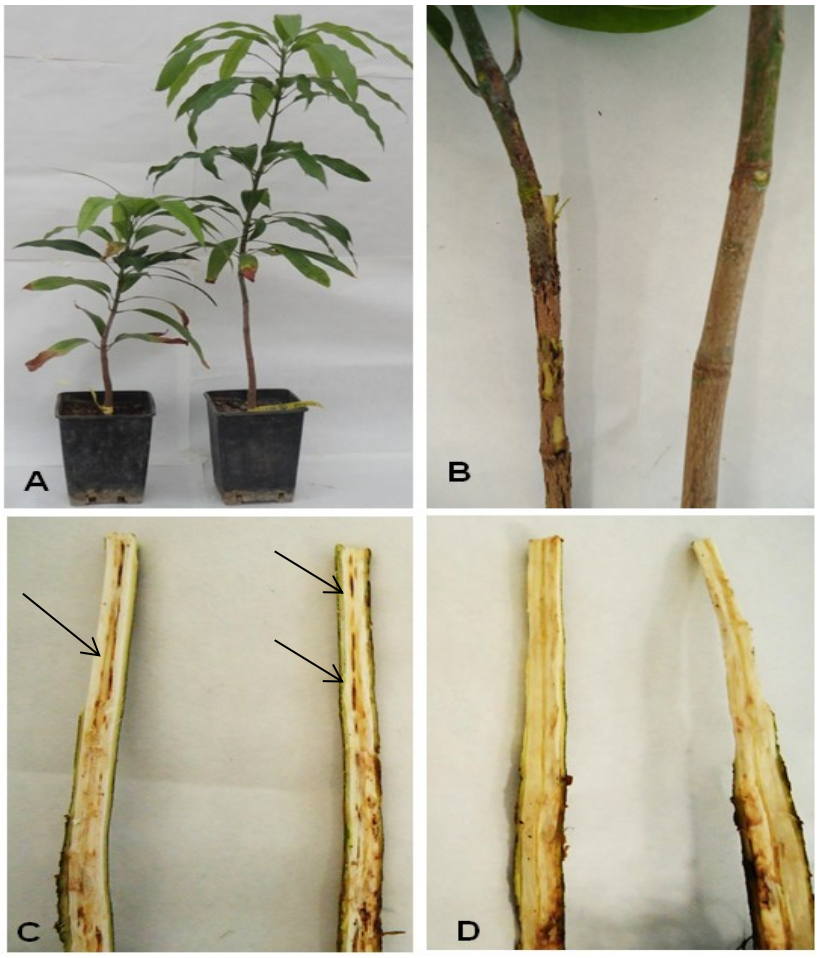

Figure 3.5 Pathogenicity of *V. dahliae* isolates on mango plants. A, typical symptoms of Verticillium infection developed as stunting of the inoculated plant (on the left) compared to normal growth of the healthy plant (on the right); B, lesions on the stem of the inoculated plant (on the left) and healthy stem (on the right); C vascular discoloration in tissues of the inoculated plant; D, stem of the healthy plant show no vascular discoloration.

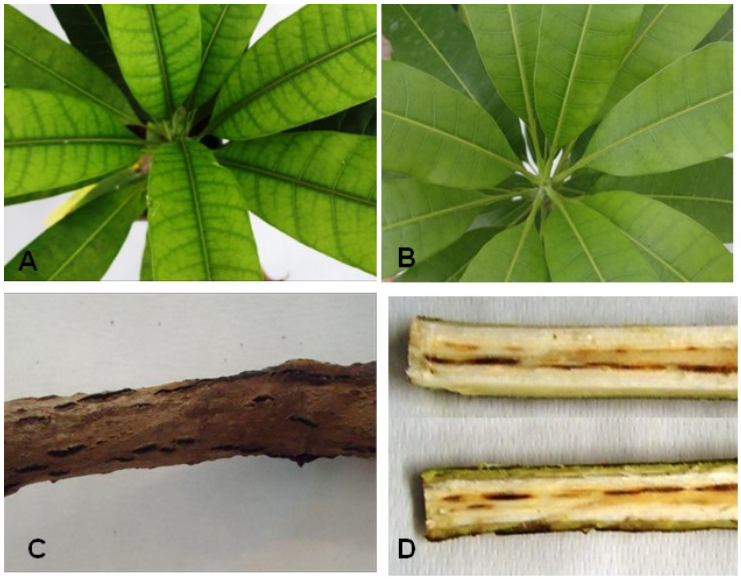

Figure 3.6 Pathogenicity of *V. dahliae* isolates Vd-1 and Vd-2 on mango. A, symptoms of chlorosis, appeared on the leaves; B, healthy green leaves; C, gummosis and cracks appeared on mango stem; D. vascular discoloration in longitudinal-sections of stem.

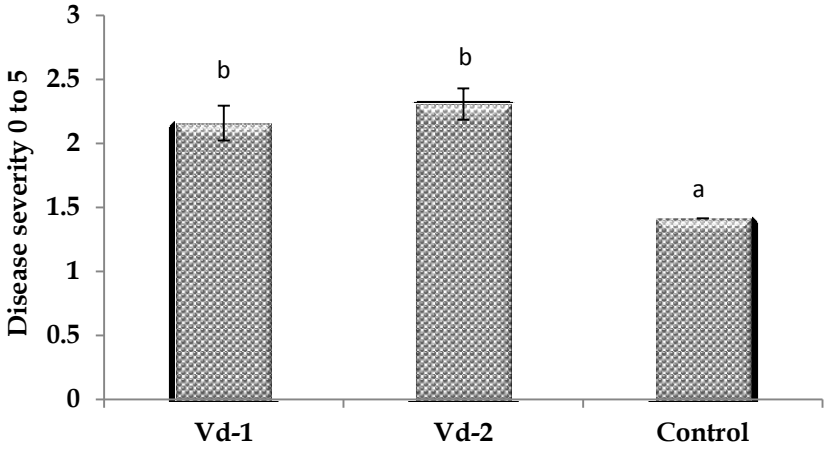

Figure 3.7 Pathogenicity of *V. dahliae* isolates Vd-1 and Vd-2 on mango plants determined as severity of vascular tissue discolored (wilt severity) on a scale of 0 to 5, 0 = no vascular discoloration, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of vascular tissues exhibited discoloration in the absence of foliar symptoms, and 5 = 100% of vascular tissues exhibited discoloration in the presence of foliar symptoms typical of Verticillium wilt. The bars represent the means (± standard error) of three replicates with three plants per isolate. Bars with the same letter were not statistically different according to Duncan- Waller *k*-ratio *t* test ($P = 0.05$). Percent severities were angular transformed [sqr (x+2)] before analysis.

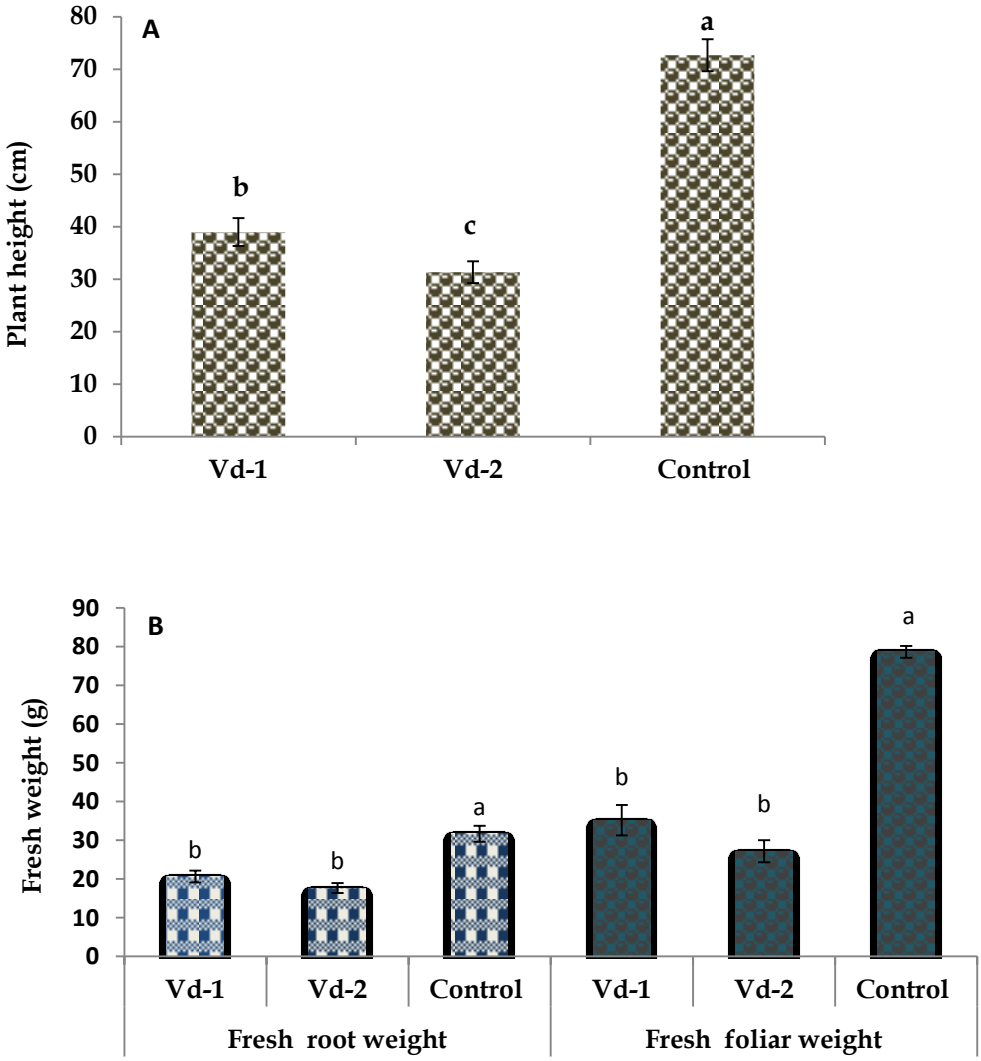

Figure 3.8 Effect of two isolates of *V. dahliae* (Vd-1 and Vd-2) on the mean of plant height (A) and fresh weight of foliar and root tissues (B) of mango 10 months after inoculation. The bars represent the means (± standard error) of three replicates per isolate. Bars with the same letter were not statistically different according to Duncan- Waller *k*-ratio *t* test ($P = 0.05$).

5. Discussion

Verticillium wilt diseases occur worldwide but are most important in temperate regions. Verticillium attacks more than 200 species of plants, including most vegetables, flowers, fruit trees, strawberries, field crops and fruit trees like olive (Agrios, 2005).

Verticillium wilt is one of the major soilborne diseases of mango therefore, our research aimed to assess its presence in the new mango groves of Sicily.

In Summer 2010 and Spring 2011 during survey for soilborne diseases of mango, typical symptoms of Verticillium wilt were observed. The disease was reported in a new mango grove of cv. Kensington Pride previously planted with olive in Catania province.

All six *Verticillium* isolates obtained from mango trees could easily be identified as *V. dahliae*, because of the verticilliate conidiophores, the conidia length and formation and morphology of microsclerotia. This result is in accordance with the studies of Zea-Bonilla *et al.* (2007) who also isolated exclusively *V. dahliae* from mango diseased trees. There is much confusion in the earlier literature concerning the causal pathogen.

The results of pathogenicity tests carried out in this study indicate that each of the two tested isolates were capable of causing disease symptoms in mango plants. Symptoms were similar to those described for wilt diseases caused by this pathogen in mango and other fruit trees species (Zea-Bonilla *et al.*, 2007; Dervis *et al.*, 2010).

In *V. dahliae*, pathogenic variability is related to several factors, including the ability to produce pathogenicity factors such as toxins, glycoproteins and protein-lipopolysaccharide complexes, which induce necrosis and wilting in host plants (Mansoori *et al.*, 1995; Palmer *et al.*, 2005).

Clear symptoms of cholrosis on mango leaves were observed, however in some cases, symptoms on the leaves did not reflect the true potential of an isolate to cause disease, because they could be induced by other stress factors that often occur in growth chambers (Alkher *et al.*, 2009). The presence of chlorosis implies that, if toxins are involved (Pu *et al.*, 2007), they possibly reach the top of the plant through the vascular system even though the progress of the pathogen is blocked early on by the host. The ability to induce more or less of these symptoms may depend on the differential expression of genes controlling pathogenicity factors in *V. dahliae* isolates (Alkher *et al.*, 2009).

On the other hand, vascular discolouration in stem cross-sections seemed to be a good criterion to discriminate highly from weakly aggressive isolates, probably because vascular discolouration is permanent and can extend beyond infected tissues. Even when plants recover quickly from the disease, by forming new vascular tissues, vascular discolouration remains visible in the old xylem tissues. External symptoms are sometimes induced by factors other than the soilborne pathogen and may be overestimated. *V. dahliae* has a wide host range and is able to cause both external and internal symptoms, sometimes similar to those induced by other biotic and abiotic stresses.

Inoculation of mango plants with *V. dahliae* (Vd-1 and Vd-2) reduced the plant height and fresh weight of foliar and roots as compared to the control. In many fruit trees, wilt inducing *V. dahliae* causes chlorosis, stunting, premature defoliation, and wilting. Similar results were obtained in filed crops, Verticillium wilt can reduce plant height, lateral branching, and dry matter accumulation in leaves, stems and roots (Qin *et al.*, 2008).

6. Conclusion

The obtained results in this work contribute to the progress in the knowledge of the distribution of *V. dahliae* in mango plantation in Sicily and would be of much interest for implementing potential disease management strategies for Verticillium wilt.

This is the first report of Verticillium wilt of mango in Sicily. The disease is still localised in province of Catania, however the disease is considered a serious threat for mango cultivation in other regions of Sicily. Diverse factors could likely contribute to the spread of disease within Sicily, configuring the disease as the most serious phytopathological problem for mango growers. More problems with Verticillium wilt are expected because of the increasing planting of mango in fields previously dedicated to susceptible crops. The presence of this disease demonstrates the need for effective control methods. At the present time most commercially grown mango cultivar is likely to be susceptible to the disease.

Management of diseases caused by soilborne pathogens is challenged by the persistence of a resident pathogen population. It is usually not possible to eradicate a pathogen that has become established in soil, so management relies on maintaining the population below a damaging threshold. Therefore, an integrated disease management strategy that fits modern sustainable agriculture criteria must be applied. Overall approach is the best strategy to effectively control Verticillium integrating biological, chemical, physical, and cultural approaches.

7. References

Agrios G. N. (2005). *Plant pathology* Elsevier-Academic Press, Amsterdam-Boston.

Alkher H., El Hadrami A., Rashid K. Y., Adam L. R. and Daayf F. (2009). Cross-pathogenicity of *Verticillium dahliae* between potato and sunflower. *European Journal of Plant Pathology*, 124(3): 505-519.

Baeza-Montanez L., Gomez-Cabrera R. and Garcia-Pedrajas M. D. (2010). First report of verticillium wilt caused by *Verticillium dahliae* on mango trees (*Mangifera indica*) in Southern Spain. *Plant Disease*, 94(3): 380-381.

Dervis S., Mercado-Blanco J., Valverde-Corredor A., Perez-Artes E. and Erten L. (2010). Verticillium wilt of olive in Turkey: A survey on disease importance, pathogen diversity and susceptibility of relevant olive cultivars. *European Journal of Plant Pathology*, 127(2): 287-301.

Hoffman C. S. and Winston F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, 57(2-3): 2-3.

Mansoori B., Milton J. M. and Smith C. J. (1995). Isolation and partial-purification of a phytotoxin related to pathogenic *Verticillium* species. *Journal of Phytopathology-Phytopathologische Zeitschrift*, 143(1): 33-36.

Marlatt R. B., Knight R. J., Jr. and Goldweber S. (1970). Verticillium wilt of mango (*Mangifera indica*) in Florida. *Plant Disease Reporter*, 54: 569-571.

Palmer C. S., Saleeba J. A. and Lyon B. R. (2005). Phytotoxicity on cotton ex-plants of an 18.5kDa protein from culture filtrates of *Verticillium dahliae*. *Physiological and Molecular Plant Pathology*, 67(6): 308-318.

Pu S., Duchscher M., El-Bebany A. F., Alkher H., Adam L. R., El Hadrami A. and Daayf F. (2007). Development of bioassays for the screening of toxin(s) produced by *Verticillium dahliae*. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie*, 29(4): 448-449.

Qin G. M., Vallad G. E. and Subbarao K. V. (2008). Characterization of *Verticillium dahliae* and *V. tricorpus* isolates from lettuce and artichoke. *Plant Disease*, 92(1): 69-77.

Xiao C. L. and Subbarao K. V. (1998). Relationships between *Verticillium dahliae* inoculum density and wilt incidence, severity, and growth of cauliflower. *Phytopathology*, 88(10): 1108-1115.

CHAPTER 4

4. PHYTOPHTHORA ROOT AND CROWN ROT OF MANGO IN SICILY (SOUTHERN ITALY)

1. Abstract

A survey on mango groves (cv Kensington Pride.), carried out in Sicily during Summer 2010 and Spring 2011, revealed the presence of symptoms of leaf wilting, chlorosis, foliage dessication and tree stunting associated to root rot and extensive dark-brown cankers in the crown. *Phytophthora cryptogea* was consistently isolated from symptomatic tissues taken from the crown and necrotic roots of mango. Pathogen identification was based on colony morphology, characterization of the sexual and asexual reproductive structures, and temperature range. *Phytophthora* colonies showed petalloid patterns on PDA and stellate patterns on V8 media. *Phytophthora* isolates produced abundant non papillate sporangia when they flooded with water. Sporangia average size was 41 µm long × 26 µm wide; they were ellipsoid to ovoid, occasionally obpyriform, non papillate and non caducous. Distinct hyphal swellings were noted while chlamydospores were observed rarely in culture. No growth was observed at 35°C. All isolates were heterothallic of mating type A2 and produced oogonia with amphigynous antheridia. In addition, the rDNA internal transcribed spacer (ITS) region was sequenced and BLAST searches showed 99% similarity with sequences of *P. cryptogea* isolates available in the GenBank. DNA sequence data of ITS, COI, LSU and 60S loci were used for phylogenetic inferences. The identification of *P. cryptogea* was further corroborated by Single-Strand Conformation Polymorphism (SSCP) analysis. Koch's postulates were fulfilled by pathogenicity tests conducted on mango plants 'Kensington Pride'. All isolates were pathogenic because the inoculated plants developed symptoms of wilt, root and crown rot 12 months after inoculation. *P. cryptogea* was recovered from the inoculated plants. No symptoms were observed and no pathogen was isolated from the plants used as negative control. This study demonstrated that *P. cryptogea* is the cause of crown and root rot on mango in Italy. To our knowledge, this is the first report of *P. cryptogea* occurring on mango in Italy and worldwide.

Keywords: *Phytophthora* root and crown rot; Mango; *P. cryptogea*

2. Introduction

Mango is affected by a number of diseases at all stages of its growth from the seedling in the nursery to the fruits during storage or transport. Diseases in the field result in the crop loss while post-harvest diseases are directly linked with the losses in export and domestic market (Ploetz and Freeman, 2009). *Phytophthora* is a major genus of soilborne plant pathogens, known to cause problems on fruits trees and considerable yield losses worldwide. It is responsible for the most serious and economically important plant diseases (Judelson and Blanco, 2005).

Many *Phytophthora* species even other genera have been associated with mango root rot in many countries. *P. palmivora* was isolated from infected mango trees showing crown and root rot symptoms in the Philippines (Tsao *et al.*, 1994). *P. palmivora* has been reported also as the causal agent of mango root rot and the death of nursery plants in Arizona USA and Thailand (Matheron and Matejka, 1988). In India, *P. parasitica* has been reported to cause leaf blight disease on mango (Prakash and Singh, 1980; Prakash and Srivastava, 1987). Recently, Phytophthora root rot was reported in Spain and *P. citricola* was isolated from mango trees that were wilt, chlorotic and had sparse canopies and cracked bark (Zea-Bonilla *et al.*, 2007).

The objectives of this research were i) to survey mango nurseries and orchards in Sicily for Phytophthora crown and root rot; ii) to identify *Phytophthora* species and iii) to determine the pathogenicity of *Phytophthora* species.

3. Materials and Methods

Survey of Phytophthora root and crown rot in Sicily

In the period Spring 2010 and Summer 2011, a total of 20 mango orchards (cv. Kensington Pride), located in four areas of Sicily, were surveyed. Symptoms of wilting and chlorosis, stunting and gradual dieback of the tree, associated with root and crown rot, were observed on fifteen-seventeen years old trees. Soil and root samples from their rizospheres were collected in each field and were processed in laboratory.

Isolation of *Phytophthora* from the soil

Once soil samples became dry, the isolation of *Phytophthora* spp. was made by the soil dilution plate method using the selective agar medium by Masago *et al.* (1977). Per each sample, ten grams of sieved soil were suspended in 100 ml of distilled water and shacked using a magnetic stirrer. After vigorous shaking, the soil slurry was kept agitated, while 1 ml portions were transferred with a wide mouth pipette to the surface of the selective agar medium plate. Ten plates were used for each soil sample and incubated for 24 hours in the dark at 19°C; after 24 h soil suspension was washed off the agar surface with a gentle stream of running water and incubated again at 20°C for 3-6 days. Starting from the third day of incubation, plates were daily checked for the presence of *Phytophthora* spp. colonies and the number of propagules per gram of soil (PPG) was calculated.

Isolation of *Phytophthora* from roots

Feeder roots infection was assessed by using the same *Phytophthora* selective medium. Roots from each sample were rinsed with tap water, dried on blotting-paper, cut into small fragments (roughly 1 cm), then placed into Petri dishes containing the selective medium, and incubated for 3-6 days at 20°C. The colonies showing the morphological characteristics of *Phytophthora* were recorded and the percentage of infected roots was evaluated.

Morphological identification of *Phytophthora* spp.

Identification of many *Phytophthora* species is relatively simple; however the morphological differences among some species are so small and some characteristics are so variable that makes species differentiation difficult task. The morphological characteristics by which most *Phytophthora* species are classically identified, based on the groups' I-VI of Waterhouse (1963), include observations on oospores, antherdium, sporangia, sporangiophores, chlamydospores and growth temperature.

Colony morphology and growth rate

All isolates were grown at 25 °C on V8-juice agar and Potato dextrose agar (PDA). Petri dishes (9 cm diameter), containing 15 ml of the test media, were seeded with 5mm diam. discs cut from the edge of a 7 days-old culture. The discs were placed upside down in the center of each plate. Colony morphology was noted after 3 and 6 days of incubation. Measurements of the growth rate were made along two lines intersecting at right angles at the center of the inoculum. Growth rate (mm d-1) was recorded on both media after 6 days. The inoculations were replicated twice in each media. Front and reverse photos of the colonies were taken at 3 and 6 days after inoculation.

Sporangial morphology

Discs (5 mm diam.), cut from the growing edge of a 7 days old culture, were grown on PDA at 20 °C in the dark, and then placed in a 9 cm Petri dish and flooded with sterile water. After incubation at 20 °C in the dark for 48–72 h, characteristic features of a number of fully mature sporangia were determined for each isolate at 400 magnifications.

Caducity determination

To determine the caducity in the isolates, a disk bearing sporangia from an old culture was inverted on a microscope slide in a drop of water and vigorously agitated with needle and then the slide was examined with a compound light microscope.

Cardinal temperature

The effect of temperature (5, 15, 20, 25, 30, and 35°C) on the growth of mycelium was tested on PDA. Each isolate was plated in triplicate and colony diameter was determined after 7 days of incubation.

Mating type

Homothallic and heterothallic species of *Phytophthora* generally produce oospores in V8 juice agar supplemented with 30 mg β-Sitosterol. Mating type was determined by pairing the isolates on cleared V8 juice agar in the dark at 25°C with A1 and A2 mating type of *P. cryptogea* and *P. drechsleri*. *Phytophthora* isolates collected from mango were also paired among themselves in all possible combinations. Reference testers (Table 4.1) were kindly provided by Prof. A. Pane (University of Catania, Italy).

Table 4.1 *Phytophthora* species used as reference isolates for mating type study.

***Phytophthora* spp.**	**International code**	**Mating type**	**Host**	**Source***
P. cryptogea	CBS 113. 19	A1	*Lycopersicon esculentum*	CBS
P. drechsleri	CBS 292. 35	A2	*Beta vulgaris* var. *altissima*	CBS

* CBS Centraalbureau voor Schimelcultures, Utrecht, the Netherlands

Molecular identification of *Phytophthora* spp.

Growing mycelium for DNA extraction

Phytophthora isolates were grown in Petri dishes containing Malt Extract Agar (MEA) media, covered with sterile cellophane sheets to facilitate collection of the mycelium. DNA was extracted using the method of Hoffman and Winston (1987), with minor modifications (See chapter 2).

PCR amplification and conditions

DNA of the internal transcribed spacers (ITS) were amplified using the universal primers ITS6 and ITS4 (Cooke *et al.*, 2000). ITS6 is a version of ITS5 (White *et al.*, 1990) modified by comparison against 18S sequences of *Phytophthora* to improve the amplification of DNA from oomycetes (Cooke & Duncan 1997). Fragments of the 28S ribosomal DNA (LSU) loci and 60S Ribosomal protein gene were amplified using, 60SL10F and 60SL10R, LROR-O and LSURint (Kroon *et al.*, 2004) primers, respectively. The region containing the

mitochondrial cytochrome c oxidase subunit I (COI) gene fragment was amplified using Oom-Lev and FM85- mod primers (Kroon *et al*., 2004). Primers used in this study, along with PCR amplification conditions are listed in Table 4.2. Amplifications were performed in C-1000 Thermal cycler (Bio Rad, USA). The final volume of mixture reactions was 25ul and the template DNA was 50 ng.

1.1.1. Gel electrophoresis and Sequencing

Amplicons were separated by electrophoresis in 1 % agarose gels in TAE buffer and stained for 10 min with Ethidium bromide (2 µg ml-1), and analyzed under UV light. PCR products were sequenced in forward and reverse orientation using the same primers for amplification. Purification of PCR products and Cycle sequencing reactions were performed at Primm SR1 sequencing service (http://www.primmbiotech.com/dna-sequencing.html), using the Big Dye system. ABI trace files were analyzed using CLC Genomics Workbench 5. Heterozygous or ambiguous sites were labelled using the IUPAC code and consensus sequences were exported for phylogenetic analysis.

Phylogenetic analysis

Single gene comparisons were applied in the study of the phylogenetic relationships. P. *cryptogea* sequences generated in this study were compared with those of other taxa obtained from GenBank. A preliminary alignment of sequences was made using ClustalX (Thompson *et al*., 1997) with subsequent visual adjustment. The alignments of each of the four regions were analyzed. The GenBank accession numbers of *Phytophthora* species used for phylogenetic studies are listed in Table 4.3.

Table 4.2. PCR primer sequences, reaction mixtures composition, and cycling conditions for the ITS, COI, LSU, and 60 ribosomal loci.

Locus	Primer name [a]	Primer sequence 5'- 3'	Size [b]	Reaction mixtures in 25µl volumes	Cycling conditions [d]	References
Internal transcribed spacer region (ITS)	ITS6	5'- GAAGGTGAAGTCGTAACAAGG-3'	900bp	5 µl 5X GoTaq buffer[c] 1.5 µl 25mM MgCl2 1 µl 40mM dNTP mix 0.5 µl 50 µM ITS4 primer 0.5 µl 50 µM of ITS6 primer 0.2 µl GoTaq Flexi DNA polymerase [c] 16.25 µl nuclease-free sterile water	94°C 3 min 35 cycles of 94°C 30 sec 51°C 30 sec 72°C 30 sec 72°C 10 min	(Cooke *et al.*, 2000)
	ITS4	5'- TCCTCCGCTTATTGATATGC-3'				
Cytochrome c oxidase subunit 1 (COI)	Oom-Lev	5'-TCAWCWMGATGGCTTTTTTCAAC-3'	727bp	5 µl 5X GoTaq buffer [c] 2 µl 25mM MgCl2 2.5 µl 40mM dNTP mix 1.5 µl 100 µM Oom primer 1.5 µl 100 µM of FM85 primer 0.2 µl GoTaq Flexi DNA polymerase [c] 11.3 µl nuclease-free sterile water	94°C 4 min 35 cycles of 94°C 30 sec 55°C 1 min 72°C 1 min 72°C 5 min	(Martin & Tooley, 2003)
	FM85-mod	5'-RRHWACKTGACTDATRATACCAAA-3'				
28S Ribosomal DNA (LSU)	LROR-O	5'- ACCCGCTGAACTYAAGC-3'	817bp	5 µl 5X GoTaq buffer [c] 2 µl 25mM MgCl2 2.9 µl 40mM dNTP mix 1.2 µl 100 µM LRORO primer 1.2 µl 100 µM of LSURint primer 0.25 µl GoTaq Flexi DNA polymerase [c] 10.9 µl nuclease-free sterile water	94°C 2 min 35 cycles of 94°C 30 sec 53°C 30 sec 72°C 2 min 72°C 5 min	(Blair *et al.*, 2008)
	LSURint	5'- TTTCCACACCCTAACACTTGC-3'				
60S Ribosomal protein	60SL10F	5'-GCTAAGTGTTACCGTTTCCAG-3'	456bp	5 µl 5X GoTaq buffer [c] 2 µl 25mM MgCl2 1.8 µl 40mM dNTP mix 1.5 µl 100 µM 60SL10F primer 1.5 µl 100 µM of 60SL10R primer 0.2 µl GoTaq Flexi DNA polymerase [c] 12 µl nuclease-free sterile water	94°C 3 min 35 cycles of 94°C 30 sec 52°C 30 sec 72°C 1 min 72°C 5 min	(Blair *et al.*, 2008)
	60SL10R	5'-ACTTCTTGGAGCCCAGCAC-3'				

[a] Primers used for PCR amplification and sequencing
[b] Average amplicon length
[c] GoTaq buffer and GoTaq Flexi DNA polymerase from Promega (Madison, WI).
[d] Cycling conducted on C-1000 Thermal cycler (Bio-Rad Laboratories, Inc, USA).

Table 4.3 Phytophthora species and origins of their isolates studied and their GenBank sequence accession numbers

Phytophthora species	Isolate Code [a]	Host	Origin	Reference [b]	GenBank accession No.			
					ITS	*COI*	*LSU*	*60S*
P. cinnamomi	P6305	Avocado	Indonesia	(Blair *et al.*, 2008)	FJ801911	HQ261272.1	EU079898.1	EU079893.1
P. citricola	P10338	Raspberry	Ireland	(Blair *et al.*, 2008)	GU259256.1	NA	EU079530.1	EU079525.1
P. citrophthora	P6310	Cacao	Indonesia	(Blair *et al.*, 2008)	FJ801913	NA	EU080542.1	EU080537.1
P. hibernalis	P3822	Citrus	Australia	(Blair *et al.*, 2008)	FJ801869	HQ261323.1	EU079518.1	EU079513.1
P. palmivora	P0255	Cacao	Costa Rica	(Blair *et al.*, 2008; Robideau *et al.*, 2011)	FJ801246	HQ261382.1	EU080343.1	EU080338.1
P. erythroseptica	P10382	Potato	USA	WPC - (Blair *et al.*, 2008)	FJ801423	HQ261301.1	EU080780.1	EU080775.1
P. drechsleri	P10331	Gerbera	USA	WPC - (Blair *et al.*, 2008)	FJ801387	HQ261300.1	EU079511.1	EU079506.1
P. cryptogea	P1088	Aster	USA	(Robideau *et al.*, 2011)	HQ261549.1	HQ261549.1	EU080451.1	EU080446.1
P. cryptogea	P16165	oil palm	Colombia	(Robideau *et al.*, 2011)	GU259130	HQ261294.1	NA	NA
P. cryptogea	P11822	Aster	USA	(Blair *et al.*, 2008; Robideau et al., 2011)	FJ801629	NA	EU080082	EU080079
P. drechsleri	IMI040500	Potato	Argentina	(Mostowfizadeh-Ghalamfarsa *et al.*, 2010)	AY659444	NA	NA	NA
P. nicotianae	P1325	Citrus	USA	WPC	GU258781	NA	NA	NA
P. citrophthora	P0318	Citrus	Australia	WPC	FJ801247	NA	NA	NA
P. cryptogea	IMI 397473	Mandeville	Italy	(Pane *et al.*, 2010)	GQ463702	NA	NA	NA
P. cryptogea	AN1	Cherry	Italy	(Vettraino *et al.*, 2008)	F661576.1	NA	NA	NA
P. cryptogea	P3848	Kiwi	USA	WPC	FJ801872	NA	NA	NA

[a] Local identification numbers from the World Phytophthora Genetic Resource Collection (P).
[b] Isolate sequences were obtained from the World Phytophthora Genetic Resource Collection; http://phytophthora.ucr.edu/databasemain.html
NA, not available

Single-strand conformation polymorphism (SSCP)

Four isolates of *P. cryptogea* and two isolates of other species (*P. palmivora* and *P. nicotianae*) were included in SSCP study. *Phytophthora* isolates were cultured and DNA was extracted using the method of Hoffman and Winston (1987). DNA amplification and SSCP analysis of the PCR products were performed as described previously by Kong *et al.* (2004) with minor modifications. PCR amplification was done using ITS-4 and ITS-6 primer pairs. Reactions were performed in a 25µl mixture volume. Each reaction contained 1µl of 50 ng/µl of genomic DNA, 5µl of 5x PCR buffer, 15.3 ml of sterile distilled water, 1µl of DNTPs, 5µl of 25 mM MgCl2, 0.5 µl each of forward and reverse primers (ITS-4 and ITS-6) and 0.2µl of GoTaq® DNA polymerase (5U/µl Promega, Madison WI-USA). The thermal cycling parameters were initial denaturation step at 94°C for 3 min followed by 35 cycles consisting of denaturation step at 94°C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 30 sec. A final extension at 72°C for 10 min was done at the end of the amplification. One microliter of individual PCR products was mixed with 9 µ l of the denaturing buffer (95% formamide, 20 mM EDTA and 0.05% bromophenol blue). After a brief spin, mixtures were heated at 95°C for 10 min then chilled on ice. Five microliters of each mixture was loaded on an 8% acrylamide: Bis (29: 1) non denaturing minigel cast using a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, polyacrylamide gels were peeled from the glass plate and soaked in 100 ml (for two gels) of 10% acetic acid for 20 min, and placed in the same amount of 1% nitric acid for 3 min. After two brief washes with 100 ml dH_2O, gels were stained in 50 ml of 2 ppm silver nitrate for 20 min then rinsed three times in 200 ml dH_2O. Gels were developed by briefly rinsing in 30 ml of 1 ppm formaldehyde in 3% sodium carbonate until desired band intensity was reached. The stain was fixed in 10% acetic acid once the SSCP patterns were visible.

Pathogenicity tests

Five representative isolates of *Phytophthora* obtained from affected mango trees were tested for pathogenicity on 6-months old mango plants (cv. Kensington Pride). The inoculum was produced by growing isolates on sterile medium-texture vermiculite moistened with V8 juice for 20 days at 24±1°C, then mixed to the soil at the rate of 1% (v/v). *Phytophthora* propagules free growing medium was used as a control. Inoculated and no inoculated plants were subjected to soil saturation for 24 h every 2 weeks, keeping each plant wet with a continuous water column above the soil surface. After flooding, plants were drained and returned to a normal irrigation regime. All plants were incubated in the greenhouse for 10 months at 25°C and 90–95% relative humidity. The inoculated plants were examined for symptoms development. The disease severity of the aerial and root symptoms was visually estimated for each plant on a 0-to-5 scale. For aerial symptoms, 0 = healthy plants and 1, 2, 3, 4, and 5 were < 20, 21 to 40, 41 to 60, 61 to 80, and >80% of the foliage showing symptoms, respectively. For root rot, 0 = all healthy roots and 1, 2, 3, 4, and 5 were < 20, 21 to 40, 41 to 60, 61 to 80, and >80% rotted roots, respectively. The fresh weight of root and foliage was determined for each plant. The experiment was arranged in completely randomized factorial design and was repeated once. Three plants were used for each isolate.

Data were subjected to the analysis of variance one way ANOVA and mean values of the lesion were compared using the Least Significant Difference (LSD) test.

4. Results

Survey of Phytophthora root and crown rot disease in Sicily

Our survey results revealed the presence of Phytophthora root and crown rot disease associated with mango trees in Sicily. *Phytophthora* propagules were recovered from different soil, crown and roots samples collected from different locations in the provinces of Messina and Palermo (Fig. 4.1).

The first signs of the disease displayed on the canopy with pale green leaves with yellow veins, wilting and chlorotic tree (Fig. 4.2). The symptoms developed on the bark as cracks and lesions, whereas root and crown rots were found at or below the ground level. The small feeder roots were in some cases absent; when present, they were black, brittle and decayed (Fig. 4.3). No mortality of trees was observed.

Isolation of *Phytophthora* spp. was consistently made on *Phytophthora* selective media from symptomatic mango plants. No *Phytophthora* propagules were recovered from the mango nursery.

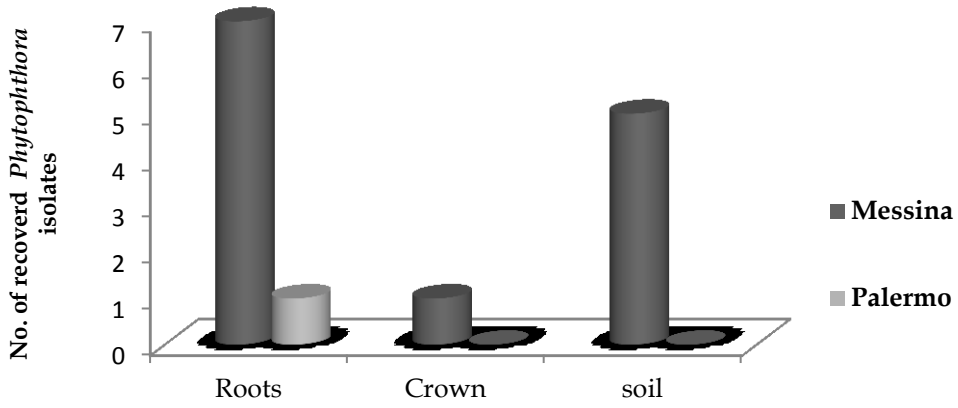

Figure 4.1 Number of *Phytophthora* isolates recovered from Messina and Palermo

Figure 4.2 Symptoms of Phytophthora root and crown rot on mango canopy appeared as pale green leaves, wilt and chlorotic trees.

Figure 4.3 Symptoms of Phytophthora root and crown rot on mango roots.

Morphological identification of *Phytophthora* spp.

a. Colony morphology on PDA and V8 media

Fourteen *Phytophthora* isolates showed different colony morphology when grown on PDA and V8 for 6 days at 25°C. Colonies on PDA was white petalloid in isolate Ph2, recovered from province of Messina (Fig. 4.4), and rosaceous with fluffy mycelium in isolates Ph13, recovered from province of Palermo (Fig. 4.5). On V8A all the colonies showed a stellate pattern (Figs. 4.4, 4.5). The linear growth of *Phytophthora* isolates was varied on PDA after 6 days and ranging from 59 to 82 mm of diameter. On V8 medium all isolates had the same diameter of 90 mm after 6 days.

b. Sporangial morphology

The sporangial morphology was observed in water after 3 days. All *Phytophthora* isolates showed similar characteristics. All isolates produced hyaline, coenocytic hyphae, branching at right angles, and developed irregular, round, angular hyphal swellings (Figs. 4.6, 4.7). Sporangia were nonpapillate ovoid to obpyriform with dimension of 41 x 26 µm (31-54 x 19-31µm) and length: breadth ratio 1.8 (1.1- 1.5), produced terminally or on sympodially branching sporangiophores. Internal and external proliferations of the sporangiophores were also observed (Figs. 4.6, 4.7).

c. Caducity determination

No caducity was observed in the sporangia of all *Phytophthora* isolates.

d. Cardinal temperature

The minimum and maximum temperatures for growth on PDA and V8 were 10 and 35°C respectively, with the optimum at 20 and 25°C. No mycelial growth was obtained at 5 and 35°C.

Figure 4.4 Colony morphology of *Phytophthora* isolate (Ph2) recovered from Messina, on PDA and V8 media after 6 days at 25°C. Top (from left to right) PDA - V8 and bottom (from left to right) PDA - V8 reverse colony.

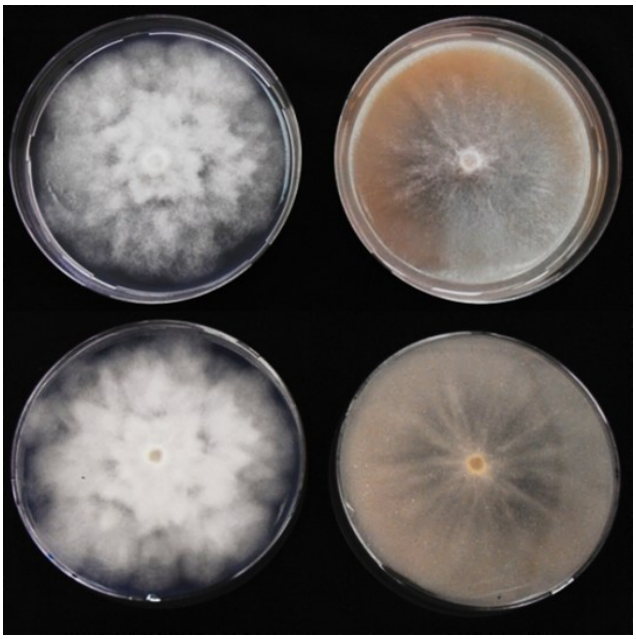

Figure 4.5 Colony morphology of *Phytophthora* isolate (Ph13) recovered from Palermo, on PDA and V8 media after 6 days at 25°C. Top (from the left to the right) PDA - V8 and bottom (from the left to the right) PDA - V8 reverse colony.

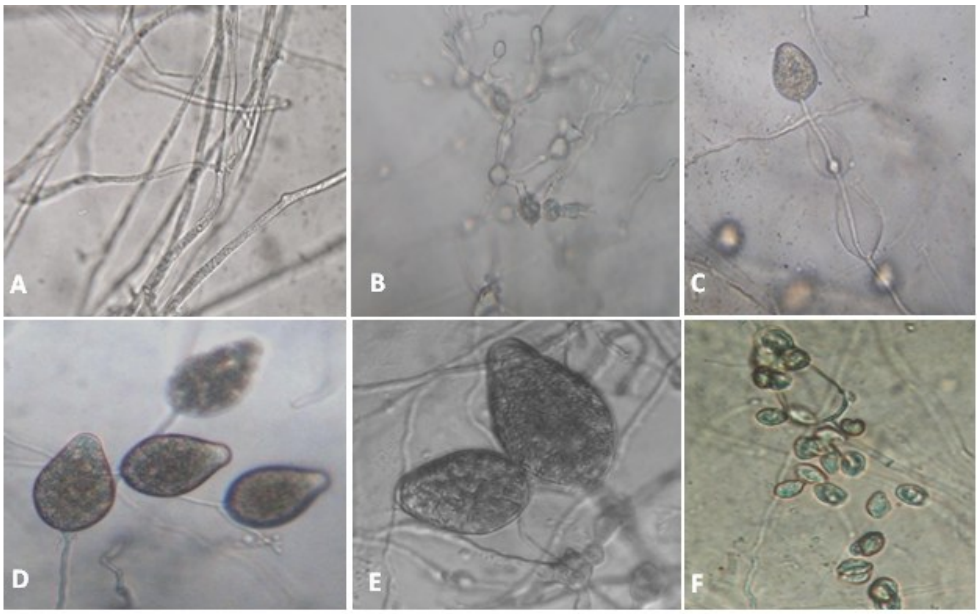

Figure 4.6 Morphological characteristics of *Phytophthora* isolate (Ph2) recovered from Messina. A, hyaline coenocytic hyphae; B, hyphal swellings; C, proliferations of the sporangiophores; D and E, nonpapillate ovoid to obpyriform; F, zoospores.

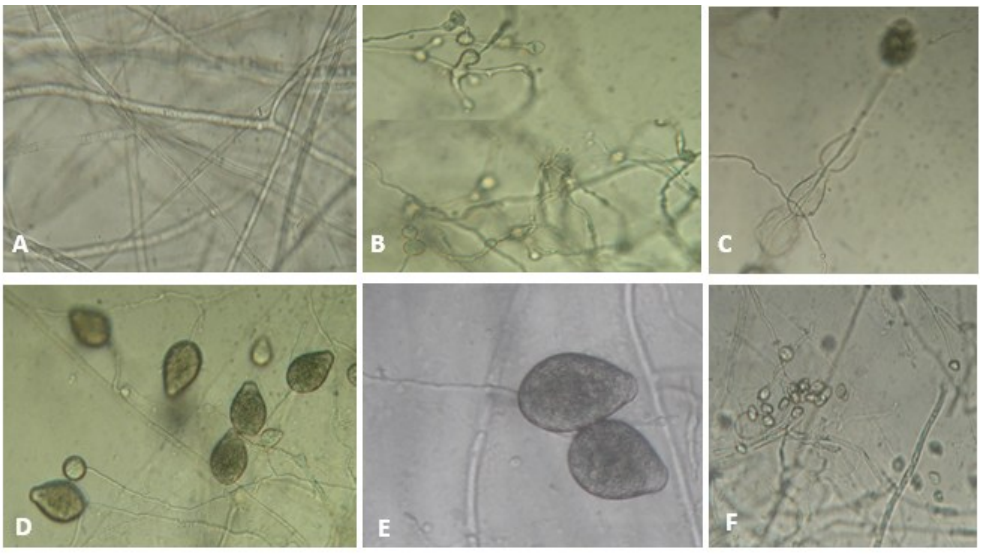

Figure 4.7 Morphological characteristics of *Phytophthora* isolate (Ph13) recovered from Palermo. A, hyaline coenocytic hyphae; B, hyphal swellings; C, proliferations of the sporangiophores; D and E, nonpapillate ovoid to obpyriform; F, zoospores.

a. Mating type

None of the isolates formed sexual structures in single culture; however 13 isolates formed oospores when paired in dual cultures with the reference isolate of *P. cryptogea* mating type A1 (CBS 113.19), and were treated as heterothallic isolates of mating type A2 (Table 4.4). Isolate Ph1 recovered from Messina did not form any sexual structures and was treated as sterile isolate.

The isolates formed smooth oogonia with amphigynous antheridia, (Figs. 4.8, 4.9), and spherical, plerotic oospores 26 μm (23-31 μm).

Table 4.4 Sexual compatibility among isolates of *P. cryptogea* infecting mango in Italy

***P. cryptogea* isolates (from mango)**	**Self-crossing**	**Compatibility types [a]**	
		A1 ***P. cryptogea*** **(CBS 113.19)**	**A2** ***P. drechsleri*** **(CBS 292.35)**
Ph1	-	-	-
Ph2 to Ph14	-	+	-

[a] Oospores were present (+) or absent (–) on amended β-Sitosterol V8 media after 20 days of incubation in the dark at 25 °C.

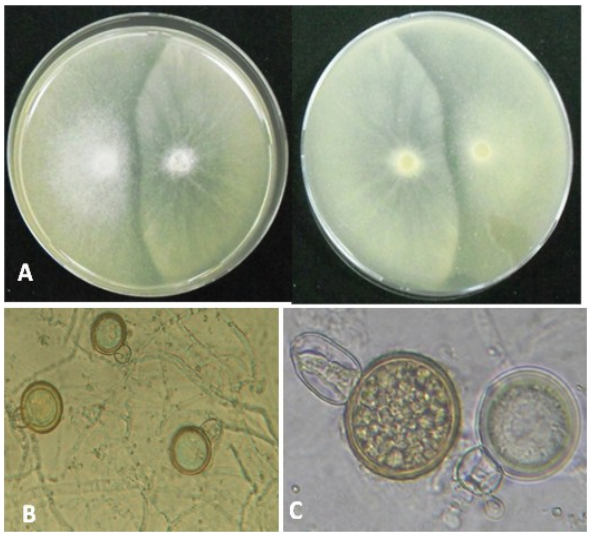

Figure 4.8 Mating of *Phytophthora* spp. A, dual culture of pairing *Phytophthora* isolate (Ph2) recovered from Messina with *P. cryptogea* (CBS 113.19 mating type A1) on amended β-Sitosterol V8 media after 20 days of incubation in the dark at 25°C. B, smooth oogonia with amphigynous antheridia. C, spherical plerotic oospores.

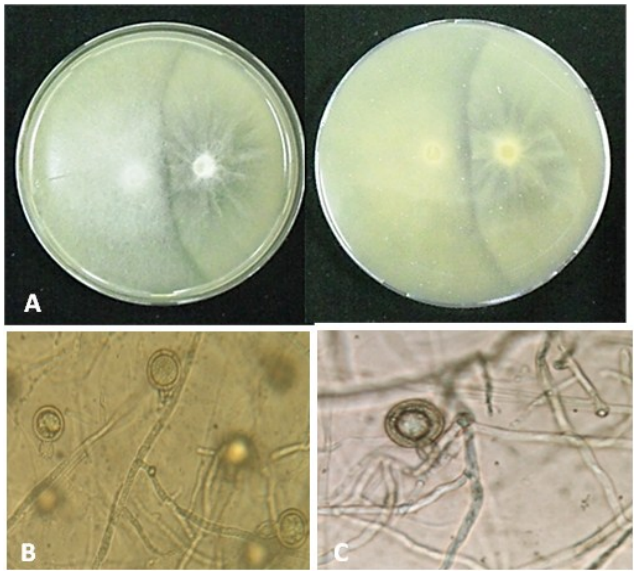

Figure 4.9 Mating of *Phytophthora* sp. A, dual culture of pairing *Phytophthora* isolate (Ph13) recovered from Palermo with *P. cryptogea* (CBS 113.19 mating type A1) on amended β-Sitosterol V8 media after 20 days of incubation in the dark at 25°C. B, smooth oogonia with amphigynous antheridia. C, spherical plerotic oospores

Molecular identification of *Phytophthora* spp.

Fourteen isolates of *Phytophthora* were molecularly identified. Four loci were successfully amplified and sequenced for phylogenetic analysis. These loci included nuclear (ITS region of the ribosomal DNA, 60S ribosomal proteinL10, the 50 portion of the 28S ribosomal DNA) and mitochondrial (COI) loci.

For ITS rDNA region, a fragment of approximately 900 bp was amplified using ITS4-ITS6 primer set (Fig. 4.10 lane 1- 14) and the same fragment of the expected size was obtained from the positive controls (Lane 15). No amplification was achieved with the negative DNA control (Lane H_2O). Amplicons of approximately 727bp were obtained with Cytochrome c oxidase subunit 1 (COI) using Oom-lev and Fm85-mod primers pair (Fig. 4.11).

PCR products of the amplified 28S Ribosomal DNA (LSU) region was about 817bp using LROR-O and LSURint primers (Fig. 4.12), while amplicons of 456bp were obtained by 60S Ribosomal protein using 60SL10F and 60SL10R primers pair (Fig. 4.13).

Phylogenetic analysis

Analysis of the five individual loci showed gene – gene concordance in the five observed lineages. The positions of *P. cryptogea* isolates collected from mango in Italy in the phylogenetic trees were typical (Figs. 4.14, 4.15, 4.16, 4.17).

All the isolates were grouped within *P. cryptogea* isolates collected from other hosts.

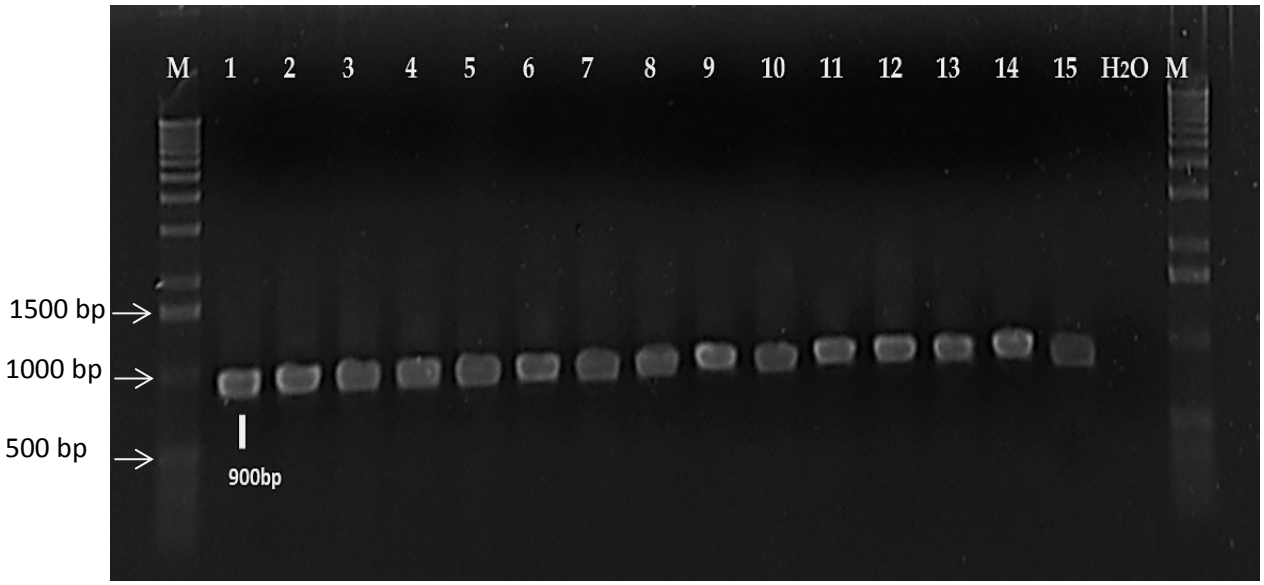

Figure 4.10 Agarose gel electrophoresis of amplified PCR products of the internal transcribed spacer (ITS- rDNA) region using ITS4 and ITS6 primers pair. *P. cryptogea* isolates from mango (Lanes 1 to 14); *P. nicotianae* IAMB160 isolate as positive control (Lane 15); Lane H_2O contains negative DNA control and Lane M contains 100-bp DNA ladder.

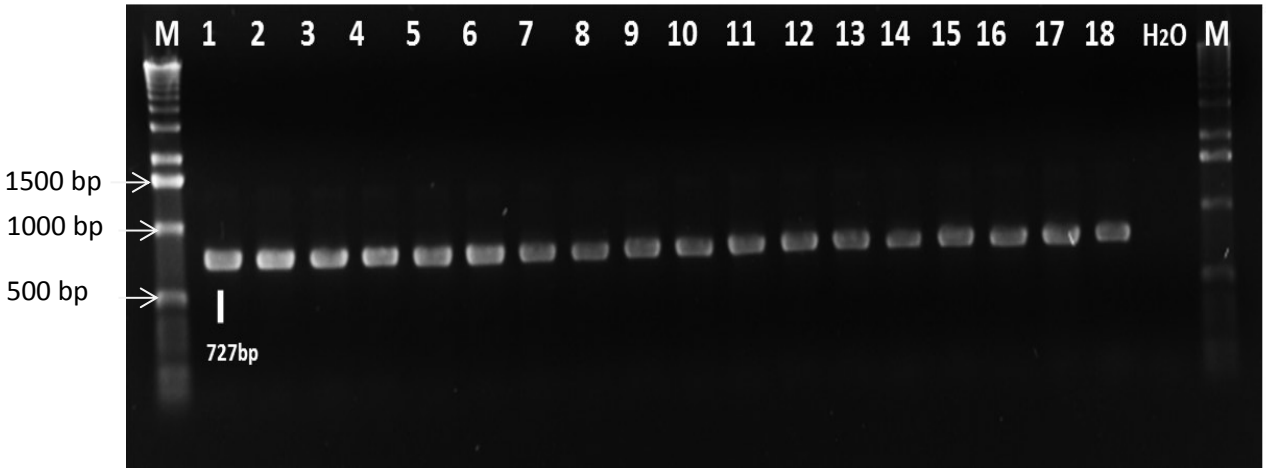

Figure 4.11 Agarose gel electrophoresis of amplified PCR products of Cytochrome c oxidase subunit 1 (COI) using Oom-lev and Fm85-mod primers pair. *P. cryptogea* isolates from mango (Lanes 1 to 14); *P. nicotianae* IAMB160 (Lane 15); *P. citrophthora* IAMB161 (Lane 16); *P. palmivora* IAMB162 (Lane 17); *P. nicotianae* IAMB163 as positive control (Lane 18); Lane H_2O contains negative DNA control and Lane M contains 100-bp DNA ladder.

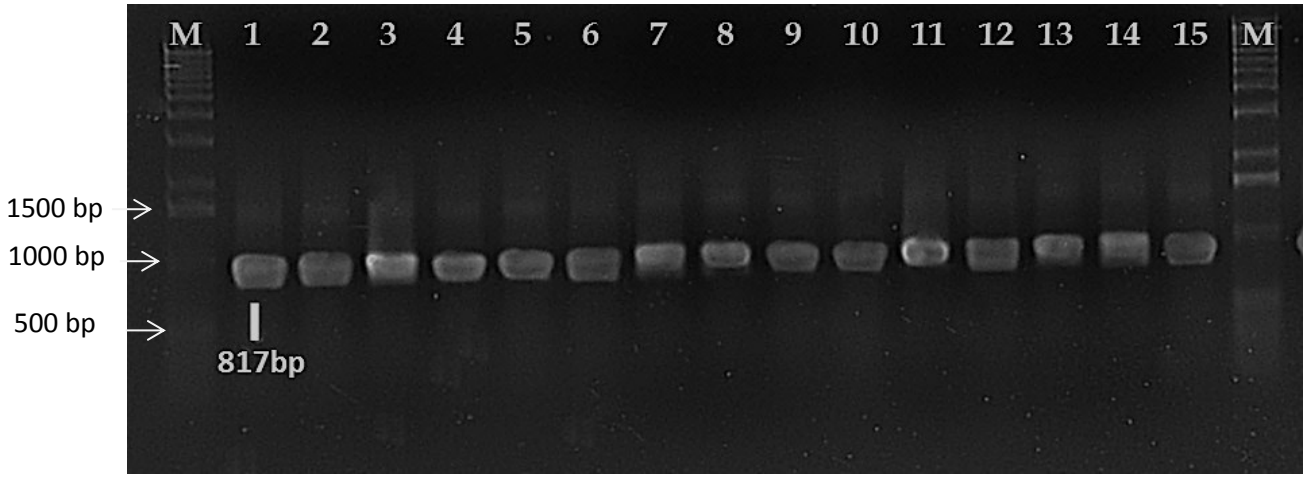

Figure 4.12 Agarose gel electrophoresis of amplified PCR products of 28S Ribosomal DNA (LSU) using LROR-O and LSURint primers pair. *P. cryptogea* isolates from mango (Lanes 1 to 14); *P. nicotianae* IAMB160 isolate as positive control (Lane 15) and Lane M contains 100-bp DNA ladder.

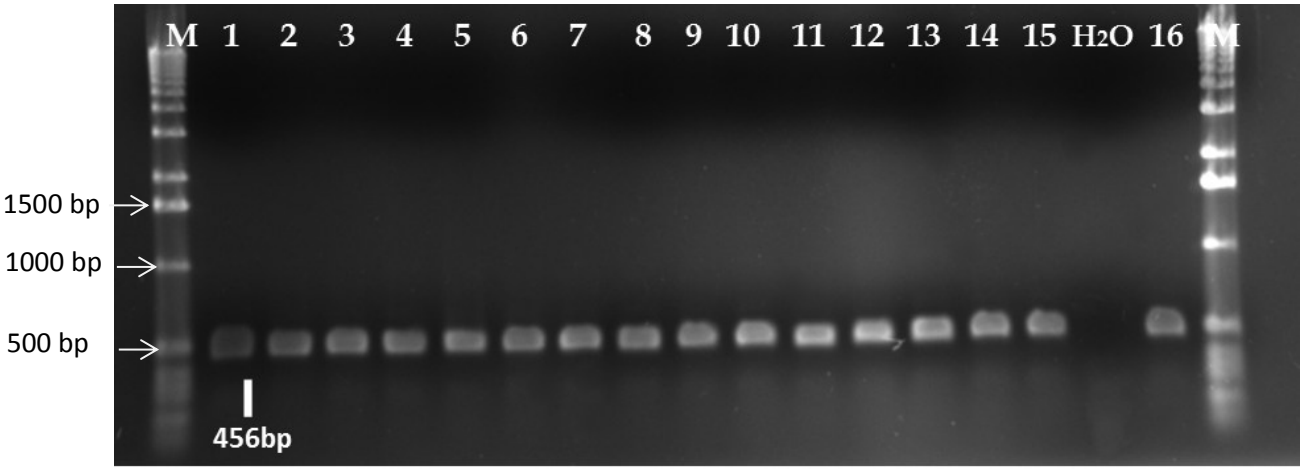

Figure 4.13 Agarose gel electrophoresis of amplified PCR products of the 60S Ribosomal protein using 60SL10F and 60SL10R primers pair. *P. cryptogea* isolates from mango (Lan1 to 14); *P. nicotianae* IAMB160 isolate as positive control (Lane 15); *P. palmivora* IAMB162 isolate as positive control (Lane 16); Lane H_2O contains negative DNA control and Lane M contains 100-bp DNA ladder.

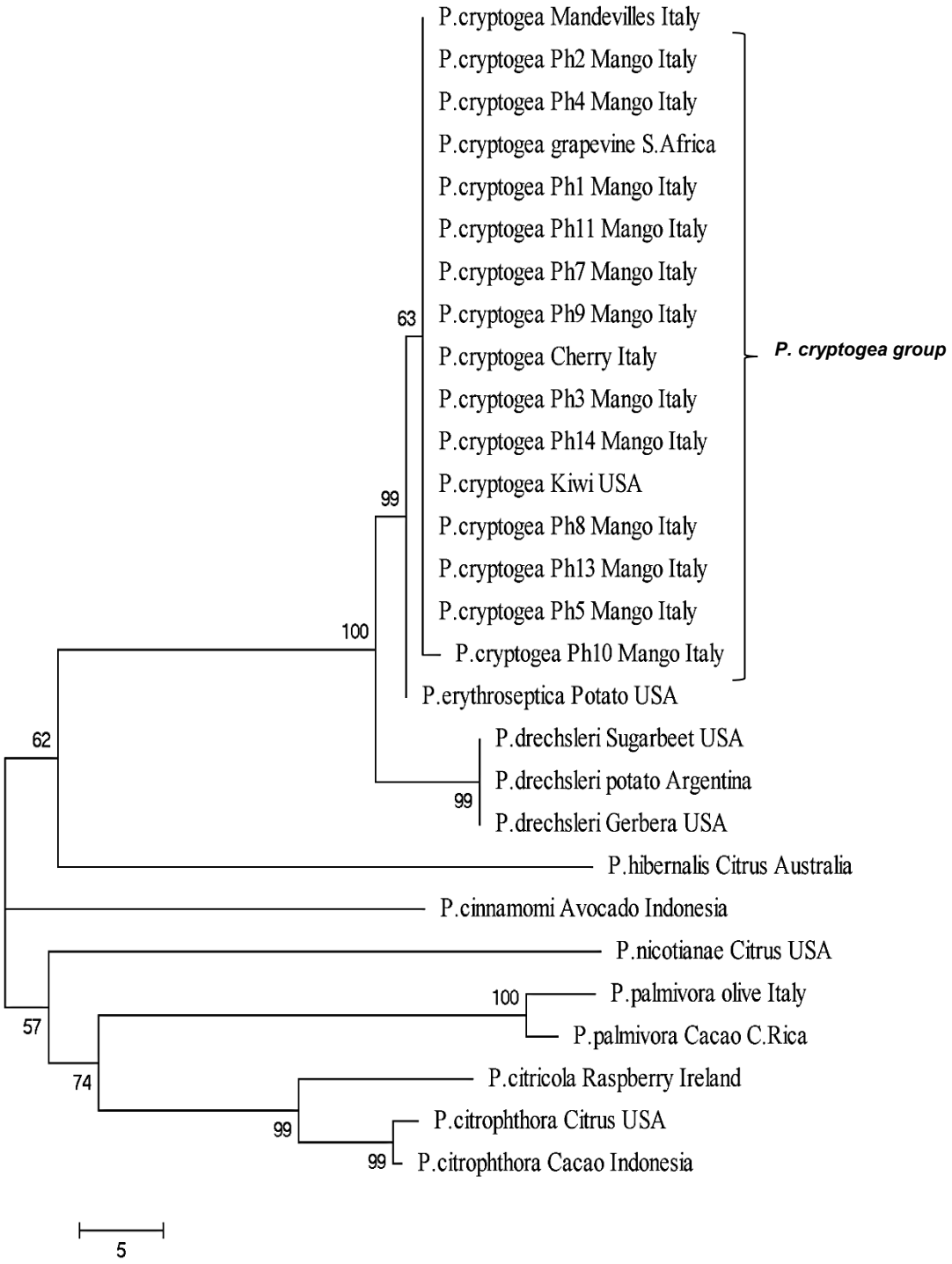

Figure 4.14 Phylogenetic relationships of 5 *P. cryptogea* isolates from mango and 6 *Phytophthora* taxa using ITS sequence data, based on maximum likelihood analysis. The numbers at the branch points indicate the percentages of bootstrap values ≥50 %.

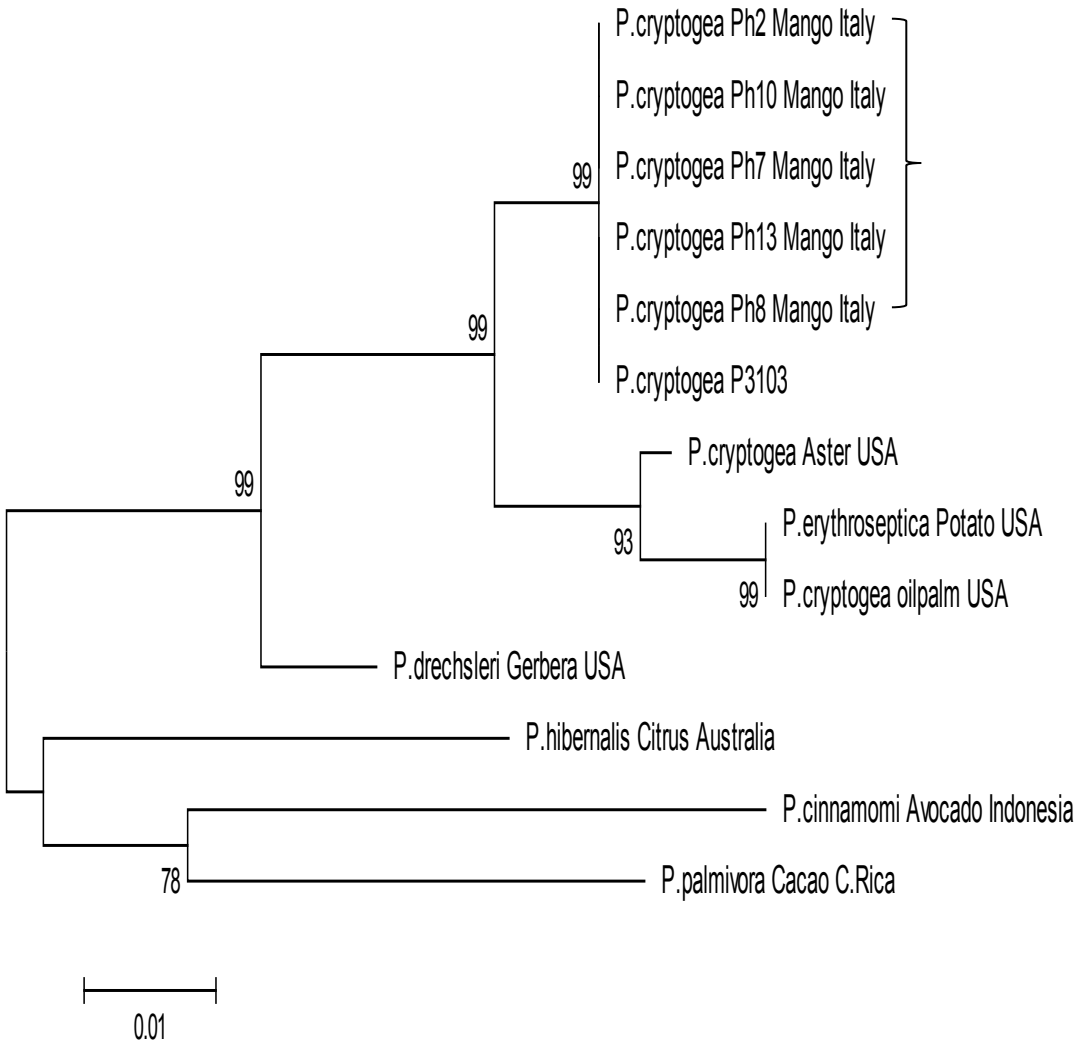

Figure 4.15 Phylogenetic relationships of 5 *P. cryptogea* isolates from mango and 6 *Phytophthora* taxa using COI sequence data, based on maximum likelihood analysis. The numbers at the branch points indicate the percentages of bootstrap values ≥50 %.

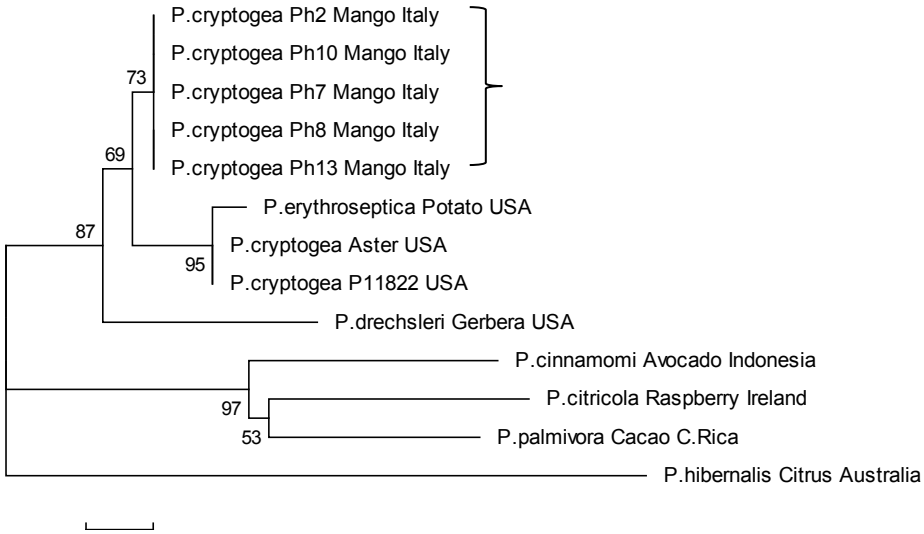

Figure 4.16 Phylogenetic relationships of 5 *P. cryptogea* isolates from mango and 6 *Phytophthora* taxa using LSU sequence data, based on maximum likelihood analysis. The numbers at the branch points indicate the percentages of bootstrap values ≥50 %.

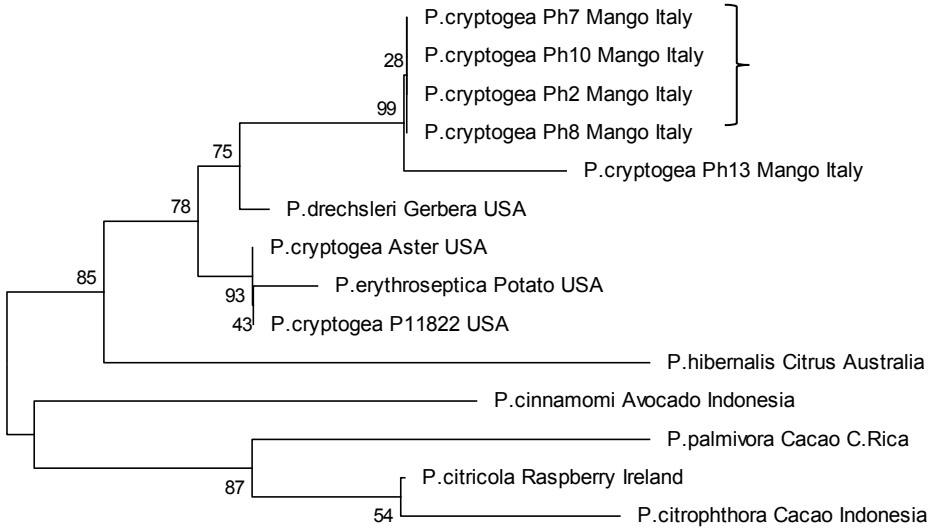

Figure 4.17 Phylogenetic relationships of 5 *P. cryptogea* isolates from mango and 6 *Phytophthora* taxa using 60S sequence data, based on maximum likelihood analysis. The numbers at the branch points indicate the percentages of bootstrap.

Single-strand conformation polymorphism (SSCP)

Four isolates of *P. cryptogea* tested for SSCP analysis had an identical banding pattern (Fig. 4.18), with two bands different in their position (Lane 3; Lane 4; Lane 5 and Lane 6) when compared with other tested species, *P. nicotianae* (Lane 1) and *P. palmivora* (Lane 2). (Fig. 4.18).

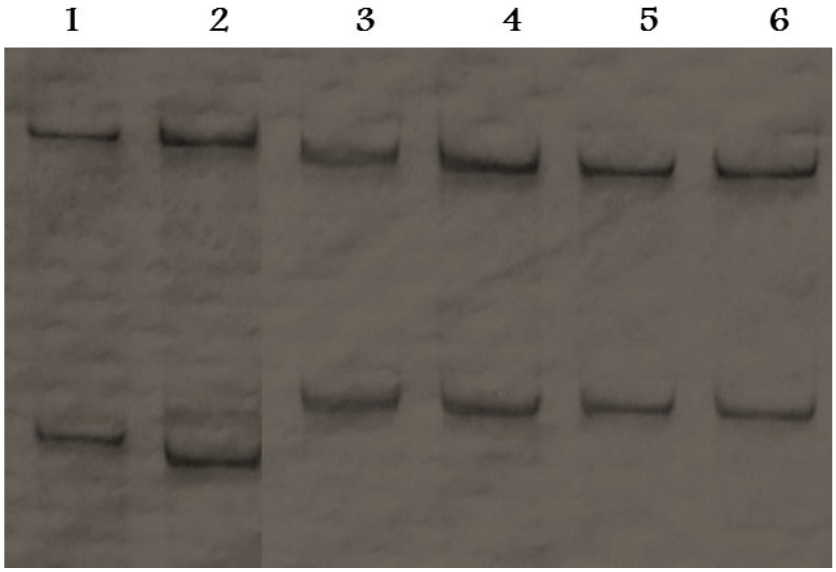

Figure 4.18 Single-strand-conformation polymorphism (SSCP) profiles of ITS for *P. nicotianae* (Lane 1); *P. palmivora* (Lane 2) and *P. cryptogrea* (Lanes 4-7).

Pathogenicity test

Five representative isolates of *Phytophthora* obtained from affected mango trees were tested for pathogenicity on 6-months old mango plants (cv. Kensington Pride).

All tested mango plants developed moderate leaf chlorosis, lesions and wilting when inoculated with *P. cryptogea* isolates. First symptoms consisted of leaf wilting and appeared after 10 months on inoculated plants. These foliage symptoms were always associated with root rot and a considerable reduction of the root mass, root weight, and foliage weight (Fig. 4.19, 4.20).

Based on the foliar and root symptoms, significant ($P < 0.05$) differences in severity were found with *P. cryptogea* isolates, with Ph10 and Ph8 as the most severe. For instance, root fresh weight was reduced significantly with a mean root rot index of 2.5, 2.6 when plants were root inoculated with isolates Ph-3 and Ph-5, respectively, whereas there was only 2.0 for isolate Ph13 (Table 4.5).

Phytophthora propagules reisolated from pathogenicity tests were confirmed as *P. cryptogea* by microscopic examination.

Figure 4.19 Root and crown rot symptoms on mango plants, 10 months after inoculation with P. cryptogea (Ph2).

Figure 4.20 Pathogenicity test of *P. cryptogea* on mango plants, 10 months of after inoculation. Disease severity of root symptoms in a 0-to-5 scale, where 0 = healthy roots and 1, 2, 3, 4, and 5 were <20, 21– 40, 41–60, 61–80, and >80%, respectively, of rotted roots.

Table 4.5 Pathogenicity tests of five isolates of *Phytophthora cryptogea* on mango plants, 10 months after inoculation.

P. cryptogea	Pathogenicity [a]				
	Plant height (cm)	Root symptoms		Foliar symptoms	
		Severity [b]	Fresh weight (g) [c]	Severity [b]	Fresh weight (g) [c]
Ph2	46.3 [ac]	2.3 [ab]	17.3 [ab]	2.29 [ab]	22.8 [ab]
Ph7	56.0 [ab]	2.3 [ab]	17.6 [ab]	2.27 [ab]	33.8 [ab]
Ph8	35.7 [c]	2.5 [ab]	15.3 [a]	2.44 [b]	22.8 [ac]
Ph10	48.0 [ab]	2.6 [b]	11.3 [a]	2.5 [a]	13.9 [c]
Ph13	59.3 [b]	2.0 [a]	22.8 [b]	1.9 [b]	38.8 [b]
Control	110.3 [d]	1.41 [c]	64.7 [c]	1.41 [c]	137.3 [d]

a Means of three replicates within a column followed by a common letter are not significantly different according to Duncan-Waller *k*-ratio *t* tests ($P = 0.05$).

[b] Percent severities were angular transformed [sqr (x+2)] before analysis. Severity was visually estimated on a 0-to-5 scale, where 0 = healthy roots or foliage and 1, 2, 3, 4, and 5 were <20, 21–40, 41–60, 61–80, and >80%, respectively, of rotted roots or foliage showing wilting, chlorosis, leaf necrosis, and stunt.

[c] Fresh weights were determined on air-dried roots and foliage.

5. Discussion

Mango is liable to infection with many soilborne and airborne diseases causing a reduction and significant losses in crop yield. Among these diseases there is Phytophthora crown and root rot disease. *Phytophthora* causes a variety of devastating diseases on many crops. Most species cause root rots, damping-off of seedlings, and rots of lower stems and roots. Some species attack only one or two species of host plants, but others may cause similar or different symptoms on many kinds of host plants. Mango has introduced into Sicily recently thus, the sanitary status of the trees is still unknown. Phytophthora root and crown rot of mango has not been reported in Sicily previously. Therefore, this study aimed to assess the presence of Phytophthora crown and root rot in mango orchards located in Sicily and to characterize *Phytophthora* species associated with the disease.

During the survey period, different types of symptoms were observed on mango trees. The most common diseases were general chlorosis, yellowing of the foliage, defoliation, stunting and crown and root rot. Similar symptoms on other fruit crops were attributed to *Phytophthora* infection (Vettraino *et al*., 2008). Colonies of *Phytophthora* spp. were consistently obtained on PARPH selective medium from symptomatic root tissues and rhizosphere soil. No *Phytophthora* propagules were recovered from mango nursery during the survey time while several isolates were recovered from commercial orchards located in Palermo and Messina. *Phytophthora* propagules were recovered from different soil, crown and roots samples.

Identification of some *Phytophthora* species is relatively simple; however the morphological differences among some species are so small and some characteristics are so variable. In this study, morphological identification was carried according to the Stamps *et al*. (1990) key, the latest revision of Waterhouse (1963), and the explanation discussed in Chapter 4 in Phytophthora Diseases Worldwide (Erwin and Ribeiro, 1996); also the species description by Gallegly and Hong (2008) was considered.

The presence of non papillated, non caducous sporangia restricts isolates identification to Group VI. The amphigynous position of antheridia, the shape and dimensions of sporangia, the presence of chlamydospres, hyphal swellings of the mycelia, the growth at 35°C and the heterothallism, all of these features are characters that

distinguish *P. cryptogea* from the other species included in the group. Differentiating *P. cryptogea* from the closely-related species *P. drechsleri* was quite difficult because of highly overlapping morphological characters. *P. drechsleri* was described on potato tubers by Tucker (1931) as being similar to *P. cryptogea* but different in its ability to grow well at 35°C. In Tucker's original comparison of *P. cryptogea* and *P. drechsleri* he proved both species being alike, but indicated that they could be separated using temperature. In this study, all the 14 *Phytophthora* isolates recovered from mango did not show any mycelial growth at 35°C, a feature that characterizes this species from *P. drechsleri.* Therefore, the fourteen isolates examined were identified as *P. cryptogea*. Similar results were obtained by Mills *et al.* (1991) as all the isolates in *P. cryptogea* groups did not grow at 35°C. These findings also are confirmed in previous studies by Cacciola *et al.* (2002), Vettraino *et al.* (2002), Cacciola *et al.* (2005), Vettraino *et al.* (2008) and Mostowfizadeh-Ghalamfarsa *et al.* (2010).

During our study, all *P. cryptogea* isolates recovered from Messina and Palermo were A2 mating type. A1 was not present which greatly reduces the opportunity for sexual reproduction to occur. Asexual reproduction appears to be the primary means of reproduction in mango field while oospores can be important survival structures.

The traditional classification, based on morphological and growth characteristics, has several limitations. Growth characteristics and optimal growth temperature are not always reliable. Morphological traits are depend on the method used for measurement (e.g. the effect of growth media or host tissue on oospore size), or may vary because of ambiguity in trait description by observers (e.g. papillate vs. semi-papillate). Additionally, growth characteristics and morphological traits are phenotypic, and groups of species sharing similar traits do not necessarily reflect evolutionary relatedness, since they may have evolved independently.

The most accurate molecular method for identification of isolates to a species level is accomplished by sequence analysis of specific loci. Sequence-based species identification methods are now considering the biggest leap in information on *Phytophthora* species so far. If the sequences for particular genes or DNA regions are identical or nearly identical, the isolates supposedly belong to the same species (Kroon *et al.*, 2004). If DNA sequences of the same region are available for many species, a

phylogeny can be made (Kroon *et al.*, 2012). Species can then be grouped in clades, consisting of a single common ancestor and all its descendants.

In this study four loci were successfully amplified and sequenced for phylogenetic analysis. These loci included nuclear (ITS region of the ribosomal DNA, 60S ribosomal proteinL10, the 50 portion of the 28S ribosomal DNA) and mitochondrial (COI) loci. These genes do not contain introns and are conserved throughout eukaryotes. All the loci grouped *P. cryptogea* isolated from mango in distinct clade with other *P. cryptogea* from other hosts.

The most common region of DNA, being used for identification of oomycetes to the species level, is the internal transcribed spacer (ITS) region of rDNA. The ITS region in oomycetes is easy to amplify for DNA sequencing in most species with the use of universal eukaryotic PCR primers (Cooke *et al*., 2000).

Cytochrome c oxidase subunit I (COI, COX1) is a mitochondrially encoded gene which is recognized as an extremely useful DNA barcode capable of accurate species identification in a very broad range of eukaryotic life forms (Robideau *et al.*, 2011). COI is the default DNA barcode approved by GenBank and the Consortium for the Barcode of Life (CBOL). COI has proven useful in phylogenetic studies of the oomycete genus.

The 28S ribosomal DNA locus been used in other studies of Peronosporomycete and Oomycete phylogeny (Voglmayr *et al.*, 2004), and in multilocus phylogeny of *Phytophthora* (Blair *et al*., 2008) and is an appropriate candidate for comparisons across genera and families due to its high level of sequence conservation.

The 60S ribosomal protein L10 locus was also consistently amplified across the genus; however, this locus provided very little phylogenetic resolution among species due to its short length (456 bp) and thus limited number of variable characters.

The single strand conformation polymorphism (SSCP) study aimed to determine if the analysis can be used for differentiation of *P. cryptogea* and other *Phytophthora* species. Although the analysis was performed with few isolates, but the results indicate that *P. cryptogea* can be easily distinguished from other species examined, especially those that may cause similar root rot symptoms on mango plants. This study provides additional evidence that SSCP analysis is a powerful tool for detection of nucleotide variations. Four

isolates of *P. cryptogea* tested had an identical SSCP banding patterns when compared with other patterns obtained with *P. palmivora* and *P. nicotianae*. Similar results were obtained by Kong *et al.* (2003), in a comprehensive analysis of 29 *Phytophthora* species (282 isolates) characterized based on SSCP technique. Compared with classical methods, SSCP analysis is an effective alternative tool for differentiating *P. cryptogea* from other *Phytophthora* species. In addition, SSCP provides a simple, rapid and reliable tool for confirming positive detections in ongoing surveys for *P. cryptogea* at nurseries.

The pathogenicity of 5 *P. cryptogea* isolates was evaluated on mango plants by soil infestation. This method gave a reliable indication of how well isolates colonized the host tissue and also provided information on host and parasite interactions at the rhizosphere level. All the tested isolates of *P. cryptogea* were pathogenic on mango cv. Kensington Pride. Symptoms were similar to those described for diseases caused by *P. cryptogea* in other hosts (Erwin, 1996), and were similar to those observed on mango trees during the survey. Symptoms include leaf wilt, moderate chlorosis, necrosis and stunt. These foliage symptoms were associated with root rot and a considerable reduction of the root mass, root weight, and foliage weight. Reisolations made from inoculated mango always yielded *P. cryptogea*, successfully fulfilling Koch's postulates.

Pathogen aggressiveness is defined as the relative ability to colonize the host and cause damage. Some differences in virulence were observed among tested isolates, with Ph10 causing more disease than others (Ph13). Disease incidence was obtained in the presence of a soil saturation period (24 h every 2 weeks) keeping each plant wet with a continuous water column above the soil surface. Therefore, the frequent and excessive irrigation regime normally used to water mango plants is probably conducive to the development of this disease. Similar results were obtained by Bowers and Mitchell (1990); and Besoain *et al.* (2005) where the disease severity of increased as the length of the flooding period increased.

Three species of *Phytophthora* have been reported to infect mango in many countries. *P. palmivora* was found in Arizona USA, the Philippines and Thailand (Kueprakone *et al.,* 1986; Matheron and Matejka, 1988; Tsao *et al.,* 1994), *P. citricola* was recently reported in Spain (Zea-Bonilla *et al.,* 2007). In India, leaf blight disease was attributed to *P. parasitica* (Prakash, 1977; 1984). In addition, the oomycete *Pythium vexans* de Bary, in Malaysia is known to be associated with the mango root rot seedlings (Lim and Khoo, 1985). However, Phytophthora root and crown rot of mango has not been reported in Sicily previously. Our results indicate that *P. cryptogea* is the causal pathogen of mango root and crown rot in Sicily. The pathogen is known as easily recovered from irrigation water and from soil where it can survive in the absence of a suitable host (Garibaldi *et al*., 2003). *P. cryptogea* was first described in Ireland as the causal pathogen of tomato foot rot (Pethybridge and Lafferty in 1919).

According to Erwin and Ribeiro (1996) *P. cryptogea* is considered one of the most important major pathogens in the genus and has the widest host range almost 150 species in 23 families. The fungus distributes widely and infects vegetables, ornamental and woody plants. In Sicily, this species has been associated with tomato crown and root rot (Pane *et al*., 2000), walnut decline (Vettraino *et al*., 2002), and was reported as the causal pathogen of root and foot rot on Lantana (Cacciola *et al*., 2005), sweet cherry (Vettraino *et al*., 2008) and on sage (Cacciola *et al*., 2002).

6. Conclusion

Our finding is important because the presence of *P. cryptogea* in mango orchards represents a threat to this host and other woody hosts as grape, almond, apple, peach and kiwi. This study demonstrated that *P. cryptogea* is the cause of mango root and crown rot disease. To our knowledge, this is the first report of *P. cryptogea* on mango in Italy and in other countries. The biology, ecology and means of fungal control on mango should be further investigated.

These results further increase our understanding in the epidemiology of *P. cryptogea* and will support the development of more effective strategies to manage *Phytophthora* diseases in nurseries and mitigate the spread of the pathogen into new agro ecosystems.

7. References

Besoain X., Latorre B. A. and Arenas C. J. (2005). Effect of the flooding period on the development of avocado (*Persea americana*) root rot caused by *Phytophthora cinnamomi*. *Ciencia e Investigacion Agraria*, 32(2): 97-103.

Blair J. E., Coffey M. D., Park S.-Y., Geiser D. M. and Kang S. (2008). A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology*, 45(3): 266-277.

Bowers J. H. and Mitchell D. J. (1990). Effect of soil-water matric potential and periodic flooding on mortality of pepper caused by *Phytophthora capsici*. *Phytopathology*, 80(12): 1447-1450.

Cacciola S. O., Chimento A., Pane A., Cooke D. E. L. and di San Lio G. M. (2005). Root and foot rot of Lantana caused by *Phytophthora cryptogea*. *Plant Disease*, 89(8): 909-909.

Cacciola S. O., Pane A., Raudino F. and Davino S. (2002). First report of root and crown rot of sage caused by *Phytophthora cryptogea* in Italy. *Plant Disease*, 86(10): 1176-1176.

Cooke D. E. L., Drenth A., Duncan J. M., Wagels G. and Brasier C. M. (2000). A Molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology*, 30(1): 17-32.

Hoffman C. S. and Winston F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, 57(2-3): 2-3.

Judelson H. S. and Blanco F. A. (2005). The spores of *Phytophthora*: Weapons of the plant destroyer. *Nature Reviews Microbiology*, 3(1): 47-58.

Kong P., Hong C., Richardson P. A. and Gallegly M. E. (2003). Single-strand-conformation polymorphism of ribosomal DNA for rapid species differentiation in genus *Phytophthora*. *Fungal genetics and biology : FG & B*, 39(3): 238-249.

Kong P., Hong C. X., Tooley P. W., Ivors K., Garbelotto M. and Richardson P. A. (2004). Rapid identification of *Phytophthora ramorum* using PCR-SSCP analysis of ribosomal DNA ITS-1. *Letters in Applied Microbiology*, 38(5): 433-439.

Kroon L. P., Bakker F. T., van den Bosch G. B., Bonants P. J. and Flier W. G. (2004). Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal genetics and biology : FG & B*, 41(8): 766-782.

Kroon L. P., Brouwer H., de Cock A. W. and Govers F. (2012). The genus *phytophthora anno*. *Phytopathology*, 102(4): 348-364.

Martin F. N. and Tooley P. W. (2003). Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia*, 95(2): 269-284.

Masago H., Yoshikawa M., Fukada M. and Nakanishi N. (1977). Selective-inhibition of *pythium* spp on a medium for direct isolation of *phytophthora* spp from soils and plants. *Phytopathology*, 67(3): 425-428.

Matheron M. E. and Matejka J. C. (1988). Phytophthora crown and root rot of nursery grown mango trees delivered to Artizona (Abs.). *Phytopathology*, 78: 1572.

Mostowfizadeh-Ghalamfarsa R., Panabieres F., Banihashemi Z. and Cooke D. E. (2010). Phylogenetic relationship of *Phytophthora cryptogea* Pethybr. & Laff and P. drechsleri Tucker. *Fungal Biol*, 114(4): 325-339.

Pane A., Faedda R., Cacciola S. O., Rizza C., Scibetta S. and di San Lio G. M. (2010). Root and basal stem rot of Mandevillas caused by *Phytophthora* spp. in Eastern Sicily. *Plant Disease*, 94(11): 1374-1375.

Ploetz R. C. and Freeman S. (ed) (2009). Foliar, floral and soilborne diseases. In: *The Mango: Botany, production and uses*. 2nd edition. CABI.

Prakash O. and Srivastava K. C. (1987). *Mango diseases and their management: a world review.* Today & Tomorrow's Printers and Publishers.

Prakash O. m. and Singh U. N. (1980). Root rot and damping off of mango seedling caused by *Rhizoctonia solani*. *Indian Journal of Mycology and Plant Pathology*, 10(1): 69.

Robideau G. P., De Cock A. W., Coffey M. D., Voglmayr H., Brouwer H., Bala K., Chitty D. W., Desaulniers N., Eggertson Q. A., Gachon C. M., Hu C. H., Kupper F. C., Rintoul T. L., Sarhan E., Verstappen E. C., Zhang Y., Bonants P. J., Ristaino J. B. and Levesque C. A. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Mol Ecol Resour*, 11(6): 1002-1011.

Singh L. B. (1960). *The Mango: Botany, cultivation, and utilization.* London, Leonard Hill (Books) Ltd; New York, Interscience Publishers, Inc.

Tsao P. H., Luzaran P. B., Delossantos A. B., Portales L. A., Gochangco A. M. and Gruber L. C. (1994). Phytophthora crown and root-rot of mango detected in Philippine nurseries. *Plant Disease*, 78(1): 100-100.

Vettraino A. M., Belisario A., Maccaroni M., Anselmi N. and Vannini A. (2002). First report of *Phytophthora cryptogea* in walnut stands in Italy. *Plant Disease*, 86(3): 328-328.

Vettraino A. M., Flamini L., Pizzichini L., Prodi A., Nipoti P., Vannini A. and Lagnese R. (2008). First report of root and collar rot by *Phylophthora cryptogea* on sweet cherry in Italy. *Plant Disease*, 92(1): 177-177.

Voglmayr H., Riethmuller A., GÖKer M., Weiss M. and Oberwinkler F. (2004). Phylogenetic relationships of *Plasmopara*, *Bremia* and other genera of downy mildew pathogens with pyriform haustoria based on Bayesian analysis of partial LSU rDNA sequence data. *Mycological Research*, 108(9): 1011-1024.

Waterhouse G. M. (1963). Key to the species of *Phytophthora* de Bary. *Mycological Papers*, 92: 22 pp.

Zea-Bonilla T., Martín-Sánchez P. M., Hermoso J. M., Carmona M. P., Segundo E. and Pérez-Jimenéz R. M. (2007). First report of *Phytophthora citricola* on *Mangifera indica* in Spain. *Plant Pathology*, 56(2): 356-356.

CHAPTER 5

5. PHYLOGENETIC ANALYSIS OF P. CRYPTOGEA, P. DRECHSLERI AND ASSOCIATED SPECIES BASED ON MITOCHONDRIAL AND NUCLEAR DNA SEQUENCES

1. Abstract

The differentiation of *P. cryptogea*, *P. erythroseptica* and *P. drechsleri* is a controversial issue. They could not be distinguished based on morphological characterization. In this study, reevaluation of worldwide collection of 140 isolates assigned to *P. cryptogea*, *P. drechsleri* and *P. erythroseptica* was carried out. Single and multiple gene phylogenetic analysis were performed on DNA sequences of nuclear (Internal Transcribed Spacers, ITS) and mitochondrial (Cytochrome c Oxidase subunit I, COI) genes. Congruence was observed between the different phylogenetic analysis results. Ten isolates of *P. drechsleri* along with two authentic isolates were formed in a distinct group with low level of variation. For *P. cryptogea* high levels of intraspecific variation were found in which two different groups were inferred. *P. cryptogea* group I (GI) clade includes 18 isolates in addition to one isolate for each of *P. cryptogea* authentic and type culture. The group was homogenous with some variations as few sequence differences reflected in sub clusters in both ITS and COI phylogenetic trees. In *P. cryptogea* group II (GII) clade, 23 isolates collected from different woody trees and diverse origins were fall altogether in this group, with some few sub clusters. *P. erythroseptica* isolates clustered into separate group that is closely related to *P. cryptogea* group II clade. The phylogenetic analysis suggests that *P. erythroseptica* is a uniform and distinct taxon. Another sub clade includes four isolates from Calla lily was formed in a close affiliation to *P. erythroseptica* with only few differences of three SNPs. A set of 17 isolates formed a group of the new provisional taxon "*Phytophthora.* sp. *kelmania*". In addition, fifty two isolates were found to be misidentified and subsequently identified as *P. asparagi, P. cajani, P. cambivora, P. cinnamomi, P. cinnamomi var. parvispora, P. gonapodyides, P. megasperma, P. melonis, P. multivesiculata, P. rubi* and other *Phytophthora* species taxa that not formally yet identified. This study provides the first nuclear (ITS) and mitochondrial (COI) phylogenetic analysis that based on using the type and authentic isolates of each species. Both markers provide an acceptable resolution for this species complex. However, more potential genetic markers for phylogenetic analyses are needed to fully evaluate the taxonomic position of this species.

Keywords: ITS, COI, *P. cryptogea*, *P. drechsleri, P. erythroseptica,* DNA barcoding.

2. Introduction

An overview of Phytophthora taxonomy and identification

Phytophthora species are responsible for serious diseases of economically important crops and an extensive damage to natural plant communities Cooke *et al.* (2000a). Some species have a narrow host range and infect one or a few plant species while others have a broad host range. According to Erwin and Ribeiro (1996), the genus consist of over 60 species. Since the publication made by Erwin and Ribeiro (1996), the number of described species has increased. Érsek and Ribeiro (2010) recently listed 100 described species of *Phytophthora*. Meanwhile, additional species have been named (some provisional), raising the total to 117 with a number of other distinct taxonomic entities in the process of formal description (Martin *et al.*, 2012). This enormous number of new species expected to increase due to many reasons; i) the availability of more sophisticated tools for species identification, ii) the large scale surveys by several researchers in forestry, agricultural and natural ecosystems, iii) the greater attention that is devoted to clarify phylogenetic relationships and define boundaries in species complexes (Martin *et al.*, 2012).

Since the first description of *Phytophthora infestans* (Mont.) de Bary type species, in 1876, and several taxonomical studies of genus *Phytophthora* have been published. Most of the publications contain dichotomous keys (Waterhouse, 1963;1970) or tabular format (Stamps, 1990), for species identification.

Grace Waterhouse, a British mycologist, was an expert in the identification of *Phytophthora* species and was an authority on the genus (Rossiter, 2009; Ristaino, 2012). Waterhouse gathered together a large collection of *Phytophthora* cultures and published a compilation of species descriptions from original papers. The well-authenticated and useable dichotomous keys to species in the genus *Phytophthora* that she developed are still in use today (Waterhouse, 1963). Her work was based on careful observation of the morphological characteristics of the pathogen including sporangia, sexual reproductive organs, chlamydospores, and hyphal and cultural characteristics. Waterhouse (1963), introduced the concept of morphological groupings (I-VI) as a utility to species identification, but not to imply a natural classification. The six groups were defined by

two distinct morphological criteria, the degree of sporangium papillation (papillate, semipapillate and non-papillate) and the mode of attachment between the antheridium and the oogonium (amphigynous and paragynous) (Oudemans and Coffey, 1991b). Stamps (1990), and Newhook *et al.* (1978) included also these groupings in their taxonomic evaluation of the genus and transferred Waterhouse's dichotomous keys into a more easily used tabular format (Martin *et al*., 2012; Ristaino, 2012). In 1990s Erwin and Ribeiro (1996) published "*Phytophthora Diseases Worldwide*" a book that is still until nowadays a highly useful bibliographic reference for morphology, taxonomy, biology and pathology of *Phytophthora* species. Cline and *et al*. (2008) have recently published a *Synopsis* online list of *Phytophthora* with accurate scientific names, host range, and supporting literature. They listed the species occurring in US and elsewhere with hyperlink to USDA SMML database (http://www.plantmanagementnetwork.org/pub/php/review/2008/phytophhora/).

The taxonomy of the genus *Phytophthora* that is based mainly on morphological characters (Tucker, 1931; Waterhouse, 1963) has been used for long time and is still in use. Unfortunately, these characters are few in number, highly variable and require an expert to observe and discriminate. Some species display overlapping variability among each other, making separation difficult (Erwin *et al*., 1983). Further taxonomic problems arise from the assignment of a type culture to act as the standard example for description of the species. A single culture cannot conceivably represent the intraspecific variability of an entire population, nor is it likely to represent the mean of this variability (Mills *et al*., 1991). Brasier (1983) pointed out that due to the limited number of morphological characters, there might be morphologically similar but physiologically distinct species within the same genus.

The method of species identification and classification began to change when DNA fingerprinting methods became more readily available (Drenth *et al*., 1993). DNA fingerprinting could be used as a tool to group isolates within a particular species in the same way as phenotypic characters (Drenth *et al*., 1994). Another frequently used method to distinguish *Phytophthora* isolates at the species level was isozyme analysis, utilizing polyacrylamide gel electrophoresis to detect different alleles for a number of enzymes (Mills *et al*., 1991; Oudemans and Coffey, 1991). These techniques provided invaluable new

insights in both population structure within species and variation between species. There are several gel-based techniques that can be used to identify isolates to a species level using amplicons generated by PCR. Ristaino *et al.* (1998) developed a PCR procedure to amplify DNA for quick identification of the economically important species from each of the six taxonomic groups in the plant pathogen genus *Phytophthora*. Ristaino *et al.* (1998) observed that amplification of the ITS region followed by digestion with restriction enzymes generated restriction profiles that could be useful for identification at the species level. The use of PCR-restriction fragment length polymorphism (RFLP) of the ITS region for species identification was also used by (Cooke *et al.*, 2000b). Recently in an attempt to simplify *Phytophthora* identification, Gallegly and Hong (2008) developed a simplified dichotomous key for identifying *Phytophthora* species based on morphology and DNA fingerprinting technique. They included single-strand conformational polymorphism (SSCP) analysis as a means to differentiate 60 species.

Recently Ristaino (2012) published lucid Key to identify 54 common *Phytophthora* species. Lucid v 3.4 is a matrix-based computerized identification key and includes important morphological and molecular characters that are useful for identification. The main morphological features included in the key are: asexual structures, sexual structures, chlamydospores, hyphae and cultural characteristics, temperature requirements, and host pathogenicity. FASTA files of the ITS and COX1 gene sequence were also included as molecular characters for each species. The key was made to provide an easily accessible tool to distinguish *Phytophthora* species.

Sequence-based species identification methods are considering considered the biggest leap in knowledge of *Phytophthora* species so far. If the sequences for particular genes or DNA regions are identical or nearly identical, the isolates supposedly belong to the same species (Kroon *et al.*, 2004a). If DNA sequences of the same region are available for dozens of species, a phylogeny can be made (Kroon *et al.*, 2012). Species can then be grouped in clades, consisting of a single common ancestor and all its descendants. A clade is, simply expressed, a branch (*Greek: klados*) of the evolutionary tree which is separated from the rest of the tree by a single cut. Any branch, however large or small, that is cut off in this way is monophyletic, or of a single origin (Kroon *et al.*, 2004).

One of the first DNA regions to be used in phylogenetic analysis was the 5.8S ribosomal RNA gene and the flanking internal transcribed spacers 1 and 2 (ITS1 and ITS2) (Lee and Taylor, 1992). The ITS regions provide suitable targets because they are relatively stable, can be easily amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (Cooke *et al.*, 2007). This region contains stretches of high homology that were used to design primers for Polymerase Chain Reaction (PCR) amplification. For almost all *Phytophthora* species, the same primers can be used (Kroon *et al.*, 2004a). ITS-based PCR primers have been also used for the detection of a number of *Phytophthora* species. Furthermore the same regions have been utilized to design specific oligonucleotide arrays for a number of species (Lievens *et al.*, 2005; Anderson *et al.*, 2006). The first extensive phylogenetic study of the *Phytophthora* genus based on ITS1 and ITS2 sequences was described by Cooke *et al.* (2000a). That study, which included 234 isolates from 50 distinct *Phytophthora* species, provided the basis for the clade nomenclature currently used to group the genus (Kroon *et al.*, 2004).

Sequence-based identification of *Phytophthora* species is now widely used, and identification using online tools such as GenBank, the *Phytophthora* database, or *Phytophthora*-ID have been developed (Park *et al.*, 2008; Grünwald *et al.*, 2010). The website of *Phytophthora* Database (www.phytophthoradb.org) includes a listing of the species, their morphological features based on the description of Erwin and Ribeiro (1996), geographic distribution, and a listing of references about the genus. In addition, the database provides the users with comprehensive phylogenetic framework for the genus and tools that could be used for molecular identification (Martin *et al.*, 2012). By July 2011, a total of 6,192 sequences from 2,593 isolates representing 107 species were posted in the database and are available for BLAST analysis for identification of unknown isolates. It is also possible to download the sequences as well as conduct some forms of analysis (Martin *et al.*, 2012).

The pitfalls of sequence-based identifications have been recently reviewed by (Kang *et al.*, 2010). Species identification cannot rely only on sequence-based identification, since some of the sequence data present in GenBank and elsewhere may not have been submitted for morphologically well-characterized *Phytophthora* species (Ristaino, 2012).

Martin *et al.* (2012), emphasized that it is important to recognize that the taxonomic classification of the genus has not yet with phylogeny; there are species complexes with closely related species where morphological features alone are not enough to define species boundaries observed from phylogenic analysis (*P. cryptogea*/*P. drechselria* complex). Until these complexes are subjected to detailed multilocus analyses using isolates representing the diversity observed in nature, identification by sequence analysis alone will continue to be problematic for some species. Comparing results from several loci is necessary to confirm species identification, especially when working with species complexes.

The advantage of the ITS approach is that the sequence of ITS1, 5.8S, and ITS2 can be readily obtained, and as a result, the ITS sequences of a large number of *Phytophthora* species are currently available in GenBank (Kroon *et al*., 2004) however, is the strict selection pressure on this genomic region, which is present in many copies within one genome. The observation that variability in ITS sequences between closely related species is low because of this selection pressure raised concerns about the applicability of ITS regions for phylogenetic inference (Kroon *et al*., 2012). The same concern raised about the specificity of some ITS-based molecular detection methods. This is due to cases where the ITS sequences are not sufficiently variable, making the design of primers to identify and detect closely related taxa very difficult or impossible (Cooke *et al*., 2007). Martin *et al.* (2012) reported that ITS region is not the optimal locus for all species, particularly those that are phylogenetically closely related such as *P. rubi* and *P. fragariae* have identical ITS sequences.

Because of the disadvantages of ITS regions, a new approach was developed for generating appropriate sequences for phylogenetic analysis of *Phytophthora* species. This approach involves the sequencing of "*housekeeping genes*" i.e. genes, either mitochondrial or nuclear, that encode proteins with known functions in the metabolism or catabolism of the organism.

The first significant example of a study using *Phytophthora* "housekeeping" genes was published by Martin and Tooley (2003a) who sequenced two mitochondrial genes, Cytochrome Oxidase I and II (*cox*1 and *cox*2) from 51 isolates representing 27 *Phytophthora* species. Additionally, a number of other nuclear loci (60S ribosomal protein L10, β-

tubulin, enolase, HS protein 90, large subunit rRNA, TigA gene fusion, translation elongation factor 1α) and mitochondrial loci (cox1, nad1, cox2, nad9, and rps10) have been sequenced for phylogenetic studies and for identification purposes within *Phytophthora* species (Martin *et al.*, 2012). More recent studies have used multiple loci from both the nuclear and mitochondrial genomes (Martin and Tooley, 2003b; Kroon *et al.*, 2004; Villa *et al.*, 2006). While these studies have been successful in establishing a number of well-supported clades within the genus, they have been unable to resolve the deeper evolutionary relationships among the clades (Blair *et al.*, 2008). In addition, some newly described *Phytophthora* species have been placed in an unresolved, basal group that appears to be outside the main radiation of the genus (Mirabolfathy *et al.*, 2001; Brasier *et al.*, 2005; Belbahri *et al.*, 2006; Dick *et al.*, 2006).

Blair *et al.* (2008) presented the genus-wide phylogeny for 82 *Phytophthora* species and also took in consideration many newly discovered species. They used seven of the most informative loci (28S Ribosomal DNA, 60S Ribosomal protein L10, Beta-tubulin, Elongation factor 1alpha, Enolase, Heat shock protein 90 and TigA gene fusion Protein). The results of phylogenetic groupings obtained by Blair *et al.* (2008) were similar to those observed in the internal transcribed spacer (ITS) analysis by Cooke *et al.* (2000a) and the multilocus analysis of Kroon *et al.* (2004), with a difference that Cooke *et al.* (2000a) reported 8 major clades (Fig. 5.1), while Blair *et al.* (2008) with the adding of more recently described species supported the genus division into 10 well-supported clades , with the 2 additional groupings (clades 9 and 10) that were basal to the prior 8 Clades (Fig. 5.2).

While the Blair *et al.*, (2008) analysis provided an excellent view of the terminal relationships among species of the ten described clades, bootstrap support was not robust enough to conclusively delineate the relationships among major clades 1 through 8 (Martin *et al.*, 2012). A recent study was done by Martin *et al.* (2012) they examined the same isolates used in Blair *et al.*, (2008), along with more newly described species using four mitochondrial loci (cox2, nad9, rps10 and secY). The results obtained were nearly identical to the nuclear phylogeny with some minor differences in the placement of some species within a clade was encountered (Fig. 5.3). Although the combined analysis with both the nuclear and mitochondrial data generated a similar tree with improved bootstrap support for some nodes, many of the basal nodes showing the relationship among the

major clades are still unresolved. Martin *et al.* (2012) observed a greater level of sequence divergence with the mitochondrial data as illustrated by the longer branch lengths compared to the nuclear data. Martin *et al.* (2012) recommended the need for further evaluation of potential new species descriptions of isolates closely affiliated with *P. citricola, P. citrophthora, P. cryptogea, P. meadii,* and *P. porri.*

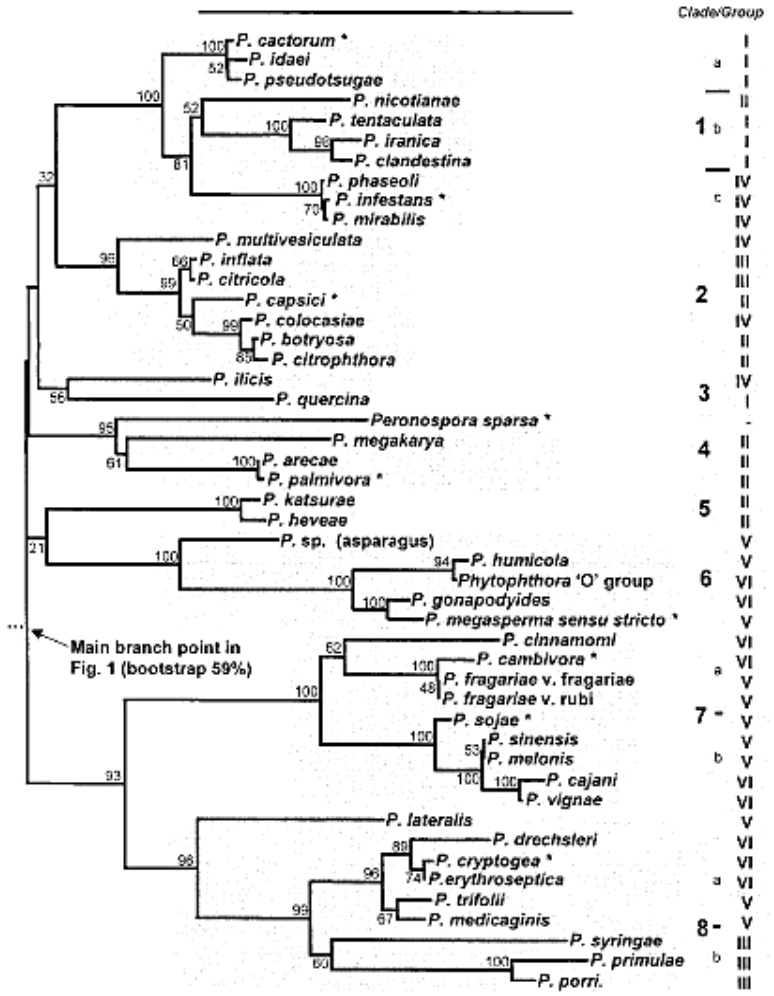

Figure 0.1 Phylogram of 47 *Phytophthora* species with *Peronospora* species (as outgroup) with 8 clades based on analysis of the combined ITS1 and ITS2 regions of the genomic ribosomal RNA according to (Cooke *et al.*, 2000a).

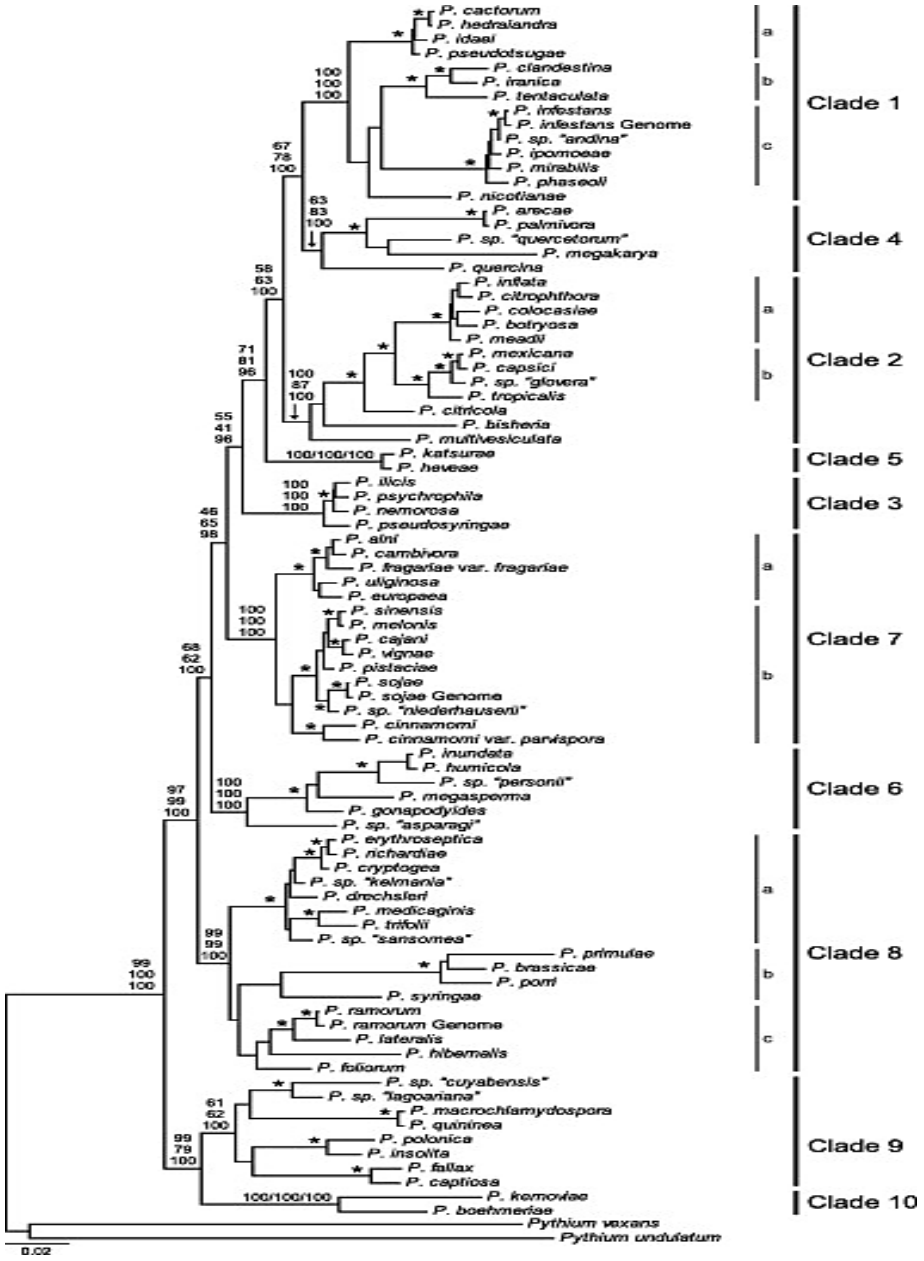

Figure 0.2 A genus wide phylogeny for *Phytophthora* with 10 clades according to Blair *et al.* (2008) using seven nuclear loci (28S Ribosomal DNA, 60S Ribosomal protein L10, Beta-tubulin, Elongation factor 1alpha, Enolase, Heat shock protein 90 and TigA gene fusion Protein).

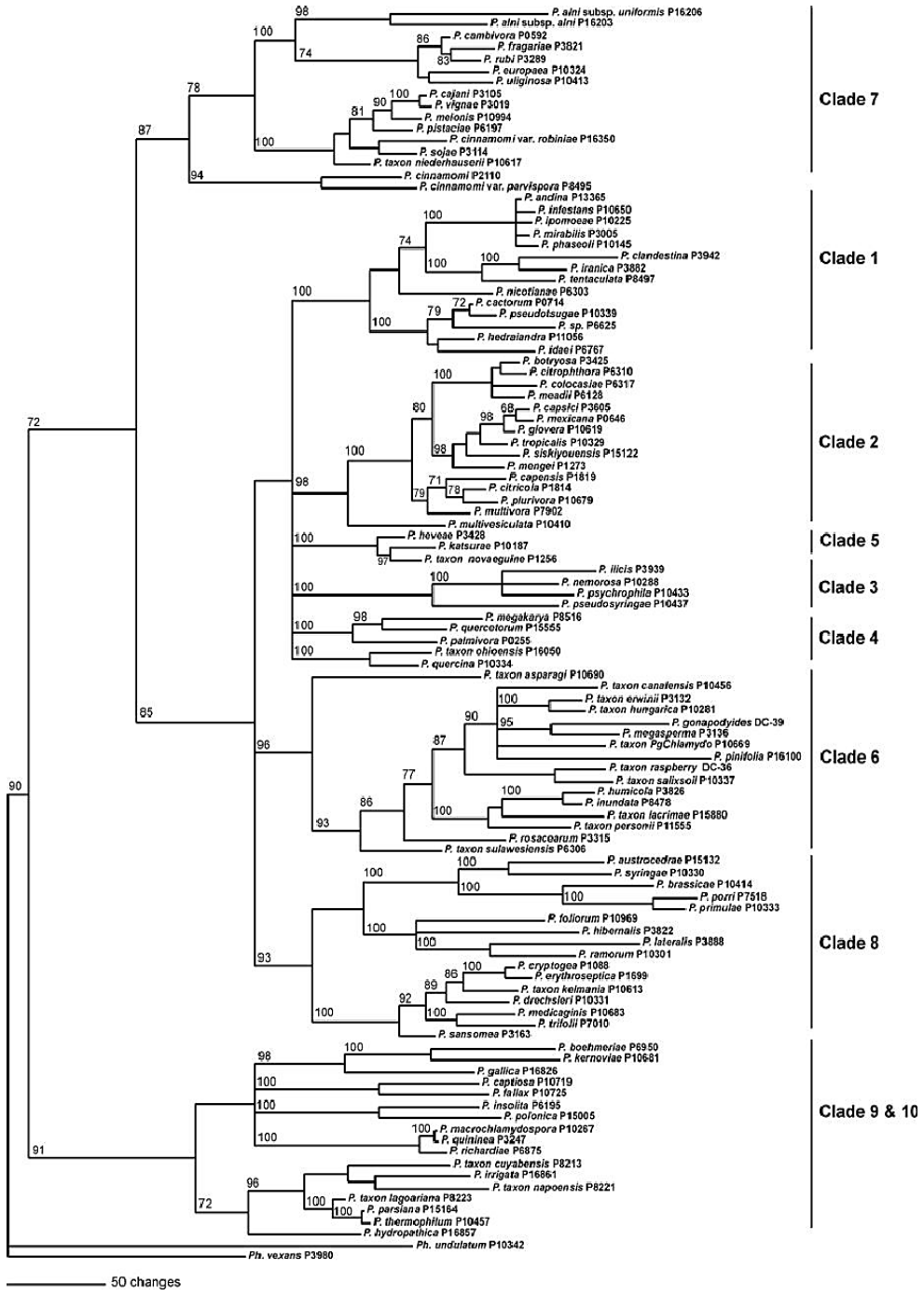

Figure 0.3 Maximum parsimony analyses of concatenated cox2, nad9, rps10, and secY genes from a range of *Phytophthora* spp. with *Phytopythium vexans* and *Ph. undulatum* as outgroups according to Martin *et al.* (2012).

DNA barcoding in fungi

Long before the term 'DNA barcoding' assumed its present meaning, mycologists were developing DNA sequence databases to facilitate fungal identification (Bruns *et al.*, 1991). The 1990s was a stormy decade in fungal systematics, as new molecular techniques challenged phenotypic taxonomy. The attitudes and feelings expressed in mycology would be familiar to supporters of DNA barcoding, which is still attacked using similar arguments (Seifert, 2009).

Given the long history of use of molecular markers (e.g., allozymes, rDNA, and mtDNA) for these purposes (Avise, 1994), there is nothing basically new in the DNA barcoding idea, except an increased scale of application and the above-mentioned standardization based on a new friendly experimental protocol and the choice of a rather universal molecular marker, at least in a well-defined taxonomic range (Seifert *et al.*, 2007).

In 2003, a group of scientists lead by Paul Hebert, researcher at the University of Guelph in Ontario, Canada, developed the use of part of one mitochondrial gene as a universal identification marker for animal species. Building upon the idea of the universal product code known as barcodes in the retail industry (Savolainen *et al.*, 2005), a few DNA nucleotides (e.g. the sequences of a short DNA fragment) may well provide an immediate diagnosis for species. Hebert and Gregory (2005) discussed the promise of DNA barcoding as a novel system designed to provide rapid, accurate, and automatable species identifications. They supposed that the method will make the Linnaean taxonomic system more accessible, with benefits to ecologists, conservationists, and the diversity of agencies charged with the control of pests, invasive species, and food safety. In addition, DNA barcoding will accelerate the leap of species discovery by allowing taxonomists to rapidly sort specimens and by highlighting divergent taxa that may represent new species.

Although the advocates of DNA barcoding say that it will regenerate biological collections and speed up species identification and inventories, its opponents argue that it will destroy traditional systematics and turn it into a service industry (Ebach and Holdrege, 2005), several papers have provided weighted analyses of the pros and cons (Moritz and Cicero, 2004; Marshall, 2005).

The basic idea of this approach is quite simple through the analysis of the variability in a single or in a few standard molecular marker(s), it is possible to discriminate biological entities (Meyer and Paulay, 2005). The original idea was to apply DNA barcoding systematically to all metazoans, by the use of one or a few (mitochondrial) markers. Rapidly, but with less comprehensible results, the idea was extended to flowering plants and fungi (Min and Hickey, 2007), and now the DNA barcoding initiative can be considered as a tool suitable for all of the tree kingdoms. Efforts in DNA barcoding development and management are coordinated by the Consortium for the Barcode of Life (CBoL; http://barcoding.si.edu/). DNA barcoding was suitable for two different purposes: i) the molecular identification of already described species and ii) the discovery of un-described species (Hebert *et al.*, 2004). DNA sequence should be as much short and easy to be produced in laboratory.

DNA barcode marker selection for fungi

The initial phylogenetic and molecular identification work on fungi began with nuclear ribosomal genes. The classic paper by White *et al.*, (1990) included universal primers still widely used for amplifying three main components of the fungal ribosomal operon: (i) the large subunit (LSU, variously referred to as the 26S or 28S, and including two variable sub regions called D1 and D2); (ii) the small subunit (SSU, or 18S) and (iii) the ITS, comprising two sections (ITS1, ITS2) that bracket the conserved 5.8S region. Because of the length limitations of manual sequencing, early studies of the fungal ITS often focused only on either the ITS1 or ITS2. The White *et al.*, (1990) primers are remarkably robust, working with the vast majority of fungi. The ITS became the default marker for species level studies of or most fungi, with the notable exception of the yeasts, where the LSU became the standard for identification. ITS is varying in length considerably among major taxonomic groups and gives superior resolution in those groups with longer amplicons.

Past and current taxonomy status of *P. cryptogea, P. drechsleri* and *P. erythroseptica* complex

The differentiating of *P. cryptogea, P. erythroseptica* and *P. drechsleri* is considered one of the most vexing and difficult taxonomic problems in the genus *Phytophthora* (Erwin *et al.*, 1983; Erwin and Ribeiro, 1996).

P. erythroseptica was first described in Ireland by Pethybridge (1913) as the causal pathogen of pink rot of potato tubers while *P. cryptogea* was described by Pethybridge and Lafferty (1919) with an isolate causing foot rot of tomato in Ireland. Pethybridge (1913) considered both species were alike in having ovoid to obpyriform, non-papillate, internally proliferating sporangia and amphigynous antheridia, but were distinguished by color of sexual organs, oospore size and the thallic nature of oospore production. Almost 10 years later, Tucker (1931) described *P. drechsleri* as an isolate of *Phytophthora* that caused rot of potato in Idaho. The early comparison between the three species was made by Tucker (1931) based on a single isolate of each species. He indicated that all the three species were morphologically similar but they could be separated by the differences in oospore size and using temperature relations as physiological characteristics. Isolates of *P. drechsleri* were able to grow well at 35^{o}C while no growth at 35^{o}C for *P. cryptogea*. *P. erythroseptica,* discriminated on the basis of yellowish appearance of oogonia, homothallism, larger oospores, and an inability to grow at 35^{o}C.

In Waterhouse (1963) Key, *P. drechsleri*, *P. cryptogea* and *P. erythroseptica* were included in Group VI, based on the common features of non-caducous, non-papillate, internally proliferating sporangia and amphigynous antheridia. Group VI also contains *P. richardiae*, *P. cinnamomi*, *P. cambivora*, *P. lateralis*, and *P. gonapodyides*. Although temperature was originally used by Tucker (1931) as a criterion to separate *P. cryptogea* from *P. drechsleri*, Waterhouse (1963) used maximal sporangial length as the primary distinguishing feature in her key with maximum growth temperature being of secondary importance. She added that *P. drechsleri* could also be distinguished by its narrower hyphal diameter, larger oospores, more elongated sporangia (larger, with a tapered base)

and occasional homothallic behavior. These additional distinctions further complicated identification procedures (Mills *et al*., 1991; Mostowfizadeh-Ghalamfarsa *et al*., 2010).

In addition, the shape of the sporangium base was used as a criterion to differentiate *P. cryptogea* from *P. drechsleri*. Klisiewicz (1977) described cultures from safflower that grew at 35°C, some of which he designated *P. drechsleri* because the sporangia were elongated to ellipsoid and the bases were sloping and others *P. cryptogea* because sporangia had rounded bases (Fig. 5.4). Even though, Klisiewicz (1977) did not present data for the population of sloping-base or rounded-base types, he stated that the majority of sporangia of *P. drechsleri* had sloping bases and sporangia of *P. cryptogea* had rounded bases. Mircetich (1976) also illustrate sporangia with sloping bases to be typical of *P. drechsleri* from cherry trees (Fig. 5.5). While isolates from bean produced sporangia with both tapered and rounded bases were designated as *P. cryptogea* (Flowers *et al.,* 1973). Ho and Jong (1986) compared 15 isolates previously identified as *P. cryptogea* and 14 isolates previously identified as *P. drechsleri* from a wide range of sources, but morphological characters, including rounded or tapered sporangial bases, were inconsistent. Moreover, the high-temperature criterion did not always correlate with the other identifying features and as a result, some isolates were described as intermediate between both species (Klisiewicz, 1977). Because of highly overlapping morphological characteristics, differentiating *P. cryptogea, P. erythroseptica* and *P. drechsleri* is considered one of the most taxonomic problem in the genus *Phytophthora* (Erwin and Ribeiro, 1996).

Many researchers casted doubt upon the validity of temperature response as the main distinguishing feature and they have proposed merging the two species because of these morphological identification difficulties, whereas others believe the species status of each is supported based on the growth at 35°C. Others simply rejected *P. drechsleri* as an acceptable species (Bumbieris, 1974). Some maintained that *P. drechsleri* should be kept as an acceptable species until more conclusive data were obtained.

Bumbieris (1974) performed a morphological and physiological study on seven isolates of *P. cryptogea* and five isolates of *P. drechsleri* from Australia and concluded that they could not be separated. He suggested merging *P. drechsleri* and *P. cryptogea* into *P. cryptogea* since this species was named earlier.

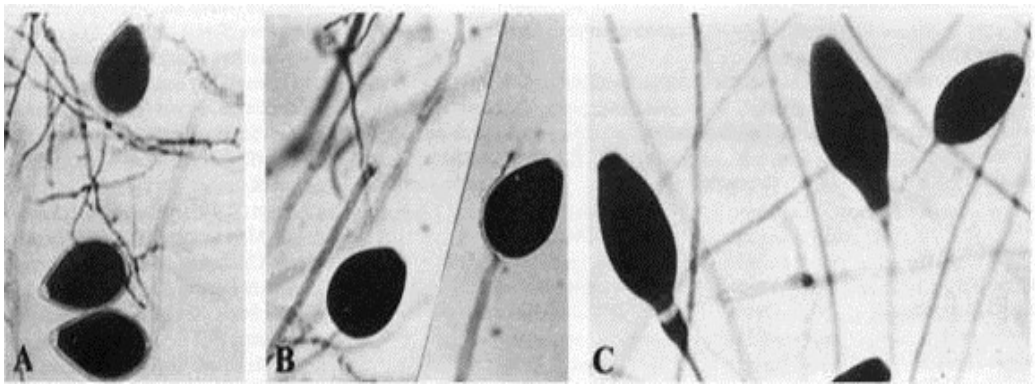

Figure 0.4 Comparison of sporangial shapes. A, *P. parasitica;* B, *P.cryptogea;* and C, *P. drechsleri* from safflower:- http://www.phytophthoradb.org/file/html_fppd/phytophthora/drechsleri/Figure_2.htm

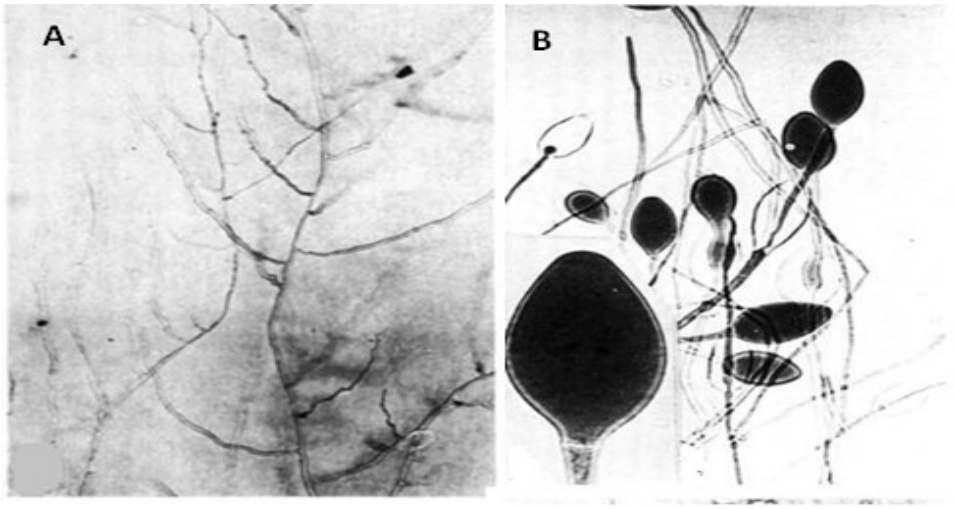

Figure 0.5 Culture characteristics of *P. drechsleri* as illustrated by Mircetich (1976). A, Hyphae of *P. drechsleri* with sparse branching at an acute angle; B, typical sporangia of *P. drechsleri.*

Halsall (1976) reported the absence of consistent serological variation between *P. cryptogea and P. drechsleri*. He agreed with the suggestion made by Bumbieris (1974) that *P. cryptogea* and *P. drechsleri* should be considered as one species. Shepherd (1978) observed similar mating behaviors between these two species and also suggested that they were conspecific. Similar conclusions were reached by Matsumoto and Sato (1979), who noted that the two isolates of each species had the same protein patterns, Ho and Jong (1986), who examined the morphology and physiology of a large number of species described as either *P. cryptogea* or *P. drechsleri*. Ho and Jong (1986), found that the sporangial shapes and sizes of these two species were varied considerably with much overlapping of characteristics. The high degree of morphological and physiological variability encountered did not allow them to discriminate between the two species. They did, however, consider the possibility of *P. drechsleri* being a variant of *P. cryptogea* that accumulated minor changes in morphological traits alongside its adaptation to higher temperatures and infection of hosts of warmer areas (Mostowfizadeh-Ghalamfarsa *et al*., 2010).

In a study by Cacciola *et al.* (1990), protein profiles obtained by polyacrylamide gel electrophoresis showed that a type culture of *P. cryptogea* and an authentic culture of *P. drechsleri* were clearly differentiated showing distinct different electrophoretic patterns of arylesterase and malate dehydrogenase isozymes of total mycelial proteins. Some Italian isolates previously identified as *P. cryptogea* or *P. drechsleri* had intermediate features between those of both species, however thus making it impossible to discriminate the two species solely on the basis of the electrophoretic patterns of mycelial proteins. An isolate from lentisk in Italy showed an identical protein profile to that of the type culture of *P. cryptogea* and was considered to be *P. cryptogea* (Magnano di San Lio, 1992). Ho and Jong (1991) reexamined more isolates of *P. drechsleri* and *P. cryptogea*, concluded that these species should not be merged.

Most of the previous studies that showed evidence for merging the two species were convincing until a study of a larger number of isolates for both species was done by (Mills *et al*., 1991). In their study based on isozyme and mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) patterns showed that 10 groups could be

differentiated within the complex. Differences between some of the groups were as great as those among certain other valid *Phytophthora* species (Mills *et al*., 1991).

Mills *et al.* (1991) concluded from their molecular studies that the *P. cryptogea -P. drechsleri* complex was too diverse to warrant a merger of the two species and postulated that there were probably seven genetic species within this group. After analysis through isozymes and mtDNA RFLP technology of 129 isolates previously identified as *P. drechsleri*, *P. cryptogea*, *P. melonis*, and *P. drechsleri* f.sp. *cajani* and selected from a wide range of geographical areas and crops.

Mills *et al.* (1991) grouped the cultures according to their genetic relatedness. ***Group A*** was homogeneous and included the *P. drechsleri* culture from potato originally described by Tucker (1931), three isolates from rotted roots of safflower that had originally been named as *P. drechsleri* but had been renamed *P. cryptogea* on the basis of the production of sporangia with tapered bases (Klisiewicz, 1977), isolates causing root rot of sugar beet and an isolate causing root rot of sweet pepper. All of these isolates grew well at 35^{o}C, and the non papillate, ovoid, ellipsoid, obpyriform sporangia were typical of the criteria for *P. drechsleri* formulated by Tucker (1931) and continued by Waterhouse (1963). A molecularly different ***group B*** contained what Mills *et al.* (1991) considered to be authentic cultures of *P. cryptogea* that did not grow at 35^{o}C but were similar morphologically to both *P. drechsleri* and *P. cryptogea*. Mills *et al.* (1991) found the criterion of rounded or tapered sporangial bases was not consistent (Fig. 5.6). Many isolates produced both rounded and tapered-base sporangia. They noted a few isolates in groups G and H that had predominately tapered sporangial bases and some isolates in groups J and K that produced mainly sporangia with tapered bases. Isolates of *P. drechsleri* f. sp. *cajani* were in ***group G*** produced sporangia with both rounded and tapered bases (Fig. 5.7). Isozyme analysis of *P. erythroseptica* revealed that it is a uniform and distinct taxon termed ***group 'Per***.

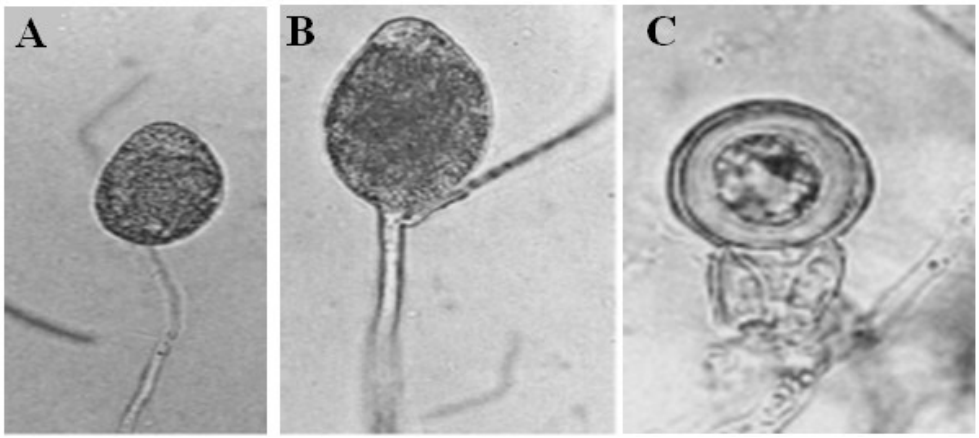

Figure 0.6 Sporangium morphology of *P. cyptogea*. (A) isolate P3104 from group D, (B) P3850 from group E and (C) Oogonium with mature oospore and amphigynous antheridium from the type culture of *P. cryptogea* (P1738) mated with *P. cinnamomi* A2 (P2411), Source: (Mills *et al.*, 1991).

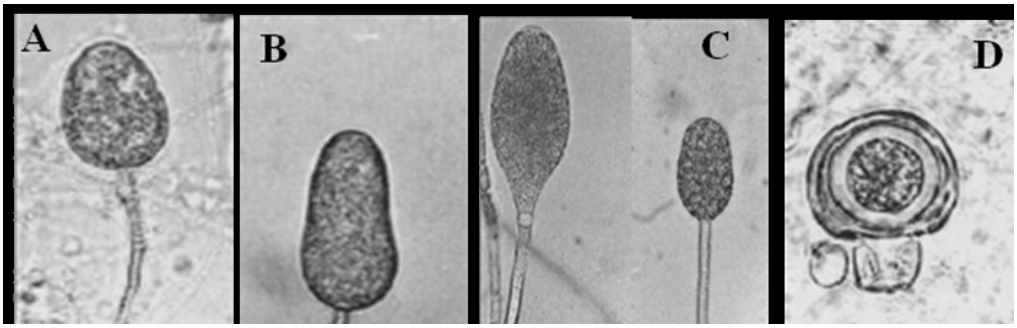

Figure 0.7 Sporangium morphology of *P. drechsleri.* (A) isolate PI087 , (B) isolate P1899 from group A. (C), tapered and rounded base of *P. drechsleri* f. sp. *cajani* isolate P1795. (D) Oogonium with mature oospore and amphigynous antheridium from an isolate of *P. drechsleri* (P1741) mated with *P. cinnamomi* A1 (P2100), Source: (Mills *et al.*, 1991).

The development of tools to analyze DNA sequence variation has allowed researchers to begin to discern the genetic relatedness of *Phytophthora* spp. This is particularly useful where morphological and biological characters are ambiguous (Olson *et al*., 2011). Phylogenetic analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) of species of *Phytophthora* placed *P. cryptogea* and *P. drechsleri* in clade 8a (Cooke *et al*., 2000a; Robideau *et al*., 2011). The study by Cooke *et al.* (2000a), *P. cryptogea* and *P. drechsleri* were consistently differentiated as distinct taxa. Martin and Tooley (2003a) used the cytochrome oxidase I and II regions of the mitochondrial DNA to conclude that *P. cryptogea* and *P. drechsleri* were distinct but closely related species. Kroon *et al.* (2004) utilized five sets of sequence data from the nuclear and mitochondrial genome to deduce that *P. cryptogea*, *P. erythroseptica*, and *P. drechsleri* form clade 8a. Kroon *et al.* (2004) found that growth characteristics and morphological traits are phenotypic, and groups of species sharing similar traits (exemplify: *P. cryptogea*, *P. erythroseptica*, and *P. drechsleri*) do not necessarily reflect evolutionary relatedness, since they may have evolved independently. Similar conclusion was made earlier by FÖRster *et al.* (2000). Their study provided evidence that the morphological characters used in *Phytophthora* taxonomy are of limited value for deducing phylogenetic relationships, because they exhibit convergent evolution.

Mirabolfathy *et al.* (2001) studied two non-papillate species of *Phytophthora* as the causal agents of pistachio gummosis in Iran. Their previous descriptions as *P. drechsleri* and *P. megasperma* was re-examined by RFLPs and sequence comparison of ITS regions of rDNA. The isolates from pistachio described as *P. drechsleri* had ITS sequences identical to *P. melonis*, *P. sinensis*, and isolates described as *P. drechsleri* from cucurbits in Iran (five isolates). They concluded that these taxa should be considered conspecific and all subsumed within *P. melonis*. lately, a multilocus phylogenetic study using seven loci supported the division of the genus *Phytophthora* into 10 well-supported clades (Blair *et al*., 2008), *P. cryptogea*, *P. erythroseptica*, and *P. drechsleri* were form clade 8a as in accord with the results obtained previously with single-locus studies (Cooke *et al*., 2000a; Kroon *et al*., 2004). Significant differences in Blair *et al.* (2008) work, was the occurrence of the undescribed taxon *P.* sp. '*kelmania*' as basal to *P. cryptogea*. In addition, the position of *P.*

richardiae in clade 8a closely related to *P. erythroseptica* and *P. cryptogea* while this species was placed in Clade 9 of Cooke *et al.* (2000a) and Clade 8e of Kroon *et al.* (2004).

Recently, Mostowfizadeh-Ghalamfarsa *et al.* (2010) evaluated a worldwide collection of isolates of *P. cryptogea* and *P. drechsleri* . They found that *P. drechsleri* and *P. cryptogea* are distinct species. Isolates of *P. drechsleri* form a monophyletic grouping with low levels of intraspecific diversity whereas *P. cryptogea* is more variable. The *P. cryptogea* complex formed 3 well-defined phylogenetic groups, with group I closely affiliated with *P. erythroseptica* and groups II and III together on a basal clade. Some isolates were intermediate between groups II and III and exhibited a greater amount of heterozygous bases than the other isolates, suggesting possible outcrossing between these groups.

In North Carolina, Olson *et al.* (2011) studied the evolutionary history of *P. cryptogea* and *P. drechsleri* isolates collected from floriculture crops in commercial greenhouses. The results suggested that divergence between *P. cryptogea* and *P. drechsleri* was recent and that speciation is still in progress.

Most of the previous phylogenetic studies were based on either sequence information of single (nuclear or mitochondrial) DNA regions or multilocus genes but covering a limited number of *P. cryptogea*, *P. erythroseptica* and *P. drechsleri* isolates. Although Mostowfizadeh-Ghalamfarsa *et al.* (2010) study was based on a multilocus analysis of *P. cryptogea* and *P. drechsleri* isolates from diverse hosts and origins but mainly from Iran, the results did not resolve heterozygous sites within the analyzed DNA sequences.

The study by Olson *et al.* (2011) was restricted for *P. cryptogea* and *P. drechsleri* isolates from floriculture crops in greenhouse. The greenhouse conditions might provide a distinctive opportunity for these two species to interact differently from those isolates come from different production system, especially *P. cryptogea* and *P. drechsleri* have overlapping floriculture host ranges. In addition, Olson e*t al*. (2011) reported that the divergence between *P. cryptogea* and *P. drechsleri* is recent and that speciation is still in progress for these species. This finding was in dissimilarity to the study by Mostowfizadeh-Ghalamfarsa *et al.* (2010) which found no evidence of gene flow and concluded that these were well-diverged species.

Overall, it has been a challenge to solve such conflicts in the *P. cryptogea*/*P. drechsleri* species complex based on morphological and biological studies. Although the new tools to analyze DNA sequence variation has allowed providing phylogenetic separation of some *Phytophthora* species, the taxonomic structure and phylogenetic positions for *P. cryptogea*/*P. drechsleri* complex are still indistinct and confused. This confusion is due in part to the number of sequences in GenBank from misidentified cultures or poorly annotated sequences as well as a lack of an ex-holotype "Type" culture in the analysis (Martin *et al.*, 2012). The need for a comprehensive analysis including the holotype strain and/or authentic isolates and wide number of isolates from different parts of the world and various hosts is also fundamental. A well resolved phylogeny of this complex is essential not only for validating diagnostic methods but also for interpreting the evolutionary history of various genetic traits of interest, understanding of the pathogenicity and origins of these taxa. Therefore, our objectives were to re- assess the status of *P. cryptogea/P. drechsleri* complex using molecular methods and phylogenetic analysis.

3. Materials and methods

Previous phylogenetic studies of *P. cryptogea/ P. drechselri* species complex have based on the use of one or few isolates to represent each species. This study was the first to use large number of isolates from different hosts and diverse geographical locations. For each species the type and/or authentic species culture was included in the analysis. Our objectives from this study were to a investigate the phylogenetic relationship of *P. cryptogea/ P. drechselri* species complex using locus oomycete *de facto* DNA barcode (ITS) with the default barcode (COI) which is officially accepted as the DNA barcode for eukaryotic groups (Robideau *et al.*, 2011).

Selection of isolates

To facilitate comparison between the data presented in this work and the analysis done by Mills *et al.* (1991), we have used the same isolates when available. In addition, a number of *Phytophthora* isolates that submitted recently into the culture collection were included. A total of 265 isolates assigned to either *Phytophthora cryptogea* or *P. drechsleri* were pre-screened first based on ITS sequences and from this, misidentified isolates were excluded and those listed in Table 5.2, were analysed further for mitochondrial analysis. All the isolates are maintained at the World Phytophthora Genetic Resources Collection, at the University of California, Riverside (WPGRC). http://phytophthora.ucr.edu). All accessions in WPGRC are preserved cryogenically under liquid nitrogen. Working cultures were maintained on either clarified or non-clarified V8 agar. At all stages of growth, cultures were checked for bacterial contamination by incubation for 24 h in Luria broth (LB). Details of the *Phytophthora* isolates examined in this study are listed in Table 10.

DNA extraction

For DNA extraction, actively growing cultures were produced in either clarified (1:2) V8 broth or pea broth and harvested after 5–7 days. About 200 mg of mycelium was rinsed with ultrapure water, placed in a 1.7 ml microcentrifuge tube and frozen by immersion in liquid nitrogen. DNA was extracted from frozen tissue using the FastDNA kit and FastPrep FP 120 instrument (MP Biomedicals Inc., Irvine, CA) according to the

manufacturer's instructions, with modifications using 1 ml of CLS-VF cell lysis solution and omitting the PPS protein precipitation solution. DNA concentration was determined using a 260/280 ratio with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc. USA). All DNA samples were stored in low TE buffer at -20°C., and are available through the (WPGRC) DNA Bank upon request (to Prof. M. Coffey).

DNA amplification and sequencing

DNA of the internal transcribed spacers (ITS) was amplified using the universal primers ITS1 and ITS4 (Cooke *et al.* 2000; White *et al.* 1990). The Oomycete-specific primers OomCoxILevup and Fm85mod modified from (Martin and Tooley, 2003a), were designed to amplify the region spanning the 727 bp from the 5'end of COI mitochondrial DNA. PCR Amplifications were performed in PTC-100® Thermal Cycler (Bio-Rad Laboratories, Inc, USA). The PCR mixture contained 10 ng of template DNA in final a reaction volume of 25 ml. Primers used in this study, along with PCR amplification conditions are listed in Table 5.1.

PCR cleanup and sequencing

PCR products were then cleaned up for sequencing using an enzymatic purification system following the manufacturer's instructions (ExoSAP-IT; USB Corporation, Cleveland, OH). For sequencing COI and ITS regions, the same primers used for PCR amplifications were used. Sequencing cycle reactions were performed at University of California Riverside genomics center on system (version 3.1 dye terminators; Applied Biosystems, Foster City, CA) and run on an ABI 3730XL DNA Analyzer, using the ABI Data Collection Program (version 2.0) and ABI Sequencing Analysis software (version 5.1.1). ABI trace files were analyzed using Sequencher version 4.6 (Gene Codes, AnnArbor, MI); heterozygous or ambiguous sites were labeled using the IUPAC code and consensus sequences were exported for phylogenetic analysis. DNA sequences will be deposited in the Barcode of Life Data Systems (BOLD) and GenBank (Work is still in progress).

Sequences editing, alignment and cluster analysis

Sequences were aligned using ClustalX under default settings (Thompson *et al.,* 1997). Alignments were visually inspected and edited manually. Preliminary phylogenetic trees were reconstructed for each marker using Neighbor-Joining with a Kimura two parameter nucleotide substitution model as implemented in MEGA version 5.05 (Kumar *et al.,* 2004).

Table 0.1 PCR primer sequences, reaction mixtures composition, and cycling conditions for the ITS and COI loci used in this study.

Locus	Primer name [a]	Primer sequence 5'- 3'	Size [b]	Reaction mixtures in 25µl volumes	Cycling conditions [d]	References
Internal transcribed spacer region (ITS)	ITS6	5'- GAAGGTGAAGTCGTAACAAGG-3'	900bp	5 µl 5X GoTaq buffer[c] 1.5 µl 25mM MgCl2 1 µl 40mM dNTP mix 0.5 µl 50 µM ITS4 primer 0.5 µl 50 µM of ITS6 primer 0.2 µl GoTaq Flexi DNA polymerase [c] 16.25 µl nuclease-free sterile water	94°C 3 min 35 cycles of 94°C 30 sec 51°C 30 sec 72°C 30 sec 72°C 10 min	(Cooke *et al.*, 2000a)
	ITS4	5'- TCCTCCGCTTATTGATATGC-3'				
Cytochrome c oxidase subunit 1 (COI)	Oom-Lev	5'-TCAWCWMGATGGCTTTTTTCAAC-3'	727bp	5 µl 5X GoTaq buffer [c] 2 µl 25mM MgCl2 2.5 µl 40mM dNTP mix 1.5 µl 100 µM Oom primer 1.5 µl 100 µM of FM85 primer 0.2 µl GoTaq Flexi DNA polymerase [c] 11.3 µl nuclease-free sterile water	94°C 4 min 35 cycles of 94°C 30 sec 55°C 1 min 72°C 1 min 72°C 5 min	(Martin and Tooley, 2003a)
	FM85-mod	5'-RRHWACKTGACTDATRATACCAAA-3'				

[a] Primers used for PCR amplification and sequencing

[b] Average amplicon length

[c] GoTaq buffer and GoTaq Flexi DNA polymerase from Promega (Madison, WI).

[d] Cycling conducted on C-1000 Thermal cycler (Bio-Rad Laboratories, Inc, USA).

Table 05.2 List of *Phytophthora* isolates used in this study

Species as Formerly identified	Isolate code WPC	International	IG(DG) (Mills, 1991).	Phylogenetic (G) species & group	Host	Origin	Mating type
P. drechsleri	P11638[Auth]	CBS 292.35	A1(A1)	*P. drechsleri*	*Beta vulgaris var altissima*	Idaho-USA(1935)	A2
P. drechsleri	P11637[Auth]		A1	*P. drechsleri*	*Beta vulgaris*		
P. drechsleri	P1904		A1	*P. drechsleri*	*B. vulgaris*	California	A1
P. erythroseptica	P3901		A1	*P. drechsleri*	*Solanum tuberosum*		A2
***Phytophthora* sp.**	P3808		diverse	*P. drechsleri*	*Vigna sinensis*	California	A1
P. drechsleri	P3857		-	*P. drechsleri*	*Capsicum annuum*	Mexico	
P. drechsleri	P10331		-	*P. drechsleri*	*Gerbera jamsonii*	N. Hampshire (2003)	
P. cryptogea	P6140		A1	*P. drechsleri*	*Cartharmus tinctorius*	California	A1
P. cryptogea	P11811		-	*P. drechsleri*			
P. drechsleri	P1741	IMI 40500, CBS 359.52	A1 (A2)	*P. drechsleri*	*L. esculentum*		A2
P. drechsleri	P1899		A2	*P. drechsleri*	*B. vulgaris*	California	A1
P. drechsleri	P3402		A2(A3)	*P. drechsleri*	*B. vulgaris*	California	A1

[Auth] = The Authentic culture, [T] =The Type isolate, **S =** sterile

? = unknown

- = isolate studied by Mills *et al*. (1991)

IG(DG), Isozyme and mitochondrial DNA groupings of isolates studied by Mills *et al*. (1991)

(G) Molecular groupings identified in this study

Table 5.2 continued

Species as Formerly identified	Isolate code WPC	Isolate code International	IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
P. cryptogea	P1088[Auth]	CBS 290.35 ATCC 46721	B1[(B1)]	*P. cryptogea* (GI)	*Aster* sp.		A1
P. cryptogea	P3085	ATCC 15402	B1	*P. cryptogea* (GI)	*Aster* sp.	California	A1
P. cryptogea	P3700		B1[(B2)]	*P. cryptogea* (GI)	*Asparagus officinalis*	California	A1
P. cryptogea	P3702		B1[(B2)]	*P. cryptogea* (GI)	*A. officinalis*	California	A1
P. erythroseptica	P1693[T]	IMI 34684	B3	*P. cryptogea* (GI)	*S. tuberosum*	Ireland	A1
P. cryptogea	P1738[T]	CBS 113.19	B4[(B3)]	*P. cryptogea* (GI)	*L. esculentum*	Ireland	A1
P. cryptogea	P3447	IMI 69664	B4[(B3)]	*P. cryptogea* (GI)	*L. esculentum*	Channel Island	H
P. cryptogea	P3448	IMI 180615	B4	*P. cryptogea* (GI)	*L. esculentum*	Ireland	A1
P. cryptogea	P1703	ATCC 36301	B5	*P. cryptogea* (GI)	*S. tuberosum*	Ohio	A2
P. cryptogea	P1739	IMI 45168	B5	*P. cryptogea* (GI)	*L. esculentum*	N. Zealand (1951)	A1
P. cryptogea	P3806	PI72 (Erwin)	B5	*P. cryptogea* (GI)	*Senecio* sp.	California	A2

Table 5.2 continued

Species as Formerly identified	Isolate code WPC	International	IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
P. cryptogea	P7239		-	*P. cryptogea* (GI)	*Spinacia oleracea*	Sweden	-
P. cryptogea	P7241		-	*P. cryptogea* (GI)	*Triticum aestivum*	Sweden	-
P. cryptogea	P7240		-	*P. cryptogea* (GI)	*T. aestivum*	Sweden	-
P. cryptogea	P7242		-	*P. cryptogea* (GI)	*T. aestivum*	Sweden	-
P. drechsleri	P1902		-	*P. cryptogea* (GI)	*Parthenium argentatum*	California	-
P. cryptogea	P6355		-	*P. cryptogea* (GI)	*Gerbera jamesonii*	Poland	-
P. richardiae	P7788		-	*P. cryptogea* (GI)	*Daucus carota*	England (1988)	-
P. cryptogea	P11825		-	*P. cryptogea* (GI)	?	?	-
P. cryptogea	P3533		-	*P. cryptogea* (GI)	*Iris* sp.	Channel Island	-
P. cryptogea	P1205	ATCC 46722	C	*P.* sp.″ *kelmania*″	*Simmondsia chinensis*	California	A1
P. cryptogea	P1254		C	*P.* sp.″ *kelmania*″	Forest soil	Papua New Guinea	A2
P. cryptogea	P1702	ATCC 34301	C [(c)]	*P.* sp.″ *kelmania*″	*Pseudotsuga menziesii*	Oregon	S

Table 5.2 continued

Species as Formerly identified	Isolate code		IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. cryptogea	P1810		C [(c)]	*P.* sp.″ *kelmania*″	*Prunus avium*	California	A2
P. cryptogea	P3134		C	*P.* sp.″ *kelmania*″	*Eucalyptus radiata*	Australia	A2
P. cryptogea	P3170		C	*P.* sp.″ *kelmania*″	*Pinus lambertiana*	Oregon	?
P. cryptogea	P3115		C	*P.* sp.″ *kelmania*″	*P. menziesii*	Oregon	S
P. cryptogea	P3200		C [(c)]	*P.* sp.″ *kelmania*″	*P. lambertiana*	Oregon	A2
P. cryptogea	P3586		C	*P.* sp.″ *kelmania*″	*Malus pumila*	California	S
P. cryptogea	P3587		C	*P.* sp.″ *kelmania*″	*Prunus avium*	California	S
P. kelmania	P10613		-	*P.* sp.″ *kelmania*″	*Abes fraseri*	North Carolina (2002)	
P. drechsleri	P1714		diverse	*P.* sp.″ *kelmania*″	*C. tinctorius*	Australia	A2
Phytophthora sp.	P3854		E	*P.* sp.″ *kelmania*″		California	
P. kelmania	P10614		-	*P.* sp.″ *kelmania*″	*Actinidia deliciosa*	California	-
P. drechsleri	P1713		diverse	*P.* sp.″ *kelmania*″	*C. tinctorius*		
P. cryptogea	P3086		-	*P.* sp.″ *kelmania*″	*Godetia* sp.	South Africa	A2
P. kelmania	P11688		-	*P.* sp.″ *kelmania*″			

Table 5.2 continued

Species as Formerly identified	Isolate code		IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. cryptogea	P3088	ATCC 28194.	E	*P. cryptogea* (GII)	*Pinus* sp.	Australia	A2
P. cryptogea	P3093	ATCC 48234	diverse	*P. cryptogea* (GII)	*Juniper chinensis*	California	A2
P. cryptogea	P1704	ATCC46999	E	*P. cryptogea* (GII)	*V. vinifera*	South Africa	A2
P. cryptogea	P1998		E	*P. cryptogea* (GII)	*Prunus dulcis*	Australia	A2
P. cryptogea	P3851		E(DE)	*P. cryptogea* (GII)	*A. deliciosa*	California	A2
P. cryptogea	P3103		D2	*P. cryptogea* (GII)	*Solanum marginaturn*	Ecuador	A2
P. cryptogea	P3089	ATCC36226	E	*P. cryptogea* (GII)	*?*	Australia	A2
P. cryptogea	P2001		E(DE)	*P. cryptogea* (GII)	*Malus sylvestris*	Australia	A2
P. cryptogea	P1999		E	*P. cryptogea* (GII)	*M. sylvestris*	Australia	A2
P. cryptogea	P3852		E(DE)	*P. cryptogea* (GII)	*A. deliciosa*	California	S
P. cryptogea	P3850		E(DE)	*P. cryptogea* (GII)	*A. deliciosa*	California	A2
P. cryptogea	P3198		E	*P. cryptogea* (GII)	*Abis nobolis*	Oregon	A2

Table 5.2 continued

Species as Formerly identified	Isolate code		IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. cryptogea	P3091	ATCC44672	E	*P. cryptogea* (GII)	Soil	Australia	A2
P. drechsleri	P3494	IMI129907	E	*P. cryptogea* (GII)	Soil	Australia	A2
P.c.*f.sp. *begoniae	P3104	ATCC52401	D(DE)	*P. cryptogea* (GII)	*Begonia elatior*	W. Germany	A2
P.c.*f.sp. *begoniae	P3145		D(DE)	*P. cryptogea* (GII)	*Begonia elatior*	W. Germany	A2
P. cryptogea	P1380		E	*P. cryptogea* (GII)	*V. vinifera*	South Africa	A2
P. cryptogea	P1947		E	*P. cryptogea* (GII)	*Pinus radiata*	South Africa	A2
P. cryptogea	P3092	ATCC46518	-	*P. cryptogea* (GII)	*Chrysanthemum*	California	A2
P. cryptogea	P19700		-	*P. cryptogea* (GII)	*Mangifera indica*	Italy (2010)	A2
P. cryptogea	P19698		-	*P. cryptogea* (GII)	*M. indica*	Italy (2010)	A2
P. cryptogea	P19696		-	*P. cryptogea* (GII)	*M. indica*	Italy (2010)	A2
P. cryptogea	P19697		-	*P. cryptogea* (GII)	*M. indica*	Italy (2010)	A2

Table 5.2 continued

Species as Formerly identified	Isolate code		IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. erythroseptica	P0340(T)		Per	*P. erythroseptica*	*Solanum tuberosum*	Australia	H
P. erythroseptica	P1698	ATCC 28766	Per	*P. erythroseptica*	*S. tuberosum*	England	H
P. erythroseptica	P1699	ATCC 36302	Per	*P. erythroseptica*	*S. tuberosum*	Ohio	
P. erythroseptica	P3452	IMI 21277 (ex CBS, 1948)	Per	*P. erythroseptica*	*S. tuberosum*	England	H
P. erythroseptica	P3454	IMI 181716	Per	*P. erythroseptica*	*S. tuberosum*	England	H
P. erythroseptica	P3938	PDA 69720-87	Per	*P. erythroseptica*	*S. tuberosum*	Pennsylvania - USA	H
P. erythroseptica	P10382		-	*P. erythroseptica*	*S. tuberosum*	Idaho- USA	-
P. erythroseptica	P8457		-	*P. erythroseptica*	*S. tuberosum*	Maine- USA	H
P. erythroseptica	P10172		-	*P. erythroseptica*	-	California- USA	-
P. erythroseptica	P11021		-	*P. erythroseptica*	*S. tuberosum*	Colorado- USA	H
P. richardiae	P3876	CBS 240.30 ATCC 46734	Prc	*P.* sp. *aff. erythroseptica*	*Zantedeschia aethiopica* (Calla Lily)	USA	
P. richardiae	P10359		-	*P.* sp. *aff. erythroseptica*	*Z. aethiopica* (Calla Lily)	Japan	----
P. richardiae	P10358		-	*P.* sp. *aff. erythroseptica*	*Z. aethiopica* (Calla Lily)	Japan	----
P. richardiae	P10811		-	*P.* sp. *aff. erythroseptica*	*Z. aethiopica* (Calla Lily)	Japan	----

Table 5.2 continued

Species as Formerly identified	Isolate code		IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. melonis	P1371		F	*P. melonis*	*Cucumis sativa*	China	A1
P. sinensis	P1475 (T)	ATCC 46538	F	*P. melonis*	*C. sativa*	China	S
P. melonis	P1746		F(F)	*P. melonis*	*C. sativa*	Taiwan	?
P. drechsleri	P3239		F(F)	*P. melonis*	*C. sativa*	China	A2
P. melonis	P3609		F	*P. melonis*	*C. melo*	Japan	A2
P. melonis	P6870(T)	ATCC 52854 CBS 582.69		*P. melonis*	*C. sativa*	Japan	?
P. sinensis	P1748		-	*P. melonis*	*C. sativa*	China	S
P. drechsleri* f. sp. *cajani	P1796		G1(G1)	*P. cajani*	*Cajanus cajan*	India	A1
P. d.* f. sp. *cajani	P1798		G1	*P. cajani*	*C. cajan*	India	A1
P. d.* f. sp. *cajani	P3105	ATCC 44388	G1(G1)	*P. cajani*	*C. cajan*	India	A1
P. d.* f. sp. *cajani	P3106	ATCC 44389	G1	*P. cajani*	*C. cajan*	India	A1
P. d.* f. sp. *cajani	P1795		G2(G2)	*P. cajani*	*C. cajan*	India	A1
P. erythroseptica	P6823		-	*P. cajani/vignae*	*Solanum lycopersicum*	Australia	?

Table 5.2 continued

Species as Formerly identified	Isolate code		IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. drechsleri	P1812		H	*P.* species	*Juglans hindsii*	California	S
P. drechsleri	P1813		H(H)	*P.* species	*Prunus avium*	California	S
P. drechsleri	P3602		H(H)	*P.* species	*Malus pumila*	Arizona	S
P. drechsleri	P3839		H	*P.* species	*M. pumila*	Arizona	S
P. drechsleri	P3849		H	*P.* species	*Actinidia deliciosa*	California	A1
P. drechsleri	P3859		H	*P.* species	*A. deliciosa*	California	A1
P. drechsleri	P3860		H(H)	*P.* species	*A. deliciosa*	California	A1
***Phytophthora* sp.**	P6823		---------		*Solanum lycopersicum*	Australia (NSW)	?
***Phytophthora* sp.**	P3199		J3	*gonapodyides*	*Pseudotsuga menziesii*	Oregon	S
P. cryptogea	P3731		J3(J4)	*megasperma*	*Prunus cerasus*	Michigan	S
P. erythroseptica	P7105		----------	*megasperma*	*Solanum tuberosum*	Australia	
P. erythroseptica	P7193	IMIN 33234	----------	*multivesiculata*	*Cymbidium* sp.	Australia (WA)	
P. multivesiculata	P10670			*multivesiculata*			
P. erythroseptica	P7254		----------	*multivesiculata*	*Cymbidium* sp.	Hawaii	

Table 5.2 continued

Species as Formerly identified	Isolate code WPC	International	IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
***Phytophthora* sp.**	P3196		K1	*P.taxon Pgchlamydo*	*Pseudotsuga menziesii*	Canada	S
P. cryptogea	P3279		K1	*P.taxon Pgchlamydo*	*Prunus cerasus*	New York	A1
P. cryptogea	P3317		K1	*P.taxon Pgchlamydo*	*P. persica*	New York	
P. cryptogea	P3320		K1	*P.taxon Pgchlamydo*	*P. persica*	Michigan	
***Phytophthora* sp.**	P3196		K1	*P.taxon Pgchlamydo*	*Pseudotsuga menziesii*	Canada	S
P. cryptogea	P3279		K1	*P.taxon Pgchlamydo*	*Prunus cerasus*	New York	A1
P. cryptogea	P3713		K1	*P.taxon Pgchlamydo*	*P. persica*	Michigan	
P. cryptogea	P3716		K1	*P.taxon Pgchlamydo*	*P. persica*	Michigan	

Table 5.2 continued

Species as Formerly identified	Isolate code		IG (DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. canalensis	P10456			*P. thermophilum*	Canal water	California	
P. erythroseptica	P3688			*P. asparagi*	*S. tuberosum*	UK (Scotland)	
P. cryptogea	P7796			" niederhauserii"	*Eustoma sp.*	Netherlands	
P. drechsleri	P7797			" niederhauserii"	*Spathiphyllum sp.*	Netherlands	
P. drechsleri	P7795			" niederhauserii"	*Eustoma sp.*	Netherlands	
P. drechsleri	P10171	MYA-4163		" niederhauserii"	Ornamental plants	Israel	A1
P. cryptogea	P7794			*P. cinnamomi* var. *parvispora*	*Codiaeum* sp.	Netherlands	
P. cryptogea	P6354			*P. cambivora*			
P. erythroseptica	P6404			*P. rubi*	Raspberry	W. Germany	
P. cryptogea	P3959			*P. cambivora*	Raspberry	Australia (V)	
P. cryptogea	P11601			*P. cinnamomi*	*Arctostaphylos viscida*	California	
P. richardiae	P6875			*macrochlamydospora*			
P. richardiae	P7789			*macrochlamydospora*	*Zantedeschia aethiopica*		
P. richardiae	P10335	IMIN 340618		*macrochlamydospora*	*Zantedeschia* sp.	Netherlands	

Table 5.2 continued

Species as Formerly identified	Isolate code		IG(DG) (Mills, 1991).	Phylogenetic species & group	Host	Origin	Mating type
	WPC	International					
P. sansomeana	P3163	117692	out	*P. sansomeana*	*Silene latifolia*	USA	
P. trifolii	P7010		out	*P. trifolii*	*Trifolium* sp.	USA	
P. medicaginis	P0127		out	*P. medicaginis*	*Medicago sativa*	Australia	
P. medicaginis	P1678		out	*P. medicaginis*	*Malus sylvestris*	USA	
P. medicaginis	P10683		out	*P. medicaginis*	*Medicago sativa*	USA	
niederhauserii	P10616		out	" niederhauserii"			
niederhauserii	P16237		out	" niederhauserii"			
niederhauserii	P10976		out	" niederhauserii"			
macrochlamydospora	P8017		out	*macrochlamydospora*			
P. macrochlamydospora	P10267		out	*macrochlamydospora*			
Pythium vexans			out				

Out= Isolates used as out of groups

4. Results

A preliminary ITS based screen was made in World Phytophthora collection (WPC). A total of 265 isolate that assigned to *P. cryptogea* (155 isolates), *P. drechsleri* (50), *P. erythroseptica* (32), *Phytophthora* sp. *kelmania* (5) and other *Phytophthora* species were screened. For further phylogenetic analysis, 140 isolates were selected. The isolates selection was aimed to use the same isolates examined previously on the base of morphological, isozyme and mitochondrial analysis by Mills *et al.* (1991), also to represent a full range of genetic diversity within the isolates.

Of 140 isolates that were selected for the phylogenetic study, 88 isolates were confirmed as *P. drechsleri*, *P. cryptogea*, *P. erythroseptica* and *P.* sp. *kelmania*. Fifty two isolates were found to be misidentified and subsequently identified as *P. asparagi, P. cajani, P. cambivora, P. cinnamomi, P. cinnamomi var. parvispora, P. gonapodyides, P. megasperma, P. melonis, P. multivesiculata, P. rubi* and other *Phytophthora* species taxa that not formally yet identified (Table 5.2).

Successful amplification and good sequencing of the nuclear and mitochondrial locus was obtained from all the isolates. For COI locus, the primers OomCoxI-Levup and Fm85mod amplified a 727 bp fragment from the 5`end of COI (Fig. 5.8). Introns were not present in any COI sequence of the species studied. For ITS region, ITS1 and ITS4 primers amplified fragment varied from 792bp to 796bp depend on the species.

Neighbor-joining phylogenetic analysis of the two nuclear and mitochondrial DNA loci revealed six different groups for all the isolates used in the study.

P. cryptogea were formed in two groups (GI and GII), while one group was observed for each of *P. drechsleri* and *P. erythroseptica* isolates.

A total of 17 *Phytophthora* isolates from woody hosts were closely matched the ITS and COI sequences of undescribed taxa *P.* sp. '*kelmania*'. In both COI and ITS phylogeny, all 17 isolates form a distinct sister group to *P. cryptogea* group II (GII). The sixth group was intermediate for those set of isolates collected from Calla lily plants.

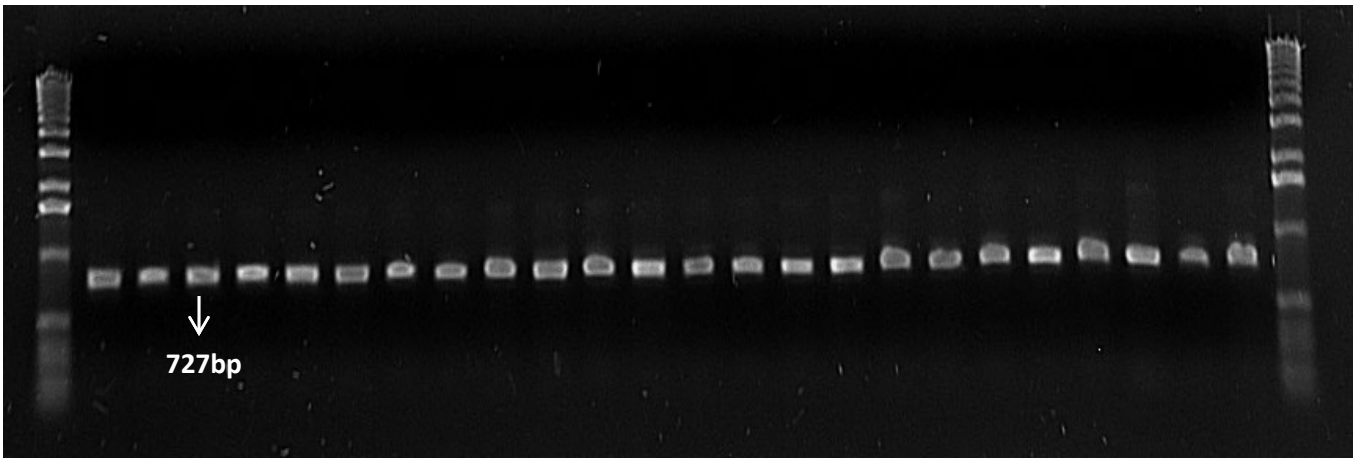

Figure 0.8 Amplified PCR products of 727bp fragment for COX1 region with OomCoxI-Levup and Fm85mod.

Neighbor-Joining phylogenetic analysis of the ITS and COI loci showed gene-gene concordance in six observed groups with some few exceptions, in case of Calla lily group. Phylogenetic analyses of ITS region showed that these isolates were most closely affiliated with *P. cryptogea* group I (GI), but in the phylogenetic analysis using COI region, there were most closely affiliated with *P. erthyroseptica* group. The position of P3902 isolates and P1902 varied according to the sequenced region.

P. drechsleri group contained the authentic isolates (P11638 and P11637) of *P. drechsleri* in addition to other 8 isolates. *P. drechsleri* clade was determined as monophyletic in both individual Neighbor-Joining phylogenetic trees with bootstrap support ranging from 83 % in case of ITS and 97 % in case of COI (Fig. 5.9). The clade was distinct from all other isolates which could consider being *P. drechsleri sensu stricto.*

The 44 isolates *P. cryptogea* formed two separate groups. In case of *P. erythroseptica* clade was rooted amongst the *P. cryptogea* group.

The isolates P10358, P10359, P10811 (from Japan) and P3876 (from USA), all recovered from Calla lily plants (*Zantedeschia* sp.), showed intermediate positions. They were close affiliation to *P. erythroseptica* in COI while more affiliated to *P. cryptogea* in case

of ITS analysis. The multiple alignments showed SNPs differences between these isolates and the isolates from the five groups (data shown in supplement).

A set of 17 isolates formed group of the new *Phytophthora* taxon *"P. sp. kelmania",* (Fig. 5.9). Double peaks in sequencing electropherograms indicated heterozygosity were observed in isolate P3586 recovered from apple in California. These were reflected as ambiguity codes in the multiple alignments

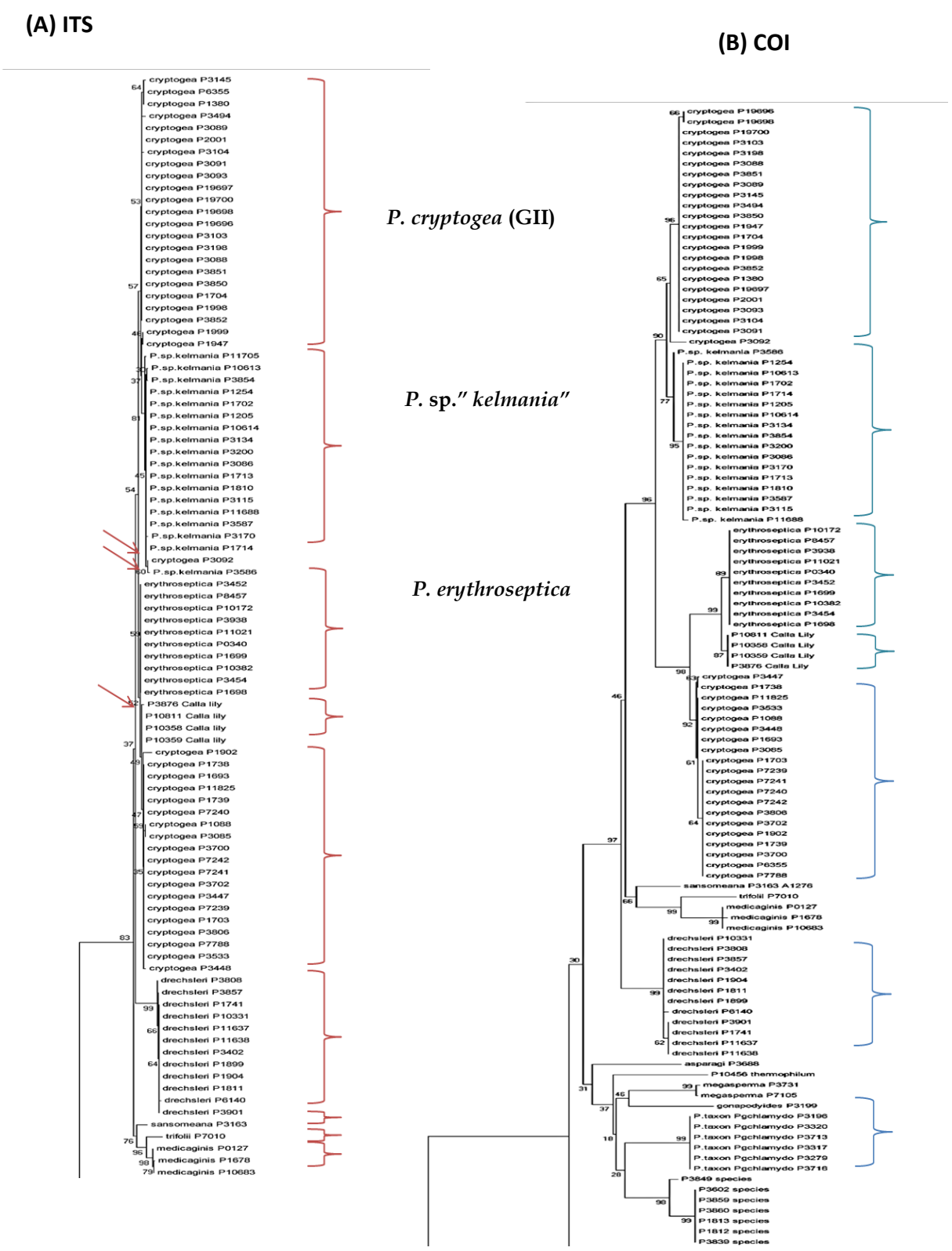

Figure 0.9 Phylogenetic relationships of *Phytophthora drechsleri*, *P. cryptogea* groups and associated species based on neighbor joining method. The numbers at the branch points indicate the percentages of bootstrap values. A, Internal Transcribed Spacers, ITS. B, mitochondrial Cytochrome c Oxidase subunit I, COI.

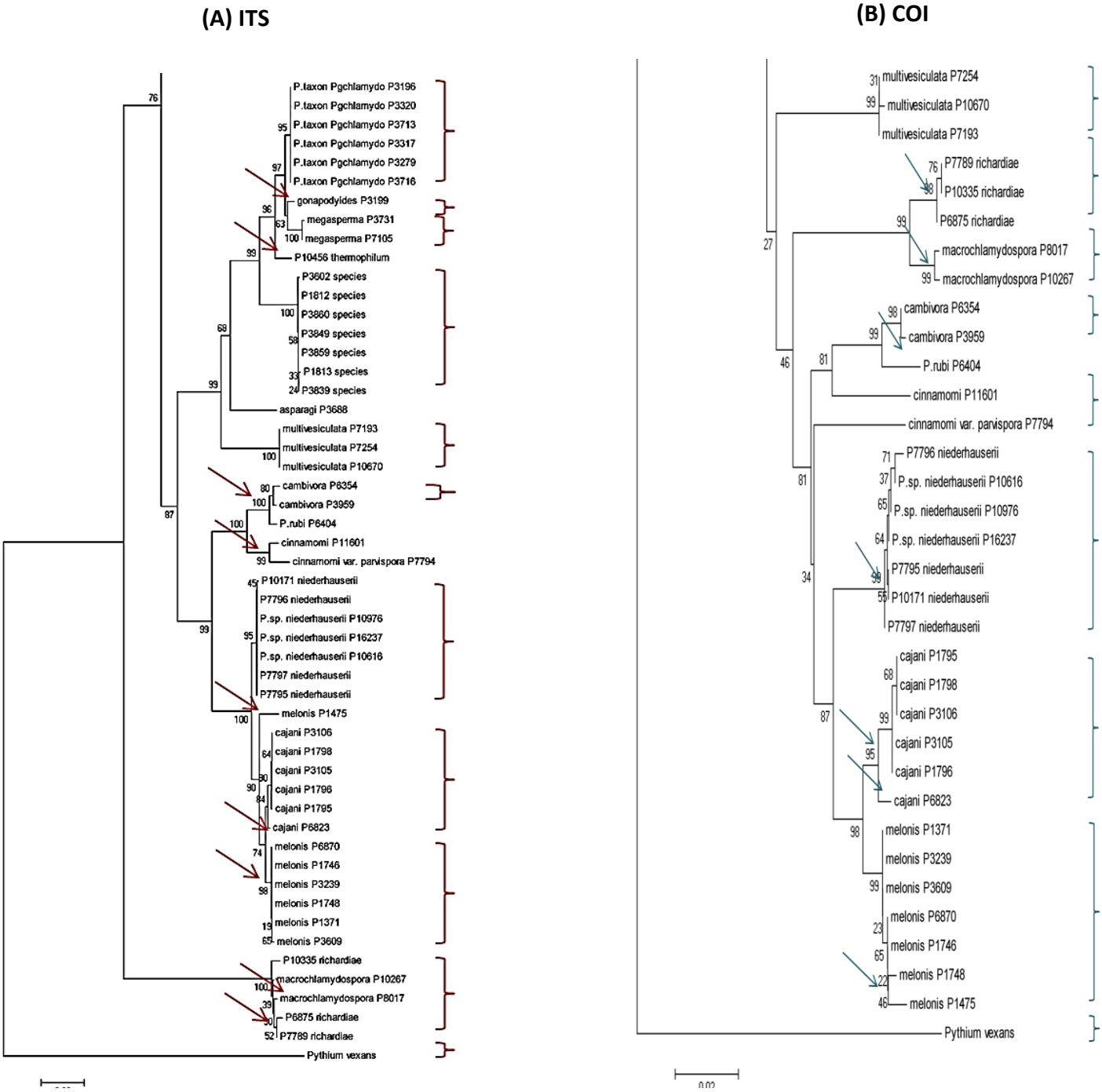

Figure 5.9 Continued.

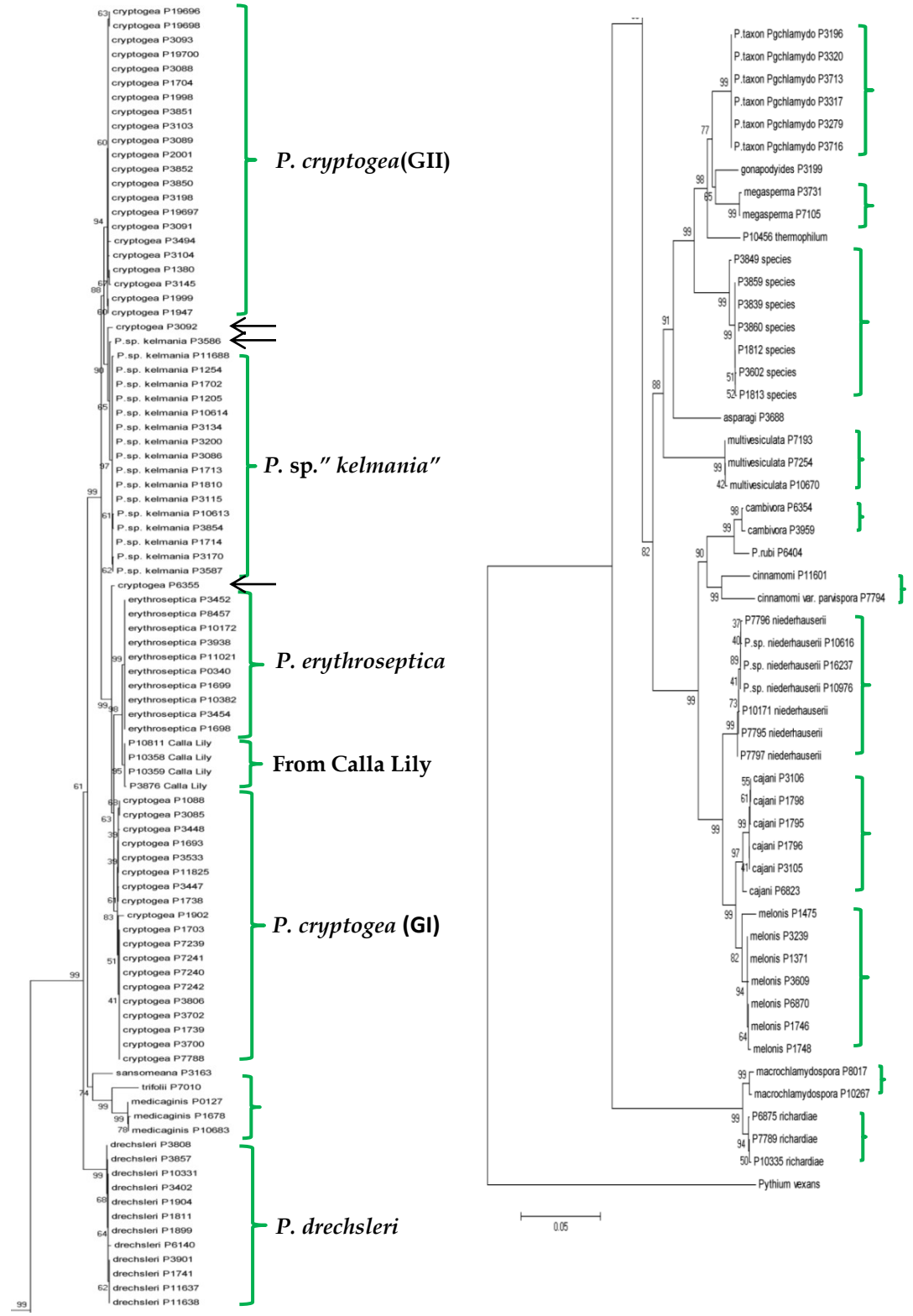

Figure 0.10 Phylogenetic relationships of Phytophthora drechsleri, P. cryptogea groups and associated species based on Neighbour Joining method of combined sequences of Internal Transcribed Spacers (ITS) and mitochondrial Cytochrome c Oxidase subunit I (COI). The numbers at the branch points indicate the percentages of bootstrap values. *Pythium vexans* sequences were used as out-group.

5. Discussion

The differentiation of *P. cryptogea*, *P. erythroseptica* and *P. drechsleri* is a controversial issue. They could not be distinguished based on morphological characterization. The traditional classification, based on morphological traits and growth characteristics, has several limitations. The problem associated with assigning isolates to either *P. drechsleri* or *P. cryptogea* is an example proves this situation (Erwin and Ribeiro, 1996). Additionally, growth characteristics and morphological traits are phenotypic, and groups of species sharing similar traits do not necessarily reflect evolutionary relatedness (Kroon *et al.*, 2004), while DNA sequence-based identification methods were able to provide phylogenetic separation for many *Phytophthora* species and to support their status as distinct taxa. It is so obvious that without advantage of molecular identification, much previous analysis was confused by misidentified isolates and inappropriate conclusions were made. Another example is the isolate P3901 that was originally described as *P. erythroseptica,* but now based on DNA sequence data and phylogenetic analysis; the isolate is placed with *P. drechsleri* group being high identical to P11638 the authentic culture of *P. drechsleri* from beet (*Beta vulgaris*). Similar confirmation was made by Mills *et al.* (1991) in which P3901 appeared in isozyme/DNA group A. In addition the isolate P3901 was found to be heterothallic and it grew well at 35°C the two features not associated with *P. erythroseptica* description (Pethybridge, 1913; Tucker, 1931).

Our study provides the first nDNA and mtDNA sequence analysis that based on using abroad range of isolates including the type and authentic isolates of each species. With the benefit of DNA sequence based methods, ITS based screen was made for isolates in WPC collection assigned before as either *P. drechsleri* or *P. cryptogea.* A total of 140 isolates were selected and were further sequenced with COI locus.

Based in our finding, high levels of intraspecific variation were found in *P. cryptogea* in which two different groups were inferred. All isolates of *P. drechsleri* were formed in another distinct group with low level of variation, similar results were found with all *P. erythroseptica* isolates. These are in contrast with other previous studies that grouped these species based on morphology and physiology (Bumbieris, 1974; Ho and Jong, 1986), however the results are in accord with Mills *et al.* (1991), who suggested that *P. cryptogea*

and *P. drechsleri* should not be merged as they do not appear to be closely related to each other and they are genetically distinct.

ITS based pre-screen showed that among the tested isolates in WPC collection, 30 isolates were confirmed to be identical with what we considered now as typical *P. drechsleri sensu* (data not shown). The phylogenetic data based on ITS and COI of 12 chosen isolates of *P. drechsleri* provided clear evidence that *P. drechsleri* is distinct taxon. In nDNA and mDNA locus, all the 12 isolates formed separate clade supported with the bootstrap analysis. In agreement with our analysis, Some of the isolates used in our study have been used by Mills *et al.* (1991) and they placed them all in isozyme/mtDNA group A, with exception of isolate P3808 that was placed in diverse group. Mostowfizadeh-Ghalamfarsa *et al.* (2010) based on multiple gene phylogenetic analysis found that isolates of *P. drechsleri* form a monophyletic grouping.

P. drechsleri clade was consistent and included the authentic isolates of *P. drechsleri* (P11637and P11638), also contained one isolate from rotted roots of safflower (P6140) that had originally been named *P. drechsleri* by Tucker (Erwin 1952) but were later renamed *P. cryptogea* due to a suggestion to merge these two species (Bumbieris, 1974). Bielenin *et al.* (1988) found that the authentic isolate of *P. drechsleri* and one of the isolates of *P. cryptogea* from safflower were indistinguishable according to total protein electrophoresis. Furthermore, they showed these isolates to be different from the authentic culture of *P. cryptogea* (P1088). In addition Mills *et al.* (1991) placed isolate P6140 into isozyme/mtDNA group A where *P. drechsleri* dominated the group. *P. drechsleri* clade included four isolates causing root rot of sugar beet from California (P1899, P3402 and P1904), and an isolate (P3857) from Mexico causing root rot of sweet pepper.

According to the work by Mills *et al.* (1991), the isolates (P1741, P1899, P3875, P3901 P6140, and authentic isolate) grew well at 35^{o}C thus fulfilling a major criterion for placement in *P. drechsleri* (Tucker, 1931). Similar results were obtained in recent study by Mostowfizadeh-Ghalamfarsa *et al.* (2010), in which 14 isolates of *P. drechsleri* included the isolate P3901 had an optimum temperature of 30^{o}C and grew well at 35^{o}C.

The isolate P10331 isolated on 2003 from gerbera plants in New Hampshire was used by Blair *et al.* (2008) in multilocus analysis and by Robideau *et al.* (2011) and it was placed in within *P. drechsleri* group.

For *P. cryptogea*, ITS based pre-screen data showed that, 43 isolates could be considered what we know now to be *P. cryptogea.* The phylogenetic analysis of the nDNA and mDNA sequenced genes demonstrated distinct sub-population within *P. cryptogea* in which two subgroups were determined (named GI and GII).

P. cryptogea group I (GI) clade was different from group II (GII). This group included the authentic culture of *P. cryptogea* P1088 (CBS 290.35 (Tucker), ATCC 46721), the type culture P1738 (CBS 113.19), isolate P1739 from tomato in New Zealand, P3085 from *Aster* sp. plants, P1703 isolated from potato in Ohio. In addition, four isolates originally from Sweden and were isolated from wheat (P7240, P7241 and P7242) and Spinach (P 7239). This group (GI) included P7788 isolates in 1988 from carrot plants in England and was assigned in WPC collection as *P. richardiae.*

An interesting side light was that this group also included what thought to be the type culture of *P. erythroseptica* P1693 (IMI 34684). This was of particular interest since the remaining isolates of *P. erythroseptica* formed a uniform group that was quite distinct from the type culture (see below in the discussion). A reasonable explanation for this observation is that at one point in the long history of this isolate (Pethybridge, 1913), it was accidentally switched with the type culture of *P. cryptogea*, which was then mislabeled as the type culture of *P. erythroseptica* and accidentally distributed to culture collections throughout the world. This example illustrates the critical shortcomings of assignment of a single type culture to represent an entire species. Another explanation could be a misidentification by those who donated the cultures.

Based in our observation, most of the isolates of *P. cryptogea* within group I (GI) were homogenous with some variations as few sequence differences reflected in sub clusters in both ITS and COI phylogenetic trees (Fig. 5.10). Such variation could be explained by the wide host range and global origin of the isolates included in the clade.
Cross referencing and comparison with common isolates used by other studies, showed that our *P. cryptogea* group I (GI) is corresponded to group "B" defined on the basis of isozymes and mtDNA by Mills *et al.* (1991). Our results are in agreement with Mills *et al.* (1991), who observed the type isolate of *P. cryptogea* was placed as member of group "B". They also reported that these isolates displayed a high degree of intragroup isozyme variability but were uniform according to mtDNA RFLP analysis. In the work by

Mostowfizadeh-Ghalamfarsa *et al.* (2010), the two isolates that could be cross referenced are P1693 and P1739 in our group (GI) that were corresponded to those in *P. cryptogea* (termed GI) in their study along with other isolates from Iran.

P. cryptogea group II (GII) is represented another sub population group of *P. cryptogea*. This group corresponded to those of "E , D and diverse" groups defined by Mills *et al.* (1991) on the basis of isozyme and mtDNA RFLPs. This group is characterized by diversity of the host range and origins. Isolates in (GII) were collected from different woody trees such as mango (Southern Italy), grape (South Africa), pine (South Africa and Australia), apple and almond (Australia), noble Fir (Oregon), Kiwi (California) and purple African nightshade (Ecuador). In addition to three isolates (P3145, P3103 and P3104) collected from Begonia in Germany by Krober (1981). Noteworthy, Kröber (1981a,b) described the forma specials *begoniae* of *P. cryptogea* on the basis of host specificity to begonia and the isolates were A2 mating type.

Based on the phylogenetic analysis for both COI and ITS, all the above isolates were fall together within our GII with some few sequences variation reflected in sub clades within the group. These results did not match with the placement of these isolates based on isozyme and mDNA RFLPs study by Mills *et al.* (1991), however, our findings are in accord with Mostowfizadeh-Ghalamfarsa *et al.* (2010).

In Mills *et al.* (1991) study, they placed our (GII) isolates in three different groups "D, E, and diverse". Group D consists of isolates of *P. cryptogea* f. sp. *begonia* recovered from Begonia in W. Germany and one isolate of *P. cryptogea* from purple African nightshade in Ecuador. Group E included isolates were from grape, pine, and apple. Although both groups "E and D" did not grow at 35°C and were isozymically similar, but Mills *et al.* (1991) placed them separately.

However, in looking at the phylogenetic analysis based on ITSI reported in Foster 2000, there is a great level of variation among *P. cryptogea/ P. drechsleri* complex in which different sub clusters were observed. A sub-cluster of three isolates (P1087, P1741 and P3402) formed a group represented what we now know as *P. drechsleri*. Another sub cluster contained two isolates (P1703 and P3700) corresponded to *P. cryptogea* (GI) in our study. An interesting note that P3145 from Begonia had position in sub-cluster with P3850 corresponded to our (GII). The phylogenetic analysis by Foster 2000 showed some slight

variations reflected in one intermediate subgroup with only one isolate (P1702) that matches the informally described *Phytophthors* sp. *kelmania*.

Based on the analysis by Mills *et al.* (1991), All the isolates in *P. cryptogea* (GI and GII), did not grow at 35°C, a feature that characterizes this species from *P. drechsleri*. Although mating type A1 dominated *P. cryptogea* (GI) with few exception of (P3806 and P1703) while A2 mating type dominated group (GII), there is no clear relationship between molecular lineage and mating type. Although no barriers for mating across molecular types of *P. cryptogea* are apparent, comprehensive reciprocal mating studies are needed to examine this more in detail (Mostowfizadeh-Ghalamfarsa *et al.*, 2010).

Based on ITS and COI phylogenetic analysis (Fig. 5.9, 5.10), all *P. erythroseptica* isolates were clustered into separate group but derived from *P. cryptogea*. The group contains 10 isolates, all are from potato tubers and collected from different regions in US, England and the type culture (P0340) from Australia. Our *P. erythroseptica* group is corresponded to the group "Per" in Mills *et al.* (1991) isozyme and mtDNA study. They concluded *P. erythroseptica* is a uniform and distinct taxon based on the isozyme analysis. Mostowfizadeh-Ghalamfarsa *et al.* (2010) suggested that *P. erythroseptica* and *P. cryptogea* are conspecific and they supported the hypothesis of *P. erythroseptica* is a secondarily derived homothallic form of *P. cryptogea*. Such a phenomenon has been reported in the case of *P. drechsleri* (Mortimer *et al.*, 1977), and other studies support the derivation of homothallic taxa from heterothallic ones (Cooke *et al.*, 2000a). Kroon *et al.* (2004) demonstrated that *P. erythroseptica* is closely related to *P. cryptogea* based on molecular analysis. Kroon *et al.* (2004) mentioned that secondary homothallics was reconstructed within some species in their phylogeny study. Such as *P. ipomoeae* in clade 1c, the homothallic isolates of *P. meadii* in clade 2, and *P. erythroseptica* in clade 8a. These results indicate that the switch from homothallism to heterothallism, and *vice versa*, has occurred quite often in the genus *Phytophthora*. This observation brings up the question of how the underlying genetic mechanism of the switch from homothallism to heterothallism (and *vice versa*) operates.

P. erythroseptica has been misidentified over the years as one of the *Phytophthora* species that causes root rot of red raspberry *(Rubus idaeu* L.*)*. Characterization of mtDNA RFLP pattern has confirmed the similarity of red raspberry isolates, formerly attributed to

P. erythroseptica, to *P. fragariae* (Forster and Coffey, 1992). Lately this pathogen has been identified and described as *P. fragariae* var. *rubi* Wilcox and Duncan var. nova (Erwin and Ribeiro, 1996). Although *P. erythroseptica* is known as a pathogen of solanaceous hosts, it also been reported to be a serious pathogen of other plants such as rice and calla lilies. In Waterhouse (1963), *P. erythroseptica* was sub classified into *P. erythroseptica* var. *erythroseptica* and in Stamps (1990) into *P. erythroseptica* var. *pisi.*

P. erythroseptica var. *pisi* was first isolated from diseased pea roots in England. This species was rejected by Waterhouse (1963) while Stamps (1990) recommend that it be retained but its acceptance is datable.

In our study, a total of seven *Phytophthora* isolates formally named as *P. richardiae* were examined by ITS and COI analysis. Three of the seven are isolated in the late 1980s from Calla lily in Japan. Two of seven are isolated from Calla lily USA and the other two are from Netherlands. The results showed that four of seven isolates,(P3876 -USA), and (P10811, P10358 and P10359 -Japan) were placed as intermediate cluster amongst *P. cryptogea* GI and GII in ITS phylogeny tree (Fig. 5.10), while in the phylogenetic data of COI there was a close affiliation to *P. erythroseptica* with only few differences of three SNPs.

Surprisingly, the other three of seven isolates, P6875 (USA), P7789 and P10335 (Netherlands) were found to be most closely related to *P. macrochlamydospora* (Table 5.2).

In Blair *et al.* (2008) multilocus study, the sequences of the cross referenced isolates (P3876 and P10359) and other isolates published as *P. richardiae* (P10811 and P7788) and recorded as *P. richardiae* in the Phytophthora database (www.phytophthoradb. org) formed group within clade 8 alongside the GI and GII isolates of *P. cryptogea.* While the Buisman isolate (P6875) corresponded to (ATCC60353) (CBS 240.30) from calla lily USA and P10335 (IMI 340618) from calla lily Netherlands, have been examined by Kroon *et al.* (2004), Cooke *et al.* (2000a), respectively, In both studies these isolates were placed in ITS-clade 9 and were found to be most closely related to *P. macrochlamydospora*. The balance of the evidence suggests that the taxon originally isolated from calla lily by Buisman was *P. richardiae* (clade 9) and subsequently other *Phytophthora* species that similar to *P. cryptogea*

has also been reported from calla lily and incorrectly named *P. richardiae* on the basis of its plant host.

Based on the observation made by Martin *et al.* (2012), they concluded that there is strong bootstrap support separating *P.* sp. *aff. erythroseptica* from *P. erythroseptica*, based in his observation from previous studies on these isolates. They concluded that further analysis is needed to clarify if they represent a new species.

The subgroups of *P. cryptogea* based on the mDNA and nDNA gene tree indicates a basal position of GII and *P.* sp. *kelmania* that are more closely related to each other and ancestral to the more distantly related GI.

P. melonis clade contains seven isolates that recovered from cucurbit plants mainly cucumber (*Cucumis sativa*) and cantaloupe (*C. melo*) in various parts of Asia (China, Japan, Taiwan and Iran). *P. melonis* group contains two isolates (P1682 and P3239) that were misidentified as *P. drechsleri.* The clade includes also the type species isolate of *P. sinensis* (P1475^{T}) and *P. melonis* (P6870^{T}). In the present data both *P. melonis* and *P. sinensis* share similar ITS and COI sequences and were formed together in the same clade. Our results are consistent with the study by Mills *et al.* (1991) and the molecular analysis of (Cooke *et al.*, 2000a). All the seven isolates were examined before by Mills *et al.* (1991). Based on the mtDNA REFLPs and isozyme analysis, these isolated were identical. They also had similar temperature/growth responses, displaying no growth at 5^{o}C and growing well at 35^{o}C.

P. melonis was first isolated by Katsura (1968) from diseased cucumber plants in Japan. Katsura (1976) reported that isolates of *P. melonis* were limited to the Cucurbitaceae. Ho (1986b) reexamined the type culture of *P. sinensis*, authentic cultures of cucumber isolates of *P. melonis* from Japan and *P. drechsleri* isolate from Iran. He found that all isolates were predominantly heterothallic but formed a few oospores in single culture, proliferation of nonpapillate sporangia occurred, antheridia were occasionally two celled, and that chlamydospores were not formed by any culture. Ho (1986b) concluded that *P. sinensis* should be included in *P. drechsleri* based on nomenclatural priority.

Previous reports of cucurbit disease are almost certainly associated with misidentified isolates of what we now know to be the unrelated *P. melonis* (Cooke *et al.*, 2000a). The results of Mills *et al.* (1991) support this as all their isolates reported as *P.*

drechsleri which caused cucumber crown rot were clustered in their group 'F' which also included all *P. melonis* isolates and were highly pathogenic to cucumber, but not tomato. Kastura (1976) described *P. melonis* as species with semipapillate sporangia. Sporangia were later found to be nonpapillate by Mills *et al.* (1991).However, the further proposal that they be synonymized with *P. drechsleri* (clade 8) on morphological and physiological grounds (Ho and Jong, 1986) is not supported by the present data.

In a study of mtDNA RFLP and isozyme pattern by Mills *et al.* (1991), however, four isolates from China and Japan that had been classified as *P. melonis* from cucurbit plants grouped with one *P. sinensis* and eight *P. drechsleri* isolates from cucurbit plants (group F). This group of isolates was distinct from isolates that had been previously identified as *P. drechsleri* and *P. cryptogea*. Since the groups were genetically distinct Mills *et al.* (1991) recommended not merging *P. melonis* into *P. drechsleri.*

More recently, Mirabolfathy *et al.* (2001) studied two non-papillate species of *Phytophthora* as the causal agents of pistachio gummosis in Iran. Their previous descriptions as *P. drechsleri* and *P. megasperma* was re-examined by RFLPs and sequence comparison of ITS regions of rDNA. The isolates from pistachio described as *P. drechsleri* had ITS sequences identical to *P. melonis*, *P. sinensis*, and isolates described as *P. drechsleri* from cucurbits in Iran (five isolates). They concluded that these taxa should be considered conspecific and all subsumed within *P. melonis*.

P. cajani group includes five isolates collected from pigeon pea (*Cajanus cajan*) in India. The species was initially named by Pal *et al.* (1970) as *P. drechsleri* var. *cajani* the cause of the stem rot disease of pigeon pea but this was later described a *Phytophthora cajani* (Amin *et al*.1978). Kannaiyan *et al*. (1980) then re-examined several isolates and renamed it *P. drechsleri* f. sp. *cajani* on the basis of morphological similarity to *P. drechsleri*. Isozyme and mtDNA RFLP analysis however identified these isolates as a group (G) distinct from the typical *P. drechsleri* isolates in group 'A' (Mills *et al,.* 1991). The isolates also found to be pathogenic only to pigeon pea and *Atylosia* spp. This result was supported by phylogenetic analysis of ITS rDNA sequences that showed *P. cajani* as a distinct and distantly related species to *P. drechsleri* (Cooke *et al.,* 2000).

The isolate P6823 that recovered from tomato *Solanum lycopersicum* in Australia had ITS sequences similarity with *P. cajani* while with COI sequence was similar to *P. vignae*.

P. vignea was first isolated from Cowpea with root and stem rot in Queensland, Australia. Widespread outbreaks of disease caused by *P. vignae* appear to be limited geographically to Australia on cowpea and to Japan on adzuki bean (*Vigna angularis*) (Notsu *et al.*, 2003)

P. megasperma was erected by Drechsler (1931), who isolated it from rotted root tissue of hollyhock. It is characterized by its large oogonia. *P. megasperma* is a broadly based species that includes a number of different biotypes from several hosts. Isolates of the different groups are morphologically similar but mtDNA FLP data indicate that the taxa may actually include more species (Förster and Coffey, 1993). In Förster and Coffey (1993) study of 129 isolates delineated nine groups, three of which have already been removed from *P. megasperma* and placed in separate taxa, *P. sojae*, *P. medicaginis* and *P. trifolii*. *P. megasperma* taxonomy is still one of the most problematic species in the genus (Förster and Coffey, 1993).

P. gonapodyides group contained only one unidentified isolate (P3199) from Oregon recovered from Douglas fire plants. The ITS and COI sequences of P3199 showed similarity with *P. gonapodyides* and the phylogeny analysis placed the isolate in separate group. This results agreed with previous studies (Mills, 1989; Brasier *et al.*, 1993). Based on the mtDNA and isozyme study, Mills (1989), placed this isolate into group J3. Brasier *et al.* (1993), found that most isolates of *P. gonapodyides* exhibited a similar protein banding pattern distinct from that of *P. cryptogea* or *P. drechsleri*, and including a characteristic band designated the PG band. *Pythiomorpha gonapodyides* is the only known synonym of *P. gonapodyides* and was originally described from submerged fruits and twigs in Denmark by Petersen (1909), and later from similar habitats in Germany and in Michigan, U.S.A. The species was transferred to *Phytophthora* by Buisman (1927), a move supported by Waterhouse (1958). Hansen *et al.,* (1988) attributed a *Phytophthora* obtained from muskegs and streams in remote forested areas of southern Alaska and Oregon, and from roots of Douglas fir in nurseries in the Pacific Northwest, to the common root and collar pathogen *P. drechsieri* Tucker. In a subsequent examination, however, this fungus was reassigned to

P. gonapodyides. In Britain the fungus is associated with root death in hardwoods. It is also a weak to moderate pathogen able to cause a severe root rot of young cuttings of raspberry. In the Pacific Northwest it is a moderate root pathogen on seedling Douglas fir. In the field, therefore *P. gonapodyides* may frequently attack the small or fine feeder roots of its woody hosts, and may often be in balance with unstressed root systems, but may sometimes contribute to rapid decline if host stress ensues, particularly following flooding or under prolonged wet soil conditions.

P. macrochlamydospora was isolated in 1974 from soybean. Since sporangia could not be produced, the culture was stored until 1990 when sporangia and chlamydospores were induced by Irwin 1991 but sex organs were not found. Irwin and Mackie (2000) revised the description of *P. macrochlamydospora* with additional isolates. They concluded that *P. macrochlamydospora* is a Group VI *Phytophthora* species, producing non-papillate sporangia, and sex organs in paired culture with *P. cambivora* A1 mating type. *P. macrochlamydospora* produces large (up to 90 mum), thick-walled chlamydospores that distinguish it from *P. colocasiae* and all other Group VI species. Stovold *et al.* (1996) re-examined a herbarium material of *Phytophthora* species recorded on soybean in New South Wales during 1985 and identified as *P. macrochlamydospora*.

6. Conclusion

The differentiation of *P. cryptogea*, *P. erythroseptica* and *P. drechsleri* is a controversial issue. They could not be distinguished based on morphological characterization. The traditional classification, based on morphological traits and growth characteristics, has several limitations. Our study provides the first nDNA and mtDNA sequence analysis that based on using abroad range of isolates including the type and authentic isolates of each species. This detailed study of the phylogenetic analyses amongst worldwide collections of *P. cryptogea*, *P. erythroseptica*, *P. drechsleri* and other associated species has resolved several issues. It is clear that misidentification of cultures has confused the taxonomy of these species and this has impacted our understanding of the pathogenicity and origins of these taxa of pathogens that remain significant plant health threats, particularly in the plant nursery industries.

Based on the phylogenetic analyses of the combined ITS and COI sequences data, we could confirm that P. drechsleri is distinct species from *P. cryptogea*. Within the tested population of *P. crypotogea* we observed two molecularly distinct groups named; GI and GII. The wide host range and broadly distributed heterothallic species such as *P. cryptogea* and *P. drechsleri* have greater opportunities for genetic exchange among and within sub-populations and this may explain the molecular diversity we observed in the current study. Both markers provide an acceptable resolution for this species complex. However, more potential genetic markers for phylogenetic analyses are needed to fully evaluate the taxonomic position of this species. It could be better to include in further studies, isolates of these clade 8 taxa from natural ecosystems to understand more about their center of diversity, ecological role, distribution and potential future threat to plant industries worldwide.

7. References

Anderson N., Szemes M., O'Brien P., de Weerdt M., Schoen C., Boender P. and Bonants P. (2006). Use of hybridization melting kinetics for detecting *Phytophthora* species using three-dimensional microarrays: demonstration of a novel concept for the differentiation of detection targets. *Mycology Research*, 110(Pt 6): 664-671.

Avise J. C. (1994). *Molecular markers, natural history and evolution*. Chapman & Hall, New York.

Belbahri L., Moralejo E., Calmin G., Oszako T., Garcia J. A., Descals E. and Lefort F. (2006). *Phytophthora polonica*, a new species isolated from declining *Alnus glutinosa* stands in Poland. *FEMS Microbiol Lett*, 261(2): 165-174.

Bielenin A., Jeffers S. N., Wilcox W. F. and Jones A. L. (1988). Separation by protein electrophoresis of 6 species of *phytophthora* associated with deciduous fruit crops. *Phytopathology*, 78(11): 1402-1408.

Blair J. E., Coffey M. D., Park S.Y., Geiser D. M. and Kang S. (2008). A multi-locus phylogeny for Phytophthora utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology*, 45(3): 266-277.

Brasier C. M., Beales P. A., Kirk S. A., Denman S. and Rose J. (2005). *Phytophthora kernoviae* sp. nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. *Mycological Research*, 109(8): 853-859.

Brasier C. M., Hamm P. B. and Hansen E. M. (1993). Cultural characters, protein patterns and unusual mating behaviour of *Phytophthora gonapodyides* isolates from Britain and North America. *Mycological Research*, 97(11): 1287-1298.

Bruns T. D., White T. J. and Taylor J. W. (1991). Fungal Molecular Systematics. *Annual Review of Ecology and Systematics*, 22(1): 525-564.

Bumbieris M. (1974). Characteristics of 2 phytophthora species. *Australian Journal of Botany*, 22(4): 655-660.

Cacciola S. O., Di San Lio G. M., Greco G. and Pane A. (1990). Electrophoretic study of three related Phytophthora species1. *EPPO Bulletin*, 20(1): 47-58.

Cline E. T. and et al. (2008). A Synopsis of *Phytophthora* with accurate scientific names, host range, and geographic distribution. *PHP Plant Health Progress*.

Cooke D. E. L., Drenth A., Duncan J. M., Wagels G. and Brasier C. M. (2000a). A Molecular Phylogeny of *Phytophthora* and Related Oomycetes. *Fungal Genetics and Biology*, 30(1): 17-32.

Cooke D. E. L., Duncan J. M., Williams N. A., Hagenaar-de Weerdt M. and Bonants P. J. M. (2000b). Identification of *Phytophthora* species on the basis of restriction enzyme fragment analysis of the internal transcribed spacer regions of ribosomal RNA. *Bulletin OEPP*, 30(3-4): 519-523.

Cooke D. E. L., Schena L. and Cacciola S. O. (2007). Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. *Journal of Plant Pathology*, 89(1): 13-28.

Dick M. A., Dobbie K., Cooke D. E. L. and Brasier C. M. (2006). *Phytophthora captiosa* sp. nov. and *P. fallax* sp. nov. causing crown dieback of Eucalyptus in New Zealand. *Mycological Research*, 110(4): 393-404.

Drechsler C. (1931). A Crown-rot of Hollyhocks Caused by *Phytophthora Megasperma* n.sp, 21:513-526. *Journal Washington Academy of Sciences.*

Drenth A., Goodwin S. B., Fry W. E. and Davidse L. C. (1993). Genotypic Diversity of Phytophthora infestans in The Netherlands Revealed by DNA Polymorphisms. *Phytopathology - new york and baltimore then st paul-*, 83(10): 1087.

Drenth A., Tas I. C. Q. and Govers F. (1994). DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. *European Journal of Plant Pathology*, 100(2): 97.

Ebach M. C. and Holdrege C. (2005). DNA barcoding is no substitute for taxonomy. *Nature*. 434(7034).

Érsek T. and Ribeiro O. (2010). Mini Review Article: An annotated list of new Phytophthora species described post 1996. *Acta Phytopathologica et Entomologica Hungarica*, 45(2): 251-266.

Erwin D. C., Bartnicki-García S., Tsao P. H. and Society A. P. (1983). *Phytophthora: its biology, taxonomy, ecology, and pathology.* American Phytopathological Society.

Erwin D. C. and Ribeiro O. K. (1996). *Phytophthora diseases worldwide.* APS Press.

Forster H. and Coffey M. D. (1992). Molecular characterization of *Phytophthora* isolates with nonpapillate sporangia causing root-rot of raspberry using mtdna restriction-fragment-length-polymorphisms. *Mycological Research*, 96: 571-577.

Förster H. and Coffey M. D. (1993). Molecular taxonomy of *Phytophthora megasperma* based on mitochondrial and nuclear DNA polymorphisms. *Mycological Research*, 97(9): 1101-1112.

Förster H., Cummings M. P. and Coffey M. D. (2000). Phylogenetic relationships of *Phytophthora* species based on ribosomal ITS I DNA sequence analysis with emphasis on Waterhouse groups V and VI. *Mycological Research*, 104(9): 1055-1061.

Gallegly M. E. and Hong C. (2008). *Phytophthora* : identifying species by morphology and DNA fingerprints. *American Phytopathological Society*, St. Paul, MN.

Grünwald N. J., Martin F. N., Larsen M. M., Sullivan C. M., Press C. M., Coffey M. D., Hansen E. M. and Parke J. L. (2010). Phytophthora-ID.org: A Sequence-based *Phytophthora* identification tool. *Plant Disease*, 95(3): 337-342.

Halsall D. M. (1976). Specificity of cytoplasmic and cell-wall antigens from four species of *Phytophthora*. *J Gen Microbiol*, 94(1): 149-158.

Hebert P. D. N. and Gregory T. R. (2005). The Promise of DNA Barcoding for Taxonomy. *Systematic Biology*, 54(5): 852-859.

Hebert P. D. N., Penton E. H., Burns J. M., Janzen D. H. and Hallwachs W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, 101(41): 14812-14817.

Ho H. H. and Jong S. C. (1986). A comparison between *Phytophthora cryptogea* and phytophthora-drechsleri. *Mycotaxon*, 27: 289-319.

Ho H. H. and Jong S. C. (1991). Species concepts of *Phytophthora cryptogea* and *Phytophthora drechsleri*. *Mycotaxon*, 40: 35-39.

Irwin J. A. G. and Mackie J. M. (2000). Sexual reproduction by *Phytophthora macrochlamydospora*: a Group VI species. *Australian Systematic Botany*, 13(6): 817-821.

Kang S., Mansfield M. A., Park B., Geiser D. M., Ivors K. L., Coffey M. D., Gruenwald N. J., Martin F. N., Levesque C. A. and Blair J. E. (2010). The Promise and pitfalls of sequence-based identification of plant-pathogenic fungi and oomycetes. *Phytopathology*, 100(8): 732-737.

Klisiewicz J. M. (1977). Identity and relative virulence of some heterothallic *phytophthora* species associated with root and stem rot of safflower. *Phytopathology Phytopathology*, 77(10).

Kroon L. P., Bakker F. T., van den Bosch G. B., Bonants P. J. and Flier W. G. (2004). Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal genetics and biology* 41(8): 766-782.

Kroon L. P., Brouwer H., de Cock A. W. and Govers F. (2012). The genus P*hytophthora anno* 2012. *Phytopathology*, 102(4): 348-364.

Kumar S., Tamura K. and Nei M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, 5(2): 150-163.

Lee S. B. and Taylor J. W. (1992). Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molecular Biology and Evolution* 9(4): 636-653.

Lievens B., Brouwer M., Vanachter A. C., Levesque C. A., Cammue B. P. and Thomma B. P. (2005). Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. *Environmental Microbiology*, 7(11): 1698-1710.

Marshall E. (2005). Taxonomy: Will DNA bar codes breathe life into classification? *Science*, 307(5712): 1037.
http://dx.doi.org/10.1126/science.307.5712.1037

Martin F. N., Abad Z. G., Balci Y. and Ivors K. (2012). Identification and detection of *Phytophthora*: Reviewing our progress, identifying our needs. *Plant Disease*, 96(8): 1080-1103.

Martin F. N. and Tooley P. W. (2003a). Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia*, 95(2): 269-284.

Martin F. N. and Tooley P. W. (2003b). Phylogenetic relationships of *Phytophthora ramorum*, *P. nemorosa*, and *P. pseudosyringae*, three species recovered from areas in California with sudden oak death. *Mycological Research*, 107(12): 1379-1391.

Meyer C. P. and Paulay G. (2005). DNA Barcoding: Error Rates Based on Comprehensive Sampling. *PLoS Biol*, 3(12): 422.

Mills S. D., Förster H. and Coffey M. D. (1991). Taxonomic structure of *Phytophthora cryptogea* and *P. drechsleri* based on isozyme and mitochondrial DNA analyses. *Mycological Research Mycological Research*, 95(1): 31-48.

Min X. J. and Hickey D. A. (2007). Assessing the effect of varying sequence length on DNA barcoding of fungi. *Molecular Ecology Notes*, 7(3): 365-373.

Mirabolfathy M., Cooke D. E. L., Duncan J. M., Williams N. A., Ershad D. and Alizadeh A. (2001). *Phytophthora pistaciae* sp. nov. and *P. melonis:* the principal causes of pistachio gummosis in Iran. *Mycological Research*, 105(10): 1166-1175.

Mircetich S. M. (1976). Phytophthora Root and Crown Rot of Cherry Trees. *Phytopathology*, 66(5).

Moritz C. and Cicero C. (2004). DNA barcoding: promise and pitfalls. *PLoS biology*, 2(10).

Mortimer A. M., Shaw D. S. and Sansome E. R. (1977). Genetical studies of secondary homothallism in *Phytophthora drechsleri*. *Archives of Microbiology*, 111(3): 255-259.

Mostowfizadeh-Ghalamfarsa R., Panabieres F., Banihashemi Z. and Cooke D. E. (2010). Phylogenetic relationship of *Phytophthora cryptogea* Pethybr. & Laff and P. drechsleri Tucker. *Fungal Biology*, 114(4): 325-339.

Newhook F. J., Waterhouse G. M. and Stamps D. J. (1978). Tabular key to the species of *Phytophthora* de Bary. *Mycological Papers*(143): 20 pp.-20 pp.

Notsu A., Kondo N., Fujita S., Murata K. and Naito S. (2003). New race of *Phytophthora vignae* f. sp. *adzukicola*, the causal agent of Phytophthora stem rot of the adzuki bean. *Journal of General Plant Pathology*, 69(1): 39-41.

Olson H. A., Carbone I. and Benson D. M. (2011). Phylogenetic history of *Phytophthora cryptogea* and *P. drechsleri* isolates from floriculture crops in North Carolina greenhouses. *Phytopathology*, 101(11): 1373-1384.

Oudemans P. and Coffey M. D. (1991a). Isozyme comparison within and among worldwide sources of 3 morphologically distinct species of *Phytophthora*. *Mycological Research*, 95: 19-30.

Oudemans P. and Coffey M. D. (1991b). A revised systematics of twelve papillate *Phytophthora* species based on isozyme analysis. *Mycological Research*, 95(9): 1025-1046.

Park J., Park B., Veeraraghavan N., Jung K., Lee Y.-H., Blair J. E., Geiser D. M., Isard S., Mansfield M. A., Nikolaeva E., Park S.-Y., Russo J., Kim S. H., Greene M., Ivors K. L., Balci Y., Peiman M., Erwin D. C., Coffey M. D., Rossman A., Farr D., Cline E., Gruenwald N. J., Luster D. G., Schrandt J., Martin F., Ribeiro O. K., Makalowska I. and Kang S. (2008). *Phytophthora* database: a forensic database supporting the identification and monitoring of Phytophthora. *Plant Disease*, 92(6): 966-972.

Pethybridge G. H. (1913). On the rotting of potato tubers by a new species of *Phytophthora* having a method of sexual reproduction hitherto undescribed. In: (eds). *Scientific Proceedings, Royal Dublin Society* 13.

Pethybridge G. H. and Lafferty H. A. (1919). A Disease of Tomato and Other Plants Caused by a New Species of *Phytophthora*. In: (eds). *Scientific Proceedings, Royal Dublin Society*. 15.

Ristaino J. B. (2012). A Lucid Key to the Corn on Species of *Phytophthora*. *Plant Disease*, 96(6): 897-903.

Ristaino J. B., Madritch M., Trout C. L. and Parra G. (1998). PCR Amplification of Ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. *Applied and Environmental Microbiology*, 64(3): 948-954.

Robideau G. P., De Cock A. W., Coffey M. D., Voglmayr H., Brouwer H., Bala K., Chitty D. W., Desaulniers N., Eggertson Q. A., Gachon C. M., Hu C. H., Kupper F. C., Rintoul T. L., Sarhan E., Verstappen E. C., Zhang Y., Bonants P. J., Ristaino J. B. and Levesque C. A. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Mol Ecol Resour*, 11(6): 1002-1011.

Rossiter M. W. (2009). Pioneering Women in Plant Pathology. *Agricultural History*, 83(3): 413-414.

Savolainen V., Cowan R. S., Vogler A. P., Roderick G. K. and Lane R. (2005). Towards writing the encyclopaedia of life: an introduction to DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360(1462): 1805-1811.

Seifert K. A. (2009). Progress towards DNA barcoding of fungi. *Mol Ecol Resour*, 9: 83-89.

Seifert K. A., Samson R. A., deWaard J. R., Houbraken J., Lévesque C. A., Moncalvo J.-M., Louis-Seize G. and Hebert P. D. N. (2007). Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *Proceedings of the National Academy of Sciences*, 104(10): 3901-3906.

Stamps D. J. (1990). Revised tabular key to the species of *Phytophthora*. *CAB International Mycological Institute*, Kew, Surrey.

Stovold G. E., Smith H. J. P. and Priest M. J. (1996). Occurrence of *Phytophthora macrochlamydospora* in New South Wales and its pathogenicity to some legume species. *Australasian Plant Pathology*, 25(2): 106-109.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. and Higgins D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, 25(24): 4876-4882.

Tucker C. M. (1931). Taxonomy of the genus *Phytophthora* de Bary. *Research Bulletin. Missouri Agricultural Experiment Station,* (153): 208 pp.-208 pp.

Villa N. O., Kageyama K., Asano T. and Suga H. (2006). Phylogenetic relationships of *Pythium* and *Phytophthora* species based on ITS rDNA, cytochrome oxidase II and beta-tubulin gene sequences. *Mycologia*, 98(3): 410-422.

Waterhouse G. M. (1963). Key to the species of *Phytophthora* de Bary. *Mycological Papers*, 92: 22 pp.

Waterhouse G. M. (1970). The genus *Phytophthora* de Bary; diagnoses (or descriptions) and figures from the original papers. *Commonwealth Mycological Institute, Kew, Eng*.

White T.J., Bruns T., Lee S., Taylor J., (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J.,White T.J. (eds). *PCR protocols: a guide to methods and applications*, pp 315-322. Academic Press, Inc., San Diego,California.

CHAPTER 6

6. QUANTITATIVE DETECTION OF *P. CRYPTOGEA*, *P. MEGASPERMA* AND *P. CITROPHTHORA*, USING MULTIPLEX REAL-TIME PCR ASSAY; BASED ON INTERGENIC SPACER REGION (IGS) SEQUENCES.

1. Abstract

Phytophthora is a genus in the Oomycota, responsible for some of the most serious and economically important plant diseases. The ITS sequences, the most used marker to identify *Phytophthora* species in many cases, are not sufficiently variable, making the design of primers to detect closely related taxa very difficult and impossible. IGS sequence analysis showed greater potential as a new epidemiological tool for diagnostic purposes. In this study, the complete sequence of IGS regions was obtained for *P. cryptogea*, *P. citrophthora* and *P. megasperma*. Species-specific primer pairs for *P. cryptogea*, and two other species (*P. megasperma* and *P. citrophthora*), were designed from the most variable fraction of IGS regions. *P. cryptogea* specific primer (Cry5F/Cry5R) amplified 79 bp short fragment, while the primers Cit3F/Cit3R and Mega10F/Mega10R amplified 144 and 121 bp fragments in *P. citrophthora* and *P. megasperma,* respectively. The above three sets of species specific primers pair were chosen to develop specific probes for the detection of the three *Phytophthora* species in Real-time PCR (*Taq*Man) assay. The preliminary obtained results showed that the designed specific species primers have high level of specificity.

Keywords: *P. cryptogea*, *P. citrophthora*, *P. megasperma*, IGS, *Taq*man Real-time PCR.

2. Introduction

Phytophthora is a major genus of soilborne plant pathogens, known to cause huge problems on fruits trees and considerable yield losses worldwide. It is responsible for the most serious and economically important plant diseases (Judelson and Blanco, 2005). Rapid and accurate identification and detection of *Phytophthora* species would improve diagnosis and prophylaxis, especially where several pathogens have the same host range but are not equally severe in the disease that they cause (Huang *et al*., 2010). Diagnosis of *Phytophthora* species can be performed using traditional mycological methods involving isolation from host material followed by morphological identification with microscopy in comparison with a published description of the organism (Hughes *et al.*, 2006). However, this relies on key taxonomic features being present and skilled mycologists to differentiate target species from the potential range of other *Phytophthora* spp.; if these taxonomic features are absent, overlooked, or misidentified, this may lead to an incorrect diagnosis (Hughes *et al*., 2006). Additionally, successful isolation may be dependent on factors such as the time of year (Waterhouse, 1963; Erwin and Ribeiro, 1996). Classical methods usually require 1-2 weeks to process samples which is inadequate for timely disease-management (Hughes *et al*., 2006).

PCR technologies open opportunities for detecting and studying phytopathogenic organisms. Most of the detection methods of fungi are based on the ITS regions (Cooke *et al*., 2000; Martin and Tooley, 2003). Hong *et al.* (1999) developed PCR primers derived from the ITS region of the nuclear encoded ribosomal RNA genes (rDNA) for the detection of *P. cryptogea - P. drechsleri* complex group in plants using conventional PCR assay. Ribosomal DNA provide attractive targets to design specific primers since they are highly stable, can be amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White *et al*.,1990). However in some cases the ITS sequences are not sufficiently variable, making the design of primers for identifying and detecting closely related taxa very difficult or impossible.

The IGS region of rDNA was chosen as a focus of the current study based in part on previous observations that the IGS sequence analysis is very useful in differentiating the species. Sugita *et al.* (2002) examined the IGS sequences of all members of the genus

Trichosporon and concluded that IGS sequence analysis was superior to ITS sequence analysis in differentiating phylogenetically closely related species.

Real-time PCR assays especially those based on *Taq*Man, are becoming increasingly preferred to gel-based PCR (Schaad and Frederick, 2002), because they are quicker, generally offer increased specificity and sensitivity and are less likely to post-PCR contamination (Gachon *et al.*, 2004).

*Taq*Man assays amplify DNA in the same way as conventional PCR but exploit a dual-labeled fluorescent probe (designed to hybridize to the DNA sequence between the forward and reverse primers) and the 5′–3′exonuclease activity of *Taq* polymerase to produce a fluorescent signal (Hughes *et al.*, 2006). As amplification occurs, the probe is cleaved in a sequence specific resulting in an increase in fluorescence proportional to the amount of product amplified. The number of cycles it takes for a sample to produce fluorescence exceeding a threshold value is referred to as the cycle threshold (Ct) value and can be used to compare amplification between samples (Hughes *et al.*, 2006).

*Taq*Man assays have recently shown promise in the detection of many of plant pathogenic fungi (Hughes *et al.*, 2006). Because the *Taq*Man amplicon is generally between 60 and 70 bp, the reaction is also more efficient than a standard PCR in which target PCR products are required to be at least 200 bp in length to permit detection by electrophoretic separation techniques. This technique eliminates the need for post-PCR processing steps such as gel electrophoresis and ethidium bromide staining of target DNA (Weller *et al.*, 2000).

The goal of this research activity is to set up a molecular approach that can provide rapid and accurate detection of *Phytophthora* spp. at an adequate level of sensitivity which allows growers to make timely disease management decisions. This assay will also be used and optimized for detection of the pathogen in plants and soils and finally, a quantitative capability of the assay will be undertaken using real-time PCR systems based on a species specific quantitative detection of *Phytophthora* spp. This assay could be an effective research tool for studying the ecology of *Phytophthora* spp. and evaluating management strategies for diseases caused by this pathogen. The timely and accurate detection of pathogens is a critical aid in the study of the epidemiology and biology of

plant diseases. In fact, the availability of a sensitive and reliable assay is essential when trying to achieve early detection of the pathogen.

The initial steps were carried out by Yaseen *et al*. (unpublished data), and Ippolito *et al*. (2004). In their studies, the complete IGS sequence of the rDNA of *P. cryptogea, P. citrophthora,* and *P. megasperma* consequently was obtained. They developed primer pairs targeting the IGS region.

In this study, the IGS sequences were used to design species-specific *Taq*Man primers and probes. The specificity of those primers, and the potential use in routine multiplex quantitative assay were also assessed.

3. Material and methods

Fungal isolates and cultures conditions

Isolates of *Phytophthora cryptogea* were obtained from mango orchards located in Southern Italy, while isolates of *P. citrophthora* and *P. megasperma* were obtained from culture collection of IAM-Bari and Department of Plant Protection and Applied Microbiology (DPPMA), University of Bari, Italy (Table 6.1). All fungal purified cultures were maintained on potato dextrose agar (PDA) slants at 20°C.

Growing mycelium

To extract total genomic DNA, *Phytophthora* isolates were grown in Petri dishes containing Malt Extract Agar (MEA) covered with sterile cellophane sheets to facilitate collection of the mycelium. Inoculation of each fungal isolate was carried out using a 7 mm mycelial plug taken from the edge of actively growing colony on PDA.

DNA extraction from fungal mycelium

DNA was extracted using the method of Hoffman and Winston (1987) to extract the total genomic DNA, 5 to 10 mg of mycelia were collected with a spatula after 5-6 days of inoculation at 24°C, suspended in 400 µl of breaking buffer and extracted with 400 µl of phenol/chloroform/ isoamilic alcohol (25:24:1) in the presence of 25 mg of acid washed class beads (425-600 µm diame*t*er) and two sterile 5 mm stainless stile ball bearing. This mixture was vortexed at 3000 rpm for 10 min, and centrifuged for 15 min for 13,000×g. The aqueous phase was mixed with an equal volume of chloroform/ isoamilic alcohol (24:1), vortexed at 3000 rpm for 2 min, centrifuged for 5 min at 13,000×g and precipitated with two volumes of 100% cold (-20°C) ethanol. The precipitated DNA was washed with 70% cold (-20°C) ethanol, dissolved in 50 µl of TE buffer (10mM Tris-HCl, 0,1 Mm EDTA, pH 7.6), quantified using a spectrophotometer and diluted to 50 ng µl-1.

Table 6.1 *Phytophthora* species used for PCR amplification of ribosomal Intergenic Spacer Region IGS regions

***Phytophthora* species**	**Group** [a]	**Isolate Code**	**Origin**	**Host**	**Source**
P. cryptogea	VI	P1	Italy	Mango	Catania univ.
P. cryptogea	VI	P2	Italy	Mango	Catania univ.
P. cryptogea	VI	P3	Italy	Mango	Catania univ.
P. cryptogea	VI	P4	Italy	Mango	Catania univ.
P. cryptogea	VI	P5	Italy	Mango	Catania univ.
P. cryptogea	VI	P6	Italy	Mango	Catania univ.
P. cryptogea	VI	P7	Italy	Mango	Catania univ.
P. cryptogea	VI	P8	Italy	Mango	Catania univ.
P. cryptogea	VI	P9	Italy	Mango	Catania univ.
P. cryptogea	VI	P10	Italy	Mango	Catania univ.
P. cryptogea	VI	P11	Italy	Mango	Catania univ.
P. cryptogea	VI	P12	Italy	Mango	Catania univ.
P. cryptogea	VI	P13	Italy	Mango	Catania univ.
P. cryptogea	VI	P14	Italy	Mango	Catania univ.
P. cryptogea	VI	Ph116	France	*Gerbera jamesonii*	Frank Panabier
P. cryptogea	VI	Ph29	Italy	Tomato	DPPMA
P. megasperma	V	Ph101	Germany	Asparagus	Katrin Kaminski
P. megasperma	V	Ph105	Germany	*Barassica napus*	Katrin Kaminski
P. megasperma	V	Ph208	Germany	Carrot	Katrin Kaminski
P. megasperma	V	Ph209	Italy	Carrot	DPPMA
P. megasperma	V	Ph210	Corsica, France	Carrot	Frank Panabier
P. megasperma	V	Ph211	Corsica, France	Carrot	Frank Panabier
P. megasperma	V	Ph212	Corsica, France	Tillie	Frank Panabier

[a] = Waterhouse group of *Phytophthora* species (Waterhouse, 1963).

Table 6.1 Continue

Species	Group [a]	Isolate Code	Origin	Host	Source
P. megasperma	V	Ph213	Corsica, France	-	Frank Panabier
P. megasperma	V	Ph214	Corsica, France	-	Frank Panabier
P. megasperma	V	Ph241	Auchencruive, Scotland	Raspberry	Duncan SCRI
P. megasperma	V	Ph237	Hereford, England	Raspberry	Duncan SCRI
P. megasperma	V	Ph234	Australia	Apple	Duncan SCRI
P. palmivora	II	Ph69	Italy	Olive	DPPMA
P. palmivora	II	Ph163	Egypt	citrus	IAM-Bari
P. citricola	III	Ph111	Egypt	citrus	IAM-Bari
P. cactorum	I	Ph118	Italy	Pear	Frank Panabier
P. nicotianae	II	Ph77	Taranto, Italy	Citrus	DPPMA
P. nicotianae	II	Ph78	Taranto, Italy	Citrus	DPPMA
P. citrophthora	II	Ph34	Scotland	Olive	Duncan SCRI
P. citrophthora	II	Ph68	Catania, Italy	Clementine	DPPMA
P. citrophthora	II	Ph71	Tartous, Syria	Citrus	DPPMA
P. citrophthora	II	Ph67	Corsica, France	Clementine	Frank Panabier
P. citrophthora	II	Ph152	Egypt	Citrus	IAM-Bari
P. citrophthora	II	Ph156	Egypt	Citrus	IAM-Bari
P. hibernalis	IV	Ph164	Italy	Citrus	IAM-Bari

PCR Amplification of Ribosomal Intergenic Spacer Region IGS regions of *P. cryptogea*, *P. citrophthora* and *P. megasperma*

The conserved regions in the IGS flanking regions (28S and 18S genes) from different *Phytophthora* species were aligned and compared to design the possible universal primers. Several primers pairs (Table 6.2), were designed at the terminal part of the 28S subunit, and at initial part of 18S subunit by Yaseen *et al*. (unpublished data).

Table 6.2 Universal primers designed and used for the amplification of IGS region within the rDNA Gene of *Phytophthora* sp.

Forward primers (5′ - 3′)	**Reverse primers (5′ - 3′)**
IG10F (AGACCGTCGTGAGACAGGTT)	IG13R (GGAACAATTGGAGGGCAAGTCTGG)
IG11F (CGTGAGCTGGGTTTAGACCGTCGT)	IG12R (GATTGGGTAATTTGCGCGCCTGCT)
IG9F (CCACAGGATAACTGGCTTG)	IG1R (GCATGGTTAATCTTTGAGACA)
IG7F (ATAATTGGTTTTTGCGGCTG)	IG3R (ATAATGAGCCATTCGCAGTTTC)

PCR amplification conditions

Primers were assayed in PCR in different combination. The PCR reaction was performed in a programmable thermal cycler (Bio-Rad), starting with 1 min denaturation at 95°C, followed by 35 cycles at 95°C for 30 s, annealing at 72°C for 5 min, and extension at 72°C for 10 min. PCR reactions were performed in a total volume of 25 µl containing 50 ng of genomic DNA, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 1.25 mM from each dNTP, 1.25 mM $MgCl_2$, 1 unit of *Taq* polymerase and 0.3 µM of each primer. Amplicons were analyzed by electrophoresis in 2% agarose gels in TAE buffer and

visualized by staining with ethidium bromide (2µg/ml) and detected by UV fluorescence, 1-kb DNA ladder (Promega) was used as a molecular size marker. Negative controls were included for each set of experiments to test for the presence of contaminants in the reagents and reaction mixtures (Ippolito *et al.*, 2002).

Cloning, Bacterial transformation and PCR amplification of the recombinant DNA and Sequencing

PCR products were separated in 0.8 % agarose gel (1X TAE buffer) and visualized by staining with ethidium bromide (2µg/ml) and detected by UV fluorescence. Staining , bands were extracted from gels eluted and cloned using a PCR®–XL-TOPO® Kit (Fig.21) according to the manufacturer's procedures (Invitrogen Life Technologies) instruction. The fragment with plasmid was electroporated in *Escherichia coli* isolate Top10 by Bio-Rad Gene Pulser™ apparatus with Electroporation conditions (voltage: 1.8 kV, capacitance: 25 µF and resistance: 400 ohms). Recombinant colonies were grown overnight at 37°C in LB agar medium supplemented with 50 ng ml^{-1} kanamycin, a selected clones were grown overnight in 3 ml LB Liquid with 50 ng ml^{-1} kanamycin. The presence of cloned inserts was verified by agarose gel after restriction enzyme digestion by Ecor1 (Promega), Plasmids were purified from *E. coli* cultures using buffers, restriction enzyme, and electrophoresis techniques according to standard protocols (Sambrook *et al.*, 1989). The target DNA fragment was sequenced in MWG s.p.a. starting by the commercial T7-M13 primers, for subsequent sequences, primers were designed from the obtained sequences step by step to get the total sequence (Ippolito *et al.*, 2004).

Data analysis

Sequence homologues were analysed in the non-redundant DNA database of the National Center for Biotechnology Information (NCBI) using BLAST 2.0 software (Altschul *et al.*, 1997). Multiple sequence alignments were performed with Clustal W program (Thompson *et al.*, 1997).

Design of species-specific primers for PCR amplification and specificity

Different primers were designed for detection of *Phytophthora* spp. by aligning the sequences obtained from IGS1 and IGS2 of *P. cryptogea*, *P. citrophthora* and *P. megasperma*), and identifying the conserved and variable regions. On the basis of divergent regions, three pairs of primers were designed for *P. cryptogea* (Cry1, Cry5 and Cry6), three for *P. citrophthora* (Cit1, Cit3 and Cit6) and four primers pair for *P. megasperma* (Mega1, Mega5, Mega7 and Mega10) as it listed in Table 6.3.

Conventional PCR was used to evaluate their specificity and absence of cross reactivity, by using other *Phytophthora* species. PCR amplifications were carried out in a total volume of 25 µl, containing 1 µl of DNA (50 ng), 19.1 of nuclease free water, 2.5 µl 10X buffer, 0.65 µl of $MgCl_2$ (50 mM), 1 µl of dNTP (1mM of each dNTP), 0.5 µl of forward primer, 0.5 µl of reverse primer, 0.25 µl of Euro*Taq* DNA polymerase (5 units/µl). A negative control was included in all experiments. Amplification conditions were set up for each species (Table 6.3). PCR products were analysed in 1% agarose gel (1X TAE buffer), and visualized by staining with ethidium bromide.

All primers were assayed for their specific detection of each species. Primers showing miss matching was excluded after different tentative of modification on annealing temperatures or $MgCl_2$ concentrations. Only one pair of primer for each species was selected to perform Real-time PCR (*Taq*Man) assay.

Table 6.3 Specific primers designed in IGS regions of *P. cryptogea, P. citrophthora* and *P. megasperma* and amplification conditions**.**

Phytophthora species	Primer	Orination	Sequence (5′ to 3′)	Tm (°C)	GC (%)	Size (bp)	Amplification conditions
P. cryptogea	**Cry1**	Forward	ACAACCGCTTAGACAACAC	57.7	47.3	134bp	95°C 5 min 35 cycle of 95°C 30 sec 56°C 30 sec 72°C 30 Sec 72°C 10 min
		Reverse	GCTAAAAATCGAAAGACGCC	62.5	45		
	Cry5	Forward	AGCGAACTGCTTTAGCAC	58.3	50	74bp	
		Reverse	ACATTTTTCGCCAGCATCC	64.9	47.3		
	Cry6	Forward	AATGCCCATTTCTCGACTTC	62.9	45	107bp	
		Reverse	ACATTTTTCGCCAGCATCC	64.9	47.3		
P. citrophthora	**Cit1**	Forward	GAAAACGGCTTAGACAACAAC	60.2	42.8	128bp	95°C 5 min 35 cycles of 95°C 30 sec 58°C 30 sec 72°C 30 sec 72°C 10 min
		Reverse	CCTACTCCAAATCGGCTCAC	63.5	55		
	Cit3	Forward	ACACGCTAAAAATAGCAGCC	60.9	45	144bp	
		Reverse	GCACAAACTTAGAAACCCTCAC	61.5	45.4		
	Cit6	Forward	ACACGCTAAAAATAGCAGCC	60.9	45	148bp	
		Reverse	CCCAGCACAAACTTAGAAACC	62.4	47.6		

Table 6.3 continue

Phytophthora species	Primer	Orination	Sequence (5′ to 3′)	Tm (°C)	GC (%)	Size (bp)	Amplification conditions
P. megasperma	**Mega1**	Forward	GCGTTTAGGAAAAGGCACAG	63.5	50	89bp	95°C 5 min 35 cycle of 95°C 30 sec 54°C 30 sec 72°C 30 Sec 72°C 10 min
		Reverse	AGAGGTGAAATCGACCCAG	61.7	52.6		
	Mega5	Forward	ACCACTATTTCTACCTGACACC	58.5	45.4	149bp	
		Reverse	CCACATAAAGTACCCATCC	60.6	47.6		
	Mega7	Forward	CGTTTAGGAAAAGGCACAGAAG	63.7	45.4	88bp	
		Reverse	AGAGGTGAAATCGACCCAG	61.7	52.6		

Development Real-Time Polymerase Chain Reaction (*Taq*Man) assay

The best species-specific primer pairs were chosen to develop specific probes for the detection of the three *Phytophthora* species in the Real-time PCR (*Taq*Man) assay. These primers and probes were constructed with the use of CLC Genomics workshop 3. Accordingly, Three different fluorophores FAM, HEX AND CY5 (Table 6.4), linked at the 5′ terminus of the probes, were designed in order to distinguish singularly and simultaneously the three species reactions to the sequences designed during the Real-time and Multiplex-Real-time PCR.

PCR amplification of (*Taq*Man) Real-time PCR

The PCR reactions were performed in 96-wells plate, for each sample reaction in the real-time PCR assay, an aliquot of 50 ng of genomic DNA was included in each 20 µL PCR reaction (2× reaction buffer [qPCR MasterMix, Biorad]; 0.5 µm of each PCR specific primers Forward and Reverse and 0.2 µm *Taq*Man probe. Each sample was examined in two technical replicates.

Amplifications consisted of an initial denaturing step at 94°C for 4 min followed by 40 cycles, each consisting of 20 sec. of denaturation at 94°C; 30 sec. of annealing extension at ranging from 55°C to 58°C. The threshold cycle (Ct) corresponded to the cycle at which the fluorescent signal exceeded the background. Ct values were estimated at a threshold value of 0.5 in all cases. Two negative water controls were included with each PCR run. Positive results were confirmed by resolving the product on 1.5% agarose gel.

Sensitivity of the Real-time RT-PCR

In order to compare the sensitivity of the newly developed *Taq*Man Real-time with the conventional PCR assay, several serial dilutions of DNA templates extracted from *Phytophthora* species pure cultures were made. The initial quantity of DNA used was 50ng. All amplification reactions were conducted in 20 µL as a final volume.

Table 6.4 Specific primers and fluorescent probe sequences used to develop species-specific assays for *Phytophthora cryptogea, P. citrophthora* and *P. megasperma*.

Target species	Primers Sequence (5 to 3′)	Probes Sequence (5′ to 3′)	Amplified fragment	fluorophore
P.	**CRY5F**	**6FAM**	**(74bp)**	**FAM**
cryptogea	AGCGAACTGCTTTAGCAC	CCTAGTGTTTAGGAAGCCCCAGGA		
	CRY5F	**BHQ1**		
	ACATTTTTCGCCAGCATCC			
P.	**CIT3F**	**HEX**	**(144bp)**	**HEX**
citrophthora	ACACGCTAAAAATAGCAGC	AATAGCGGTTCGGGACGGTTACATGAAG		
	CIT3R	BHQ1		
	GCACAAACTTAGAAACCCTCA			
P.	**MEGA10F**	**CY5**	**(121bp)**	**CY5**
megasperma	CTAGTGAAACCATTTGCATTCC	CACGGAATAGCGCAACAAAAAAGTAGC AGCA		
	MEGA10R	**BHQ2**		
	GTTAAGCAGCCTCAAGCTC			

4. Results

Design of species-specific primers for PCR amplification and specificity

The most variable fractions in IGS regions of *P. cryptogea*, *P. citrophthora,* and *P. megasperma* were selected to design species-specific primers (Fig. 6.1). The amplification conditions were optimized for each primer pairs. PCR products obtained from all designed primer pairs showed the expected sizes according to their priming sizes on the sequence. On the basis of divergent regions, three couples of primers were designed for *P. cryptogea* (Cry1, Cry5 and Cry6), three for *P. citrophthora* (Cit1, Cit3 and Cit6) and four primers pair for *P. megasperma* (Mega1, Mega5, Mega7 and Mega 10).

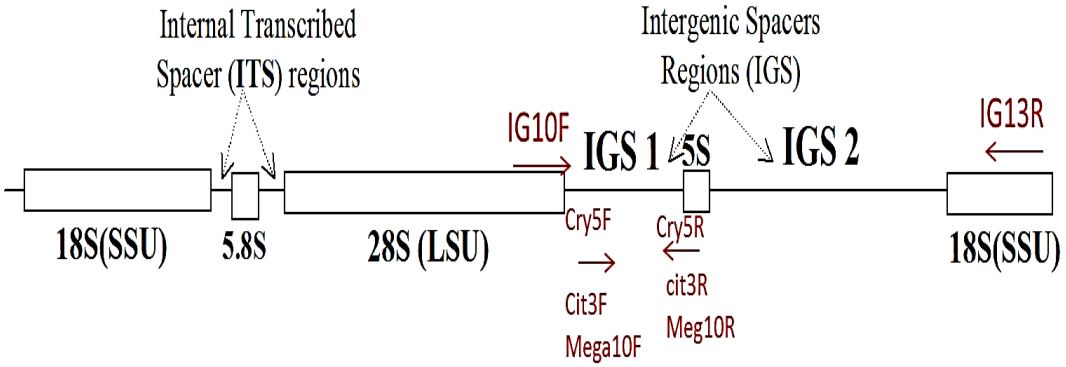

Figure 6.1 Diagrammatic representation of the organization of ribosomal DNA repeat unit and localization of specific primers pairs in IGS regions of *P. cryptogea*, *P. citrophthora* and *P. megasperma.*

***P. cryptogea* specific primers**

PCR products obtained from all designed primer pairs for *P. cryptogea* showed the expected sizes according to their priming sizes on the sequence. Primer pair of Cry5F/Cry5R was found to give a clear band producing a specific amplicon 79bp for *P. cryptogea* isolates, and no signal was obtained from the negative control (Fig. 6.2).

***P. citrophthora* specific primers**

The primer pair Cit3F/Cit3R, specific for *P. citrophthora*, produced a fragment as the expected size (144bp) amplicon. No amplification was obtained with the negative DNA control (Fig. 6.3).

***P. megasperma* specific primers**

Nine isolates *P. megasperma* were amplified using the primer pair Mega10F/Mega10R, specific for *P. megasperma*. A fragment of approximately 121 bp was amplified (Fig. 6.4. lane 1- 9). No amplification was achieved with the negative DNA control (lane 10).

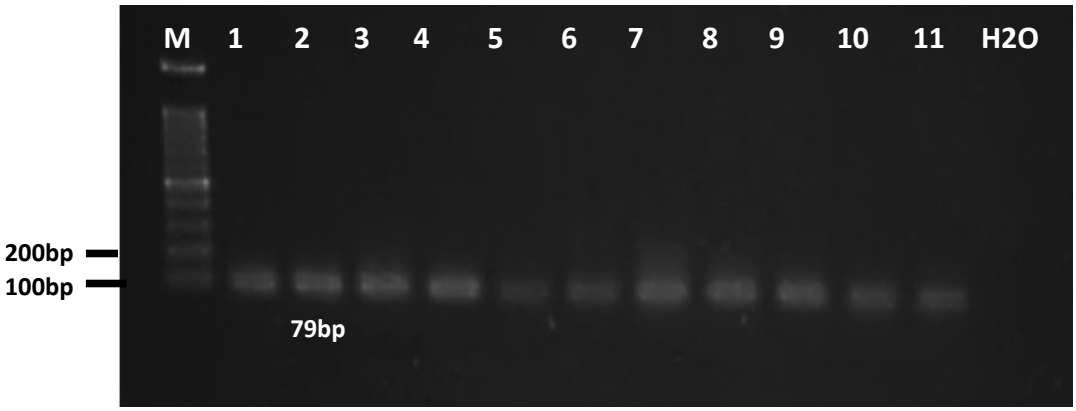

Figure 6.2 Agarose gel electrophoresis of amplification products obtained with the primers CryF5- CryR5 amplifying a fragment of 79 from different genomic DNA of *P. cryptogea* isolates. Lane 1 to 11 contain amplified product from pure cultures of *P. cryptogea* isolates obtained from mango (P1to P11), Lane H_2O contain negative DNA control and Lane M contain 100-bp DNA ladder.

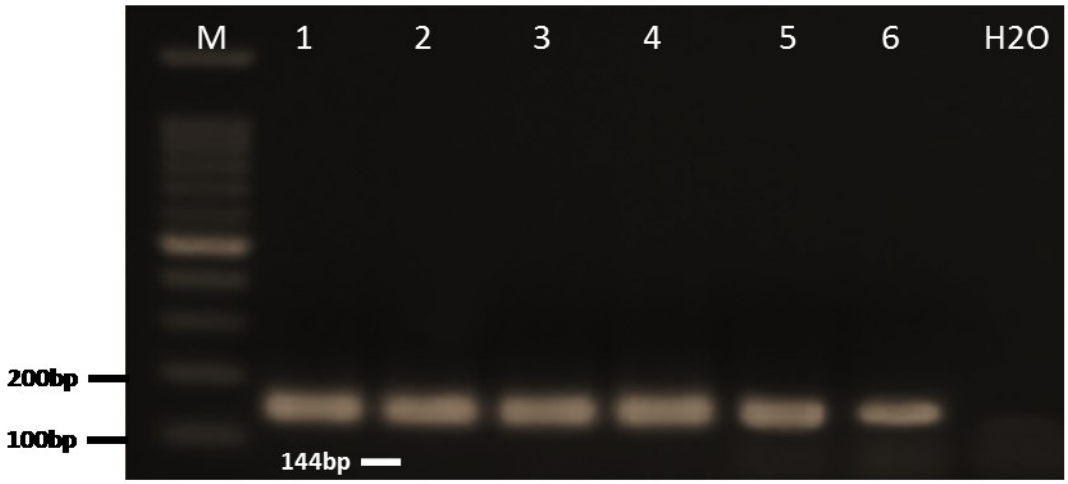

Figure 6.3 Agarose gel electrophoresis of amplification products obtained with the primers Cit3F- Cit3R amplifying a fragment of 144 from different genomic DNA of *Phytophthora citrophthora* isolates. Lane 1 contain amplified product from a pure culture of *P. citrophthora* isolate Ph34, lane 2, (*P. citrophthora* isolate Ph62), Lane 3, (*P. citrophthora* isolate Ph153), Lane 4 *(P. citrophthora* isolate Ph71), Lane 5 (*P. citrophthora* isolate Ph156), Lane 6 (*P. citrophthora* isolate Ph 156), Lane 7 contain negative DNA control and Lane M contain 100-bp DNA ladder.

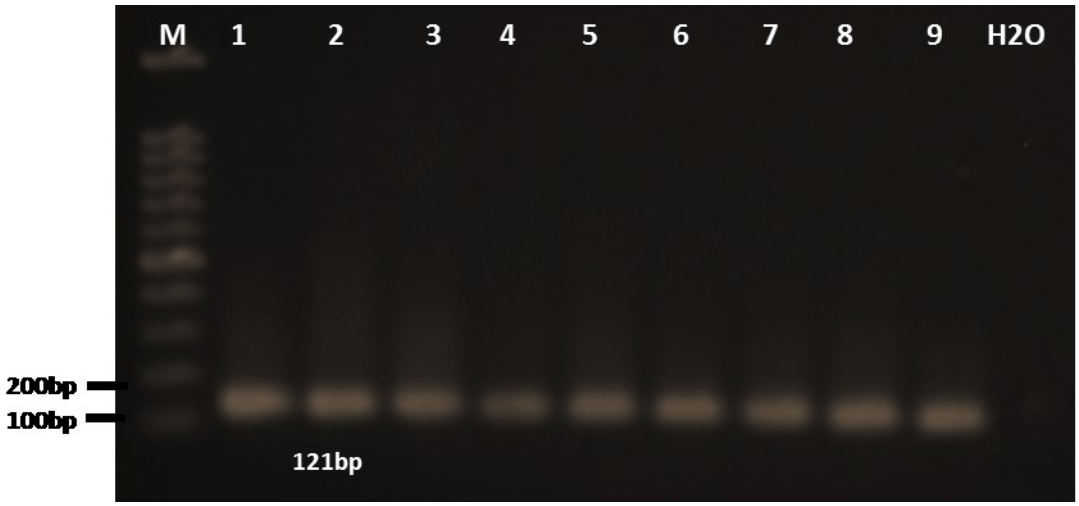

Figure 6.4 Agarose gel electrophoresis of amplification products obtained with the primers Mega10F- Mega10R amplifying a fragment of 121 from different genomic DNA of *Phytophthora megaperma* isolates. Lane 1 contain amplified product from a pure culture of *P. megasperma* isolate Ph209, Lan2, (*P. megasperma* isolate Ph210) Lane 4 *(P. megasperma* isolate Ph239), Lane 5 (*P. megasperma* isolate Ph241), Lane 6 (*P. megasperma* isolate Ph238), Lane 7 (*P. megasperma* isolate Ph208), Lane 8 (*P. megasperma* isolate Ph101), Lane 9 (*P. megasperma* isolate Ph105), Lane 10 contain negative DNA control and Lane M contain 100-bp DNA ladder.

Primers specificity

The specificity of primer pairs Cry5F/Cry5R, Cit3F/Cit3R and Mega10F/ Mega10R, were evaluated using different isolates of *Phytophthora* species (Table 6.1). The PCR products obtained were of the expected sizes producing 79, 144 and 121bp amplicons from all tested isolates of *P. cryptogea*, *P. citrophthora* and *P. megasperma* respectively, whereas no amplifications were obtained from the other tested *Phytophthora* species (Data not shown).

Design and assessment of primers and probes specificity

Specificity of all primers and probes was preliminarily assessed by means of Basic Local Alignment Search Tool (BLAST) analyses to explore all of the available DNA sequence data in international databases.

All primers and probes showed the desired level of specificity. Using amplification mixtures containing all specific primers and probes a particular increase in fluorescence at the expected wavelength was obtained for all the isolates of each of the three target pathogens. Fluorescence remained below the threshold values for the water controls (negative) and the non-target *Phytophthora* species.

The specificity of primers and the probe specific to *P. cryptogea* was evaluated using a number of *Phytophthora* species in PCR reactions. A high level of *P. cryptogea* specificity was observed using the primers Cry5F - Cry5R and the Cry5-FAM probe when tested against other species of *Phytophthora* at a concentration of 50 ng of DNA with an annealing temperature of 57°C. Only *P. cryptogea* showed a Ct value of less than 30 cycles with other species exhibiting no detection after 30 cycles (Fig. 6.5 -1). Primer and the FAM probe worked successfully at 55°C, but at 59°C, amplification became inconsistent (data not shown).

Primer Cit3F Cit3R and Cit3-Hex probe specifically detected all isolates of *P. citrophthora* when tested at an annealing temperature of 57°C, and did not amplify any of the other *Phytophthora* species (Fig. 6.5 -2), when tested at concentration of 50 ng of DNA, including 4 isolates of *P. cryptogea* and *P. megasperma*.

P. megasperma specific primers (Mega10F-Mega10R), together with their *Taq*Man labeled probe with Cy5, confirmed their specificity (Fig. 6.5 -3) giving high fluorescence with DNA of obtained from *P. megasperma*. No amplification was achieved with any of the other species.

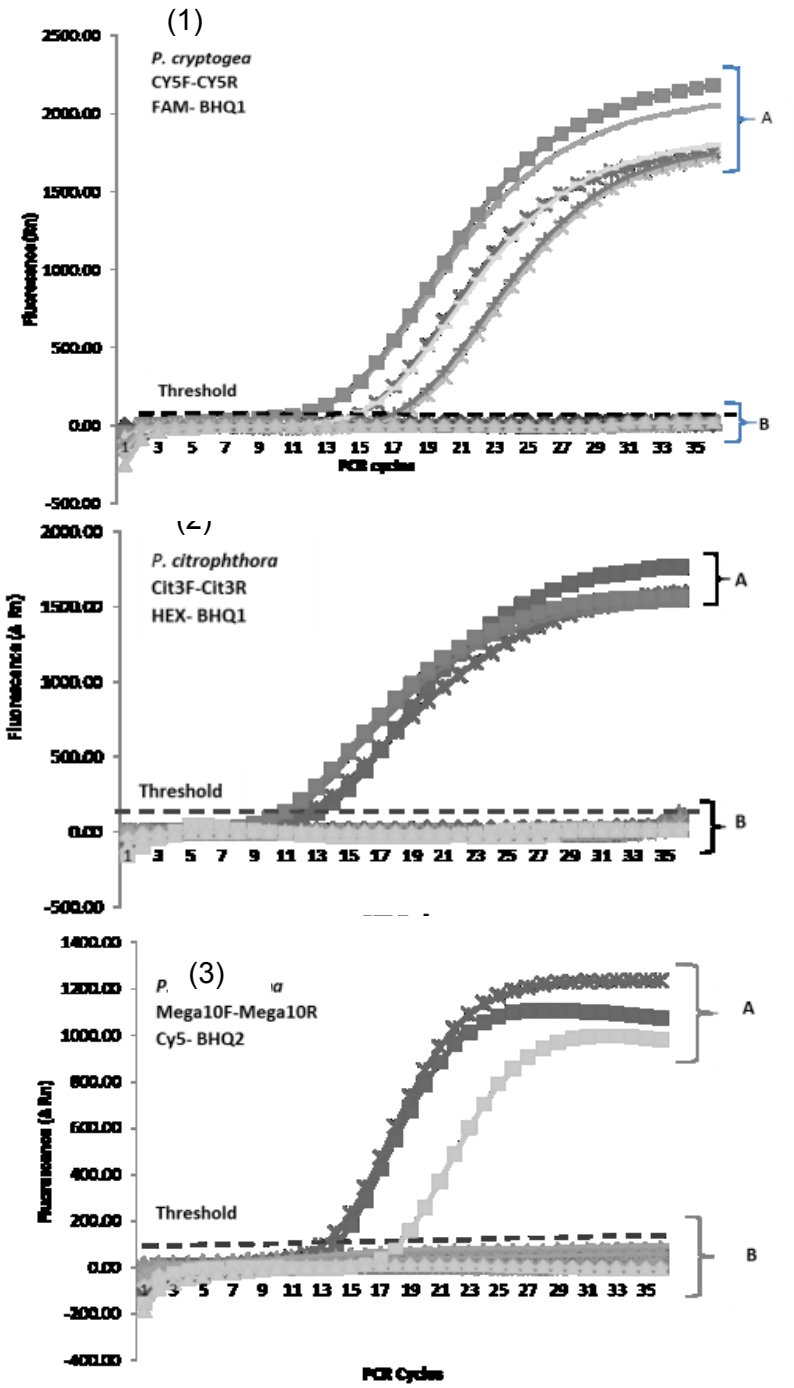

Figure 6.5 Real-time amplification profiles of test conducted to assess specificity of *Phytophthora* IGS probe in *Taq*Man Real-Time PCR using DNA from *P. cryptogea* (1); *P. citrophthora* (2) and *P. megasperma* (3) isolates. A = target *Phytophthora* species; B = other *Phytophthora* species and Negative control.

5. Discussion

The development of the PCR technique for amplification of DNA has revolutionized molecular diagnostics and enabled highly specific pathogen detection from small amounts of plant tissue. With properly designed amplification primers, highly specific diagnostic assays can be developed using conventional PCR techniques. One disadvantage however, is the need for running samples on an agarose gel to visualize the amplified band diagnostic of the pathogen in question, which can increase the time required for sample analysis, does not allow for quantification of target DNA, and is an impediment to high throughput sample processing. Greater sensitivity and rapid sample processing can be obtained with real-time PCR, where a dye included in the amplification mixture fluoresces in proportion to the amount of DNA template that has been amplified. This fluorescence is quantified by the thermal cycler during the amplification. The ability to use 96 well plates also enables analysis of high samples (Martin *et al*., 2012).

Many molecular detection methods are now available for a number of *Phytophthora* species which are known to cause diseases in many hosts (Schena *et al*., 2006). These diagnostic assays were focused on the detection of particular species and therefore are not suitable to assess what *Phytophthora* species might be present in mixed forest and natural ecosystem samples. Furthermore most of the above detection methods are based on the Internal Transcribed Spacer (ITS) regions. The nuclear-encoded ribosomal RNA genes (rDNA) provide attractive targets to design specific primers since they are highly stable, can be amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White *et al*., 1990). However in some cases the ITS sequences are not sufficiently variable, making the design of primers to identify and detect closely related taxa very difficult or impossible (Schena *et al*., 2006).

Important *Phytophthora* pathogens such as *P. nemorosa, P. ilicis*, *P. psychrophila* and *P. pseudosyringae* have very similar ITS regions sequences and the design of effective and robust specific primer sets is very challenging (Martin and Tooley, 2003). Similarly *P. alni*, *P. cambivora*, *P. fragariae*, and *P. europaea* are phylogenetically closely related and challenging to distinguish by ITS sequences (Martin *et al*., 2012).

The use of the IGS as alternative to the ITS regions to differentiate closely related species has been suggested by several other authors (Ippolito *et al*., 2004; Cooke *et al*., 2007). A short fragment of the IGS2 region, amplified and sequenced from 28 different *Phytophthora* species, was appropriate as target to differentiate some closely related species (Schena *et al*., 2006). Although IGS regions show great potential, they are still little used as target sequences for the development of species-specific molecular tools, probably due to the lack of effective universal primers, their remarkable length (2000-5000 bp), and the presence of sub repeating elements causing difficulties in their amplifying and sequencing (Grummt, 1999).

The whole IGS regions of *P. cryptogea*, *P. citrophthora* and *P. megasperma*, were successfully amplified, sequenced, and characterized Yaseen *et al*. (unpublished data). The developed method allowed designing strictly species-specific primers (Cry5F-Cry5R, Cit3F-Cit3R, and Mega10F-Mega10R); together with their specifics tested probes were able to discriminate the three species of *Phytophthora*. In particular, the specificity of the three primer pairs was tested with other species of *Phytophthora* from different geographical areas.

6. Conclusion

PCR-based *Taq*Man assays have recently shown promise in the detection of a variety of plant pathogenic fungi. In this study, the possibility to set up a molecular approach to provide an accurate detection of *P. cryptogea* was investigated. Species-specific primer pairs for *P. cryptogea* and two other species (*P. megasperma* and *P. citrophthora*), were designed from the most variable fraction of IGS regions, and then were chosen to develop specific probes for the detection of the three *Phytophthora* species in the Real-time PCR (*Taq*Man) assay. The work is still in progress however, the obtained results showed that the designed primers have high level of specificity. They may be particularly useful for routine detection and identification of Phytophthora infection. Testing the sensitivity of multiplex PCR protocol and detection of *Phytophthora* species in soil samples and natural infections plants are still under investigations.

7. References

Altschul S. F., Madden T. L., Schaeffer A. A. and Zhang J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research.* 25(17): 3389.

Cooke D. E. L., Drenth A., Duncan J. M., Wagels G. and Brasier C. M. (2000). A Molecular Phylogeny of *Phytophthora* and Related Oomycetes. *Fungal Genetics and Biology*, 30(1): 17-32.

Cooke D. E. L., Schena L. and Cacciola S. O. (2007). Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. *Journal of Plant Pathology*, 89(1): 13-28.

Erwin D. C. and Ribeiro O. K. (1996). *Phytophthora diseases worldwide.*APS Press.

Gachon C., Mingam A. and Charrier B. (2004). Real-time PCR: what relevance to plant studies? *Journal of Experimental Botany*, 55(402): 1445-1454.

Grummt I. (1999). Regulation of mammalian ribosomal gene transcription by RNA polymerase I. *Progress in nucleic acid research and molecular biology*, 62: 109-154.

Hoffman C. S. and Winston F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, 57(2-3): 267-272.

Hong S., Jee H., Lee S. and Go S. (1999). Restriction fragment length polymorphism of PCR amplified ribosomal DNA among Korean isolates of *Phytophthora*. *Plant Pathology Journal*, 15(4): 228-235.

Huang J., Wu J., Li C., Xiao C. and Wang G. (2010). Detection of *Phytophthora nicotianae* in Soil with Real-time Quantitative PCR. *Journal of Phytopathology*, 158(1): 15-21.

Hughes K. J. D., Tomlinson J. A., Griffin R. L., Boonham N., Inman A. J. and Lane C. R. (2006). Development of a One-Step Real-Time Polymerase Chain Reaction Assay for Diagnosis of *Phytophthora ramorum*. *Phytopathology*, 96(9): 975-981.

Ippolito A., Schena L. and Nigro F. (2002). Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils by nested PCR. *European Journal of Plant Pathology*, 108(9): 855-868.

Ippolito A., Schena L., Nigro F., Ligorio V. S. and Yaseen T. (2004). Real-time detection of *Phytophthora nicotianae* and *P.citrophthora* in citrus roots and soil. *European Journal of Plant Pathology*, 110 (8): 833-843.

Judelson H. S. and Blanco F. A. (2005). The spores of Phytophthora: weapons of the plant destroyer. *Nature Reviews Microbiology*, 3(1): 47-58.

Martin F. N., Abad Z. G., Balci Y. and Ivors K. (2012). Identification and Detection of *Phytophthora*: Reviewing our progress, identifying our needs. *Plant Disease*, 96(8): 1080-1103.

Martin F. N. and Tooley P. W. (2003). Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia*, 95(2): 269-284.

Sambrook J., Fritsch E. F. and Maniatis T. (1989). *Molecular cloning : a laboratory manual*.Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Schaad N. W. and Frederick R. D. (2002). Real-time PCR and its application for rapid plant disease diagnostics. *Canadian journal of plant pathology*, 24: 250-258.

Schena L., Hughes K. J. and Cooke D. E. (2006). Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. *Molecular Plant Pathology*, 7(5): 365-379.

Sugita T., Nakajima M., Ikeda R., Matsushima T. and Shinoda T. (2002). Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species. *Journal of Clinical Microbiology*, 40(5): 1826-1830.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. and Higgins D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25(24): 4876-4882.

Waterhouse G. M. (1963). Key to the species of Phytophthora de Bary. *Mycological Papers*, 92: 22 pp.

Weller S. A., Elphinstone J. G., Smith N. C., Boonham N. and Stead D. E. (2000). Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied and Environmental Microbiology*, 66(7): 2853-2858.

White T.J., Bruns T., Lee S., Taylor J., (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J.,White T.J. (eds). *PCR protocols: a guide to methods and applications*, pp 315-322. Academic Press, Inc., San Diego,California.

Yaseen T., Nigro F., Schena L., and Ippolito A. (unpublished data). The Complete Sequence of intergenic spacer region (IGS) of nuclear rDNA and specific PCR based detection of *Phytophthora megasperm*. Submitted to *European Journal of Plant Pathology.*