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STUDIES ON THE FUNGAL DISEASES OF MANGO WITH PARTICULAR REFERENCE TO DISEASES CAUSED BY *BOTRYOSPHAERIA* SPECIES

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DEDICATIONS AND ACKNOWLEDGMENTS

I dedicate this thesis to the following:

My wife thanks for your eternal love, patience and support during the accomplishment of this thesis.
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ABSTRACT

Mango (*Mangifera indica*) is a promising tropical fruit crop in Italy. In the near future, it is expected that commercial and backyard plantings of mango trees will increase being a gainful crop. Cultivation of mango is concentrated in four provinces (Catania, Palermo, Messina and Ragusa) located in the southern Italy (Sicily). Since the introduction of mango cultivation into Italy in 1990, little information is available regarding its problematical diseases. Thus, the present work represents the first attempt to assess the presence and the diversity of fungal species associated with different symptoms patterns observed on mango trees during a survey conducted in 2009-2010. Several fungi have been isolated and tentatively identified up to genus level. The most encountered isolated fungal taxa were *Alternaria* spp., *Botryosphaeria* spp., *Colletotrichum* spp. and *Pestalotiopsis* spp. All these fungi except *Botryosphaeria* spp. were identified based on conidia and culture characters and tested for pathogenicity on mango detached leaves cv. Kensington Pride. The isolated fungi showed to be pathogenic with different degrees in virulence. Other fungi were occasionally isolated with low frequency such as *Botrytis* sp., *Macrophoma* spp., *Phoma* spp., and *Stemphylium* spp.

The present work focused mostly on the Botryosphaeriaceae associated with dieback disease in Italy and in Egypt. In the present thesis, incorporation of morphology and DNA sequence data of ITS and TEF-1α revealed diverse array of *Botryosphaeria* spp. associated with mango dieback: *Neofusicoccum parvum, N. australe*, were the dominant species while, *N. vitifusiforme* and other two novel species *Neofusicoccum* sp. 3 and *Neofusicoccum* sp. 18 were less frequently isolated and associated with dieback disease in Italy. The two novel species of *Neofusicoccum* were phylogenetically and morphologically distinct from the other species. On the other hand, *Lasiodiplodia theobromae* and *L. pseudotheobromae* were the most prevalent species associated with mango dieback in Egypt. The two new described species *Lasiodiplodia* sp. 8 and *Lasiodiplodia* sp. 10 were less frequently isolated from mango in Egypt. All tested isolates of *Neofusicoccum* spp. and *Lasiodiplodia* spp. except the new species were pathogenic to apple fruits as well as mango cv. Kensington Pride seedlings. Additional studies, including, extensive survey and sampling in different geographical are required to understand the ecology of the new species and to determine their role to cause diseases to mango. Furthermore, critical intervention by developing control strategies such as chemical, biological and agricultural methods must be taken in concern.

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Abbreviations	
AL	*Alternaria*
BC	Before century
bp	Bas pair
BPP	Bayesian posterior probability
BS	Bootstrap support
C°	Celsius degree
CBS	Centraalbureau voor Schimmelcultures
cm	Centimeter
Co	*Colletotrichum*
cv	Cultivar
DMSO	Dimethyl sulfoxide
DNA	Deoxyribuneoclic acid
dNTPs	Deoxyribonucleotide triphosphate
EtOH	Ethanol
gL^{-1}	Gram per liter
HKY+G and GTR+G	Models used to calculate the command block by MrModel test program
HWB	Hot water brushing
ITS	Internal transcript spacer region
Kbp	Kilo base pair
KCL	Potassium chloride
L/W	Length/width ratio
LSD	Least significant difference
MCMC	Markov Chain Monte Carlo
Mg	Magnesium
mg	Milligram (s)
ml	Milliliter (s)
mM	Millimolar
mm	Millimeter (s)
µl	Microliter(s)
µg	Microgram (s)
µm	Micro millimeter (s)
MP	Maximum parsimony
NCBI	National center for biotechnology information
OA	Oat meal agar
PCA	Potato carrot agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
Pest	*Pestalotiopsis*

ppm	Part per million
RAPD	Random amplified polymorphic DNA
RH	Relative humidity
rRNA	Ribosomal RNA
SD	Standard deviation
sec	Second (s)
TEF-1α	Translation elongation factor region
UV	Ultra violet light
WA	Water agar

CHAPTER .1

MANGO AND THE IMPACT OF FUNGAL DISEASES WITH SPECIFIC REFERENCE TO DISEASES CAUSED BY *BOTRYOSPHAERIA* SPP.

INTRODUCTION

Definition. Mango (*Mangifera indica* L. family Anacardiaceae) is a tropical and subtropical fruit tree, belongs to the genus *Mangifera* that contains several species of tropical fruiting trees (Bally, 2006). The most-cultivated *Mangifera* species, *M. indica* (mango) has its origin in India and Myanmar (Litz, 2009). The genus *Mangifera* contains other species such as, *M. caesia*, *M. foetida*, *M. kemang*, *M. altissima*, *M. pajang*, *M. odorata*, *M. minor,* and *M. similis* that bear edible fruit however, most of the fruit trees that commonly known as mangos belong to the species *Mangifera indica* (Litz, 2009). The other edible *Mangifera* species generally have lower quality fruit and commonly referred to as wild mangos (Bally, 2006). Mango fruit in high demand and fetches a good price all over the world (Prusky, 1996), and has become a major fruit crop particularly in Asia where it has been considered the 'king of fruit' (Bally, 2006).

History. Based on the ancient accounts of travelers and written historical records, it was believed for several years that mango must have originated in India and spread outwards from there to the South-east Asia and subsequently to the New World and Africa (Litz, 2009). In addition, De Candolle (1884) demonstrated that mango cultivation appeared to have begun at least 4000 years ago and it is impossible to doubt that it is a native of South Asia with the greatest number of species found in Borneo, Java, Sumatra, and the Malay Peninsula. In India, mango is a very important cultural and religious symbol. Among Hindus, mango leaves are ritually used for floral decorations in religious ceremonies and marriages. Most of the mango cultivars such as Alphonso, Dashehari, Langra, Rani Pasand and other cultivars have originated in India and have maintained under cultivation for several years by vegetative propagation (Litz, 2009). In the early period of its cultivation, mango trees produced a low value fruit (small with thin flesh), which after selections for many hundreds years have resulted in great variation in shape and size (Litz, 2009). Ripe mango is well-known for its very sweet and unique taste, and its high water content makes it refreshing to eat (Anonymous, 2009).

Geographical distribution. Since mango seeds are recalcitrant and cannot survive for more than few days or weeks, mango germplasm in the early days must have been transported as ripe fruit, seedling, or later on as grafted plants.

The wide spread of mangoes and their cultivation probably did not occur until the beginning of the European voyages of discovery and colonization in the 15th and 16th centuries (Litz, 2009). It was believed that mango has introduced from its origin (Asia) into Africa (Mozambique and Angola) through Persia and Arabia in the 10th century by Arab traders. Later, mango introduced into Brazil from Africa by the Portuguese, then mango introduced across the Pacific Ocean into their new world colonies through the pacific trading ports of Mexico and Panama (Litz, 2009). Today mango is cultivated in tropical and warmer subtropical climates in Asia, Africa, Australia, and the Americas with more than 1,000 known cultivars, it's been said to be the most commonly eaten fresh fruit worldwide. The earliest recorded introduction into Hawai was prior to 1825; however, the most introductions to the Pacific islands have occurred over the past 100 years. *M. gedebe*, *M. minor* and *M. mucronulata* are found in the Solomon Islands and *M. minor* in Micronesia, but these do not fruit or the fruit is inedible. Mango trees are able to adapt to various environmental conditions that are normally not conductive to growth of other fruit trees (Wolstenholme and Whiley, 1995).

Mango production. Currently mango ranked in the fifth total production after four major important fruit crops worldwide, after Musa (bananas and plantains) 105,815,354 tons, Citrus 105,440,168 tons, grapes 65,584,233 tons and apples 59,444,377 tons (FAOSTAT, 2006). Mango production has been increased from 16,903,407 ton in 1990 to 28,221,510 ton in 2005. Production of mango from India represent, 51% of the total production of the world's mango, however, India's production had declined to 38% by 2005, but is still the leader in mango production by 10,800,000 ton, and then China with (3,450,000 ton), Thailand (1,800,000 ton), Pakistan (1,673,900 ton), Mexico (1,600,000 ton), Indonesia (1,478,204 ton), Brazil (1,000,000 ton) and Philippines (950,000 ton). Among these, Mexico, India and Brazil are the major exporting countries with 212,505 ton, 156,222 ton and 11,181 ton respectively (Litz, 2009). The top ten mango producing countries sharing in the current global production (in %) and the area under cultivation (in hectares) are shown in (Fig. 1A, B) (Anonymous, 2011).

Diseases influence. During all the stages of its life cycle, mango tree affected by over 140 pathogens causing different levels of damage from the seedlings in the nursery to the fruit in storage (Prakash, 2004). The majority of plant organs, such as the trunk, branches, twigs, leaves, panicles are affected. These pathogens can express different kinds of diseases symptoms (Haggag, 2010). The changes in the environmental conditions however, often can play an important role either in reducing the tree's ability to elicit an active defense response to pathogen infection and invasion (Schoeneweiss, 1984), or in reducing the productivity of the tree in association with the diseases and disorder problems (Prakash, 2004). Mango trees, therefore, can face different levels of stress in different environments, which together with varying levels of pathogen's inoculum pressure, can trigger symptoms development and result in disease expression (Finnemore, 2000).

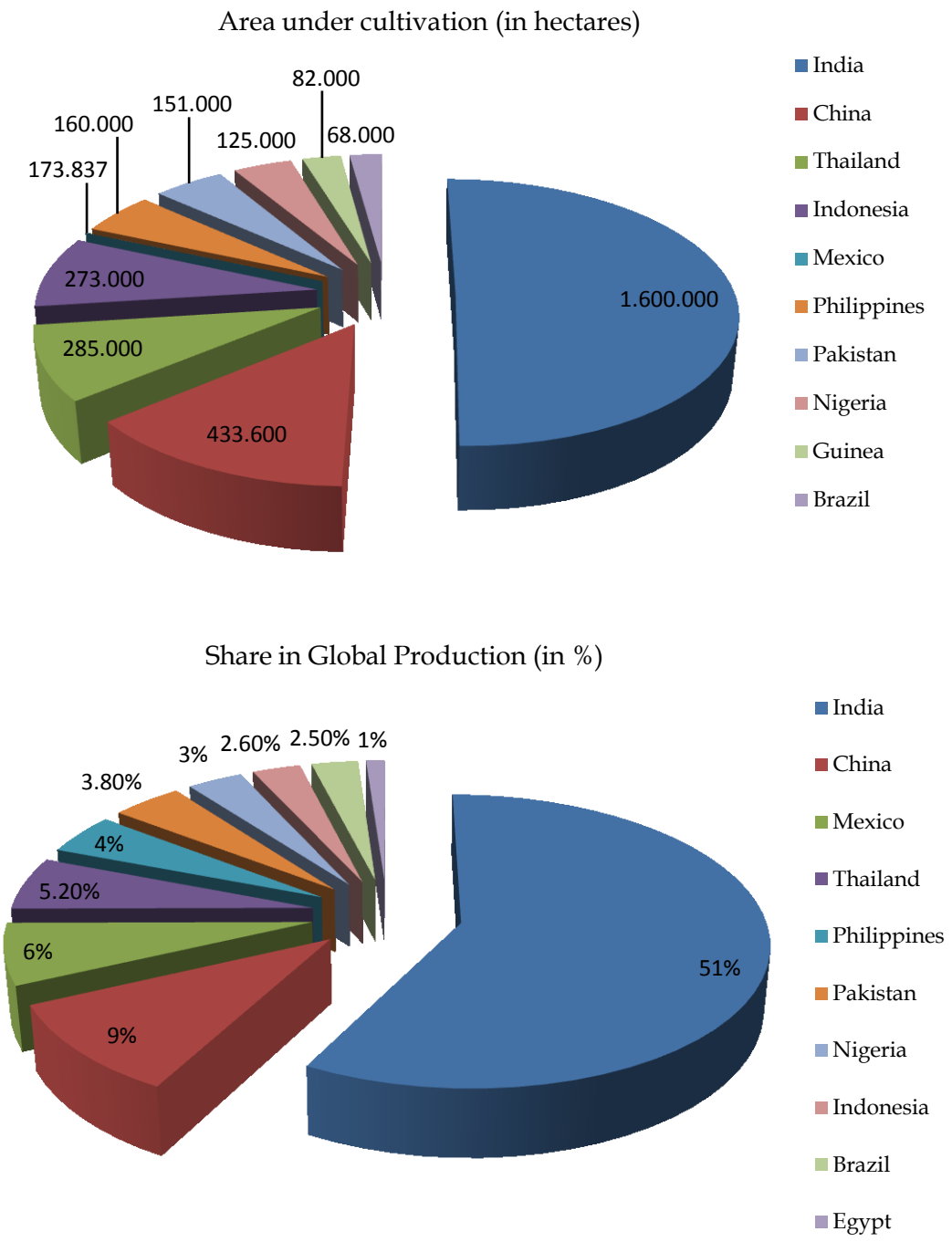


Figure 1 Mango cultivated area in hectares (A), and sharing percentage in the global production (B) of top 10 countries in the world (Anonymous, 2011).

HISTORICAL REVIEW

MANGO DISEASES AND THEIR IMPACT

Almost every part of mango trees; stem, branch, twig, root, leaf, petiole, flower and fruit are affected by various diseases (Ploetz, 2003). These diseases manifest themselves as several kinds of symptoms such as rots, dieback, mildew, necrosis, scab, blotch, stem bleeding, wilt, spots, canker, sooty mould, malformation, unknown etiology and disorders. Some of these diseases have become limiting factor in mango cultivation (Prakash, 2004).

The diseases of mango crop could play an important role in causing yield losses along with other problems in the agronomic management practices. The occurrence and severity of these diseases were attributed mostly to the environmental conditions (Ploetz, 2004). For example, in the regions where the humidity is high anthracnose is the most threaten along with other diseases, like bacterial black spot, blossom blight and stem-end rot. Whereas, in the arid areas other diseases such as, Alternaria rot (Black spot), malformation, powdery mildew and the decline disease are the most important (Ploetz, 2004). Therefore, mango tree is a host of several pathogens that affect every part of the tree, seedlings and grafted plants in the nursery (Ploetz, 2003). Several fungi have been reported to cause diseases on mango such as; *Colletotricum gloeosporioides* (Penz.) Penz & Sacc that causes mango anthracnose disease, *Alternaria alternata* (Fr: Fr.) Keissl and *A. tenuissima* (Kunze: Fr.) Wilshire that cause Alternaria leaf spot disease. Furthermore, *Botrydiplodia theobromae* Pat, *Phoma mangiferae* and *Dothiorella* spp. were reported as responsible for stem-end rot and dieback (Dodd et al, 1997; Okigbo and Osuinde, 2003). Other macroscopic fungi have frequently isolated from mango trees associated with sudden death disease including; *Ceratocystis fimbriata*, *A. alternata*, *Cladosporium* spp., *C. gloeosporioides*, *D. dominicana*, *Fusarium* spp., *Lasiodiplodia theobromae*, *Penicillium* spp., *Pestalotiopsis* spp. and *Phomopsis* spp. (Ploetz, 2004). Therefore, mango as many of fruit trees can be under stress by different threats either biotic or abiotic, which can influence on the healthy status of the tree (Prakash, 2004). In this study, we focused only on the biotic factors, particularly the fungal diseases.

THE MAJOR DISEASES OF MANGO

BOTRYOSPHAERIA DISEASES ON MANGO

Among a wide diversity of the destructive pathogens that interfere with mango trees are *Botryosphaeria* spp., which considered as the most threaten of these fungi (Johnson, 1992). *Botryosphaeria* spp. has been known as saprophytic and successful opportunistic endophytic fungi but occasionally cause extensive disease symptoms on a variety of woody hosts especially when their hosts are under stress or exposing to unfavorable environmental conditions (Schoeneweiss, 1984: Slippers and Wingfiled, 2007). Recently, several studies have demonstrated that such species of Botryosphaeria infect a wide range of hosts (Burgess et al, 2006; Pavlic et al, 2008; Slippers et al, 2004). They can attack different parts of mango tree and fruit, resulting in pre and post-harvest diseases. These opportunistic fungi colonize the twigs and the branches causing twig dieback and extensive cankers of the branches moving down causing canker of the trunk (Slippers and Wingfield, 2007). Infection of the fruit at early stage remains latent until fruit ripen, in that case the development of the pathogen in the fruit lead to a soft brown rot giving the typical symptoms of stem-end rot (Johnson, 1992; Lonsdale, 1993).

PRE-HARVEST DISEASES

TIP DIEBACK

A twig dieback or decline disease of mango is a complex phenomenon and considered as a serious problem in various mango-producing countries (Jacobs, 2002). Symptoms associated with mango decline are diverse and include: dieback of terminal shoots with or without defoliation, gummosis on branches and trunk, vascular discolouration, chlorosis and necrosis of leaves margins (Ploetz et al, 1996; Ploetz, 2004). From the most encountered *Botryosphaeria* anamorphs, that cause significant losses annually and considered, as an important and primary cause of mango twig dieback in Australia, is *D. dominicana* (Dravas, 1991; Johnson et al, 1991). In addition, Dravas (1993) demonstrated that *D. dominicana* was associated with branch dieback in South Africa and has reported in the United States as a pathogen of mango for the first time (Ploetz et al, 1996). Whereas, *D. mangiferae* found on mango trees worldwide especially in Australia and Thailand (Johnson et al, 1991). In addition, *D. aromatica* has reported from mango however,

it is known as a pathogen of avocado (Johnson, 1992). Furthermore, *L. theobromae* is a common and wide spread pathogen of decline symptoms on mango; it has reported to cause a serious dieback of mango in the Jaipur district of India (Verma and Singh, 1970), the Sonsonate area of El Salvador (Acuna and Waite, 1977) and in Egypt (Ragab et al, 1071). *L. theobromae* was also associated with a trunk canker disease of mango in Indonesia (Muller, 1940), and causes a gummosis and dieback of mango in Puerto Rico (Alvarez-García and López-García, 1971). Recently, *Neofusicoccum parvum* = (*D. dominicana*) has been reported as mango dieback pathogen in Peru (Javier-Alva et al, 2009) and in Brazil (de Olivera Costa et al, 2010).

The etiology of this disease remained confused for several years due to the different causal agents that have been associated with this disease. Ramos et al, (1991) have isolated fungi from mango trees that showed tip die back symptoms, such as *B. ribis* Gross & Duggar (anamorph: *Fusicoccum* sp. Corda) and less commonly a *Diplodia* sp. which caused tip die back after artificial inoculation. Furthermore, Smith and Scudder (1951) found that *Diplodia* sp. associated with dieback of mango but did not confirm the pathogenicity of the fungus experimentally. Whereas, in Florida *Diplodia theobromae* and *F. aesculi* were responsible for symptoms associated with mango decline on 'Keit' and 'Tommy Atkins' trees in association with other isolated fungi (Ploetz et al, 1996). The authors demonstrated that both *D. theobromae* and *F. aesculi* were most virulent and manifested significant necrosis, gummosis and vascular discolouration. Other anamorphs of *Botryosphaeria* species *Diplodia* or *Fusicoccum* and *B. dothidea* (anamorph: *F. aesculi*) have been reported also as a causal agents of fruit rot and decline of mango (Ploetz, 2004).

BLOSSOM BLIGHT

The whole tree parts can be infected with *Botryosphaeria* species. Whereas, the inflorescences are the most extensively colonized by *Botryosphaeria* species causing blossom blight especially during the rainy seasons (Darvas, 1991a). Only *F. aesculi* and *F. parvum* have reported as blossom blight agents in South Africa and they were associated with mango decline and stem-end rot in several different areas (Ploetz, 2004). The symptoms of blossom blight starts as a minute black spots resulting in wilting of the inflorescences, which later enlarge and

coalesce to cause shrinking and drying of the axes (Lonsdale, 1992; Lonsdale, 1993). The disease severity depends on environmental conditions in combination with any stress on the tree during inflorescence growth (Lonsdale, 1993).

POST-HARVEST DISEASES

STEM-END ROT

The marketability of mango fruit has been depended mainly on the fruit quality of which fruit must be free from diseases (stem-end rot and other diseases) and physiological disorders which all together lead to losses in the quantity (yield) and market value (quality) of mango fruit (Kapse et al, 2009). The losses of post-harvest diseases can be attributed to several reasons, including physiological changes, physical damage, chemical injury or residues and pathological effect (Swart, 1999). The most economically important post-harvest decay of mango in various countries is stem-end rot (Johnson 1992; Johnson and Sangchote, 1994). Stem-end rot has reported from all major mango-growing regions of the world (Jacobs, 2002). The 'stem-end rot' term has been used to describe lesions that develop at the pedicle end of the fruit after harvest and lead to complete fruit decay (Johnson et al, 1991). The lesions on the rotted fruit appear as water socked tissue start from the stem ends or infected areas on the fruit body that rapidly darken and coalesce to form irregular lesions. Later, on the surface of the lesions white mycelium of the pathogen may be seen protruding from the pedicle end of fruit. Sometimes, water drops can release from the necrotic area as well as the raised fruiting bodies of the pathogen can be observed on the surface (Darvas, 1991b; Johnson 1992; Lonsdale, 1993).

TAXONOMY OF *BOTRYOSPHAERIA* SPECIES ASSOCIATED WITH MANGO DISEASES

Kingdom: Fungi
Division: Ascomycota
Class: Dothideomycetes
Order: Botryosphaeriales
Family: Botryosphaeriaceae
Genus: *Botryosphaeria* (Crous et al, 2009)

Botryosphaeria parva Pennycook & Samuels, Mycotaxon 24: 455. 1985.

Anamorph: ***Fusicoccum parvum*** Crous, Slippers & A.J.L. Phillips, Studies in Mycology, 55: 248. 2006.

Synonym: *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, Studies in Mycology, 55: 248. 2006.

This anamorph (*F. parvum*) has been identified previously as *D. dominicana* and is one of the most common pathogens of mango causing fruit stem-end rot, dieback and blossom blight in Australia (Slippers et al, 2005). This anamorph isolated from mango in Australia was identical to those reported from the type *F. parvum* by Slippers et al, (2004). This specie, produce on PDA initially fluffy intense white aerial mycelium, later become dark olivaceaous grey to black, on the reverse side display olivaceaous to iron grey colour (Jacobs, 2002; Phillips et al, 2002), and can be differentiated from other *Botryosphaeria* fungi on mango by conidial characteristics. The conidia have typical characteristics: they are aseptate, hyaline, granular, broadly ellipsoid to fusoid with average 17-19 × 5-6 µm. The older discharged conidia sometimes become 1-2 septate and light brown with darker middle cell (Slippers et al, 2004). However, some isolates of both *F. parvum* and *F. luteum* occasionally produce micro-conidia; confusion can be exist between them, but *F. parvum* can be distinguished easily from *F. luteum* by the absence of yellow pigment colour in culture. Moreover, can be confused also with *F. ribis*, but the conidia of *F. parvum* frequently become brown and develop 1-2 septate as they mature and the middle cell is sometimes light to dark brown (Phillips, 2007).

Botryosphaeria ribis Grossenb. & Dugg., Tech. Bull. N.Y. Agric. Exp. St. 18: 128. 1911.

Anamorph: ***Fusicoccum ribis*** Slippers, Crous, M.J. Wingf., Mycologia, 96: 96. 2004.

Synonym: ***Neofusicoccum ribis*** (Grossenb. & Dugg.) Crous, Slippers & A.J.L Phillips, Studies in Mycology 55: 249. 2006.

N. ribis was isolated and described for the first time from its original host *Ribes* spp. in Florida (Grossenbacher and Dugger, 1911) and has reported for the first time as a causal agent of tip dieback of mango (Ramos et al, 1991). The fungus initially produce on PDA white cottony mycelium later turn grey to black with age and produce pigmented hyphae with swollen hyphal cells or chlamydospores. The dark mycelium is septate with segments of about 20 µm long, and sometimes has hyphal knots. Pycnidia form on sterilized poplar twigs on water agar, they are superficial globose, mostly solitary or in aggregates covered with pale olivaceous appendage-like hyphae (Phillips, 2007). Conidia are hyaline, aseptate and rarely septet with age, fusiform, have truncate to rounded base, with average size 20.8 × 5.5 µm. *N. ribis* and *N. parvum* are closely related species that belong to the Botryosphaeriaceae, Ascomycetes, Botryosphaeriales (Crous et al., 2006). Differentiation between *N. parvum* and *N. ribis* is difficult and unreliable and confusion can exist due to the overlap in the morphological characteristics of their teleomorphs and anamorphs (Pennycook and Samuels, 1985; Phillips, 2007). These difficulties were resolved in a study of Slippers et al, (2004) who relied on the combined multiple gene sequence data to delimite the anamorph *F. ribis* from *N. parvum*. Therefore, the ideal differentiation among the closely related species should base on the using of multiple gene region sequence (Slippers et al, 2004).

Botryosphaeria rhodina *Botryosphaeria rhodina* Berk. & M.A. Curtis) von Arx, Gen. Fungi Sporulating in Culture (Lehr.): 143. 1970.
=*Physalospora rhodina* Berk. & M.A. Curtis, Grevillea 17: 92. 1889.
Anamorphs: *Botryodiplodia theobromae* Pat. 1892. = *Lasiodiplodia tubericola* Ell. & Everh. 1896.
Synonym: *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl., Bull. Trimest. Soc. Mycol. Fr. 25: 57. 1909.

B. rhodina (anamorph *L. theobromae*) is a common and cosmopolitan endophytic pathogen that has a worldwide distribution and can be found on more than 500 tree species in tropical and subtropical regions (Burgess et al, 2006; Mohali et al, 2005). *L. theobromae* can be distinguished by producing very fluffy

white aerial mycelium that grows very fast to cover the entire medium surface within two days. The mycelium rapidly turns pale to dark olivaceaous on the front side and on the reverse side of Petri dishes; the colour becomes iron to very dark grey colour with age. Pycnidia simple or in aggregates appeared scattered and immersed in the mycelium mat, and covered with smooth hyphae (Jacobs, 2002). Conidia ooze from pycnidia as cirri, they are initially hyaline aseptate, with granular contents, ovoid to ellipsoid, thick-walled, and when mature becomes brown septate with one septum measuring 20-30 µm length × 10-15 µm width (Jacobs, 2002; Khanzada et al, 2004; Ploetz, 2004). The distinct criterion of this genus is the vertical striations on the mature conidia surface (Alves et al, 2008; Burgess et al, 2006; Punithalingam, 1976). Recently, several studies have revealed cryptic species in the *Lasiodiplodia* genus complex based on multiple gene sequences along with the morphological characters (Abdollahzaedh et al, 2010; Alves et al, 2008; Damm et al, 2007; Pavlic et al, 2004).

Botryosphaeria lutea A.J.L. Phillips, Sydowia, 54: 59. 2002.
Anamorph: *Fusicoccum luteum* Pennycook & Samuels, Mycotaxon, 24: 456. 1985.
Synonym: *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, Studies in Mycology, 55: 248. 2006.

F. luteum (teleomorph *B. lutea*) occurs on mango and seemed to be common in Australia (Johnson, 1992), and it was isolated from Kiwifruit in New Zeeland (Pennycook and Samuels, 1985). The main distinct feature used for separation of this species from other *Botryosphaeria* species like, *F. aesculi* and *N. parvum* is the production of yellow pigment colour on PDA, Oat Meal Agar (OA) and Potato Carrot Agar (PCA) media. This colour becomes intense after 3 days at 25°C and by 6-7 days could no longer seen in the media. The colonies by time turn grey to dark grey colour and on the reverse side display dark grey. On OA, the fungus produces unilocular pycnidia, partially immersed in the medium, globose, usually papillate and covered with olive green appendage-like hyphae. Conidia oozing from the ostioles between 5-7 days at 23°C, they are hyaline, thin-walled, aseptate fusiform to elliptical with a truncate or rounded base. Conidia size average 19.7 ± 1.8 × 5.6 ± 0.6 µm. Some isolates produce micro-conidia that are rod-shaped have either truncate or rounded end with size average 3-5 × 1-2 µm.

At germination, conidia develop one or two septa but remain hyaline (Phillips et al, 2002).

Botryosphaeria australis Slippers, Crous, & M.J. Wingf., Mycologia, 96: 1035. 2004.

Anamorph: *Fusicoccum australe,* Slippers, Crous, & M.J. Wingf, Mycologia, 96: 1035. 2004.

Synonym: *Neofusicoccum australe* (Slippers, Crous, & M.J. Wingf.) Crous, Slippers & A.J.L Phillips, Studies in Mycology, 55: 248. 2006.

N. australe was firstly isolated from *Acacia* spp. in Australia and described as new species by Slippers et al, (2004) based on the multiple gene sequences and morphological data. It produces yellow pigment colour in the media as also was reported for *N. luteum*. However, *N. asutrale* can be distinguished from *N. luteum* by its longer conidia. Moreover, *N. luteum* produces much lighter yellow pigment in culture than *N. australe* at 25ºC. Unlike *N. luteum*, no yellow colour was produced between 25-30ºC (Slippers et al, 2004). The discrimination among the closely related species based on single gene genealogies is insufficient, especially to distinguish cryptic species of *Botryosphaeria* (Slippers et al, 2005). For example, ITS sequence data alone was unable to give a distinct discrimination in some species complexes in previous studies like, *B. ribis/B. parva* (Slippers et al, 2004) and between *B. lutea/B. australis* (Deneman et al, 2003; Smith and Stanosz, 2001). Recently, phylogenetic inference study by Slippers et al, (2004) have demonstrated that *N. asutrale* is phylogenetically distinct from *N. luteum* based on the multiple gene (ITS, EF-1α and β-tubulin) sequences data.

Neofusicoccum mangiferae (Syd. & P. Syd.) Crous, Slippers & A.J.L. Phillips, Studies in Mycology, 55: 248. 2006.

Anamorph: *Fusicoccum mangiferae* (Syd. & P. Syd.) Johnson, Slippers & M.J. Wingf., Mycologia 97: 106. 2005.

Basionym: *Dothiorella mangiferae* Syd. & P. Syd., Ann. Mycol. 14:192. 1916.

≡ *Nattrassia mangiferae* (Syd. & P. Syd.) B. Sutton & Dyko, Mycol. Res. 93: 484. 1989.

Teleomorph: *Botryosphaeria* sp.

This species was isolated from mango in Australia and previously identified as *D. mangifera* (= *Nattrassia mangiferae*) by Johnson (1992). Sydow et al, (1916) described *D. mangifera* from mango and observed only the aseptate conidia. Sutton and Dyko (1989) have re-examined the type material and reported the presence of the 1-2 septate pigmented conidia. The confusion due to the misidentifications of Botryosphaeriaceae still exist and this confusion attributes mainly to overlaping of the morphological characters (e.g. conidial septation and colour) from nature as well as from culture (Johnson, 1992; Slippers et al, 2004; Slippers et al, 2005). Now these names has adopted to *F. mangiferae* a new epithet was proposed by Slippers et al, (2005) who re-examined these isolates and found that they are identical to those of *F. mangiferae*. Recently, Crous et al, (2006) reduced *Fusicoccum* to synonym *Neofusicoccum* based on the DNA sequence data of 28S rDNA. This species can be confused with *N. parvum* due to the obvious similarities of septation and pigmentation of the conidia when becomes septate with 1-2 septum. The most distinct features can discriminate *N. mangiferae* from *N. parvum* is the conidia of *N. mangiferae* are shorter in length average (~13-14 µm) and small length/width ratio (2-2.5). *N. mangiferae* produces vegetative, toruloid cells in culture as well as in nature and occasionally fluffy grey coloured aerial mycelium (Slippers et al, 2005).

Neofusicoccum vitifusiforme (Niekerk & Crous) Crous, Slippers & AJL Phillips, Studies in Mycology, 55: 249. 2006.

Synonym: *Fusicoccum vitifusiforme* Niekerk & Crous, Mycologia, 96: 793. 2004.

F. vitifusiforme was reported for the first time on grapevine (*Vitis vinifera* L.) in South Africa and described as new species on the basis of morphological and DNA sequence data (van Niekerk, et al, 2004). This species is closely related to *F. australe* and *F. luteum* since it has the fusiform shape of conidia. It can be discriminated easily from the later two species basing on the production of pigment colour in culture and conidia length. *F. vitifusiforme* does not produce pigment colour whereas *F. australe* and *F. luteum* produce pigment colour with a little difference in the degree of the colour (Slippers et al, 2004). The conidia of *F. vitifusiforme* are shorter (up to 22 µm in length) than those of *F. australe* (18-30 µm) and F. *luteum* (15-30 µm) (van Niekerk, et al, 2004). The fungus has been reported as a pathogen on olive fruit in Italy (Lazzizera et al, 2008), on grapevine in

Arkansas and Missouri (Urbez-Torres et al, 2011) and on *Prunus* spp. in South Africa (Damm et al, 2007).

EPIDEMIOLOGY

Studying and understanding the infection processes as well as the epidemiology of the pathogens can allow us to establish an effective control approaches (Johnson and Sangchote, 1994) and to uphold quarantine regulations (Slippers et al, 2005). The mode of *Botryosphaeria* entry into mango tissues is unknown well. Natural openings and wounds caused by pruning and insects considered the most methods of infection (Johnson, 1992; Mass and Uecker, 1984). *Botrysophaeria* spp. can occur endophytically in healthy plant tissue, plant debris and soil (Mass and Uecker, 1984). Invasion through natural openings lead to localized infections that appear as sunken to necrotic lesions and occasionally gum exudations release from the trunks and limbs (Jacobs, 2002). The pathogen is also able to invade the vascular system moving down the stem but with slow lateral movement (Ramos et al, 1991). Under stress conditions such as, mineral deficiency, hail, drought, freezing and other environmental factors, mango trees usually have no ability to resiste or tolerate the infection with *Botryosphaeria* spp. and the disease symptoms develop rapidly (Ramos et al, 1991; Schoeneweiss, 1984). During later stages of infection, fruiting structures of *Botryosphaeria* spp. are often produced on affected mango organs (Jacobs, 2002), and once the ostioles open, conidia are easily discharge and spread by raindrops splashing, wind, pruning tools and become in direct contact with the healthy host tissue (Mass and Uecker, 1984; Sutton, 1981).

CONTROL STRATEGIES

Once Botryosphaeriaceae have introduced into a new area, they are difficult to control (Swart and Wingfield, 1991). They are able to infect a wide range of plants. Chemical control of such fungi is extremely difficult on a large scale, whereas removal or treatment of diseased parts of trees is possible in intensively managed orchards (Flowers et al, 2001). This together with the orchard sanitation can help to reduce disease incidence by reducing the inoculum density of the pathogen (Brown-Rytlewski and McManus, 2000; Stanosz et al, 2005). Over time, new symptoms are likely to continue appearing from other endophytic

infections so the control is not absolute. Breeding for resistance might be possible, but resistance is likely easily to overcome by high gene flow and sexual reproduction. Therefore, prevention of the entry of such pathogenic species and genotypes is the best approach to control Botryosphaeriaceae pathogens. The development of control approach for the economically important pre- and post-harvest diseases caused by these fungi need a focus on the pathogen epidemiology. These fungi exist endophytically in mango tree and spread systemically through the vascular system expressing pre and post-harvest symptoms. Therefore, controlling of such pathogens could be achieved during two stages:

PRE-HARVEST

Quiescent infection of Botryosphaeriaceae fungi can be influenced by fungicides treatments; orchard sanitation, cultivar resistance, climate and tree age (Cooke et al, 1998; Johnson et al, 1992). Therefore, some pre-harvest control measures for example: using resistant or tolerant cultivars, reducing wounds as well as fungal inoculum density needed and aim in reducing such infections (Jacobs, 2002). Chemical fungicides such as Iprodione, Imazalil, Prochloraz, Manganese chloride were proven to have sufficient level of effectiveness against *Botryosphaeria* spp. (Johnson, 1992). In addition, Khanzada et al, (2005) have found that the three treatments with 15 days intervals with Carbendazim (2 gL^{-1}), Thiophanate-methyl (1.43 gL^{-1}) and Fosetyl aluminium (1.25 gL^{-1}) fungicides were effective in reducing the infection of mango trees with *L. theobromae* and the treated trees showed increasing in the vegetative growth after each treatment.

POST-HARVEST

The combination between physical (hot water) and chemical (fungicides) methods in controlling post-harvest diseases of mango is still widely used and proven to be effective against pre and post-harvest diseases of mango (Kapse et al, 2009; Swart et al, 2009a; Swart et al, 2009b). In a study conducted by Swart et al, (2009a) on evaluating the effect of Fludioxonil and Prochloraz on soft brown rot and stem-end rot of mango; heated solutions (50°C) of Fludioxonil at (450 and 300 ppm) and Prochloraz (405 ppm) gave the best control of stem end rot. In addition, Sangchote (1991) has found that treatment of mango fruit with 500 ppm of

Benomyl at 52°C for 5 minutes provided effective control of stem end rot caused by *B. theobromae*, and to ensure the high effectiveness, fruit must be treated immediately after harvest.

ANTHRACNOSE

Anthracnose is a serious fungal disease of flowers, fruit, and leaves, and considered from the devastating post-harvest diseases to mango in humid growing areas. The disease incidence can reach almost 100% in fruit produced under wet or very humid conditions (Arauz, 2000). Wet conditions during flowering promote anthracnose development after the fruit reaches approximately 4 cm in diameter; the fruit's natural defense mechanisms protect it from anthracnose by inducing the fungus into a quiescent period (Johnson, 1994). Mango anthracnose is caused by *Colletotrichum* spp. teleomoph, *Glomerella cingulata* (Stoneman) Spauld & H. Schrenk (Fitzell and Peak, 1984). This genus includes the most important two species (*C. acutatum* and *C. gloeosporioides*) that are considered as threatens for wide range of hosts (Martinez et al, 2009), and they can cause losses on mango up to 35% of the harvested fruit (Páez, 1995). *C. gloeosporioides* is responsible for many diseases, also referred to as "anthracnose," on many tropical fruits including banana, avocado, papaya, coffee, passion fruit and others (Nelson, 2008). Management of mango anthracnose can be achieved either in the field or after harvest by different means; I) cultivar resistance can be considered: however, the majority of mango commercial cultivars are susceptible to anthracnose pathogens, but some of them are less susceptible than others such as Keitt less than Kent while, Kensington Pride is considered as moderately resistant to the disease. II) Different fungicides can apply under field conditions such as, Mancozeb, Ferbam and Copper fungicides are well known and recommended for controlling the disease, but less effective than dithiocarbamate fungicides. III) Chemical and thermal combination has been widely proven to be effective together; Imazalil and Prochloraz in combination with hot water 50 to 55°C for 3-15 min have been recommended (Akem, 2006). This combination is still the most effective for post-harvest diseases of mango (Fivaz, 2009; Kapse et al, 2009; Swart et al, 2009a; Swart et al, 2009b).

ALTERNARIA BLACK SPOT

Among several post-harvest diseases Alternaria rot or black spot, is mostly significant in arid environmental conditions (Prakash, 2004). The disease caused by *A. alternata*, and several commercial mango verities are susceptible to this pathogen (Ploetz, 2004). It was reported that the pathogen infect fruit mainly thought lenticels before harvest, and the infection remain quiescent until fruit ripening to resume again during prolonged storage (Prusky et al, 2002). *A. alternata* is frequently isolated from affected mango fruits as well as leaves and panicles and reported to cause diseases to wide range of hosts such as, apple, tomato, blueberry, mango and others (Hu et al., 1995; Prusky et al., 1983). Although, this fungus is cosmopolitan and has been reported from numerous host plants, the disease on mango appears to be limited to arid environments, for example it is recognized as a cause of heavy losses in South Africa (Ploetz, 2004), and considered as greater pre and post-harvest problem than anthracnose in Israel (Prakash, 2004). It would seem that environmental conditions in these areas favor *A. alternata* over other pathogens. In Senegal Diedhiou et al, (2007) demonstrated that *Alternaria* spp. *B. theobromae*, *Dothiorella* sp., *Aspergillus niger* and other unidentified fungi were responsible for mango rotting during first harvesting. Moreover, Abdalla et al, (2003) found that the most encoutered fungi recovered from Egyptian mango fruits during storage were *B. theobromae*, *C. gloeosporioides*, *A. alternata* and *Botrytis cinerea*. Most of mango commercial cultivars are susceptible. Therefore, the effective control of such disease can be done before harvest by three applications of some fungicides (Maneb) after fruit set with 2 weeks (Ploetz, 2003). Whereas, after harvest the disease can be effectively controlled with combined hot water spray and fruit brushing (hot water brushing-HWB) treatment for 15–20 sec with 225 μg ml^{-1} Prochloraz with a high relative quiescent infection, while at low incidence of quiescent infection treatment with low efficacy fungicides like Chlorine might be sufficient (Prusky et al, 2002).

POWDERY MILDEW

Mango powdery mildew caused by *Oidium mangiferae* Berthet was reported for the first time in Brazil by (Berthet, 1914) and in Hawai (1983). It was present in India before 1874; but Wagle reported it in 1928. The fungus has minor importance when recorded earlier but now has become increasingly a critical

threat to mango cultivation in India (Prakash, 2004). The disease has reported also in other countries: Israel, Lebanon (Asia), New South Wales (Zaire), Queensland and New Caledonia (Australia), Congo, Egypt, Ethiopia, Kenya, Malawi, Mozambique, Mauritius, Reunion, Tanzania, Zambia, Zimbabwe, South Africa (Africa), USA (California and Florida), Mexico, Jamaica, Costa Rica, Guatemala (Central America), Brazil Venezuela, Colombia, Peru (South America) (Prakash et al, 1996).

The fungus is a sporadic but very severe disease on mango leaves, panicles, and young fruits (Ploetz, 2004). Mango powdery mildew is an easily recognizable problem; the symptoms are very apparent and are diagnostic. Most of plant parts can be affected, infected panicles may set few or no fruit and the infected small fruit may abort. Young leaves are more susceptible than older one, symptoms on leaves appeared as white powdery coating, (fungal structure) form on both sides of leaf blade, underside infection mostly restricted to the midrib, the affected parts become purplish and necrotic (Ploetz, 2004). Up to 90 percent of losses can occur due to its effect on fruit set and development. The most critical phase of the disease during flowering however, infection can occurs before flowering or fruit set. Therefore, its recommended that growers monitor the development of the inflorescences and to apply fungicides when panicles begin to change colour and continue every 3 weeks until panicle susceptibility decreased at the end of fruit set (Ploetz, 2003; Prakash, 2004). Removing out of the old inflorescences of the previous seasons could reduce the initial inoculum (Schoeman et al, 1995).

MALFORMATION

Malformation is one of the most devastating diseases to mango cultivations in many countries especially India and Egypt (Plotez, 2003). This name of the disease refers to the abnormal growth of inflorescences as well as vegetative buds that occurs on the affected trees, other names also used for description of the symptoms of the disease such as, 'bunchy top' and 'witches broom' (Ploetz, 2004). The disease can lead to high losses since the inflorescences do not set fruit (Ploetz, 2004; Youssef et al, 2007). The malformed panicles produce up to three times the normal number of flowers giving them the abnormal appearance. Moreover, the number of male flowers increase and the

hermaphroditic flowers that are produced are either sterile or abort even if it fertilized. The causal agent of this disease was uncertain. However, it has been confirmed for the first time that the causal agent is a fungus by Summanwar et al, (1966) and Varma et al, (1974), who have isolated it from mango in India as *Fusarium moniliforme* which known later as *F. subglutinans* causing the vegetative and floral malformation appearance. This fungus has been isolated also from mango malformation in Egypt (Ibrahim et al, 1975), Florida (Ploetz and Gregory, 1993), Israel (Freeman et al, 1999), Oman (Kvas et al, 2008) and South Africa (Manicom, 1989). *F. oxysporum* also considered as a causal agent of mango malformation since it has been isolated from all plant parts, therefore, the disease can be caused by several species of *Fusarium* (Prakash, 2004).

The epidemiology of the disease is unclear; the spread of the disease relies on the movement of plant materials inside infected nursery, while within trees still unknown (Ploetz, 2003). Since the presence of mango bud mite, *Aceria mangiferae* Sayed abundantly in the malformed vegetative buds of mango trees, it suggests that the mite could play an important role in the dissemination and facilitating the entrance of the fungus into plant tissues through the wounded sites created after feeding on the vegetative tissues (Ploetz, 2003). Controlling of such disease when introduced into orchard is difficult but some approaches could be effective in reducing the level inoculum such as, removing the infected tissues from the trees and must burned outside the field. This action can reduce the disease significantly if practiced for 2-3 years. Thereafter, the disease can be under control. Nurseries should not establish in infected orchards (Ploetz, 2003). New orchards should be established with healthy plant materials taken from pathogen free-nursery stock.

GREY LEAF SPOT

Pestalotiopsis is a complex genus contains 205 species all characterised based on conidia characteristics such as size, septation, colour and presence or absence of appendages (Keith et al, 2006). This genus has been reported on several tropical hosts such as mango (Karakaya, 2001; Ko et al, 2007) and Guava (Keith et al, 2006). Among the *Pestalotiopsis* species, *P. mangiferae* is a parasitic fungus causing a leaf spot disease on mango which has been reported to cause a serious grey blight of mango in India (Verma et al, 1996; Verma et al, 1991), in Taiwan (Ko

et al, 2007), in Malysia (Williams and Liu, 1976). Other *Pestalotiopsis* species were founded associated to leaf spot described on mango such as *P. mangifolia* (Guba) in Malawi (Peregrine and Siddiqi, 1972). The fungus produces irregular grey spots on leaves, branches, panicles and fruit, ranges from few millimeters to several centimeters in diameter (Anonymous, 2009). Fungicides used for controlling other leave diseases such as Copper oxychloride and Dithiocarbamates can achieve management of the disease (Prakash, 2004).

MACROPHOMA LEAF BLIGHT

The fungus *Macrophoma mangiferae* Hingorani & Sharma causes leaf and stem blight and post-harvest rot of mango (Hingorani et al, 1960). It occurs in India and Nigeria in mango producing areas and has been intercepted from Mexico (Farr et al, 2006; Okigbo and Osuinde, 2003). The primary host is mango (Hingorani et al, 1960), although it also weakly infects *Ficus carica*, *Eryobotrya japonica*, *Eugenia jambolina*, and *Vitis vinifera*. *M. mangiferae* infects leaves, stems and causes a storage rot of mango fruit (Hingorani et al, 1960). The disease can be controlled in the field using Lime sulfur or Dithane fungicides along with the orchards sanitation by removing the infected parts to reduce the disease inoculums (Hingorani et al, 1960).

MINOR DISEASES OF MANGO

Other diseases of minor importance on mango have been reported worldwide. Of them, Sooty Mould or Black Mildew caused by *Meliola mangiferae* Earle and *Capnodium mangiferae* (Cke. & Borwn), Scab caused by *Elsinoe mangiferae* (Bitancourt & Jenkins), Phoma Blight caused by *Phoma glomerata* Corda, Woll. & Hochap, Pink Disease caused by *Erythricium salmonicolour* (Berk. & Broome), Angular Leaf Spot caused by *Plagulae epiphyllous*, Wilt caused by *Verticillium albo-atrum* (Reinke & Berth), Sclerotium Rot caused by *Sclerotium rolfsii* Sacc. and *S. delphinii*, Cercospora Leaf Spot caused by *Cercospora mangiferae* Koorders, and *C. mangiferaeindicae* (Munjal, Lal & Chona) (Prakash, 2004).

THESIS AIMS

It was quite evident from our survey during August, September, October and November 2009 and April, 2010 in different geographical locations in Sicily-Italy that, mango plantations suffer from one or more diseases attributed to unknown causal agents associating with different symptom patterns. From the results of the initial isolation and identification, we found that *Botryosphaeria* spp. was the most prevalent isolated fungi associated with typical dieback symptoms. Species of this genus complex represent important pathogens of fruit trees and are associated with fruit rot, leaf spot, cankers, branch dieback and even causes tree death (Khanzada et al, 2004; Ploetz, 2004)**.** Such fungi considered as threaten pathogens to mango production system worldwide (Sakalidis et al, 2011; Slippers et al, 2005; Slippers and Wingfield, 2007). The present situation of mango orchards in Sicily (Southern Italy), can actually express the urgent need of much more interest and quick involvement to understand the ecology of such fungi. Since, dieback disease was the main problem in mango orchards our work therefore, focused mainly on the isolation and characterisation of *Botryosphaeria* spp. associated with mango dieback disease in Italy with a comparison case of study in Egypt. Therefore, the aims of this study were:

Objective 1 Assessment of mango diseases in different locations in Italy.

Approaches:

A) Field survey, symptoms observations and collection of plant materials.
B) Isolation and morphological characterisation of the causal agents associated with mango diseases in Italy (Sicily).
C) Testing of the isolated fungi for their pathogenicity on mango.

Objective 2 Morphological and molecular characterisation of *Botryosphaeria* spp. associated with mango dieback disease in Italy.

Approaches:

A) Identification and taxonomy of *Botryosphaeria* spp. based on conidia and culture characters.

B) Molecular analysis to determine the genetic identity of *Botryosphaeria* spp. based on ITS and TEF-1α data sequences.
C) Temperature effects on the mycelia growth of Botryosphaeria spp.

Objective 3 Isolation, morphological and molecular characterisation of *Botryosphaeria* spp. associated with mango die back disease in Egypt.

Approaches:

A) Isolation of fungi associated with mango dieback disease.
B) Morphological characterisation of *Botryosphaeria* spp. based on conidia and culture characters.
C) Molecular analysis to determine the genetic identity of *Botryosphaeria* isolates based on ITS and TEF-1α data sequences.
D) Temperature effects on the mycelia growth of Botryosphaeria spp.

Objective 4 Pathological studies on the Botryosphaeriaceae associated with mango in Italy and Egypt.

Approaches:

A) Initial screening of representative *Botryosphaeria* spp. for pathogenicity on apple fruit cv. Granny Smith.
B) Pathogenicity tests on mango seedlings cv. Kensington Pride.

CHAPTER .2

ASSESSMENT OF MANGO DISEASES IN ITALY

INTRODUCTION

Mango suffers from several diseases at all its stages of development. All tree parts trunks, branches, twigs, leaves, flowers and fruits can be attacked by different pathogens (Muhammad, 2008). The causal agents of these diseases can express several kinds of symptoms such as: fruit rot, dieback, necrosis, scab, blotch, spots, canker, and malformation. Some of these diseases have become limiting factor in mango production in some world regions (Prakash, 2004). Such as, Black rot or Alternaria leaf spot caused by *A. alternata*, Keissl considered the most devastating diseases threaten to mango production in Israel (Prakash, 2004), and has been increasingly reported as a post-harvest disease (Prusky et al, 1993).

Botryosphaeria fungi have also considered as important pathogens with effect on mango productivity either as pre or as post-harvest pathogens in many countries (Jacobs, 2002; Javier-Alva et al, 2009; Johnson, 1992; Khanzada et al, 2004; Ramos et al, 1991). Mango decline syndrome includes many different diseases under several names such as, blight, gummosis, canker, twig blight, tip dieback and stem bleeding (Khanzada et al, 2004). These symptoms can be attributed to several fungi of which, *Botryosphaeria* anamorphs like, *Diplodia* or *Fusicoccum, D. theobromae* and *F. aesculi* causes one or more of the mentioned above symptoms (Ploetz, 2004). Both causal agents *F. aesculi* and *D. theobromae* are predominant post-harvest pathogens in corporation with other fungi that less frequently cause stem-end rot such as, *C. gloeosporioides*, *F. mangiferae*, *Phomopsis mangiferae* and *P. mangiferae* (Ploetz, 2004).

To overcome the threats of such diseases, accurate identification of their causal agents needed in order to set an integrated management strategy. Thus, the work in this chapter aimed to isolate and to identify the unknown causal agents associated with various symptoms observed during the survey carried out in mango plantations in Sicily (Italy).

MATERIALS AND METHODS

Field observations and collection of plant materials

Survey of mango plantations was conducted during August, September, October and November 2009 and April 2010 in Sicily (South-Italy). A total of 11 mango orchards in 4 provinces (2 orchards in Catania, 4 orchards in Messina, 4 orchards in Palermo and one in Ragusa), were examined and surveyed for mango diseases (Fig. 2). A total of 141 vegetative samples from different trees parts (Twigs, Branches, Leaves, Bark and Fruits) manifesting various disease symptoms due to unknown causal agents, were collected and placed into plastic bags and conserved at 4°C until processing.

Isolation

Plant materials were surface disinfected by sequential washing in 70 % EtOH (30 s or 1 min), house hold bleach (NaOCl 5 %) (1 min), and then rinsed in distilled sterilized water and dried in sterile filter paper (Slippers and Wingfield, 2007). Small pieces between the healthy tissues and infected one were cut into (2 – 4 mm^2) and placed in 9-cm-diameters Petri dishes contains Potato Dextrose Agar medium (PDA) amended with Streptomycin sulfate ($0.1g/L^{-1}$) to inhibit any bacterial growth. Plates were incubated at 25°C in the dark until the fungi growth appeared. Pure cultures were obtained by a excising a hyphal tip from colony margins emerging from the tissue pieces onto fresh PDA and incubated at the same conditions (Espinoza et al, 2009). The frequency of the isolated fungi from the plated pieces of collected samples was calculated using a formula described by Masyahit et al (2009).

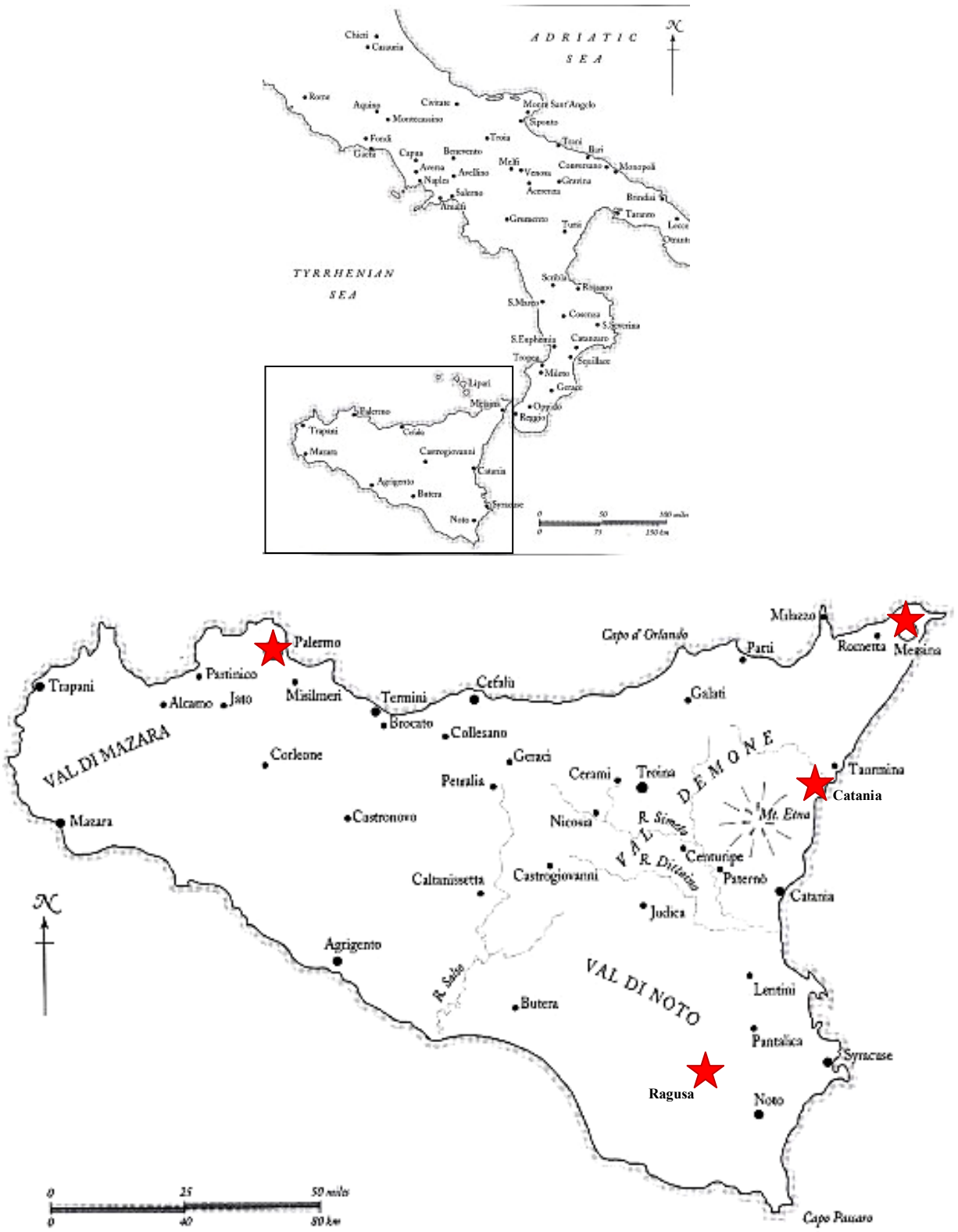


Figure 2 Illustration map of the southern part of Italy (Sicily) showing the monitored sites (Palermo, Messina, Catania and Ragusa) marked by red stars.

Morphological characterisation

All the hyphal tip purified isolates were divided into groups based on their growth patterns: mycelium growth appearance (aerial, fluffy dense or appressed) and colony colour on the front as well as the reverse side of plates. All isolates representing different colony types were maintained in pure culture on PDA medium at 25±2°C and conserved for further studies. Characteristics of fungal structures of representative isolates from each group were observed under light microscope at 100X and 40X magnifications. All the conidial characteristics (shape, size; length and width, colour, presence or absence as well as number of septa and presence or absence as well as number of appendages) used for description and identification were measured for about 30 conidia of each representative isolate. Statistical analysis was performed in order to determine the mean ± standard deviation (SD) of conidia length and width as well as other structures using one-way ANOVA (StatSoft, Inc. 2004) and conidia were photographed with (Olympus Digital Camera-Japan). Cultures morphology was recorded after incubation at 25±2°C for 7-12 days in the dark. Various taxonomic references were used to identify all fungal isolates obtained from mango in this study up to genus level (Barnett and Hunter, 1998; Freeman et al, 1998; Inoue and Nasu, 2000; Rivera-Vargas et al, 2006).

Pathogenicity tests

Twenty-one isolates (Pest-2, Pest-3, Pest-4, Pest-5, Pest-6, Pest-7, Pest-9, Pest-10, Pest-11, Pest-12, Pest-13, Pest-14, Pest-15, Pest-16, Pest-17, Pest-18, Pest-20, Pest-21, Pest-22, Pest-25, and P-26) represented *Pestalotiopsis* spp. Eighteen isolates (Al-2, Al-3, Al-5, Al-8, Al-11, Al-13, Al-15, Al-26, Al-28, Al-29, Al-31, Al-32, Al-36, Al-37, Al-43, Al-50, Al-53 and Al-54) represented a various morphological types of *Alternaria* spp. and eight representative isolates (Co-4, Co-12, Co-21, Co-24, Co-26, Co-28, Co-34 and Co-35) of *Colletotrichum* spp. were tested for pathogenicity on detached mango leaves cv. Kensington Pride under controlled laboratory conditions. Healthy leaves of the same size and age were selected for the inoculation. Leaves after surface disinfected by 10 % house bleach solution (3-5 % sodium hypochlorite) for 2 min then were washed in sterile distilled water and left to dry in the air. Two leaves were used for each isolate. Leaves were wounded in 3 points on the upper surface of leave midrib by pressing slightly using a sterile

needle tip. A 6-mm of mycelium plugs from the actively grown margins of each isolate were placed on the wounded sites. Control leaves were inoculated with only PDA sterile plugs. Inoculated leaves placed in the trays were introduced in plastic bags (50 × 70 cm [length × width]) containing wetted paper towels to maintain high relative humidity, and incubated at 25°C for 5-7 days and observed daily for symptoms development.

RESULTS

Field observations and collection of plant materials

Almost all the monitored mango plantations are cultivated with cv. Kensington Pride along with very few other varieties such as, R_2 E_2, Maya, Keitt, Kent, Irwin and Tommy Atkins being cv. Kensington Pride is the most cultivated variety. Several symptoms patterns appeared on various tree organs were mostly common observed on cv. Kensington Pride rather than on the other varieties.

Dieback and decline symptoms. They were the most frequently observed symptoms in almost all the surveyed mango orchards. The symptoms were more distinct on the apical parts of the trees (twigs and branches). In the affected trees, twigs die from the tips back into branches, canker and necrosis of the young twigs extending outwards to leaf blade. Small to large brown to black lesions coalesces to form large patches and lead to withering all the leaves or remain attached to the died twigs giving the trees the scorched appearance (Fig. 3 A, B, E). Under severe infection, the affected branches die one after another resulting in the death of most of the tree parts (Fig. 3 C). Cross section in the infected branches, twigs and stem showed brown streaks along the internal vascular tissues (Fig. 3 D). On the early infection, during fruit set the small fruit can become completely mummified and remain attached to the twig. On the mature fruit, small black skinny patches by time coalesce to form large necrotic areas that extend into the tissue pulp (Fig. 3 F).

Alternaria black spot symptoms. Symptoms were observed mainly on the old leaves and varied from small to large dark-brown to black lesions that have darker margins and brown depressed necrotic tissues center, appeared mostly on the leaf margins and extended along the leaf blade giving the appearance of zigzag. Lesions often coalesced to form large dark brown patches lead to upward leaf roll (Fig. 4A, B).

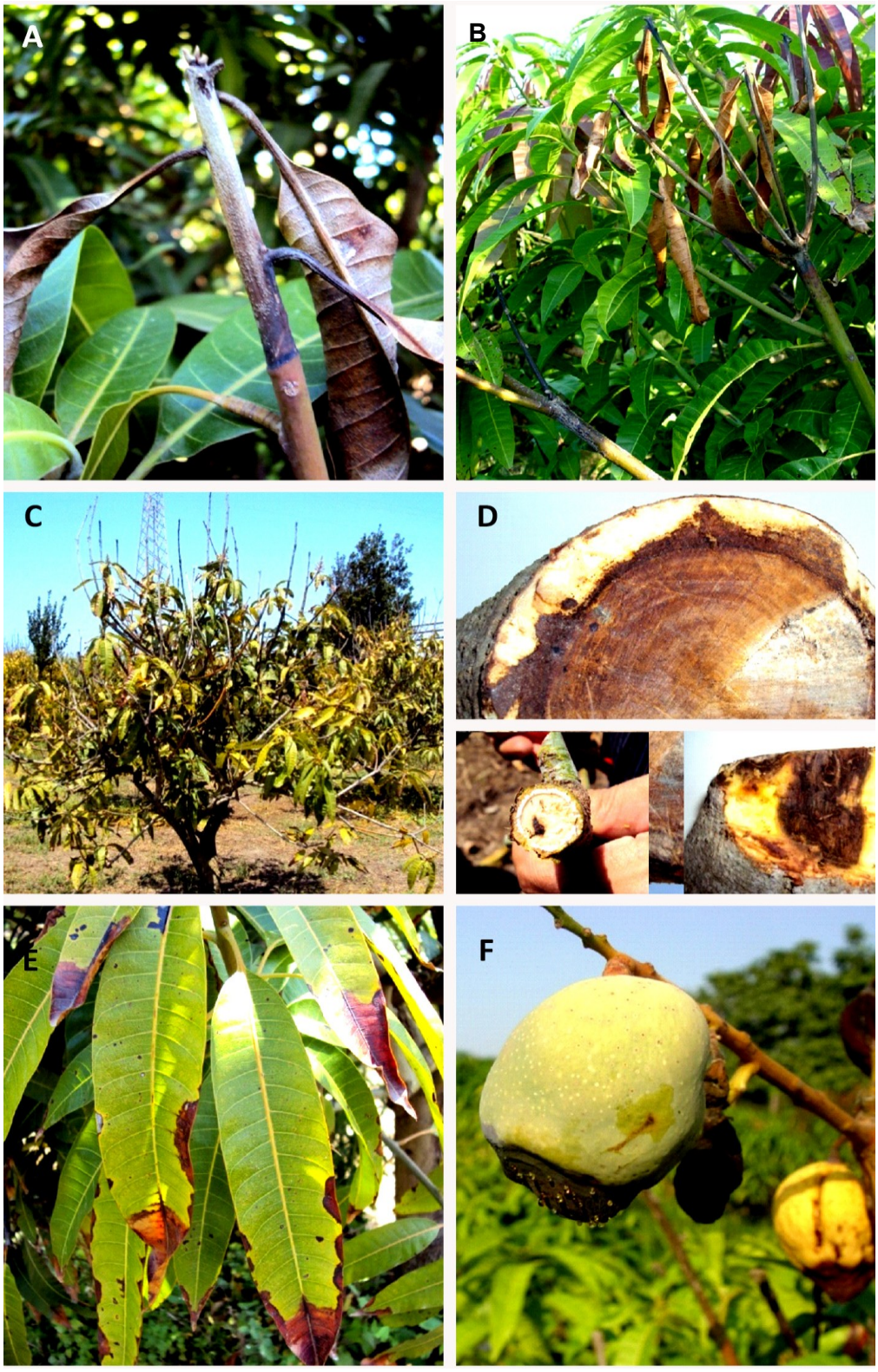


Figure 3 A, B Dieback symptoms on the young twigs and branches starting from the tip extended downward. **C,** in severe infection most of the apical parts die and dry leading to death of the whole tree, **D**, cross sections of stem and branches showing the brown vascular discolouration under cambium tissues, **E,** brown to black lesions on the leaf margins of the affected leaves. **F,** mummified small fruits on the early stage and dark brown patches on the big fruit lead to soft rot of the internal pulp.

Figure 4 Symptoms of Alternaria leaf spot caused by *Alternaria* spp. in the field. A and B, dark brown to black lesions along leaf margins giving the zigzag shape, C and D, coalescing of lesions causing upwards rolling of leaf margins (arrows).

Grey leaf spot symptoms. Plants manifested small grey lesions to large grey patches that have slightly raised dark margins with several black acervuli observed on the grey necrotic area (Fig. 5 A, B). These symptoms appeared mainly on the old leaves twigs, branches, and panicles (Fig. 5 C, D) and rarely observed on the young leaves.

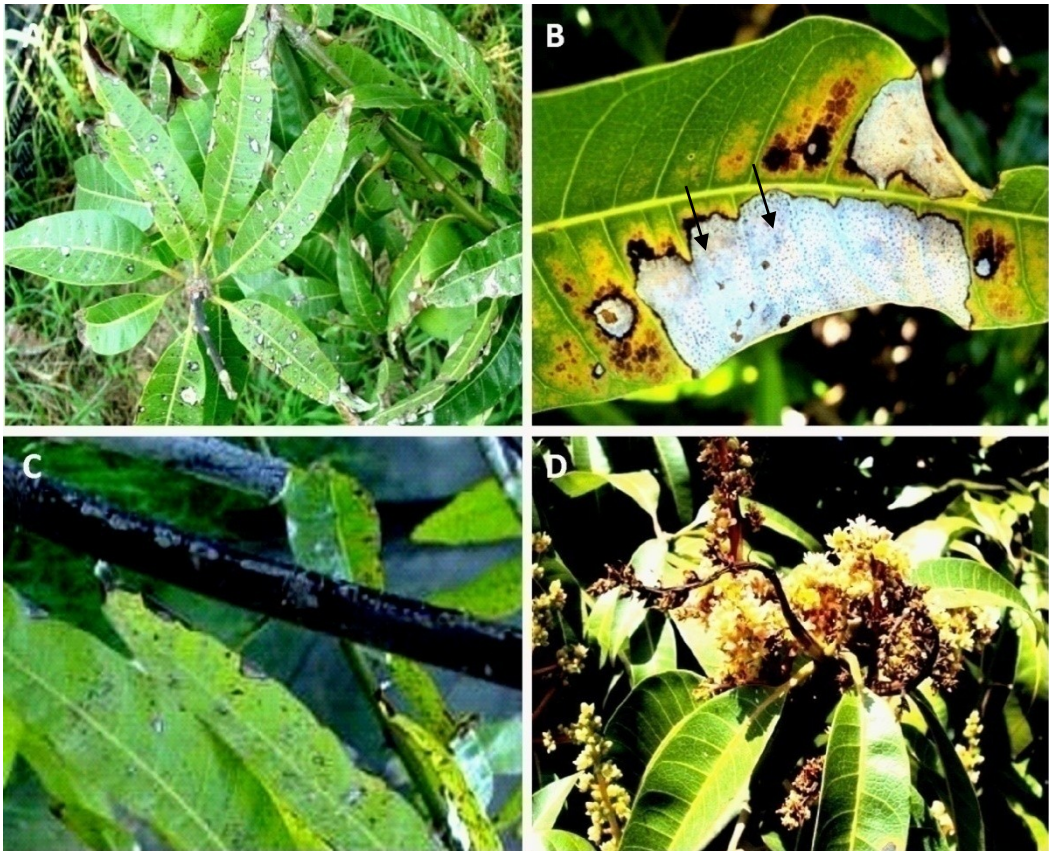


Figure 5 Grey leaf spot symptoms observed in the field. A, Small to large grey necrotic lesions with black margins B, coalesced to form larger grey patches and on the necrotic tissues abundant black acervuli were formed (see arrow). C, large white to grey specks on the secondary branches (arrow) D, light brown lesions extended to the main and secondary axis of the panicles causing blossom blight symptoms.

Anthracnose symptoms. These symptoms were more clearly visible on the young shoots of mango trees as small irregular brown to black spots scattered randomly on the young leaves that can enlarge to form necrotic black patches areas (Fig. 6A, B, C, D), giving leaves the scorch appearance. These necrotic tissues often cracks and falls out of leaves to form holes on the leaf blade. On the twigs, black speaks started from the apical part along the twig surface. Orange masses of conidia were observed on the necrotic tissues released from semi-immersed fruiting structures (acervuli). Sunken dark, prominent decay spots can extend deeply resulting in the decay of fruit pulp (Fig. 6F).

Figure 6 Symptoms of anthracnose disease observed in the surveyed fields. A,B Tiny black spots scattered on the young leaf blade have irregular margins coalescing to form large necrotic black patches that start from the leaf margins to the apical part covering most of the leaf blade. C, D, blighting of leaves giving the scorching appearance. E, dark necrotic patches lead to dying the apical shoot, orange masses of conidia released from semi-immersed acervuli observed on the necrotic area. F, brown to dark sunken and depressed lesion on the fruit.

Incidence and initial identification of isolated fungi

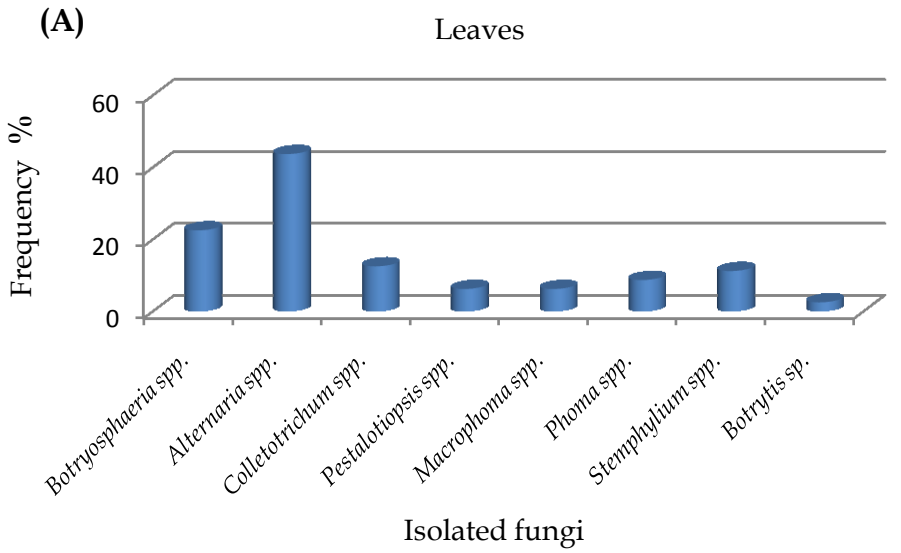
After isolation and initial identification based on the morphological structures, a diverse array of fungi was recovered from various symptomatic tissues. Of them, *Alternaria* spp., *Botrytis* spp. *Botryosphaeria* spp., *Colletotrichum* spp., *Macrophoma* spp., *Pestalotiopsis* spp., *Phoma* spp. and *Stemphylium* spp. were isolated from different mango trees organs (leaves, twigs, branches, fruits, panicles and barks).

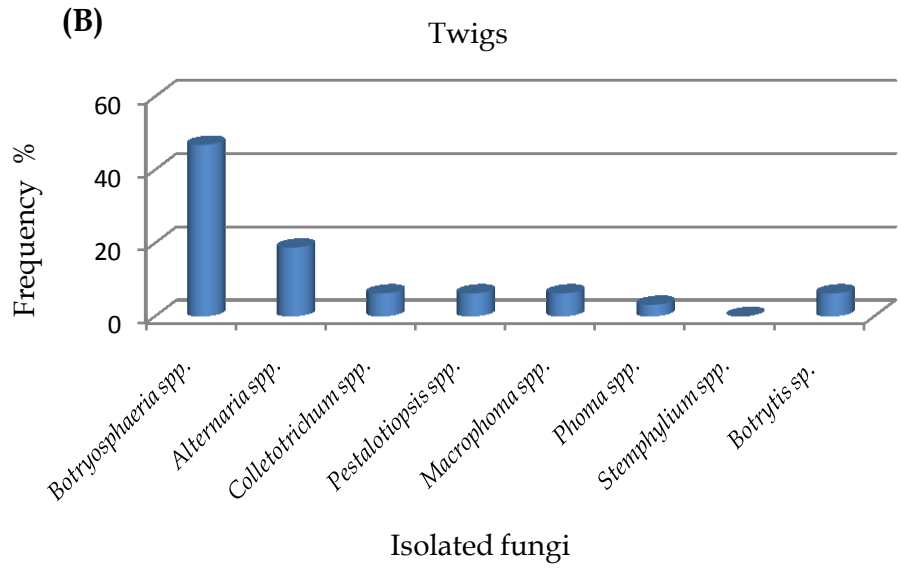
The incidence of the isolated fungi (Table. 1) varied according to plant organs as well as to monitored location. In almost all, the surveyed orchards *Alternaria* spp. and *Botryosphaeria* spp. were the most prevalent and isolated fungi (Table. 1). *Alternaria* spp. was the most frequently fungus associated with symptoms illustrated in (Fig. 4). The fungus was highly recovered from all monitored locations with high recovery from Messina (76.6%). The fungus was isolated from most of the tree parts; leaves (43.7 %), twigs (18.7 %), panicles (25 %) and branches (11.1 %) respectively (Fig. 7A, B, D, C). *Botryosphaeria* spp. was the major and most prevalent fungus associated with twig and branches die back symptoms (Fig. 3 A, B, C, D, F). The fungus was isolated from all mango plantations with high incidence in Catania (62.5%) and isolated from all tree parts except panicles; fruit (100 %), branches (50 %), twigs (46.8 %) and leaves (22.5 %) (Fig. 7 A, B, C, E). *Colletotrichum* spp. was associated with distinct typical symptoms of anthracnose (Fig. 6). The fungus was abundantly isolated from only three locations (Catania, Palermo and Messina) with high incidence in Palermo (21.1%), and isolated frequently from leaves (12.5 %), twigs (6.2 %) and with low frequency from branches (5.5 %) (Fig. 7A, B, C). *Pestalotiopsis* spp. was found in all mango plantations associated mostly with grey leaf spot symptoms and frequently isolated from panicles (12.5 %), leaves (6.2 %), and twigs (6.2 %), with low incidence in branches (5.5 %) (Fig. 7A, B, C). The other fungi were occasionally co-isolated with the dominant fungi mainly from leaves with low frequencies (Table .1).

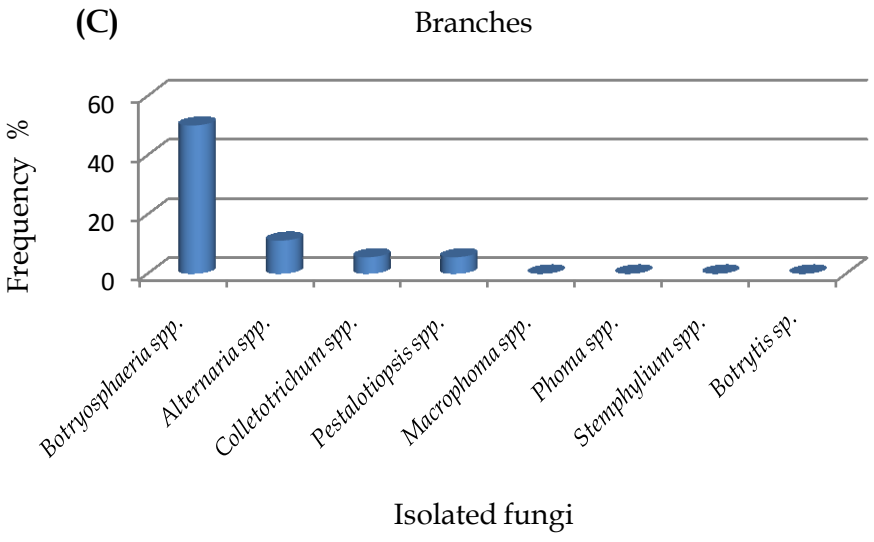
Table 1 The incidence of the isolated fungi from various mango trees organs recovered from four provinces (Messina, Catania, Palermo and Ragusa) located in south Italy (Sicily) during a survey conducted in 2009 and 2010.

Isolated fungi	Incidence % of recovered fungi in 2 locations surveyed in 2009		Incidence % of recovered fungi in 3 locations surveyed in 2010		
	Messina[a]	Catania [b]	Palermo[c]	Messina[d]	Ragusa[e]
Botryosphaeria spp.	15.3	62.5	17.3	34.6	11.1
Alternaria spp.	76.6	29.1	36.5	14.2	44.4
Colletotrichum spp.	0	4.1	21.1	8.1	0
Pestalotiopsis spp.	7.6	16.6	1.9	6.1	22.2
Macrophoma spp.	53.8	0	0	0	0
Phoma spp.	2.3	0	1.9	12.2	0
Stemphylium spp.	7.6	0	0	10.2	33.3
Botrytis sp.	0	0	9.6	4	22.2

The incidence (%) of fungi isolated from various tree organs (leaves, twigs, branches, fruits and barks), collected from 11 mango orchards located in 4 regions in Sicily. A variable number of samples according to surveyed orchards ([a])13 samples, [b] (24) samples, [c] (46) samples, [d] (49) and [e] (9) samples. Data represent mean percentage % from the number of samples yielded the isolated fungi divided by total number of collected samples; the collected samples were mainly cv. Kensington Pride.


(A)
Leaves
Frequency %
60
40
20
0
Botryosphaeria spp.
Alternaria spp.
Colletotrichum spp.
Pestalotiopsis spp.
Macrophoma spp.
Phoma spp.
Stemphylium spp.
Botrytis sp.
Isolated fungi


(B)
Twigs
Frequency %
60
40
20
0
Botryosphaeria spp.
Alternaria spp.
Colletotrichum spp.
Pestalotiopsis spp.
Macrophoma spp.
Phoma spp.
Stemphylium spp.
Botrytis sp.
Isolated fungi


(C)
Branches
Frequency %
60
40
20
0
Botryosphaeria spp.
Alternaria spp.
Colletotrichum spp.
Pestalotiopsis spp.
Macrophoma spp.
Phoma spp.
Stemphylium spp.
Botrytis sp.
Isolated fungi

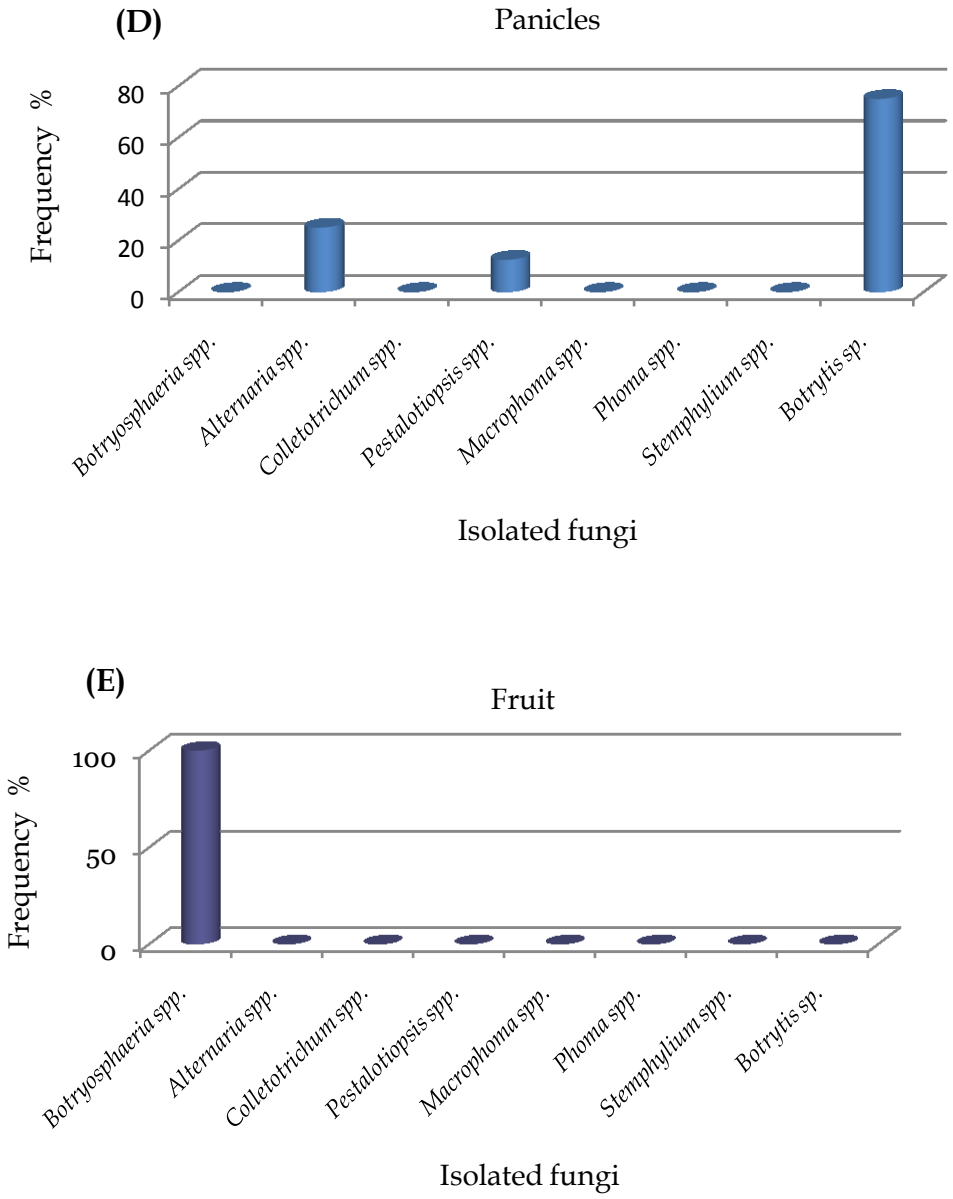


Figure 7 The frequency (%) of isolated fungi according to mango trees organs; A, Leaves, B, Twigs, C, Branches, D, Panicles and E, Fruit of cv. Kensington Pride manifested different symptoms patterns in the field. Data within columns are the mean percentage (%) calculated from the number of samples yielding the isolated fungi divided on the total number of samples for each plant part.

Morphological characterisation of the isolated fungi

***Alternaria* spp.**

Alternaria spp. were identified based on their colony and conidia characteristics. Culture morphology was observed on PDA medium after 10 days of incubation in the dark at 25±2°C. The fungus initially produced raised white cottony dense mycelium, which later showed variability in colour by age, from pale grey-olivaceaous to light brown with light margins (Fig. 8A), or dark brown aerial dense mycelium and some very small grey raised tufts on the surface (Fig. 8 E), dark grey to light brown cottony dense mycelium (Fig. 8 I). On the reverse side of the plates the colony colour varied was dark-olivaceaous (Fig. 8 B) to dark brown (Fig. 8 F, J).

Conidia were dark, mostly obclavate to byriform with size average ranging from (22.5 ± 3 - 35.6 ± 7.3) × (10.8 ± 1.3 - 13.3 ± 2.4) µm mean length × width. Mean ± standard deviation of 30 arbitrarily selected conidia. Conidia often have short cylindrical or conical beak varied in length from 3.6 ± 2.6 to 6.6 ± 2.8 µm and sometimes beakless, smooth or have some rounded warts creating a slightly rough surface, septate with abundant transverse (3-8) and longitudinal (0-4) septa (Fig. 8 C, G, K). Conidia produced on simple or branched olivaceaous-brown septate conidiophores that showed a variation in length from 27.3 ± 11.3 to 48.6 ± 28.1 µm bearing either solitary or in short chains conidia (Fig. 8 D, H, L). The microscopic observations of the above characteristics revealed *Alternaria* spp. that coincided with those described in by Barnett and Hunter (1998) and by Inoue and Nasu (2000).

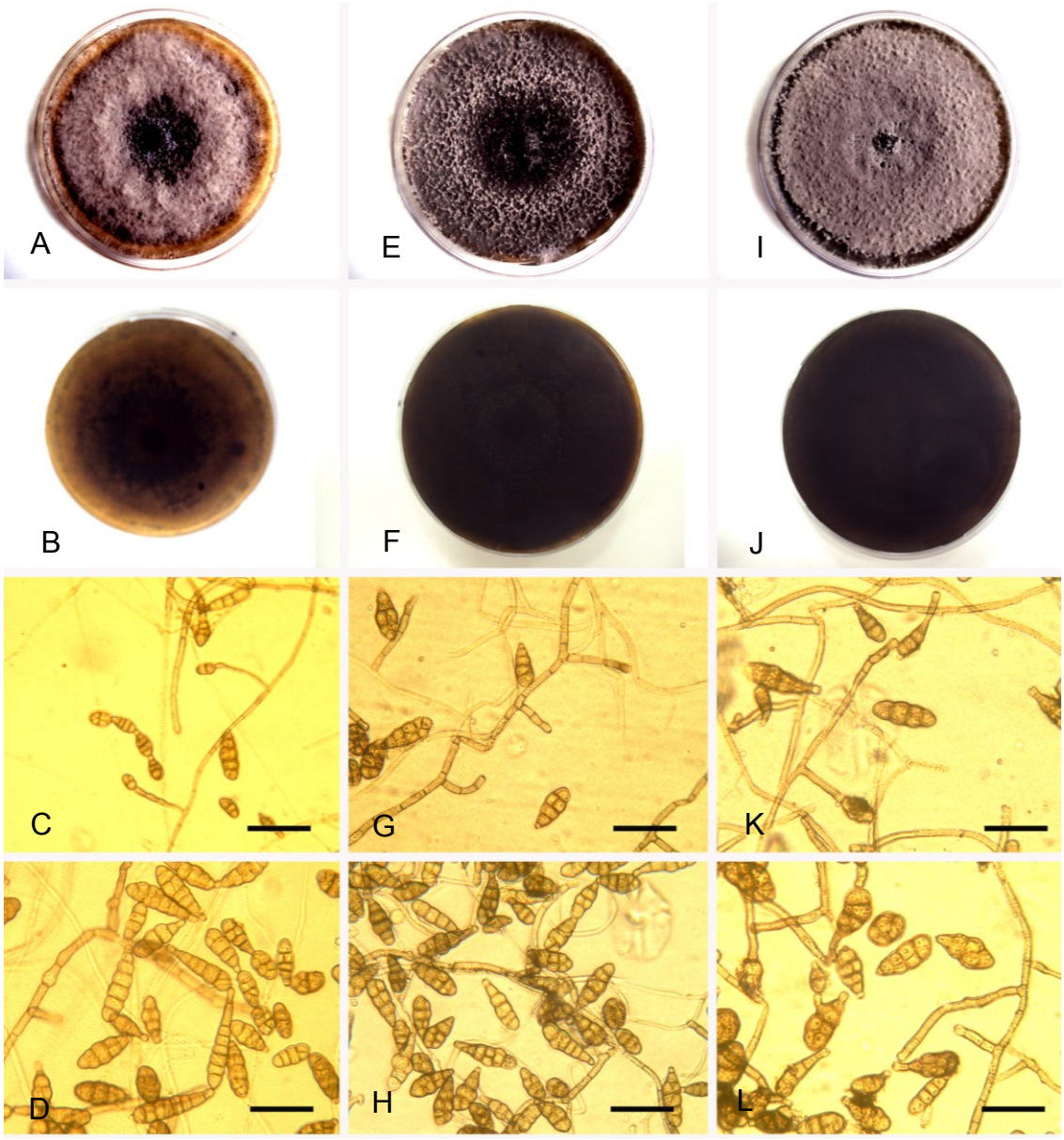


Figure 8 Morphological characteristics of colony (front and reverse side), conidia and conidiophores of three isolates of *Alternari*a spp. A, B, C, and D (AL-3). E, F, G and H (Al-13). I, J, K and L (Al-17) of *Alternaria* spp. isolated from mango. Scale bars = 10 µm.

***Colletotrichum* spp.**

Colletotrichum species isolated from mango were identified based on culture and conidia morphology. Cultures of *Colletotrichum* spp. showed variability in morphology and different types of colony were observed; on the front side of PDA white cottony dense raised mycelium with obvious concentric rings (Fig. 9 A, B). White-creamy flat to effuse mycelium with no obvious sporulation which later tuned to beige was observed for some isolates (Fig. 9D, E). White-grey raised mycelium turned dark mouse-grey with abundant orange masses of conidia released from scattered acervuli semi-immersed in the mycelium was also observed in some isolates (Fig. 9G, H). White-creamy mycelium that turned light grey in the center of colony with obvious sporulation appeared as orange masses of conidia released from semi-immersed acervuli (Fig. 9J, K).

Conidia showed also variation in shape and diameters and various shapes of conidia were observed; hyaline fusiform have an acute or sharp end from only one side conidia (Fig. 9C, I) measuring 13 ±1.9 × 5.8 ±1 µm mean length × width. Hyaline cylindrical to ellipsoid conidia with rounded or obtuse ends in both sides measuring 14.1 ± 1.4 × 7.5 ± 0.5 µm mean length × width (Fig. 9F, L). The conidia characteristics matched those of *Colletotrichum* spp. reported in Barnett and Hunter (1998) and Rivera-Vergas et al (2006).

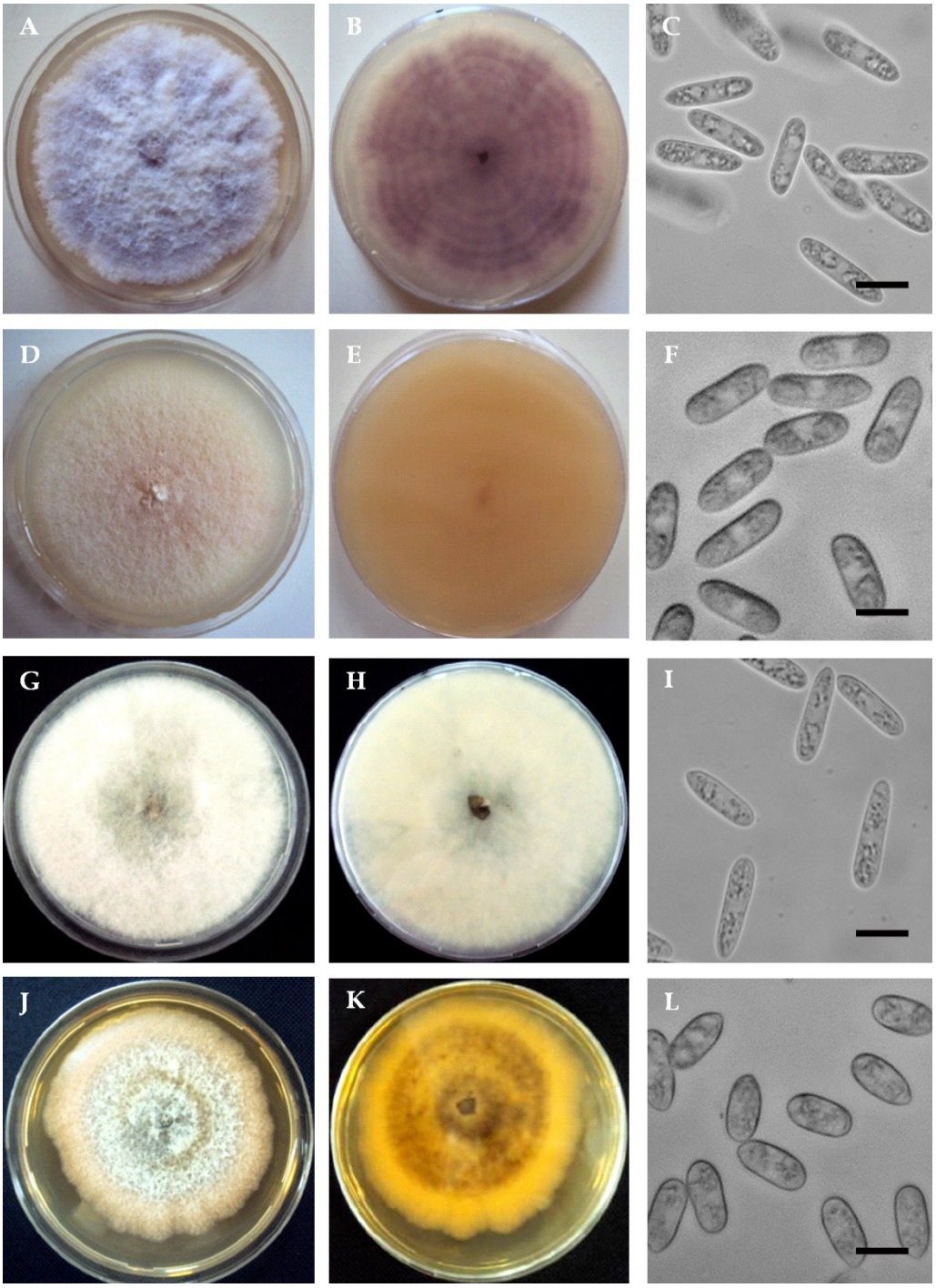


Figure 9 A, B, D, E, G, H, J, K, culture morphology of *Colletotrichum* spp. on PDA after 8 days of incubation at 25°C. C, F, I, L, the commonly observed conidia for *Colletotrichum* spp. Scale bars = 10 µm.

***Pestalotiopsis* spp.**

Twenty-one isolates of *Pestalotiopsis* spp. were identified up to genus level based on the conidia characteristics, five-celled conidia, of which apical and basal cells were hyaline, and the three median cells were from olivaceous to light or dark brown (Fig. 10C, F, I, L). *Pestalotiopsis* isolates showed little variation in culture morphology on the PDA medium after 9 days of incubation at 25°C in the dark. White to creamy mycelium on the front side of Petri plate and on the reverse side pale luteous colour in the center of colony diffused beyond colony margins (Fig. 10A, B). White greenish mycelium with some pale orange to light brown tufts and on the reverse side of colony pale saffron colour was more distinct (Fig. 10E, D). White to creamy dense mycelium has circular growth appearance (Fig. 10G) and on the reverse side, pale luteous to saffron colour diffused in the medium was observed (Fig. 10H). White cottony dense raised mycelium has zonate appearance (Fig. 10J), on the reverse side of colonies, olivaceous colour was observed and the zonation was more distinct (Fig. 10K).

All isolates produced black acervuli after exposing to the natural light for approximately 10-days. Conidia showed little variations in diameters; ranging from (21.9 ± 1.4 - 26.6 ± 1.3) × (6.4 ± 0 - 9.6) µm mean length × width. The apical cell bear two to four hyaline apical appendages measuring 11.5 to 33.9 µm mean length. The basal cell bear only one hyaline, straight and in some isolates slightly curved appendage measuring 5 to 8.2 µm mean length (Fig. 10C, F, I, L). Morphological features of conidia and culture resembled *Pestalotiopsis* spp. and matched those described by Espinoza et al, (2009) and Keith et al, (2006).

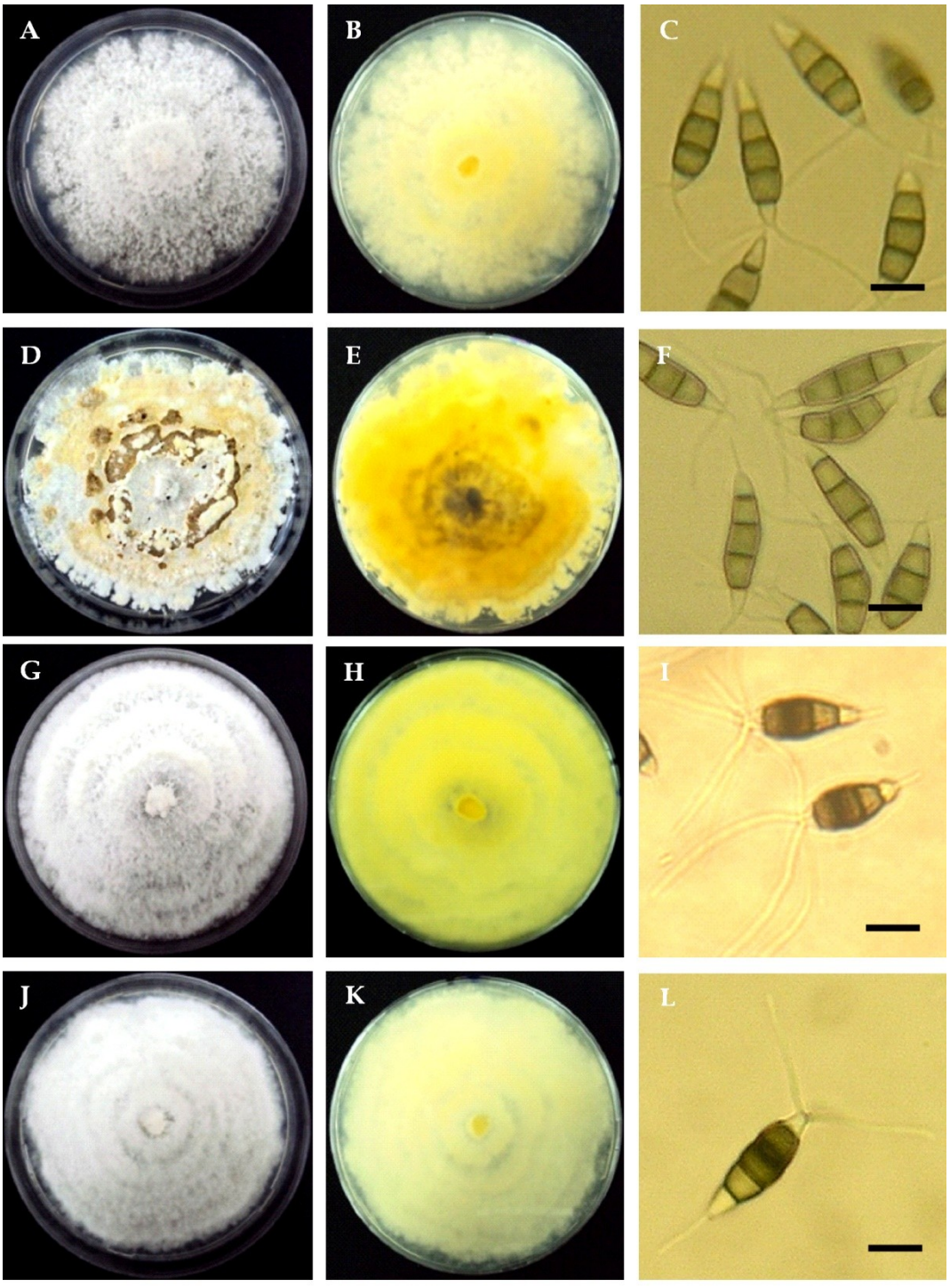


Figure 10 Colony and conidia characteristics of representatives *Pestalotiopsis* isolates on PDA after 9 days at 25°C. (A, B, D, E, G, H, J, K,), culture morphology of *Pestalotiopsis* spp. (C, F, I, L), commonly observed conidia from *Pestalotiopsis* spp. Scale bars = 10 µm.

Characterisation of other associated fungi

The other occasionally isolated fungi such as; *Botrytis* sp., *Macrophoma* spp., *Phoma* spp., and *Stemphylium* spp., were tentatively identified up to genus level based on conidia morphology.

Pathogenicity tests

***Alternaria* spp.**

All *Alternaria* spp. isolates were pathogenic to mango leaves. No symptoms were observed on leaves inoculated with sterile PDA plugs. The first symptoms observed on leaves appeared after 3 days of inoculation and were identical to those observed in the field. Symptoms initially started as small irregular brown lesions and later enlarged along leaf veins. Lesions were surrounded with yellow hallow and masses of brown to black conidia were formed on the surface of the necrotic tissues (Fig. 11). However, a significant (P=0.017) variation were obtained in lesion diameters among tested isolates but, these differences in lesions sizes were not so great, and almost all isolates revealed similar average score of lesion diameters (Fig. 12). Koch's postulates were confirmed and *Alternaria* species were re-isolated from the lesions successfully and their morphology coincided with those used for initial inoculation (data not shown).

***Colletotrichum* spp.**

Pathogenicity test confirmed that all *Colletotrichum* spp. isolates were pathogenic on mango leaves, and produced symptoms identical to those observed in the field (Fig. 13). Tiny brown lesions initially developed around the inoculation site after 4 days, extended outward to form large necrotic brown lesions with black margins; on the surface of necrotic lesions orange conidial masses were observed 6 to 8 days after inoculation (Fig. 13). No symptoms developed on the control leaves. High significant (P=0.001) difference was observed according to LSD test in lesion diameters induced by tested isolates of which, (Co-35) caused significantly (P<0.05) larger lesion diameter (22.5 mm) on detached mango leaves than the other isolates (Fig. 14). Isolate (Co-28) produced lesion diameter (13.5 mm) larger than the rest of isolates. It seemed that isolates (Co-35, Co-28, Co-21, Co-34, Co-24, Co-26, Co-12 and Co-12) obtained from leaves

produced larger lesions than (Co-4) obtained from branches (Fig. 14). *Colletotrichum* species were recovered from artificially inoculated detached mango leaves thus; Koch's postulates were successfully fulfilled.

***Pestalotiopsis* spp.**

To fulfill Koch's postulates the tested isolates of *Pestalotiopsis* spp. proven to be pathogenic to mango leaves. Symptoms consisted of light to dark brown necrotic lesions with irregular borders (Fig. 15), that appeared 4-days after inoculation and continued to develop. Lesions sizes varied according to the virulence degree of the isolates. Significant differences were obtained among isolates. No symptoms were observed on the control leaves. The most severe isolates were (Pest-13, 11, 12, 10 and 17) which developed lesions diameters larger than the rest of isolates (45. 29.8, 28.8, 17.1 and 16.1 mm) respectively, and extended along the midrib coalescing to form large brown patches giving the leaves the scorch appearance (Fig. 16). On the opposite, the other *Pestalotiopsis* isolates developed lesions not significantly (P <0.05) different in size among each other. On the surface of the necrotic area small brown to black fruiting structures (acervuli) were formed which contained the fungus conidia (Fig. 15D). Re-isolation from the artificially inoculated leaves consistently yielded *Pestalotiopsis* spp.

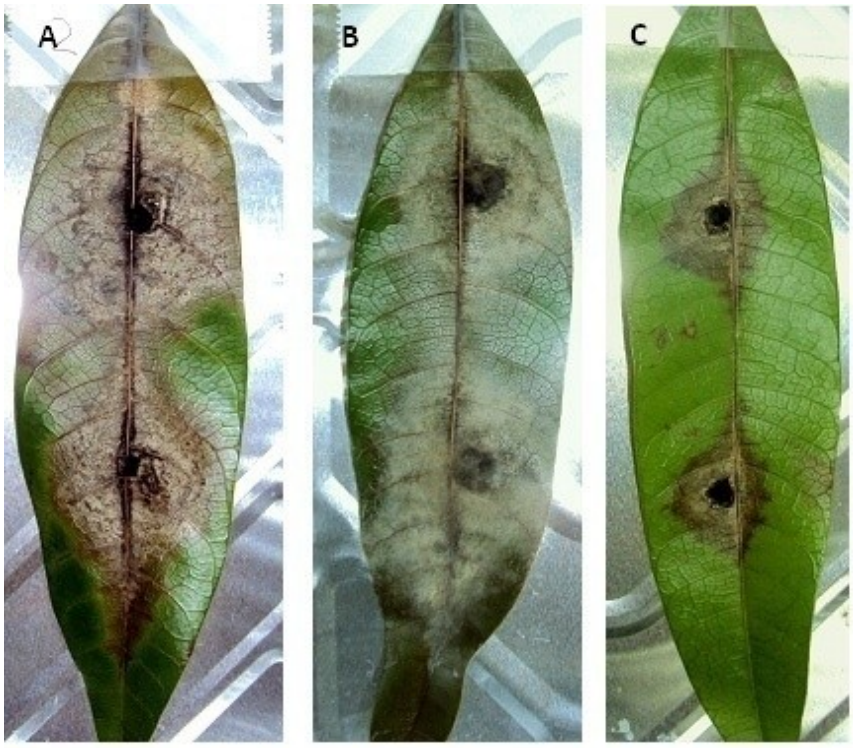


Figure 11 Symptoms of Alternaria brown leaf spot developed on the detached mango leaves after 7 days of inoculation. Different levels of symptoms appeared showing the variability in lesion diameters produced by three isolates A, Al-2 B, Al-29 and C, Al-54 of *Alternaria* spp.

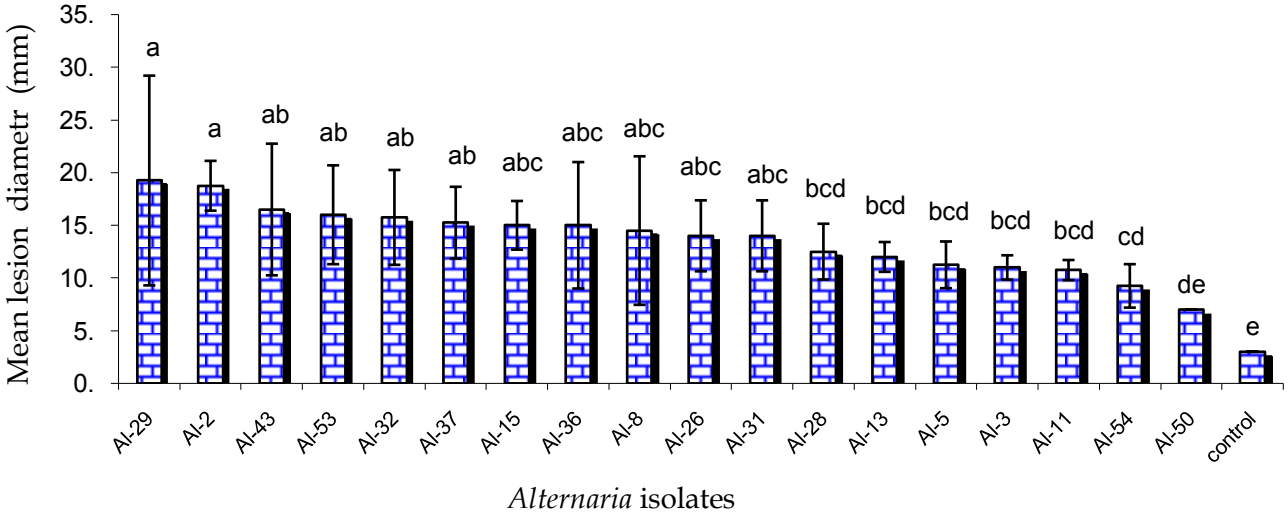


Figure 12 Mean lesion diameters (y-axis) induced by eighteen representative isolates of *Alternaria* spp. (x-axis) on detached mango leaves cv. Kensington Pride after 7 days of incubation at 25°C. Values from each isolate are the mean of n=4 replicates ± (SD). Values on the same columns followed by the same letters are not significantly different according to LSD test ($P < 0.05$).

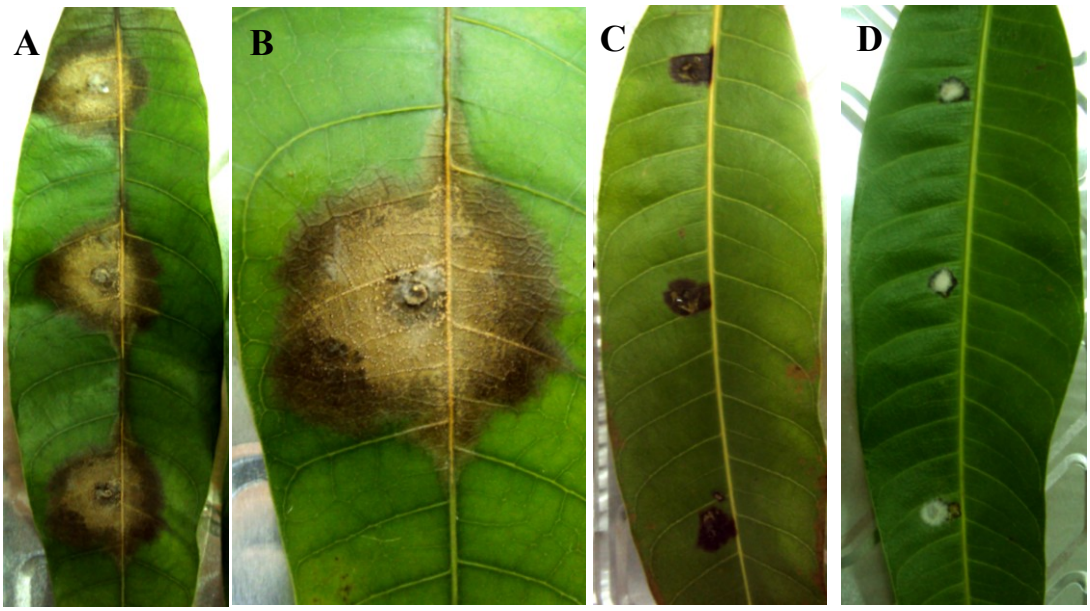


Figure 13 Symptoms reproduced by selected isolates of *Colletotrichum* spp. on detached mango leaves after 8-days of incubation at 25° C. A, sever symptoms developed by the isolate Co-35. B, magnified photo of the lesion showing orange masses of fungus conidia formed on the necrotic tissues. C, weak symptoms developed by the Co-34 isolate. D, small lesions developed by the Co-4 isolate showing the weakness of the fungus to reproduce the symptoms observed in the field.

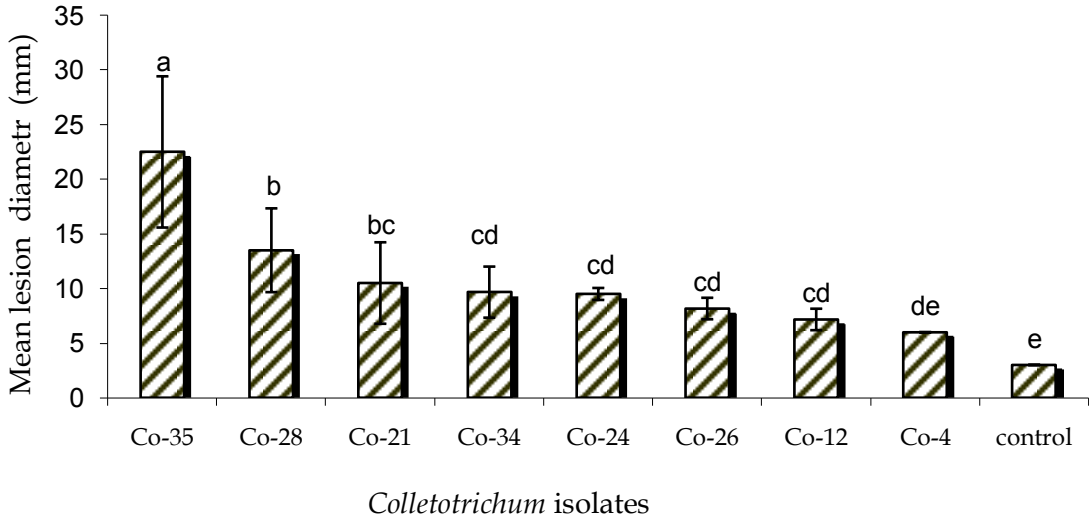


Figure 14 Pathogenicity tests of *Colletotrichum* spp. isolates. Data represent the mean lesion diameters (mm) produced by the tested eight isolates on detached mango leaves after 8 days of incubation at 25°C. Columns bearing the same letters are not significantly different according to the LSD test ($P < 0.05$).

Figure 15 Symptoms of grey leaf spot disease on mango detached leaves after 9 days of inoculation with *Pestalotiopsis* species. Developed symptoms varied from: A, small irregular light to dark brown lesions around inoculation sites. B, enlarged to form larger lesions along the main leaf vein. C, lesions expanded to coalesce forming brown patches along leaf blade giving rise to the scorch appearance. D, brown to black scattered acervuli formed on the center of the necrotic tissues (see arrow).

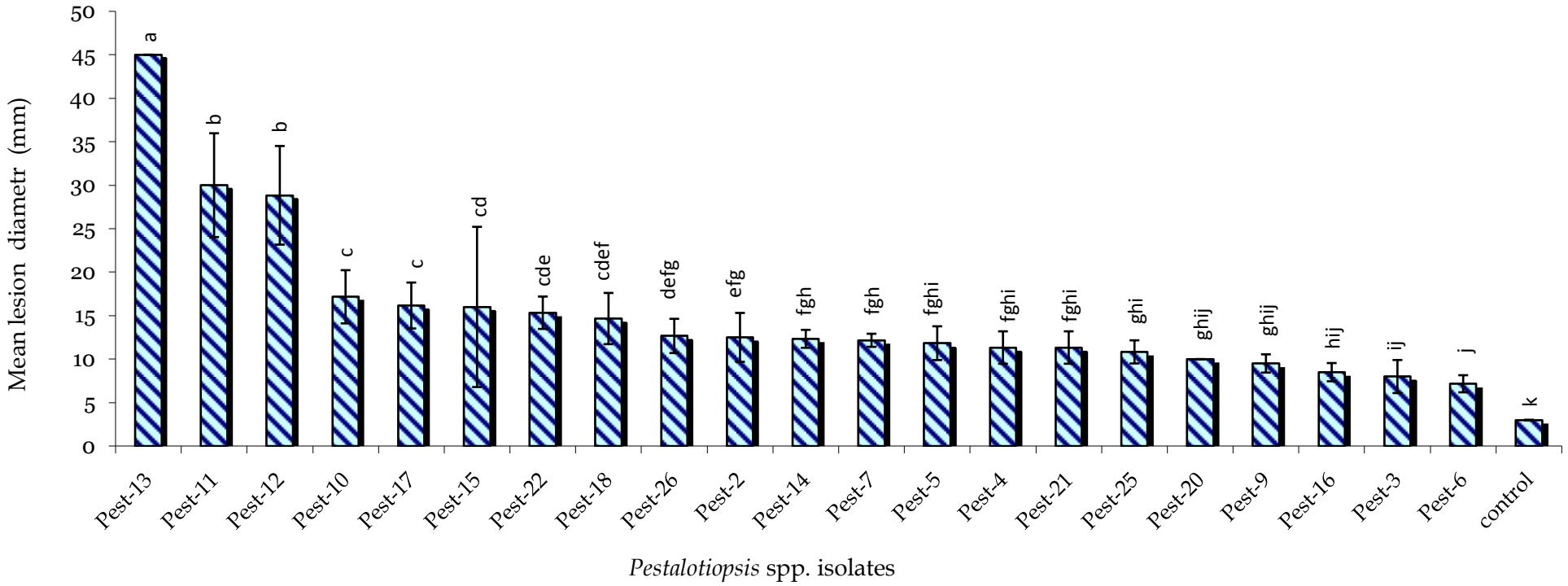


Figure 16 Lesions diameters (y-axis) developed by 21 isolates of *Pestalotiopsis* spp. (x-axis) inoculated on detached mango leaves cv. Kensington Pride after 7 days of incubation at 25ºC. Values from each isolate are the mean of n=6 replicates ± (SD). Columns bearing the same letters are not significantly different according to the Least Significant Difference (LSD) test ($P < 0.05$).

DISCUSSION

Mango has introduced into Italy in 1990 by some small growers (the first introduction of mango cv. Kensington Pride in Catania-Italy) (http://www.mangodisicilia.com/chisiamo.htm). After that, mango cultivation has been spread and extended to the around provinces in Sicily such as: Messina, Ragusa and Palermo of which the largest cultivated area was seen in Messina. The most cultivated varieties were; Kensignton Pride, R_2 E_2, Maya, Kent, Irwin, Keitt and Tommy Atkins being cv. Kensington Pride the most prevalent cultivated variety in all areas.

Much less, efforts have made on the assessment of the diseases problems of mango in Italy. Thus, the present work represents the first attempt to assess the presence and the diversity of fungal species associated with diverse symptoms patterns observed on mango trees during a survey conducted in 2009-2010. In all the eleven surveyed mango orchards, various patterns of symptoms due to unknown causal agents were observed on the whole tree. The most prevalent symptoms were dying and drying of the twigs and branches started from the apical parts of the trees moving downward to the main branches and stem causing typical symptoms of die back disease. In a severe cases trees dying and the cross section though the affected twigs, branches and stem showed dark brown to black discolouration under cambium tissues; dark brown to black lesions appeared on anywhere on the leaf lamina. In previous studies, similar symptoms were reported to be caused by several species of Botryosphaeriaceae worldwide (Jacobs, 2000; Johnson, 1992; Khanzada et al, 2004a, b, 2005; Ni et al, 2010; Ramos et al, 1991). In our investigations, the distinct symptoms of dieback and decline observed in mango fields were mainly attributed to one or more member of Botryosphaeriaceae reported and well described in this study (See Chapter 3 for full details).

Botryosphaeria spp. was abundantly isolated from symptomatic and asymptomatic plant materials. They easily distinguished by their grey to dark-green fast growing aerial mycelium on isolation media (Slippers and Wingfield, 2007) (See Chapter .3 for Isolation and characterisation of *Botryosphaeria* spp). The endophytic nature of such fungi makes their distribution very fast since the asymptomatic plant material does not exhibits any external symptoms (Slippers

and Wingfield, 2007), and can live endophytically in healthy plant material for an extended period of time. Smith et al, (1996) have reported that seeds of *Pinus* have latent infection with *L. theobromae* and *Diplodia pinea*. These latent infections of seed have been implicated in the movement of theses pathogens around the world (Burgess et al, 2004). Slippers et al (2004) have demonstrated that *Botryosphaeria australis* is native to Australia based on the geographical distribution and known hosts of this taxon. In addition, Johnson et al, (1991) have also indicated that *N. parvum* previously described, as (*D. dominicana*) is a primary casual agent of mango twig dieback in Australia. The infection sources of these fungi are unknown. Most or almost all cultivated mango varieties in Sicily-Italy have been imported either as seeds or as plant materials from Australia (personal communication). These previous findings lead us to suggest that *N. parvum* and *N. australe* might be introduced into Italy with the introduction of exotic mango plant materials (seeds or seedlings) from Australia.

Other hypothesis could give us a clear idea regarding the infection sources of Botryosphaeriaceae in mango plantations is that most of the mango orchards were neglected and in close proximity to various fruit trees which could serve as source of inoculum or as alternative hosts to *Botryosphaeria* fungi (personal observation). This aspect of the epidemiology of Botryosphaeriaceae deserve intensive study in the future to understand the ecology and the movement of such fungi in order to establish integrated and restricted control strategies.

Heavy infection of Alternaria black spot disease was also observed in almost all the monitored orchards. Alternaria black spot disease is mostly common in arid environments (Ploetz, 2004) and represents an important fruit disease especially in Israel (Prusky et al, 2002). This disease is caused by *A. alternata* and *A. tenuissima* (Prakash, 2004). The fungus is not a host-specific and can infect several plant hosts. The disease symptoms were observed throughout the year and were frequently and easily recognizable on the old leaves as well as on the young leaves and branches; small to large irregular dark brown to black lesions have black margins surrounded with yellow hallow. Infected and died leaves, twigs and branches are considered as significant inoculums sources (Ploetz, 2004). In the monitored mango fields the pruned plant materials were observed fallen on the orchard ground and they represent a sufficient inoculum sources for other infection cycles. *Alternaria* spp. were isolated most often from

leaves and identified based on the colony and conidia morphology up to the genus level. Considering the diversity among *Alternaria* spp. in terms of conidial shape, sizes and colony morphology, there have been no attempt to characterise them by molecular techniques. *Alternaria* spp. can be found associated with foliar diseases on numerous hosts with the fact that they isolated from all surveyed mango orchards as well as all isolates testes for pathogenicity were highly pathogenic to detached mango leaves suggesting that they could have either direct or indirect potential impact on mango productivity in the future. *Alternaria* spp. was not isolated from mango fruit, but they might pose serious effect on mango in the future. Additional studies are needed to understand the taxonomical status of *Alternaria* spp. and to determine their impact on mango plantations in Italy.

Anthracnose symptoms were also observed on different tree parts including leaves, twigs, fruits and branches. They can be differentiated easily from those of Alternaria black spot. Alternaria symptoms initially appear on the old leaves and the lesions centers are depressed, darker and harder than the lesions of anthracnose (Ploetz, 2004). While the symptoms of anthracnose begin most often on the young leaves as tiny dark-brown to black lesions have irregular margins and are not limited to leaf veins (Estrada et al, 2000). Under favorable environmental conditions 20-30°C and 100% relative humidity the small lesions can expand to form large patches leading to the complete drying of the leaf blade (Akem, 2006).

Infected leaves, twigs, branches and mummified fruits are considered as primarily significant sources of inoculum in the field (Fitzell and Peak, 1984; Akem, 2006). This was the case of our study, since the orange masses of conidia releasing from the acervuli were observed on the necrotic area of some infected branches in the field. The fungus is polycyclic on these organs and conidia can move to other healthy organs by rain splashing or by other means to cause secondary infections in the field (Akem, 2006;Aruz, 2000). *Colletotrichum* spp. was highly recovered from distinct symptoms of anthracnose disease and was morphologically characterised up to genus level. So far, species of *Colletotrichum* that have been reported to cause diseases on mango are *C. gloeosporioidoes* and *C. acutatum* (Aruz, 2000; Martinez et al, 2009; Nelson, 2008; Rivera-Vargas et al, 2006; Sangeetha and Rawal, 2008;).

In this work, there was a great variation in colony morphology among isolates. These variations were not adequate for reliable differentiation between *Colletotrichum* species, since the colony morphology and colour vary due to the influence of environmental factors, composition of media and the existence of intermediate forms (Freeman et al, 1998). Although, colony morphology and colour, conidia shape and size are unsatisfactory criteria to identify species of *Colletortrichum* (Freeman et al, 1998), they are still widely used to reach to the species level in combination with other advanced molecular tools (Photita et al, 2005; Than et al, 2008). Indeed, there was no attempt to classify *Colletotrichum* isolates into distinct species by molecular methods, since anthracnose disease seemed to be a secondary problem comparing to dieback and decline disease observed in the field. Further identification studies needed to clarify the taxonomic placements of such fungi.

Significant variability in pathogenicity was observed among *Colletotrichum* isolates obtained from different plant parts as well as from different locations. However, some isolates were weak pathogenic to detached mango leaves; other isolates were significantly more pathogenic and produced greater large lesion diameters. In this study, *Colletotrichum* isolates obtained from branches were weak pathogens to detached leaves whereas those obtained from leaves were more aggressive. Similar results were obtained in a study of Ureña-Padilla et al, (2001), who also reported that *C. acutatum* isolated from diseased fruit was unable to induce symptoms when inoculated on strawberry crown tissue. Furthermore, Garrido et al, (2008) also found that *C. gloeosporioides* strains isolated from infected plant material were not pathogenic when re-inoculated onto fruit or leaf tissue.

Grey leaf spot disease symptoms were frequently observed on the old leaves, branches, and panicles. *Pestalotiopsis* spp. was consistently isolated from samples manifesting distinct symptoms of grey leaf spot disease and from asymptomatic bark samples. The disease caused by several *Pestalotiopsis* spp. and it was difficult to distinguish the differences in symptoms associated with the isolated species in the field. In a study by Keith et al (2006), McQuilken, and Hopkins (2004), several species of *Pestalotiopsis* were not host-specific and caused similar symptoms on various plant organs. Furthermore, Taguchi et al (2001), has demonstrated through pathogenicity experiments and host range studies that

Pestalotiopsis spp. are generally non host-specific and can induce diseases on several host plants.

In this study, the sources of inoculum were clearly unknown. Grasses and other herbal plants were intensively grown under mango trees and few other fruit trees were in close proximity to mango trees, suggesting that they serve as sources of infection. In a study by Keith et al, (2006), *Pestalotiopsis* spp. were commonly isolated from wild guava, tea that were close to Guava fields thus, could serve as sources of inoculum. It was difficult to separate *Pestalotiopsis* spp. up to species based on conidia and colony morphology since these characteristics vary within isolates and even within isolates of the same species especially after frequently culturing on media. Thus, it is difficult to use them as reliable character for taxonomic and classification at the species level (Karakaya, 2001; Keith et al, 2006). This was our case of study all isolates were very similar in terms of the morphological structures and cultures of all isolates were initially white to creamy and the colour become darker with age. Furthermore, most of the *Pestalotiopsis* isolates produced black acervuli in media and their amount varied. Therefore, the case of our study and the past cases provide good evidence that morphological criteria solely are insufficient to distinguish species complex of *Pestalotiopsis* (Keith et al, 2006).

Koch's postulates were fulfilled: all isolates were proven to be pathogenic to detached mango leaves with high significance $P<0.05$ variations in infection degree. Previous inoculation experiments and host range studies have indicated that *Pestalotiopsis* spp. generally are not host-specific and can infect a wide range of plants (Steyaert, 1953; Suto and Kobayashi, 1993; Taguchi et al, 2001). Likewise, in a study of Hopkins and McQuilken, (2000), pathogenicity and host range tests indicated that isolates of *P. sydowiana* (Bres.) were not host specific and could infect other ornamental plants than their original hosts. In our study, six isolates of *Pestalotiopsis* spp. were co-isolated as saprophytes from decayed bark infected with *Armillaria* spp. This finding is in agreement with those of Metz et al, (2000) who has isolated *P. microspora* (Speg.) Batista & Peres as a saprophyte from bark and decaying plant material and as endophyte from stem leaves flowers and fruit of tropical and subtropical rainforest plants.

CHAPTER .3

MORPHOLOGY AND PHYLOGENY OF *BOTRYOSPHAERIA* SPP. ASSOCIATED WITH MANGO DIEBACK IN ITALY

INTRODUCTION

More than 2000 anamorphs have been linked to *Botryosphaeria*, of which include *Botryodiplodia*, *Diplodia*, *Dothiorella*, *Fusicoccum*, *Lasiodiplodia*, *Macrophoma* and *Sphaeropsis* are the most common (Denman et al, 2000). The current taxonomical situation of the *Botryosphaeria* anamorphic forms belongs to this family is still unclear, due to the overlapping of the morphological characteristics (Mohali et al, 2007). Therefore, the present several synonyms can express and reflect the confusion exist over their taxonomic and phylogenetic status for many years (Cardoso and Wilkinson, 2008). However, these morphological characters of the *Botryosphaeria* anamorphs are still considered as useful criterion for identification of these fungi that include: conidial size, shape, colour and septation (Deneman et al, 2000; Pennycook and Samuels, 1985).

Despite of conidia characteristics are often variable within species and change with age of the conidia (Pennycook and Samuels, 1985) but, these features can play an important role in the separation and placement of such species up to genus level (Taylor et al, 2009). Recently,various DNA molecular tools such as (RAPD) (single sequence repeat markers SSR), amplification and sequencing of different gene regions have been extensively used I n combination with morphological characteristics to overcome the misidentification and clarifying the taxonomic confusion of *Botrosphaeria* species (Burgess et al, 2006; Phillips et al, 2002; Slippers and Wingfield, 2007; Slippers et al, 2004a,b).

The aim of this study was to characterise *Botryosphaeria* spp. associated with mango dieback in Italy. Combination between morphological and molecular data used to compare isolates obtained from this study with those reported worldwide on mango and other hosts. Sequence data of ITS and TEF-1α regions was used for molecular analysis.

MATERIALS AND METHODS

Fungal isolation and incidence

The isolation carried out as previously described in (Chapter 2) from the symptomatic plant organs that displayed different types of symptoms attributed to Botryosphaeriaceae. The putative isolates resembling the morphology of Botryosphaeriaceae typically have fluffy, fast-growing, white turning olive green–grey to dark grey mycelium and grey to dark blue pigmentation on the reverse side of Petri dishes within a few days (Taylor et al, 2009; Slippers and Wingfield, 2007), were sub-cultured and maintained on PDA at 25±2°C for further characterisation . The previous characteristics used for initially separation of these taxa from other several isolated fungi. Incidence of isolated *Botryosphaeria* spp. was recorded according to dominant species, infected plant organs and locality. *Botryosphaeria* isolates obtained in this study have been deposited in the culture collection of the Department of Agri-Food and Environmental Managment Systems, University of Catania. Isolates used for morphological and molecular characterisation have been deposited in the culture collection of CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands.

Morphological characterisation

To induce sporulation, a plug of mycelium was removed by a cork borer (6-mm diam) from representative putative *Botryosphaeria* isolates and plated on 2% Water agar medium (WA: 20g agar/L) supplemented with sterilized pine needles. The plates were incubated at 25 ± 2°C under near Ultra violet (UV) light for up to 2-3 weeks (Jacobs, 2002; Pavlic et al, 2008). The plates were observed weekly for the formation of pycnidia on the needles. Morphological features of conidia (shape, septation, content, colour, wall thickness and ornamentation) were recorded as well as conidia size (length and width) of 50 conidia of each representative isolate, while for the other structures measurments were made for at least 30 at 100X magnification of light microscope (Nikon Eclipse 80*i* - Germany). Conidia and the other structures were mounted in 100% lactic acid and photographed using Nikon (DXM 1200) digital camera. Vertical sections through pycnidia were made using a Leica microtome (CM 1100) and mounted in lactic acid. Measurements are given as the extremes (minimum and maximum) in brackets and the mean of overall measurements minus or plus the standard

deviation (SD). Colony characteristics and mycelium appearance were described according to Crous et al (2009) and the colour in the media (on the front and reverse sides) of Petri dishes after 7-days on PDA in the dark at 25°C were described according to Rayner (1970).

Temperatures effect on the mycelial growth of *Neofusicoccum* spp.

Isolates selected for morphological characterisation were also used to determine the temperature effect on the colony growth. Three plates for each isolate were inoculated with 6-mm plugs from the actively margins of 5-days-old cultures in the center of the 85-mm PDA Petri-dishes and incubated at 6 different temperatures (10, 15, 20, 25, 30 and 35° C) in the dark. Colony diameters were determined after 1, 2, 3 and 4-days and the data were converted to radial growth in millimeters. The trial was repeated once and the original data were used without any modifications.

Data analysis

Statistical analysis was performed in order to determine the Mean size ± SD (standard deviation) of length and width of 50 conidia using one- way-ANOVA. Variations in colony diameters were recorded after subjecting data to one-way-ANOVA at confidence level 95% and $P<0.05$. Mean values of the colony diameters (in mm) were compared by using the Least Significant Difference (LSD) test ($P< 0.05$) (Stat Soft, Inc. 2004).

Molecular characterisation

DNA isolation

Approximately 50 mg of fungal mycelium from each isolate was scraped using sterile blade from the surface of 8-10 d-old cultures and transferred into extraction eppendorf tubes (Burgess et al, 2006). Total DNA extraction from all *Botryosphaeria* isolates was done using Ultraclean™ Microbial DNA Isolation Kit (MO-BIO Laboratories, Inc, Carlsbad, USA) following the manufacturer's protocol.

PCR amplification

After the DNA extraction, two loci were amplified. The 1st loci; amplification of the internal transcribed spacer region (ITS) of the rRNA including the 3' end of 18S (small subunit), the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second internal transcribed spacer (ITS2) and the 5' end of 28S large subunit (LSU) RNA region using primer pairs V9G-(F) and ITS4-(R) (Table 2). The 2rd loci: amplification of part of the translation elongation factor 1-alpha gene region (TEF-1α) using primers EF1-728 (F) and EF2 (R) (Table 2). For some *Botryosphaeria* isolates the amplification with the EF1-728 (F) and EF2 (R) was almost impossible thus, new set of primers EF1-688F and EF1-1251R designed by Alves et al, (2008) was used for both PCR amplification and sequencing (Table 2). Each PCR reaction contained final concentrations of 0.5 U/µL of *Taq* DNA polymerase (BIOLINE, San Diego, USA), 1X buffer (BIOLINE, San Diego, USA) and 2-2.5 mM of Mg CL_2 (BIOLINE, San Diego, USA), 0.4-0.6 mM of each dNTP's and 0.12-0.2 µm of each primer made up to a final volume of 12.5 µL with sterile deionised water. PCR was conducted in Bio-Rad iCycler thermal cycler. PCR conditions included the following steps: an initial step of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at (95°C for 30 sec), annealing at (52°C for 30 sec) and elongation step at (72°C for 1 min) and ended with a final elongation step at 72°C for 7 min.

PCR products were separated in 1% (w/v) agarose gels (Ultra Pure™ agarose, Invitrogen, Spain) in 1 × Tris-Acetate-EDTA (TAE) buffer. To visualize the DNA, 3µL of GelRed™ nucleic acid stain (Biotium, Inc. Hayward, USA) contained loading buffer was added to PCR product. Molecular weight of PCR amplicons were estimated using 1 Kbp ladder (Bioline, Life Science ® Company, San Diego Zoo, USA.)

DNA sequencing and phylogentic analysis

The amplified fragments of the ITS gene region were sequenced in both directions using internal primers ITS 5 (White et al, 1990) and ITS4 , whereas the TEF-1α gene region was sequenced in both directions using the same primers pairs for amplification. Sequencing reactions were performed using Big Dye terminator sequencing kit v. 3.1 (Perkin-Elmer Applied Bio Systems, Foster City, CA, USA) following the manufacturer's instructions and run in ABI PRISM™

3100 DNA automated sequencer (Perkin-Elmer Applied BioSystems, Foster City, CA, USA).

The sequences generated in this study (Table .4) together with other sequences obtained from GenBank (Table .3) were aligned using MAFFT v. 6.0 (Katoh et al, 2005). The aligned sequences were manually checked and corrected where necessary. Nucleotide substitution models were determined for each gene region using MrModelTest v.2.2 (Nylander, 2004). The model HKY+G was selected in MrModelTest 2.2 for both ITS and TEF sequence data sets. Sequences for each gene region were individually analysed for conflict using 70% reciprocal NJ (Neigbour-Joining) bootstrap analysis and the topology of the resulted trees were compared visually for inconsistency (Gueidan et al, 2007).

Bayesian analyses were performed using MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003) using the Markov Chain Monte Carlo (MCMC) (Larget and Simon, 1999) algorithm to generate trees with Bayesian probability values. Four chains were run simultaneously started from random tree topology and ended at 1,000,000 generations, and trees were saved every 100 generation. The burn in value was graphically estimated from the likelihood scores and therefore, the first 1000 trees were discarded from the analysis as the burn-in phase. The consensus tree was constructed from the remaining 9001 trees using TreeView (Page 1996). Trees were rooted using *Guignardia mangiferae* (CBS115051) and *G. citricarpa* (CBS102374) (Table .3).

All the sequence datasets were also analysed to determine possible phylogenetic relationship among taxa using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2001). Maximum parsimony (MP) tests were conducted using the heuristic research option with random stepwise addition in 1,000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithms, and random taxon addition sequences for the construction of maximum parsimony trees. Branches of zero length were collapsed and all multiple equally parsimonious trees were saved. MAXTREES was set to 10000. In the analysis, all characters were unordered and had equal weight; gaps were treated as missing data. Measures calculated for parsimony included tree length (TL), consistency index (CI), rescaled consistency index (RC), retention index (RI)

and the homoplasy index (HI). Bootstrap support values were evaluated using 1000 bootstrap replicates (Hillis and Bull, 1993).

Table 2 Degenerate Oligonucleotide primers used for PCR amplification and sequencing

Gene region	Primer name	Primer sequence	References
ITS	V9G	5'-TTAAGTCCCTGCCCTTTGTA-3'	de Hoog & Gerrits van den Ende, 1998
	ITS4	5´TCCTCCGCTTATTGATATGC 3´	White et al, 1990
	ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	White et al, 1990
TEF-1α	EF2	5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'	O'Donnell et al, 1998
	EF1-728	5'-CATCGAGAAGTTCGAGAAGG-3'	Carbone and Kohn, 1999
	EF1-688	5'-CGGTCACTTGATCTACAAGTGC-3'	Alves et al, 2008
	EF1-1251	5'-CCTCGAACTCACCAGTACCG-3'	Alves et al, 2008

Table 3 *Botryosphaeria* isolates retrieved from GenBank used for phylogenetic analysis.

Species Identity	Culture No.	Location	Host	Collector	Gen bank accession No.	
					ITS	TEF-1α
Neofusicoccum parvum	CMW27135	China	*Eucalyptus* sp.	M.J. Wingfield	HQ332205	HQ332221
N. parvum	CMW27125	China	*Eucalyptus* sp.	M.J. Wingfield	HQ332204	HQ332220
N. parvum	CMW24704	China	*Eucalyptus* sp.	M.J. Wingfield & X.D. Zhou	HQ332201	HQ332217
N. parvum	CMW27110	China	*Eucalyptus* sp.	M.J. Wingfield	HQ332202	HQ332218
N. parvum	CMW27111	China	*Eucalyptus* sp.	M.J. Wingfield	HQ332203	HQ332219
N. parvum	CMW994 TYPE*	New Zealand	*Malus sylvestris*	B. Slippers	AF243395	AY236883
N australe	CMW6837 TYPE	Australia	*Acacia* sp.	B. Slippers	AY339262	AY339270
N. australe	Y264-21-1	Spain	*Vitis vinifera*	M.T.Martin	JF437920	JF437924
N. australe	UCR1110	USA	*Citrus* sp.	A.O. Adesemoye	JF271758	JF271793
N. australe	UCR1099	USA	*Citrus* sp.	A.O. Adesemoye	JF271756	JF271791
N. vitifusiforme	STE-U5050	South Africa	*V. vinifera*	J.M van Niekerk	AY343382	AY343344
N. vitifusiforme	STE-U5252 TYPE	South Africa	*V. vinifera*	J.M van Niekerk	AY343383	AY343343
N. vitifusiforme	CAP227	Italy	*Olea europaea*	C. Lazzizera	EF638785	EF638744
N. vitifusiforme	STE-U 5820	South Africa	*Prunus salicina*	U. Damm	EF445347	EF445389
N. viticlavatum	STE-U 5044 TYPE	South Africa	*V. vinifera*	F. Halleen	AY343381	AY343342
N. viticlavatum	STE-U 5041	South Africa	*V. vinifera*	F. Halleen	AY343380	AY343341
N. mangiferae	CMW7024 TYPE	Australia	*Mangifera indica*	G.I. Johnson	AY615186	AY615173
N. mangiferae	CMW7797	Australia	*M. indica*	G.I. Johnson	AY615188	AY615175

Table .3 Continued

Species Identity	Culture No.	Location	Host	Collector	Gen bank accession No.	
					ITS	TEF-1α
N. luteum	CBS110299 TYPE	Portugal	*V. vinifera*	A.J.L Phillips	AY259091	AY573217
N. luteum	CBS110497	Portugal	*V. vinifera*	A.J.L Phillips	EU673311	EU673277
N. luteum	CMW30169	Zambia	*Eugenia* sp.	D. Chungu & J. Roux	FJ747639	FJ871113
N. luteum	CMW30168	Zambia	*Dissotis* sp.	D. Chungu & J. Roux	FJ747638	FJ871112
B. dothidea	CMW9075	New Zealand	*Populus* sp.	G.J. Samuels	AY236950	AY236899
B. dothidea	CMW8000	Switzerland	*Prunus* sp.	B. Slippers	AY236949	AY236898
B. dothidea	CAP232	Palermo-Italy	*O. europaea*	G. Lazzizera	EF638747	EF638727
B. dothidea	CAP233	Palermo-Italy	*O. europaea*	G. Lazzizera	EF638748	EF638728
N. umdonicola	CMW14058 TYPE	South Africa	*Syzygium cordatum*	D. Pavlic	EU821904	EU821874
N. ribis	CMW7772 TYPE	USA	*Ribes* sp.	B. Slippers	AY236935	AY236877
N. ribis	CMW7054	USA	*Ribes* sp.	N.E. Stevens	AF241177	AY236879
N. ribis	CBS122553	Panama	*Theobroma cacao*	E. Rojas, L. Mejı´a & Z. Maynard	EU683673	EU683654
N. ribis	CMW7773	USA	*Ribes* sp.	B. Slippers/G. Hdler	AY236936	AY236907
N. cordaticola	CMW13992 TYPE	South Africa	*S. cordatum*	D. Pavlic	EU821898	EU821868
N. kwambonambiense	CMW14023 TYPE	South Africa	*S. cordatum*	D. Pavlic	EU821900	EU821870
Guignardia mangiferae	CBS 115051	Brazil	*Spondias mombin*	K.F. Rodriques	FJ538325	FJ538383
G. citricarpa	CBS 102374	Brazil	*C. aurantium*	Unknown	FJ538313	FJ538371

Table .3 continued

Species Identity	Culture No.	Location	Host	Collector	Gen bank accession No.	
					ITS	TEF-1α
Neoscytalidium dimidiatum	CBS499.66	Mali	*M. indica*	Unknown	FM211432	EU144063
Ne. dimidiatum	CBS204.33	Egypt	*Prunus* sp.	Unknown	FM211429	EU144064
Ne. novaehollandiae	CBS122072	Australia	*Adansonia gibbosa*	Unknown	EF585535	EF585581
Ne. novaehollandiae	CBS122610	Australia	*A. synchronicia*	Unknown	EF585536	EF585578
Pseudofusicoccum adansoniae	CBS122053	Australia	*A. synchronicia*	Unknown	EF585525	EF585569
P. adansoniae	CBS122054	Australia	*Eucalyptus* sp.	Unknown	EF585532	EF585570
P. kimberleyense	CBS122060	Australia	*A. gibbosa*	Unknown	EU144058	E U144073
P. kimberleyense	CBS122061	Australia	*Ficus opposita*	Unknown	EU144059	EU144074
N. eucalypticola	CMW6539	Australia	*E. grandis*	Unknown	AY615141	AY615133
N. eucalypticola	CMW6543	Australia	*Eucalyptus* sp.	Unknown	AY615140	AY615132
L. theobromae	CBS112874	South Africa	*V. vinifera*	F. Halleen	EF622075	EF622055
L. theobromae	CBS559.70	Unknown	*Zea mays*	H.A. van der Aa	EF622073	EF622053
L. theobromae	CBS111530 TYPE	Unknown	Unknown	Unknown	EF622074	EF622054
L. theobromae	CMW24702	China	*Eucalyptus* sp.	M.J. Wingfield & X.D. Zhou	HQ332194	HQ332210
L. theobromae	CMW24701	China	*Eucalyptus* sp.	M.J. Wingfield & X.D. Zhou	HQ332193	HQ332209
L. theobromae	MUCC709	Australia	*Lysiphyllum cunninghamii*	M.L Sakalidis	GU199367	GU199393
L. pseudotheobromae	CBS116459 TYPE	Costa Rica	*Gmelina arborea*	J. Carranza-Velásquez	EF622077	EF622057
L. pseudotheobromae	CMW24700	China	*Eucalyptus* sp.	M.J. Wingfield & X.D. Zhou	HQ332192	HQ332208
L. pseudotheobromae	CMW24699	China	*Eucalyptus* sp.	M. J. Wingfield & X.D. Zhou	HQ332191	HQ332207

Table .3 continued

Species Identity	Culture No.	Location	Host	Collector	Gen bank accession No.	
					ITS	TEF-1α
L. pseudotheobromae	CMW22667	South Africa	*Pterocarpus angolensis*	J Mehl & J. Roux	FJ888471	FJ888449
L. pseudotheobromae	CBS121773	Namibia	*A. mellifera*	F.J.J.,van der Walt	EU 101311	EU 101356
L. pseudotheobromae	IRAN1518C	Iran	*Citrus* sp.	J. Abdollahzadeh & A. Javadi	GU 973874	GU 973866
L. crassispora	CMW13488 TYPE	Venezuela	*E. urophylla*	S. Mohali	DQ 103552	DQ 103559
L. crassispora	WAC12533	Australia	*S. album*	T.I Burgess/B. Dell	DQ 103550	DQ 103557
L. crassispora	WAC12534	Australia	*S. album*	T.I Burgess/B. Dell	DQ 103551	DQ 103558
L. rubropurpurea	WAC12536 TYPE	Queensland	*E. grandis*	T.I Burgess/G. Pegg	DQ 103554	DQ 103572
L. rubropurpurea	WAC12537	Queensland	*E. grandis*	T.I Burgess/G. Pegg	DQ 103555	DQ 103573
L. rubropurpurea	WAC12535	Queensland	*E. grandis*	T.I Burgess/G. Pegg	DQ 103553	DQ 103571
L. venezuelensis	CMW13513 TYPE	Venezuela	*A. mangium*	S. Mohali	DQ 103549	DQ 103570
L. venezuelensis	WAC12540	Venezuela	*A. mangium*	S. Mohali	DQ 103548	DQ 103569
L. gonubiensis	CBS115812 TYPE	South Africa	*S. cordatum*	D. Pavlic	DQ 458892	DQ 458877
L. gonubiensis	CMW14078	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ 103567
L. parva	CBS356.59	Sri Lanka	*T. cacao*	A. Riggenbach	EF 622082	EF 622062
L. parva	CBS494.78	Colombia	Cassava-field soil	O. Rangel	EF 622084	EF 622064
L. plurivora	STE-U 4583 TYPE	South Africa	*V. vinifera*	Halleen	AY 343482	EF 445396
L. plurivora	STE-U 5803	South Africa	*Peunus salicina*	U. Damm F.	EF 445362	EF 445395
L. citricola	IRAN1521C	Iran	*Citrus* sp.	A. Shekari	GU 945353	GU945339

Table .3 continued

Species Identity	Culture No.	Location	Host	Collector	Gen bank accession No.	
					ITS	TEF-1α
L. citricola	IRAN1522C	Iran	*Citrus* sp.	J. Abdollahzadeh & A. Javadi	GU 945354	GU 945340
L. gilanensis	IRAN1501C	Iran	Unknown	J. Abdollahzadeh & A. Javadi	GU 945352	GU 945341
L. gilanensis	IRAN1523C	Iran	Unknown	J. Abdollahzadeh & A. Javadi	GU 945351	GU 945342
L. hormozganensis	IRAN1498C	Iran	*M. indica*	J. Abdollahzadeh & A. Javadi	GU 945356	GU 945344
L. hormozganensis	IRAN1500C	Iran	*Olea* sp.	J. Abdollahzadeh & A. Javadi	GU 945355	GU 945343
L. iraniensis	IRAN921C	Iran	*M. indica*	N. Khezrinejad	GU 945346	GU 945334
L. iraniensis	IRAN1517C	Iran	*Citrus* sp.	J. Abdollahzadeh & A. Javadi	GU 945349	GU 945337
Diplodia mutila	CBS 112553 TYPE	Portugal	*V. vinifera*	A.J.L Phillips	AY 259093	AY 573219
D. mutila	CBS230.30	USA	*Phoenix dactylifera*	L.L. Huillier	DQ 458886	DQ 458869
D. corticola	CBS 112549 TYPE	Portugal	*Quercus suber*	A . Alves	AY 259100	AY 573227
D. corticola	CBS 112545	Spain	*Q. suber*	M.E Sanchez & A. Trapero	AY259089	AY573226
D. corticola	CBS 112546	Spain	*Q. ilex*	M. E. Sa´nchez, A. Trapero	AY259090	EU673310
Diplodia sp	CBS124134	Unknown	Unknown	A.J.L. Phillips	HM036528	GQ923851
Diplodia sp	CAA147	Unknown	Unknown	A.J.L. Phillips	GQ923857	GQ923825

Culture collections: CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria; CBS 5 Centraal Bureau voor Scimmelcultures, Utrecht, Netherlands; CAA= A. Alves, Universidade de Aveiro, Portugal; STE-U= Department of Plant Pathology, University of Stellenbosch, South Africa; CAP= culture collection of A.J.L. Phillips, Lisbon, Portugal. WAC= Department of Agriculture Western Australia, Plant Pathogen Collection.

Table 4 *Neofusicoccum* and *Lasiodiplodia* isolates obtained in this study from mango in Italy and Egypt

Species Identity	Culture No.	Location	Host	Collector	GenBank accession No.	
					ITS	TEF-1α
N. parvum	N-F1	Italy	*M. indica*	A.M.Ismail	JN814429	JN814472
N. parvum	N-F5	Italy	*M. indica*	A.M.Ismail	JN814430	JN814473
N. parvum	NF-13	Italy	*M. indica*	A.M.Ismail	JN814431	JN814474
N. parvum	NF-24	Italy	*M. indica*	A.M.Ismail	JN814432	JN814475
N. parvum	NF-36	Italy	*M. indica*	A.M.Ismail	JN814433	JN814476
N. parvum	NF-38	Italy	*M. indica*	A.M.Ismail	JN814434	JN814477
N. parvum	NF-85	Italy	*M. indica*	A.M.Ismail	JN814435	JN814478
N. parvum	NF-33	Italy	*M. indica*	A.M.Ismail	JN814436	JN814479
N. parvum	NF-34	Italy	*M. indica*	A.M.Ismail	JN814437	JN814480
N. parvum	NF-40	Italy	*M. indica*	A.M.Ismail	JN814438	JN814481
N. parvum	NF-60	Italy	*M. indica*	A.M.Ismail	JN814439	JN814482
N. parvum	NF-44	Italy	*M. indica*	A.M.Ismail	JN814440	JN814483
N. parvum	NF-66	Italy	*M. indica*	A.M.Ismail	JN814441	JN814484
N. parvum	NF-37	Italy	*M. indica*	A.M.Ismail	N814442	JN814485
N. parvum	NF-84	Italy	*M. indica*	A.M.Ismail	JN814443	JN814486
N. parvum	NF-58	Italy	*M. indica*	A.M.Ismail	JN814444	JN814487
N. parvum	NF-52	Italy	*M. indica*	A.M.Ismail	JN814445	JN814488
N. parvum	NF-51	Italy	*M. indica*	A.M.Ismail	JN814446	JN814489
N. parvum	NF-67	Italy	*M. indica*	A.M.Ismail	JN814447	JN814490
N. parvum	NF-6	Italy	*M. indica*	A.M.Ismail	JN814448	JN814491

Table 4 Continued

Species Identity	Culture No.	Location	Host	Collector	GenBank accession No.	
					ITS	TEF-1α
N. parvum	NF-28	Italy	*M. indica*	A.M.Ismail	JN814449	JN814492
N. parvum	NF-62	Italy	*M. indica*	A.M.Ismail	JN814450	JN814493
N. parvum	NF-4	Italy	*M. indica*	A.M.Ismail	JN814451	JN814494
N. parvum	NF-69	Italy	*M. indica*	A.M.Ismail	JN814452	JN814495
N. parvum	NF-17	Italy	*M. indica*	A.M.Ismail	JN814453	JN814496
N. parvum	NF-10	Italy	*M. indica*	A.M.Ismail	JN814454	JN814497
N. parvum	NF-50	Italy	*M. indica*	A.M.Ismail	JN814455	JN814498
N. parvum	NF-82	Italy	*M. indica*	A.M.Ismail	JN814456	JN814499
N. parvum	NF-29	Italy	*M. indica*	A.M.Ismail	JN814457	JN814500
N. parvum	NF-32	Italy	*M. indica*	A.M.Ismail	JN814458	JN814501
N. parvum	NF-31	Italy	*M. indica*	A.M.Ismail	JN814459	JN814502
N. parvum	NF-20	Italy	*M. indica*	A.M.Ismail	JN814460	JN814503
Neofusicoccum sp.26	NF-26	Italy	*M. indica*	A.M.Ismail	JN814461	JN814504
Neofusicoccum sp. 18	NF-18 TYPE	Italy	*M. indica*	A.M.Ismail	JN814462	JN814505
Neofusicoccum sp. 3	NF-3 TYPE	Italy	*M. indica*	A.M.Ismail	JN814463	JN814506
N. australe	NF-2	Italy	*M. indica*	A.M.Ismail	JN814464	JN814507
N. australe	NF-22	Italy	*M. indica*	A.M.Ismail	JN814465	JN814508
N. australe	NF-70	Italy	*M. indica*	A.M.Ismail	JN814466	JN814509
N. australe	NF-76	Italy	*M. indica*	A.M.Ismail	JN814467	JN814510
N. australe	NF-73	Italy	*M. indica*	A.M.Ismail	JN814468	JN814511
N. australe	NF-77	Italy	*M. indica*	A.M.Ismail	JN814469	JN814512
N. vitifusiforme	NF-71	Italy	*M. indica*	A.M.Ismail	JN814470	JN814513

Table 4 Continued

Species Identity	Culture No.	Location	Host	Collector	GenBank accession No.	
					ITS	TEF-1α
N. vitifusiforme	NF-74	Italy	*M. indica*	A.M.Ismail	JN814471	JN814514
L. pseudotheobromae	BOT-1	Egypt	*M. indica*	A.M.Ismail	JN814375	JN814402
L. pseudotheobromae	BOT-13	Egypt	*M. indica*	A.M.Ismail	JN814377	JN814404
L. pseudotheobromae	BOT-14	Egypt	*M. indica*	A.M.Ismail	JN814378	JN814405
L. pseudotheobromae	BOT-16	Egypt	*M. indica*	A.M.Ismail	JN814379	JN814406
L. pseudotheobromae	BOT-28	Egypt	*M. indica*	A.M.Ismail	JN814380	JN814407
L. pseudotheobromae	BOT-18	Egypt	*M. indica*	A.M.Ismail	JN814381	JN814408
L. pseudotheobromae	BOT-2	Egypt	*M. indica*	A.M.Ismail	JN814382	JN814409
L. pseudotheobromae	BOT-11	Egypt	*M. indica*	A.M.Ismail	JN814383	JN814410
L. pseudotheobromae	BOT-3	Egypt	*M. indica*	A.M.Ismail	JN814384	JN814411
L. pseudotheobromae	BOT-17	Egypt	*M. indica*	A.M.Ismail	JN814385	JN814412
L. pseudotheobromae	BOT-12	Egypt	*M. indica*	A.M.Ismail	JN814386	JN814413
L. pseudotheobromae	BOT-24	Egypt	*M. indica*	A.M.Ismail	JN814387	JN814414
L. pseudotheobromae	BOT-26	Egypt	*M. indica*	A.M.Ismail	JN814388	JN814415
L. pseudotheobromae	BOT-27	Egypt	*M. indica*	A.M.Ismail	JN814389	JN814416
L. pseudotheobromae	BOT-22	Egypt	*M. indica*	A.M.Ismail	JN814390	JN814417
L. pseudotheobromae	BOT-15	Egypt	*M. indica*	A.M.Ismail	JN814391	JN814418
L. pseudotheobromae	BOT-25	Egypt	*M. indica*	A.M.Ismail	JN814393	JN814420
L. pseudotheobromae	BOT-21	Egypt	*M. indica*	A.M.Ismail	JN814394	JN814421
L. theobromae	BOT-5	Egypt	*M. indica*	A.M.Ismail	JN814376	JN814403
L. theobromae	BOT-9	Egypt	*M. indica*	A.M.Ismail	JN814392	JN814419

L. theobromae	BOT-4	Egypt	*M. indica*	A.M.Ismail	JN814395	JN814422
L. theobromae	BOT-7	Egypt	*M. indica*	A.M.Ismail	JN814396	JN814423
L. theobromae	BOT-6	Egypt	*M. indica*	A.M.Ismail	JN814399	JN814426
L. theobromae	BOT-23	Egypt	*M. indica*	A.M.Ismail	JN814400	JN814427
Lasiodiplodia sp. 8	BOT-8 TYPE	Egypt	*M. indica*	A.M.Ismail	JN814398	JN814425
Lasiodiplodia sp. 10	BOT-10 TYPE	Egypt	*M. indica*	A.M.Ismail	JN814397	JN814424
Lasiodiplodia sp. 29	BOT-29	Egypt	*M. indica*	A.M.Ismail	JN814401	JN814428

RESULTS

Isolation and incidence

In total, 78 isolates of Botryosphaeriaceae were recovered from 4 sites (Messina, Catania , Palermo and Ragusa) located in southern region of Italy (Sicily) of which 41 isolates originated from Catania, 27 isolated from Messina, 6 from Palermo and only two isolates from Ragusa (Fig. 17A). The majority of isolates were recovered from twigs (26), branches (19), leaves (18), fruits (9), woods (4) and panicles (2) (Fig. 17B). Four species of Botryosphaeriaceae were isolated of which *N. parvum* was the most dominant species that represented 85.8 % of the collected isolates followed by *N. australe* (7.6%), *N. vitifusiforme* (2.5%) and *Neofusicoccum* spp. (3.8%) (Fig. 17C).

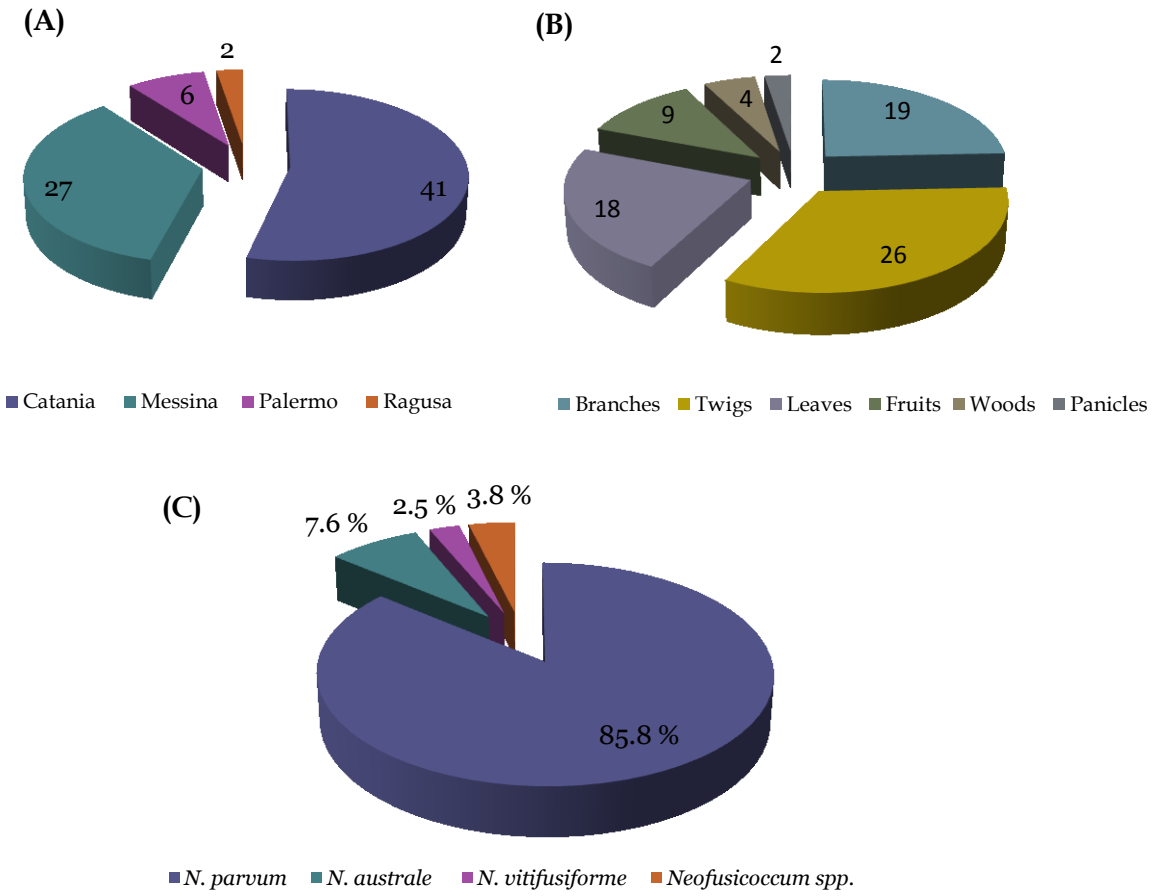


Figure 17 Incidence of *Botryosphaeria* spp. recovered from mango in Italy per locality (A), plant organ (B) and per species (C).

PCR amplification

PCR products of the amplified ITS rDNA region were approximately 700 bp using V9G and ITS4 primer set and amplicons of ~550 bp for TEF-1α amplified by EF1-728 and EF2 primers set, while amplicons of ~700bp were obtained by EF1-688 and EF1-1251 primer set (Fig. 18). The last primers pairs were used for amplification and sequencing with some *Lasiosiplodia* isolates and gave the best sequence results.

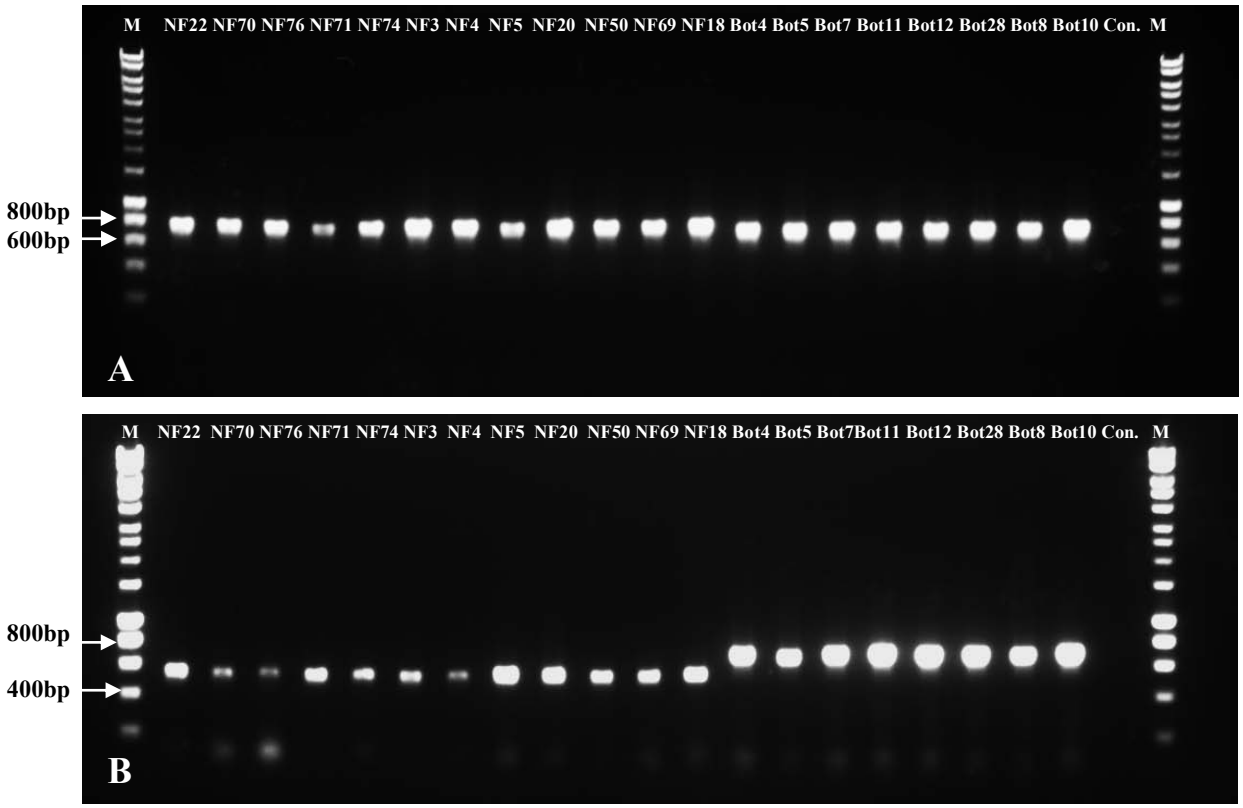


Figure 18 Amplified PCR products of A, ITS regione using V9G and ITS4 primer pairs and B, TEF-1α using EF1-728 and EF2 primers set for the first 12 isolates (from left side of gel B), while the other 6 isolates amplified using EF1-688 and EF1-1251 primers set. The selected isolates representing the nine species of *Botryosphaeria* are indicated across the top of gel. 1-Kb ladder was used and molecular weights are shown on the left of the gel. The isolates were in the same order for both ITS and EF-1 α; M, marker; Lan1, NF-22; Lan2, NF-70; Lan3, NF-76; Lan4, NF-71; Lan5, NF-74; Lan6, NF-3; Lan7, NF4; Lan8, NF-5; Lan9, NF-20; Lan10, NF50; Lan11, NF-69; Lan12, NF18; Lan13, BOT-4; Lan14, BOT-5; Lan15, BOT-7; Lan16, BOT-11; Lan17, BOT-12; Lan18, BOT-28; Lan19, BOT-8; Lan20, BOT-10.

Phylogenetic analysis of *Botryosphaeria* spp.

The 70% reciprocal NJ bootstrap analysis results showed no conflict in tree topology and the data set was congruent. The combined data set of ITS and EF-1α contained 87 taxa of which 43 representative sequences selected from 78 total *Botryosphaeria* isolates were used for phylogenetic analysis with further 44 sequences including the outgroup species retrieved from the GenBank. Approximately 150 characters including gaps from the ambiguous ends for both regions were excluded from each sequence. The combined dataset contained 909 characters of which 536 were constant, whereas 95 variable characters were uninformative and 278 were parasimony informative. Heurisitic search revealed one most parsimonious trees (tree length = 670 steps, CI=0.743, RI=0.917, RC= 0.682 and HI=0.257). The obtained trees differed only in the arrangement of isolates within the lateral clades while their overall topology was the same and were topologicaly identical to the 50% majority-rule consensus tree shown in (Fig. 19).

The combined sequences of ITS and EF-1α representing the three *Neofusicoccum* species were fell in five distinct clades. The first clade contains the majority of *N. parvum* isolates represented by strains NF-20, NF-10, NF69, NF-4, NF62, NF-28, NF-6, NF51, NF-52, NF-58, NF-84, NF-66, NF-60. NF-40, NF-34, NF-33, NF-85, NF-36, NF-24, NF13, NF-5 and NF-1 grouped with *N. parvum/ribis* complex isolates containing *N. parvum* strain CMW994 culture ext-type supported with only bootstrap value 60%. The rest of *N. parvum* isolates formed three sub-clades, of which the first subclade contained isolates NF-38, NF-17, NF67 and NF37 supported with BS value 51% abd BPP value 0.84. The second subclade contained three isolates of *N. parvum* NF-50, NF-31 and NF-32 supported with BS value 52% and BPP value 0.61. While the third subclade contained two isolates of *N. parvum,* NF-82 and NF-29 supported only with bootstrap value 55%. The second clade contained two isolates of *Neofusicoccum* sp. represtend by isolates NF-18 and NF-26 supported only with bootstrap value 68%. *N. australe* isolates were grouped in the third clade supported with BS value 69% and BPP value 0.91. Two isolates represented by strains UCR 1099 and UCR 1110 formed a well subclade supported with BS value 91% and BPP value 0.99. Four isolates of *N. australe* represented by NF-70, NF-73, NF-76 and NF-77 clustered with strain Y-264-21-1 supported with BS/BPP values (52%/0.71), while the other two isolates

NF-2 and NF-22 of *N. australe* formed a well-supported separate subcluster supported with with BS/BPP values 77%/1.0. The fourth clade contained isolates of *N. vitifusiforme* supported with bootstrap value 52%, of which the only two isolates (NF-71 and NF-74) obtained in this study formed a well-supported subclade with BS/BPP values (94%/1.0). The second subclade contained strains STEU-5050 and STEU-5252 supported with BS/BPP values (95%/1.0). The fifth clade contained only a single isolate *Neofusicoccum* sp. NF-3 with no bootstrap value.

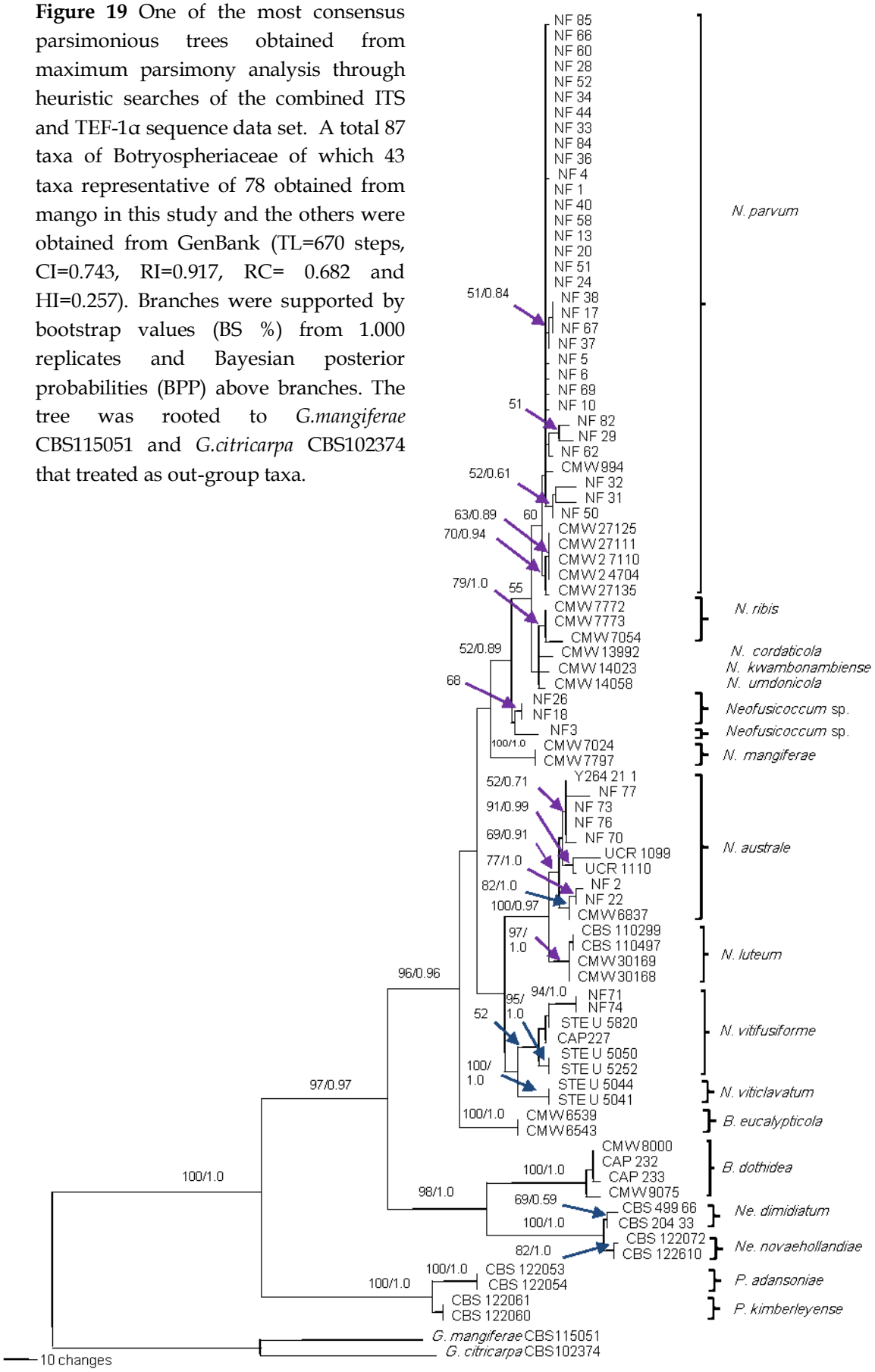


Figure 19 One of the most consensus parsimonious trees obtained from maximum parsimony analysis through heuristic searches of the combined ITS and TEF-1α sequence data set. A total 87 taxa of Botryospheriaceae of which 43 taxa representative of 78 obtained from mango in this study and the others were obtained from GenBank (TL=670 steps, CI=0.743, RI=0.917, RC= 0.682 and HI=0.257). Branches were supported by bootstrap values (BS %) from 1.000 replicates and Bayesian posterior probabilities (BPP) above branches. The tree was rooted to *G.mangiferae* CBS115051 and *G.citricarpa* CBS102374 that treated as out-group taxa.

TAXONOMY

Neofusicoccum parvum

Conidiomata (Fig. 20a) (formed on WA on pine needles within 10-15 days). Pycnidia were solitary, mostly in aggregates, globose to subglobose covered with dense, grey, hairy mycelium, semi-immersed in the pine needles tissues, become erumpent when mature, mostly multiloculate, dark-grey to black, the outer layers composed of 5-10 thick-walled dark brown *textura angularis* cell layers, followed by hyaline thin-walled cells towards the center of pycnidia (Fig. 20).

Conidiogenous cells (Fig. 20c,d) holoblastic, hyaline, cylindrical, proliferating percurrently several times near the apex with up to 1-2 proliferations, 4.5-19.3 µm long, 1.5-2.7 µm wide. *Conidia* hyaline, thin-walled, contains granular content, smooth, aseptate, ellipsoid, to slightly fusoid, base truncate, slightly tapered at apex, wider in middle, measuring (14.3-) 15.4 – 17.6 (-19.3) × (5.0-) 5.4- 6.2 (-6.6), mean of 50 conidia ± SD = 16.5 ± 1.1 µm long, 5.8 ± 0.4 µm wide, L/W ratio = 2.8.

Cultural characteristics (Fig. 20g, h) colonies with abundant dense convex with papillate surface mycelium mat, occasional columns of aerial mycelium reaching the lid, initially white, becomes glaucous grey (29′′′f) to greenish grey (33′′′i) on the upper surface, greenish grey (33′′′i) on the reverse, the colour becomes darker to be dark slate blue (39′′′k) with age. Colonies reached the edge of the Petri plate 85-mm after 3-days in the dark at 25-30°C. Cardinal temperature requirements for growth; minimum 10°C, maximum 35°C, optimum 25-30°C (Fig. 25).

Teleomorph: *Botryosphaeria parva* (not seen in this study)
Host: *Mangiferae indica*
Known distribution: South Italy-Sicily (Province of Catania)
Specimen examined South Italy, Catania: from twigs showed dieback symptoms 13 Dec 2009, NF-5 = CBS 130995.

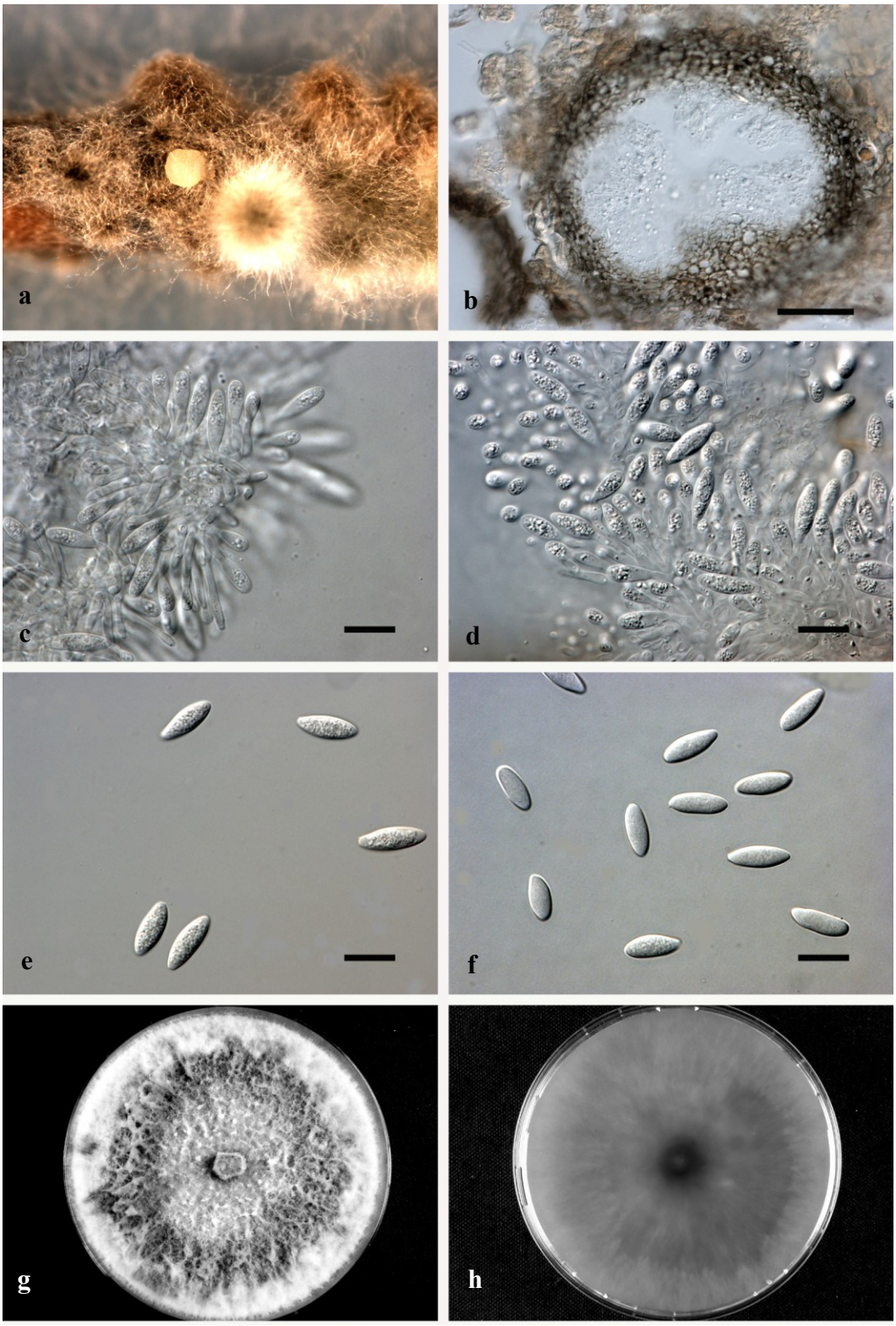


Figure 20 *Neofusicoccum parvum* NF-5 = CBS130995. (a) pycnidia formed on pine needles on WA; (b) vertical section through pycnidia; (c, d) hyaline conidiogenous cells; (e, f) hyaline conidia with granular contents. Colony morphology: (g) front sides; (h) reverse side. - Scale bars; (b) = 20 µm; (c, d, e, f) = 10 µm.

Neofusicoccum vitifusiforme

Conidiomata: (Fig. 21a) (formed on WA on pine needles within 10-15 days). Pycnidia mostly solitary or in aggregates, globose to subglobose covered with dens, grey, hairy mycelium, semi-immersed in the tissues, ultimately become erumpent when mature, uniloculate to multiloculate, dark-grey to black, vertical section through pycnidium showed that the outer layers composed of 4-6 thick-walled dark brown cell layers of *textura angularis* while, the inner cells are hyaline (Fig. 21b).

*Conidiogenous cell*s (Fig. 21c, d) holoblastic, hyaline, subcylindrical, 2-8.9 µm long, 1.9-3.3 µm wide, proliferating percurrently with up to 1-4 annellations or phialidic with minute periclinical thickens. *Conidia* (Fig. 21e, f) initially hyaline, smooth with granular content, thin-walled, fusiform to ellipsoid, slightly widest in the upper third with subtruncuate base and flattened to obtuse apex, becoming septate with 1-2 septum when mature, (-17.6) 19.2-21.8 (-23.6) × (4-) 5.1-6.1 (-6.9) mean of 50 conidia ± SD = 20.5 ± 1.3 µm long × 5.6 ± 0.5 µm wide, L/W ratio= 3.6.

Cultural characteristics (Fig. 21g, h) Colonies with sparse dense, effuse mycelium mat, initially white, becomes olivaceous buff (21‘‘‘d), to greenish olivaceous (23‘‘‘i) on the upper surface, pistachio green (32‘‘‘i) on the reverse, the colony forms slight concentric circles and the colour becomes dark green from the colony center towards the edge with age. Colonies reached the edge of the Petri plate 85-mm on PDA after 4-days in the dark at 25-30°C. Cardinal temperatures requirements for growth; minimum 10°C, maximum 35°C, optimum 25-30°C (Fig. 25).

Teleomorph: Unknown *Botryosphaeria* sp. (not seen in this study)

Host: *Mangiferae indica*

Known distribution: South Italy (Province of Palermo)

Specimen examined South Italy, Province of Palermo, Partinico-Tobia: isolated from twigs showed dieback symptoms 14 May 2010, NF-74=CBS 130998.

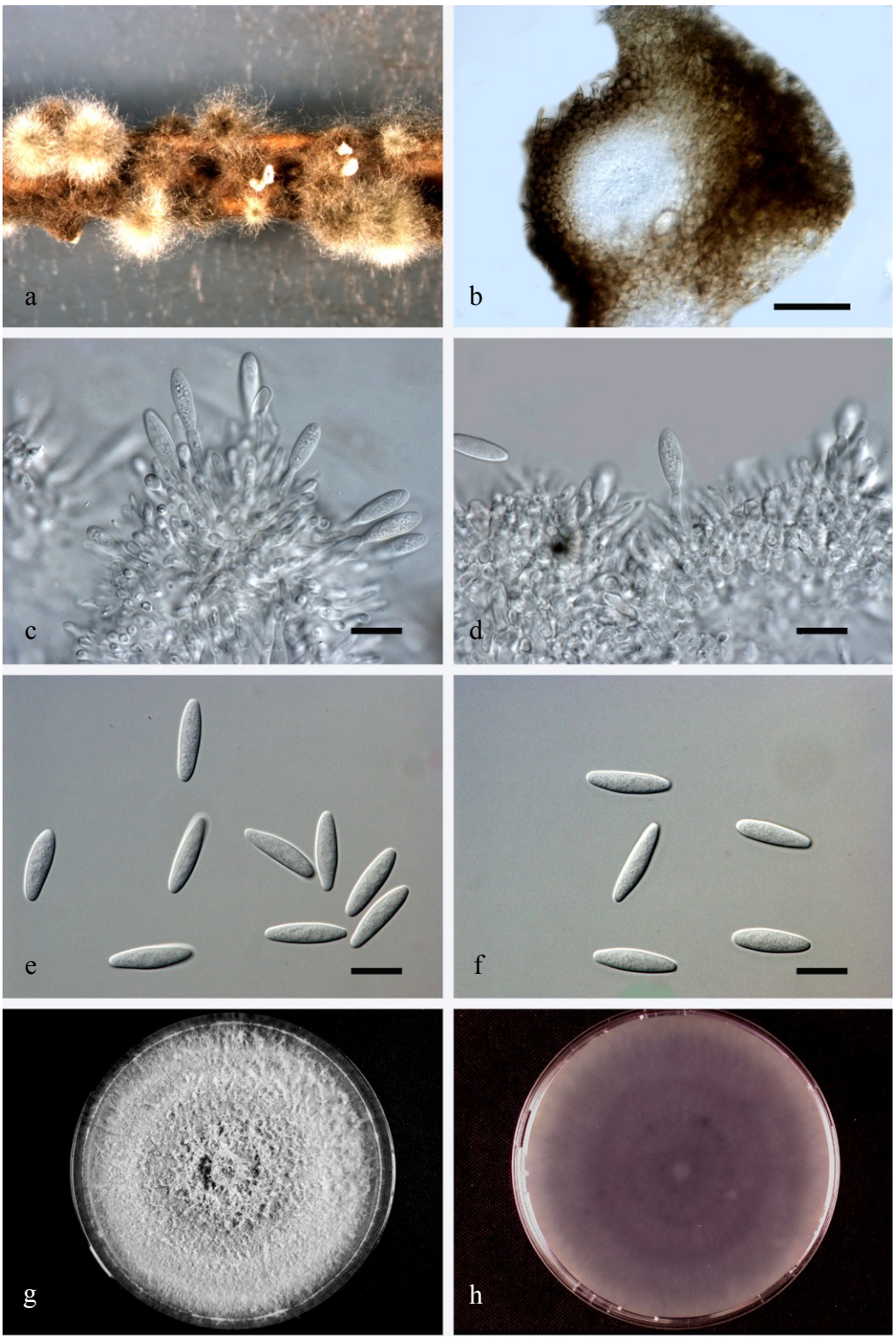


Figure 21 *Neofusicoccum vitifusiforme* NF-74 = CBS 130998; (a) pycnidia formed on pine needles on WA; (b) vertical section through pycnidia; (c, d) conidiogenous cells; (e,f) hyaline fusiform conidia;. Culture morphology: (g) front sides of colony; (h) reverse side. – Scale bars; (b) =20 µm; (c, d, e, f) = 10 µm.

Neofusicoccum australe

Conidiomata (Fig. 22a) (produced on WA on pine needles within 10-20 days), pycnidia solitary, subglobose to ellipsoidal covered with hairy mycelium, embedded in the needle tissues, semi-immersed, unilocular and sometimes multilocular with central ostiole, vertical sections through pycnidia, outer layers consisted of 4-6 dark brown thick walled cells of *textura angularis,* followed by hyaline layers towards the center (Fig. 22b).

Conidiogenous cells (Fig. 22c, d) hyaline, holoblastic, cylindrical to subcylindrical and phialidic, proliferating percurrently with around 1-3 proliferations, 8.7 - 16.2 µm long and 1.8 - 3.7 µm wide. *Conidia* (Fig. 22e) hyaline, thin-walled, smooth, contains granular contents, aspetate, fusiform, with truncate base and flattened apex, widest in the upper third, becomes light brown, forming 1-3 septum before germination (Fig. 22f), measuring (17.3-) 19.7- 22.9 (-24.5) × (4.5-) 5.6 – 6.2 (-6.8) µm, mean of 50 conidia ± SD = 21.3 ± 1.6 µm long, 5.9 ± 0.3 µm wide, L/W ratio = 3.6.

Cultural characteristics (Fig. 22g, h) Colonies with dense convex with papillate surface mycelium mat, with sparse aerial mycelium near the edge, initially white becomes glaucous grey (29‴f) to greenish grey (33‴i) on the front side of Petri dish, dark slate blue (39‴k) on the reverse, the colony colour becomes darker with age. Colonies reached the edge of the Petri plate 85-mm after 4-days in the dark at 25 °C. Cardinal temperatures requirements for growth: minimum 10°C, maximum 35°C, optimum 25°C (Fig. 25).

Teleomorph: *Botryosphaeria australis* (not seen in this study)
Host: *Mangiferae indica*
Known distribution: South Italy-Sicily (Province of Ragusa)
Specimen examined South Italy, Province of Ragusa, Acate-Santa Marina: isolated from twigs showed dieback symptoms 3 May 2010, NF-76=CBS 130997.

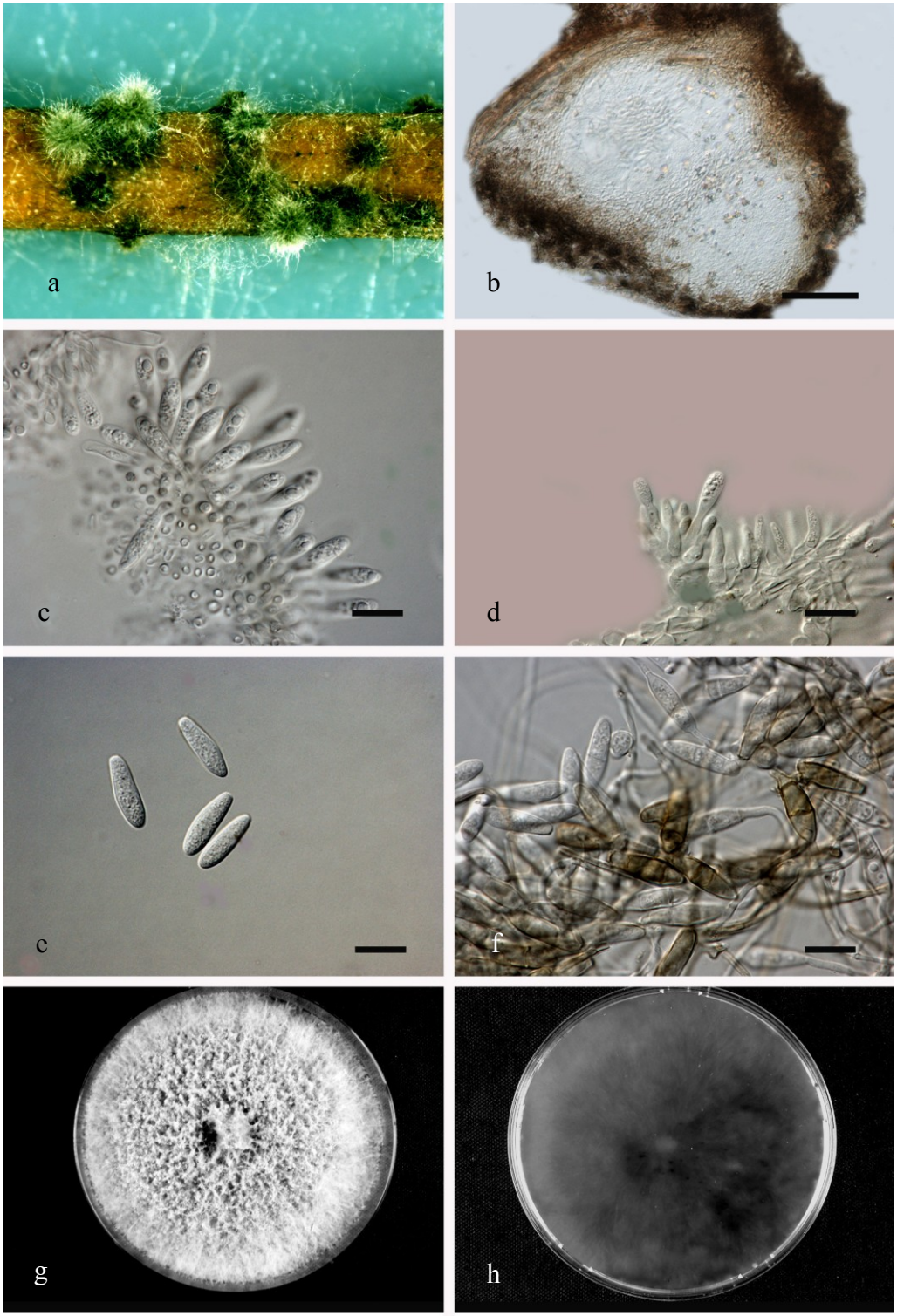


Figure 22 *Neofusicoccum australe* NF-76 = CBS130997. (a) pycnidia formed on pine needles on WA; (b) vertical section through pycnidia; (c, d) hyaline conidiogenous cells; (e) hyaline conidia with granular contents; (f) light brown 1-3 septate conidia before germination. Colony morphology: (g) front side; (h) reverse side. Scale bars; (b) = 20 µm; (c, d, e, f) = 10 µm.

***Neofusicoccum* sp. 3**

Conidiomata (Fig. 23a) (formed on pine needles on WA within 10-15). Pycnidia are solitary or in aggregates, globose to subglobose, covered with dense, dark grey to brown hairy mycelium, semi-immersed in the tissue, sometimes papillate, mostly multilocular, vertical section through pycnidia, the outer layers composed of 4-8 brown thick-walled cells layers of *textura angularis*, followed by several hyaline thin-walled cells towards the center of pycnidia (Fig. 23b).

Conidiogenous cells (Fig. 23c, d) holoblastic, hyaline, cylindrical, somewhat ampulliform and phialidic with periclinical thickening or proliferating percurrently with 1-3 annelations measuring 4.4 - 12.2 µm long and 1.9 - 4.1 µm wide. *Conidia* (Fig.23e,f) hyaline, fusoid to ellipsoid, smooth, thin-walled, aseptate, contain granular contents, tapered at apex or at both sides, base subtruncate, rarely become septate before germination, measuring (17.2-) 18 – 20.1 (-21.7) × (4.5-) 5.9 – 6.7 (-7.3) µm, mean of 50 conidia ± SD = 19 ± 1 µm long, 6.3 ± 0.4 µm wide, L/W ratio = 3.

Cultural characteristics (Fig. 23g, h) Colonies with sparse, appressed mycelium mat, with occasional sparse columns of aerial mycelium in the center of colony reach to cover lid. Initially white becomes lavender grey (45′′′′′f) on the upper surface of colony, leaden grey (45′′′′′i) on the reverse side, the colony colour becomes darker from the center toward the margins with age. Colonies reached the edge of the Petri plate 85-mm after 4-days in the dark at 25°C. Cardinal temperatures requirements for growth: minimum 10°C, maximum 35°C, optimum 25°C (Fig. 25).

Teleomorph: Unknown *Botryosphaeria* sp. (not seen in this study)

Host: *Mangiferae indica*

Known distribution: South Italy-Sicily (Province of Catania)

Specimen examined South Italy-Province of Catania, Catania: isolated from branch showed dieback symptoms 3 Dec 2009, Holotype, NF-3= CBS130993.

Based on the combined ITS and TEF-1α data set, *Neofusicoccum* sp. 3 is phylogeneticaly distinct from the other *Neofusicoccum* species and closely related to *Neofusicoccum* sp. 18. However, in this study *Neofusicoccum* sp. 3, *Neofusicoccum*

sp. 18 and *N. parvum* were reported to produce yellow pigmentation on PDA at 25°C after 2-days, but they can be clearly distinguished based on their conidia dimension as well as L/W ratio. Conidia dimension of *Neofusicoccum* sp. 3 are longer and wider (19 ± 1 µm × 6.3 ± 0.4 µm) than *Neofusicoccum* sp. 18 (15.9 ± 1.4 × 5.7 ± 0.3 µm) and *N. parvum* (16.5 ± 1.1 ×5.8 ± 0.4 µm). The L/W ratio of *Neofusicoccum* sp. 3 (3) is higher than of *N. parvum* (2.8) and *Neofusicoccum* sp. 18 (2.7). Furthermore, the optimum temperature required for *Neofusicoccum* sp. 3 to reach the Petri plate 85-mm after 4-days is 25°C, and exhibited very little growth at 35°C. In contrast, *Neofusicoccum* sp. 18 and *N. parvum* reached the Petri plate 85-mm after 3-days at 25-30°C and they did not exhibit growth at 35°C.

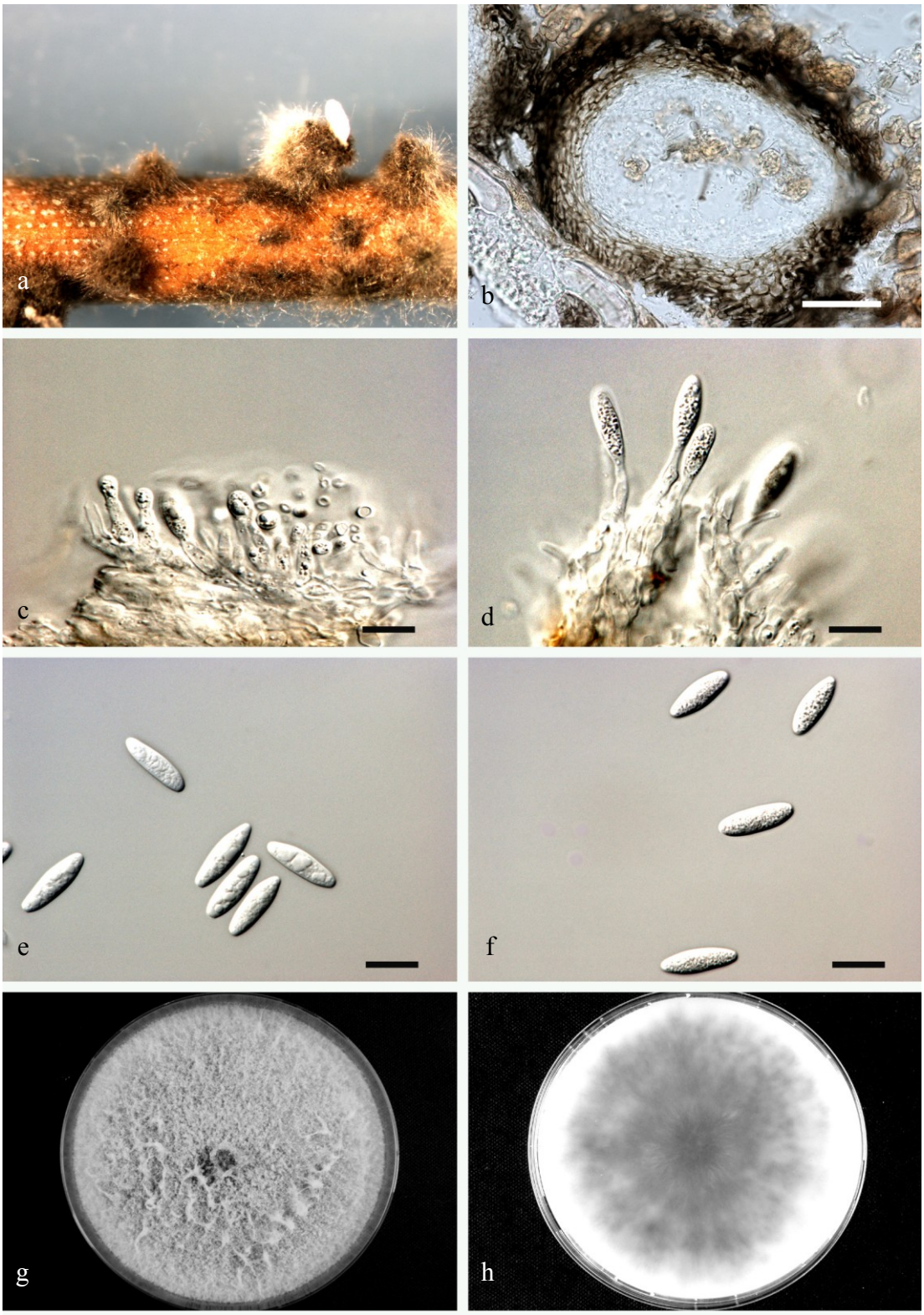


Figure 23 *Neofusicoccum* sp. 3 holotype NF-3 = CBS130993. (a) pycnidia formed on pine needles on WA; (b) vertical section through pycnidia; (c, d) conidiogenous cells; (e, f) hyaline immature conidia. Culture morphology: (g) front sides of colony; (h) reverse side. – Scale bars; (b) =20 µm; (c, d, e, f) = 10 µm.

***Neofusicoccum* sp. 18**

Conidiomata (Fig. 24a) (formed on pine needles on WA within 10-12 days). Pycnidia were solitary, mostly in aggregates, globose to subglobose, covered with dense dark grey hairy mycelium, semi-immersed in the needle tissues, become erumpent when mature oozing conidia, mostly multiloculate, vertical sections through conidiomata showing the outer layers composed of 7-10 dark brown thick-walled cells of *textura angularis*, followed by thin-walled hyaline cells towards the center of pycnidia (Fig. 24b).

Conidiogenous cells (Fig. 24c, d) holoblastic, hyaline, discrete, cylindrical, and proliferating percurrently many times at apex with about 1-4 minute proliferations measuring 8.1-17.6 µm long and 1.6-3.2 µm wide. *Conidia* (Fig.24e,f) hyaline, aseptate, fusiform, sometimes irregularly in shape, thin-walled, smooth, contains granular contents, mostly tapered at both apex and base and sometimes subtruncate at base, much wider in the middle than apex and base measuring (13.1-) 14.5 – 17.3 (-18.8) × (5-) 5.4 – 6 (-6.2) µm. Mean of 50 conidia ± SD = 15.9 ± 1.4 µm long, 5.7 ± 0.3 µm wide, L/W ratio = 2.7.

Cultural characteristics (Fig. 24g, h) Colonies with cottony, abundant dense umbonate mycelium mat, with abundant columns of aerial mycelium reaching the lid, initially white becomes pale olivaceous grey (21‴‴d) on the upper surface, olivaceous grey (21‴‴i) to iron grey (23‴‴k) on the reverse, the colour becomes darker with age. Colonies reached the edge of the Petri plate 85-mm after 3-days in the dark at 25 °C. Cardinal temperatures requirements for growth: minimum 10°C, maximum 35°C, optimum 25 - 30°C (Fig. 25).

Teleomorph: Unknown *Botryosphaeria* sp. (not seen in this study)
Host: *Mangiferae indica*
Known distribution: South Italy-Sicily, Province of Catania, Fiumefreddo
Specimen examined South Italy- Province of Catania, Catania: isolated from fruit showing stem-end rot symptoms 13 Dec 2009, holotype, NF-18 = CBS 130994.

Neofusicoccum sp. 18 (NF-18) is phylogenetically closely related to *Neofusicoccum* sp. 3 but it could be clearly distinguished based on the morphological characters. Conidia dimensions of *Neofusicoccum* sp. 18 are shorter and narrower (15.9 ±1.4 ×

5.7 ± 0.3 µm) than those of *Neofusicoccum* sp. 3 (19 ± 1 × 6.3 ± 0.4 µm), and *Neofusicoccum* sp. 18 have L/W ratio (2.7) lower than *Neofusicoccum* sp. 3 L/W (2.8). Furthermore, conidiogenous cells of *Neofusicoccum* sp. 18 are markedly longer up to 17.6 µm, whereas the conidiogenous cells of *Neofusicoccum* sp. 3 are up to 12.2 µm, and wider at base than apex for *Neofusicoccum* sp. 3, but as the same as apex for *Neofusicoccum* sp. 18. Despite the fact that *Neofusicoccum* sp. 18 is closely related to *N. parvum* in terms of production of yellow pigmentation in culture, having similar culture morphology and the same temperature requirements for growth. but, *Neofusicoccum* sp. 18 is still phylogeneticaly distinct and differ in having shorter conidia length 15.9 ± 1.4 µm than *N. parvum* 16.5 ±1.1 µm, and also having shorter conidiogenous cells 17.6 µm than *N. parvum* 19.3 µm.

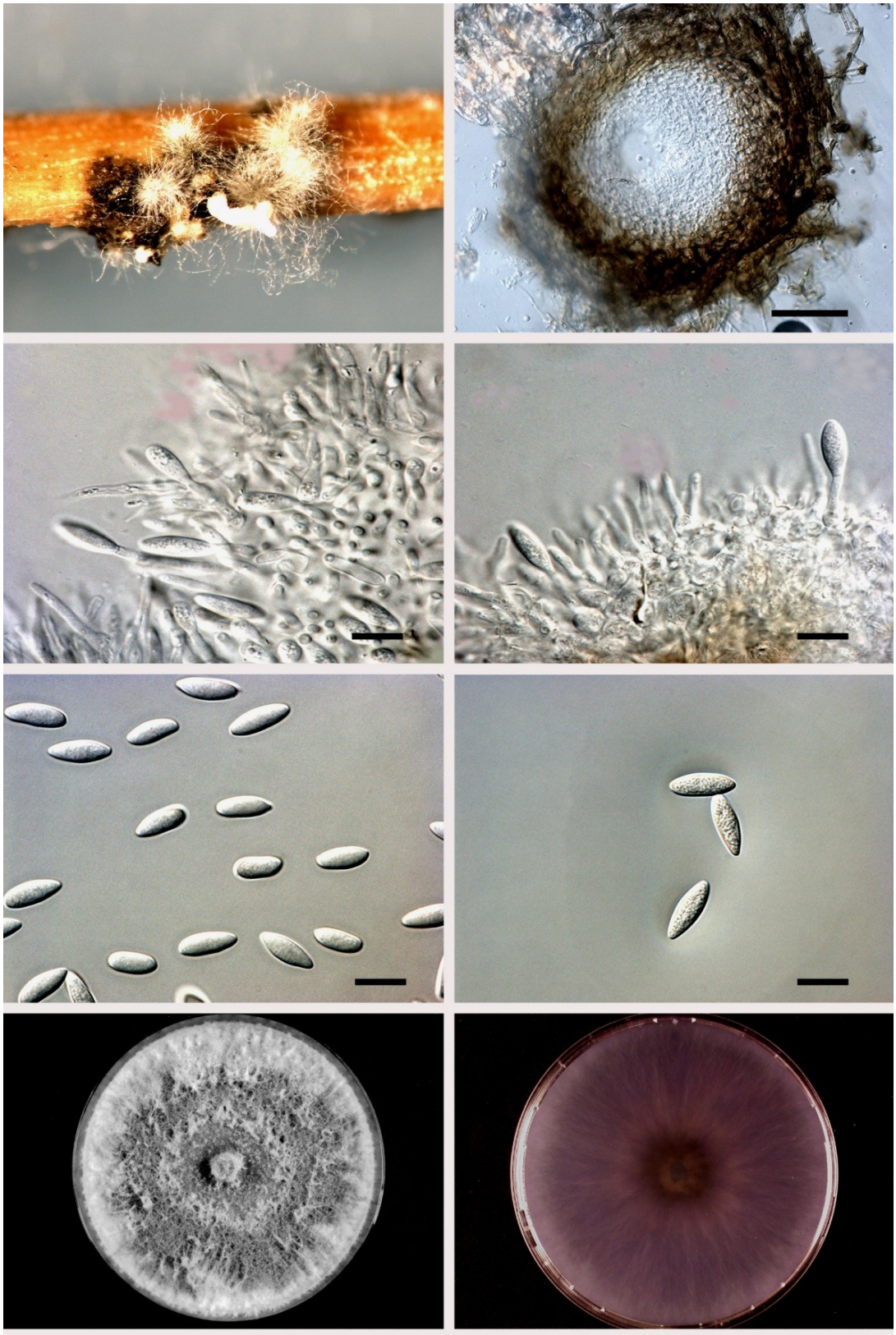


Figure 24 *Neofusicoccum* sp. 18 holotype, NF-18 = CBS 130994. (a) pycnidia formed on pine needles on WA; (b) vertical section through pycnidia; (c, d) conidiogenous cells; (e, f) hyaline immature conidia. Culture morphology: (g) front sides of colony; (h) reverse side. – Scale bars; (b) =20 µm; (c, d, e, f) = 10 µm.

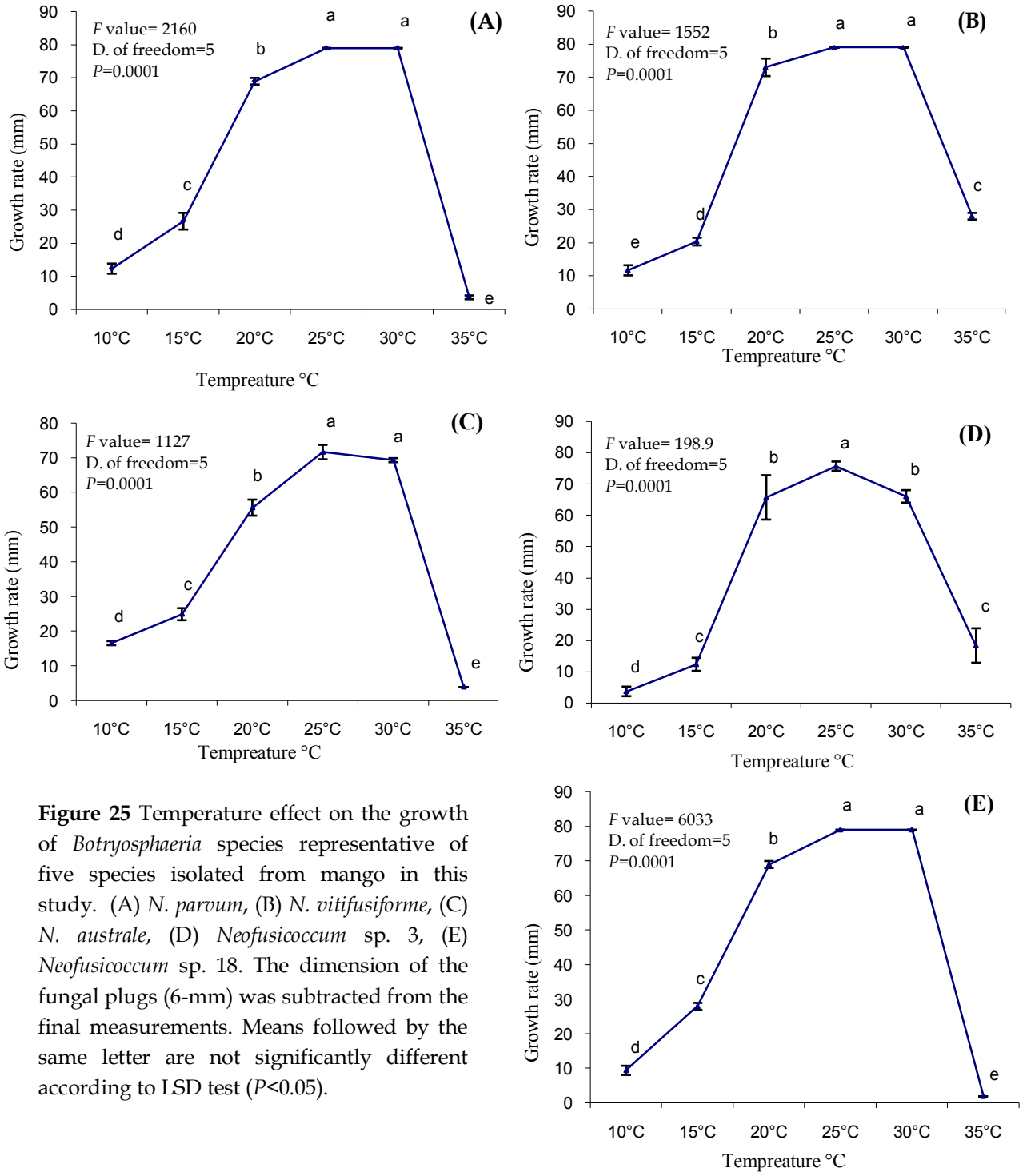


Figure 25 Temperature effect on the growth of *Botryosphaeria* species representative of five species isolated from mango in this study. (A) *N. parvum*, (B) *N. vitifusiforme*, (C) *N. australe*, (D) *Neofusicoccum* sp. 3, (E) *Neofusicoccum* sp. 18. The dimension of the fungal plugs (6-mm) was subtracted from the final measurements. Means followed by the same letter are not significantly different according to LSD test ($P<0.05$).

DISCUSSION

The present study represents the first consideration and attempt to identify possible *Botryosphaeria* spp. associated with *M. indica* in Italy. Five species occurring on mango were identified, of which two are described as new species of *Neofusicoccum* sp. (NF-3 and NF-18). The three well-known species *N. parvum*, *N. australe* and *N. vitifusiforme* were identified based on the combined DNA sequences and morphological features data. These species have been reported worldwide as pathogens of broad range of hosts (Begoude et al, 2009; Damm et al, 2007; Sakalidis et al, 2011; Slippers et al, 2004a; Slippers et al, 2005; van Niekerk et al, 2004). However, this is the first report of theses species on mango in Italy.

Neofusicoccum parvum has been reported on mango in Australia (Johnson, 1992), in South Africa (Jacobs, 2002), in Peru (Javier-Alva et al, 2009) and in Brazil (de Oliveira Costa et al, 2010). This is the first report of *N. parvum* on mango in Italy. In the present study, *N. parvum* was the most commonly isolated species from branches, twigs, leaves, internal wood and fruits exhibited various symptoms patterns and the fungus was associated mainly with dieback symptoms. The fungus found in almost all monitored areas (Catania, Messina and Palermo), but its relative prevalence differed in each location. This specie was previously reported on mango as *D. dominicana* (Johnson, 1992). Later, in a study conducted by Slippers et al, (2005) they have proposed a new name for this species to be *F. parvum* after re-identification based on morphology and multiple gene phylogenies.

In a recent phylogenetic study conducted by Crous et al, (2006), several species of Botryosphaeriaceae with *Fusicoccum,* anamorphs were reduced in a new genus synonym *Neofusicoccum*. Single gene is insufficient for phylogenetic study for definitive distinguishing closely related species of Botryosphaeriaceae such as *N. ribis/parvum* complex and more than one gene should be used (Lazzizera et al, 2008; Slippers et al, 2004a). *N. parvum* was identified in this study on the basis of morphology and combined DNA sequence of ITS and TEF-1α data set. All *N. parvum* strains formed a large clade of which 23 strains were clustered with CMW994 culture ex-type isolated from *Malus sylestris* from New Zeland. The other strains formed three different subclades supported with low bootstrap values. The differences within strains of the same species could be due to genetic

variation, which might attributed to the different environmental conditions in different geographical areas where theses strains were isolated. The conidia morphology did not differ greatly and reassembled those reported in previous studies by several authors (de Oliveira Costa et al, 2010; Jacobs, 2002; Slippers et al, 2004a; Slippers et al, 2005). However, in our study, the septate conidia were not seen as reported by the last two authors. Moreover, it was surprising to observe the yellow pigmentation on PDA after 2-3 days for most of *N. parvum* strains at 25°C in the dark, as this characteristic was reported firstly for *N. luteum* (Phillips et al, 2002) and *N. australe* (Slippers et al, 2004b). Recently, Qiu et al, (2011) reported this characteristic for *N. parvum* isolates associated with grapevine decline in Australia. This finding strongly supported our results that *N. parvum* seemed to have the same characteristic as *N. australe* and *N. luteum*.

Slippers et al (2004b) described *Neofusicoccum australe* and its teleomorph *B. australis* for the first time as new species. In the same study of Slippers et al, (2004b), the re-examination of taxons previously identified as *B. lutea* revealed that *B. australis* occurs on native Australian hosts, namely a *Banksia* sp. and a *Euaclyptus* sp. and from *Protea* sp. in South Africa and on *Pistachio* in Italy. Basing on the known hosts and geographical distribution, the authors claimed that this taxon appears to be native to the southern Hemisphere (Australia). The fungus has been reported to cause diseases on *Prunus* spp. in South Africa (Damm et al, 2007), on olive in Italy (Lazzizera et al, 2008), on grapevine in South Africa (van Niekerk et al, 2004) and on grapevine in Australia (Taylor et al, 2005). This species has never reported on mango worldwide and thus, this is the first report of *N. australe* on mango in Italy and worldwide. *N. australe* was the second dominant species isolated from twigs and branchs of mango showing typical dieback symptoms. The fungus was isolated from only three sites (Ragusa, Palermo and Catania). In our study, *N. australe* isolates produced light yellow pigment in PDA medium after 2-days at 25°C and the colour was distinct in the second day, but no longer seen after 3-days at 25°C. This characteristic was reported firstely for *N. luteum*, but the colour for the later is darker which considered as useful tool to distinguish this taxon from other *Botryosphaeria* spp. (Phillips et al, 2002). Since both *N. luteum* and *N. australe* produce such pigment in the artificial media, the discrimination between them became difficult as they share the same morphological characteristic in culture.

According to several cases of study (Abdollahzadeh et al, 2010; Alves et al, 2008; Pavlic et al, 2008; Slippers et al, 2004b) single gene is insufficient and multiple genes are needed to clarify the diversity and phylogenetic relationship between *Botryosphaeria* spp. especially for cryptic species such as *Lasiodiplodia* spp., *B. ribis*/*B. pava* complex and *B. australis*/*B. luteum*. In the present study, combined sequences of ITS and TEF-1α gene regions were integrated with conidia and culture morphology to descriminate between *Botrysopheria* species associated with mango. Isolates of *N. australe* obtained from mango in our study were risded within strains isolated from *Acacia* sp., *Vitis vinifera* and *Citrus* sp. in the clade of *N. australe* supported with low BS/BPP values. *N. australe* is closely related to *N. vitifusiforme* as it has fusiform shape of conidia, and conidia sizes range are somewhat similar (17.3 – 24.5), (17.6 – 23.6) respectively, but the latter specie does not produce yellow pigment in culture.

Neofusicoccum vitifusiforme was reported and recently described as new species on grapevine in South Africa by van Niekerk et al, (2004) on the basis of DNA sequence of ITS and TEF1-α, morphology and pathology data. The fungus has been recently reported on other hosts: on *Prunus* spp. in South Africa (Damm et al, 2007), on olive in Italy (Lazzizera et al, 2008), on blueberry in China (Kong et al, 2010) but had not been isolated previously from mango in Italy. In the present study, *N. vitifusiforme* was reported for the first time on mango. The fungus was isolated from mango leaves and twigs with low frequency from only one location (Palermo). The low incidence together with the fact that the only two isolates of *N. vitifusifome* induced the smallest lesions on mango provide an indication that it has a little importance on mango and doesn't contribute significantly to mango dieback disease (see chapter 5). Our findings were in agreement with those of Begoude et al, (2009) who indicated that, *Lasiodiplodia mahajagana* was not a primary pathogen due to its low incidence as well as low virulence on *Terminalia catappa.* Furthermore, similar results have also reported by Jacobs (2002), who considered that *F. bacilliforme* is a weak pathogen to mango plants because of the small lesions produced and its low isolation frequency. However, the *N. vitifusiforme* clade supported with low bootstrap value, the two isolates of *N. vitifusiforme* obtained from mango clustered separately in subclade with strains isolated from *Prunus* sp. in South Africa and from olive in Italy. Conidia morphology of *N. vitifusiforme* is similar to *N. australe* and *N. luteum* as they share

the same fusiform shape; however, *N. vitifusiforme* is easily distinguishable on culture by not producing yellow pigment in media as well reported for *N. australe* and *N. luteum* (Slippers et al, 2004b). Furthermore, the conidia of *N. vitifusiforme* are shorter up to 22 µm in length (van Niekerk et al, 2004). This was our case of study, no yellow pigment was observed and conidia length was almost close to the range reported by Lazzizera et al, (2008) which reached up to 23.6 µm in length.

The present study revealed two new species identified effectively based on the integration of DNA sequence and morphological data. The low incidence of *Neofusicoccum* sp. 3 and *Neofusicoccum* sp. 18 may give indication that they are of minor importance on mango since they were isolated in association with the other dominant species. Furthere studies e.g extensive survey and pathological studies are needed to clarify the ecology and to highlight their relative role to cause diseases on mango.

CHAPTER .4

MORPHOLOGY AND PHYLOGENY OF BOTRYOSPHAERIA SPP. ASSOCIATED WITH MANGO DIEBACK IN EGYPT

INTRODUCTION

Mango is a popular fruit tree in Egypt and is cultivated almost along the whole Nile valley and in the desert areas (Abdalla et al, 2007). It has reported to be introduced from Bombay-India to Egypt under Mohammed Ali in 1825, and most mangos cultivars said to be originated from India (El Tomi 1953). Most of Egyptian varieties are polyembryonic bearing fruit that are sweet with strong spicy flavor and less in fiber of which the most common are alphonso, pairi, zebda, mabroka, balady, and succary (El-Soukkary et al, 2000; Knight 1993). Species in the Botryosphaeriaceae, particularly *L. theobromae* have been reported as pathogens for mango worldwide associated commonly with cankers and die back symptoms (Abdollahzadeh et al, 2010; de Oliveira Costa et al, 2010; Khanzada et al, 2004a,b; Jacobs, 2002).

L. theobromae is an important member of Botryosphaeriaceae associated with various diseases on numerous hosts (Punithalingam, 1980; Slippers and Wingfield 2007). It was reported as the main causal agent of fruit rot, stem end rot, brown rot of panicles and tip die back diseases on mango in Egypt (Abdalla et al, 2003; Ragab et al, 1971). It seems to be geographically predominant in tropics and subtropics areas (Burgess et al, 2006; Punithalingam, 1980). The taxonomic status of *L. theobromae*/*B. rhodina* remained unclear for several years, and this confusion ended with the monograph of Punithalingam (1976) who reduced most species to synonym *L.theobromae*. Since *L. theobromae* is a species complex containing different cryptic species, morphological characters of colony and conidia are insufficient solely to rely on in separation them. Thus, recent extensive phylogenetic studies on the basis of multiple gene sequences played very significant role in delimiting new distinct species from *Lasiodiplodia* (Abdollahzadeh et al, 2010; Alves et al, 2008; Begoude et al, 2009; Burgess et al, 2006; Damm et al, 2007; Pavlic et al, 2004, 2008).

A part of the previous report of *L. theobromae* on mango in Egypt by Ragab et al, (1971), little information is available regarding the etiology of Botryosphaeriaceae on mango in Egypt. The present work therefore, represents the first attempt to study the variability between collection of isolates previously identified as *L. theobromae* or /*Botryodiplodia theobromae* on the basis of morphology and DNA sequence of ITS and TEF1-α data.

MATERIALS AND METHODS

Fungal isolation

In Feb 2010, a routine survey was conducted in different geographical areas in Egypt where mango is cultivated. A total 27 isolates reassembled Botryosphaeriaceae-fungi were obtained after isolation from freshly symptomatic plant materials showed twig, branch die back and black lesions symptoms on leaves. The isolation and pure culture processes were the same as described in (chapter 2).

Morphological characterisation

(See Chapter 3)

Temperatures effect on the mycelial growth of *Lasiodiplodia* spp.

(See Chapter 3)

Molecular characterisation

(See Chapter 3)

RESULTS

Isolates

A total of 27 isolates reassembling Botryosphaeriaceae (growing fast on medium, a greenish brown to dark greyish blue mycelium) (Slippers and Wingfield, 2007), were obtained from three locations in Egypt, 13 originated from branches, 11 from leaves and 3 from twigs. All isolates were included in the phylogenetic study. Isolates used for morphological characterisation and growth study were deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands.

Phylogeny of *Botryosphaeria* spp.

PCR amplification

Amplicons of approximately 700 bp for ITS were generated using V9G and ITS4 and ~550bp for TEF-1α were obtained by EF1-728F and EF2 primers pairs. While, amplicons of 700bp were obtained by EF1-688 and EF1-1252 primer set (Fig .18)

Phylogenetic analysis

The combined data set contained 27 taxa representing the obtained isolates in this study with further 37 sequences including the outgroup taxa. Approximately 371 characters including gaps from the ambiguous ends were excluded from each sequence of both regions. The combined data set contained 929 characters including gaps, of which 603 were constant, 89 were variable and parsimony uninformative and 237 were parsimony informative. Maximum parsimony analysis after heuristic search resulted in one parsimonius tree (TL= 588 steps, CI= 0.74, RI=0.885, RC= 0.655 and HI=0.260) presented in Fig. 26. The topology of the tree generated from MP analysis was congruent with the 50% majority-rule consensus tree. *Lasiodiplodia* species isolated from mango in Egypt fell in four distinct clades.

The majority of isolates BOT-1, BOT-2, BOT-3, BOT-11, BOT-12, BOT-13, BOT-14, BOT-15, BOT-16, BOT-17, BOT-18, BOT-21, BOT-22, BOT-24, BOT-25, BOT-26, BOT-27 and BOT-28 were clustered in the largest 1st clade supported with BS value 76% and BPP value of 1.0. this clade contained isolates of *L. pesuodtheobromae* represented strains culture ex-type CBS 116459, CBS 121773, CMW 22667, CMW 24699, CMW 24700 and IRAN 1581C,. The 2nd clade contained only single isolate (BOT-8) of *Lasiodiplodia* sp. that formed a separated clade with no bootstrap support. The 3rd clade contained two isolates of *Lasiodiplodia* sp. BOT-10 and BOT-29 from *M. indica* higly supported with BS value 88% and BPP value 0.97. Six isolates BOT-4, BOT-5, BOT-6, BOT-7, BOT-9 and BOT-23 of *L. theobromae* were clustered in the 4th clade with strains CMW 24701, CMW 24702 and (CBS 111530 culture ex-type) supported with values of (BS/BPP:84/1.0).

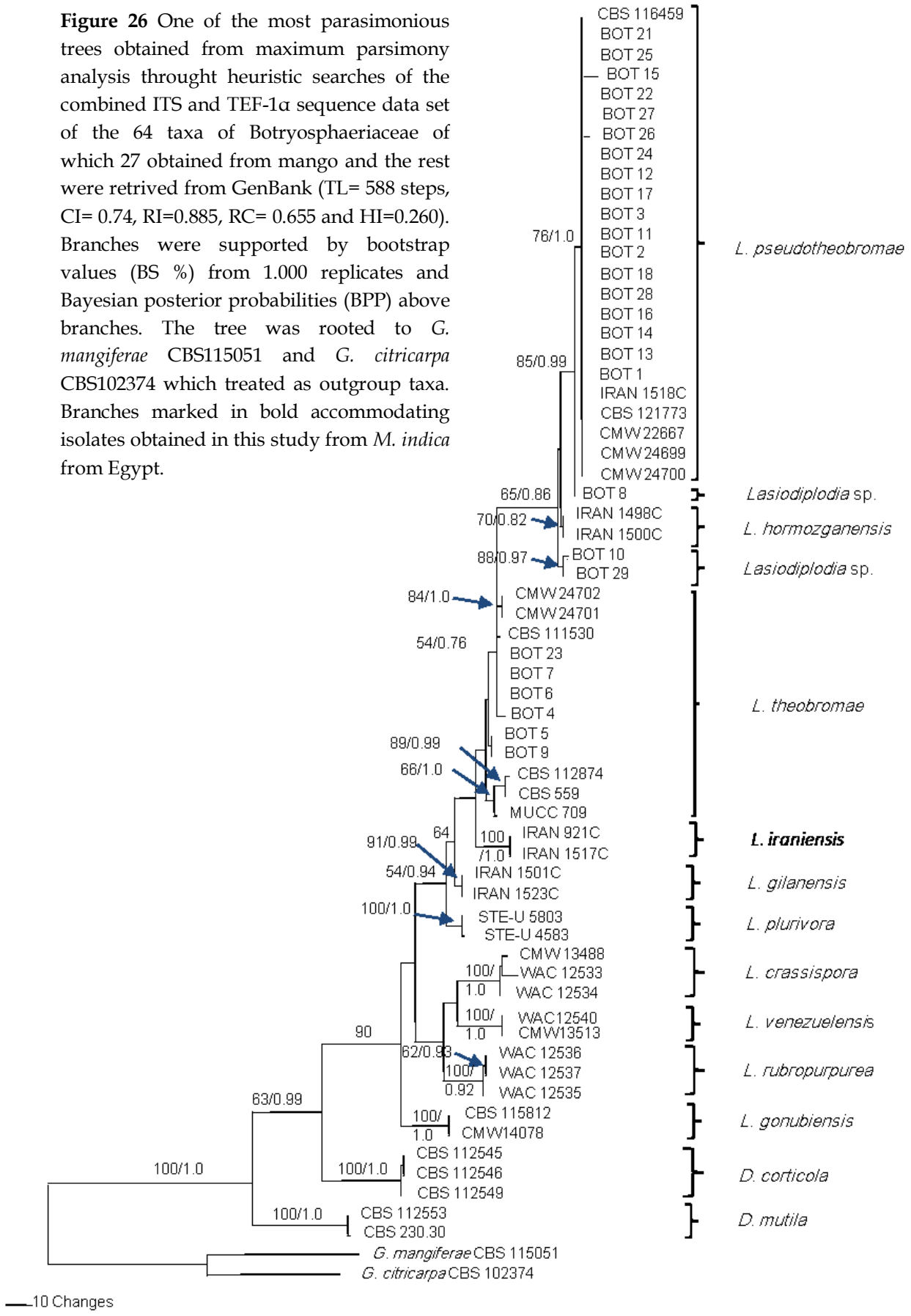


Figure 26 One of the most parasimonious trees obtained from maximum parsimony analysis throught heuristic searches of the combined ITS and TEF-1α sequence data set of the 64 taxa of Botryosphaeriaceae of which 27 obtained from mango and the rest were retrived from GenBank (TL= 588 steps, CI= 0.74, RI=0.885, RC= 0.655 and HI=0.260). Branches were supported by bootstrap values (BS %) from 1.000 replicates and Bayesian posterior probabilities (BPP) above branches. The tree was rooted to *G. mangiferae* CBS115051 and *G. citricarpa* CBS102374 which treated as outgroup taxa. Branches marked in bold accommodating isolates obtained in this study from *M. indica* from Egypt.

TAXONOMY

Lasiodiplodia theobromae

Conidiomata (Fig. 27a) (stromatic pycnidial produced on WA on pine needles within 10-12 days). Pycnidia were superficial, erumpent when mature, dark brown to black covered with dense mycelium, solitary or in aggregates, ovoid to ampulliform, most often uniloculate, papillate with central ostiole, thick walled of 3-5 dark brown *textura angularis* layers, followed by thin-walled and hyaline cells towards the pycnidia center (Fig. 27b).

Paraphyses (Fig. 27c) hyaline, cylindrical, aseptate, broader at base, rounded at apex, up to 44 µm long, 1.9-3.4 µm wide developed between conidiogenous cells. *Conidiogenous cells* (Fig. 27d,e) holoblastic, thin-walled, hyaline, cylindrical, rounded at apex, proliferating to form minute annelations, 5.5-12.2 × 2.4-4.6 µm. *Conidia* (Fig. 27f) release from pycnidia as cirri, initially hyaline, smooth, thick-walled aseptate, ellipsoid to ovoid to somewhat irregular in shape, contains granular contents, rounded to truncate at base, and sometimes tapered at apex. Conidia become dark brown septet with 1-septum, with longitudinal accumulation of melanin on the inner conidia surface giving it a striate appearance (Fig. 27g, h, I, j), measuring (17.6-) 21.3-26.1 (-30) × (9.0-) 11.2-15.4 (-17.9), mean of 50 conidia ± SD= 23.7 ± 2.4 µm long × 13.3 ± 2.1 µm wide, L/W ratio = 1.7.

Cultural characteristics (Fig. 27k, l) colonies with sparse to moderately dense raised aerial mycelium mat, occasionally columns of aerial mycelium reach to the lid, initially white become smoke-grey (21′′′′f) to greenish grey (33′′′′i) on the front side of plate. The colour was greenish grey (33′′′′i) at the reverse side within 7-days in the dark at 25^{o} C, the colony colour become dark slate blue (39′′′′k) with age. Colonies reached the edge of the Petri plate 85-mm after 2-days in the dark at 30°C. Cardinal temperature requirements for growth; minimum 10°C, maximum 35°C, optimum 30°C (Fig. 31).

Teleomorph: *Botryosphaeria rhodina* (not seen in this study)

Host: *Mangiferae indica*

Known distribution: Egypt (Sharkia Province)

Specimen examined Egypt: Sharkia Province, isolated from dark brown lesions on mango leaves 2 Feb 2010, BOT-4 = CBS 130989.

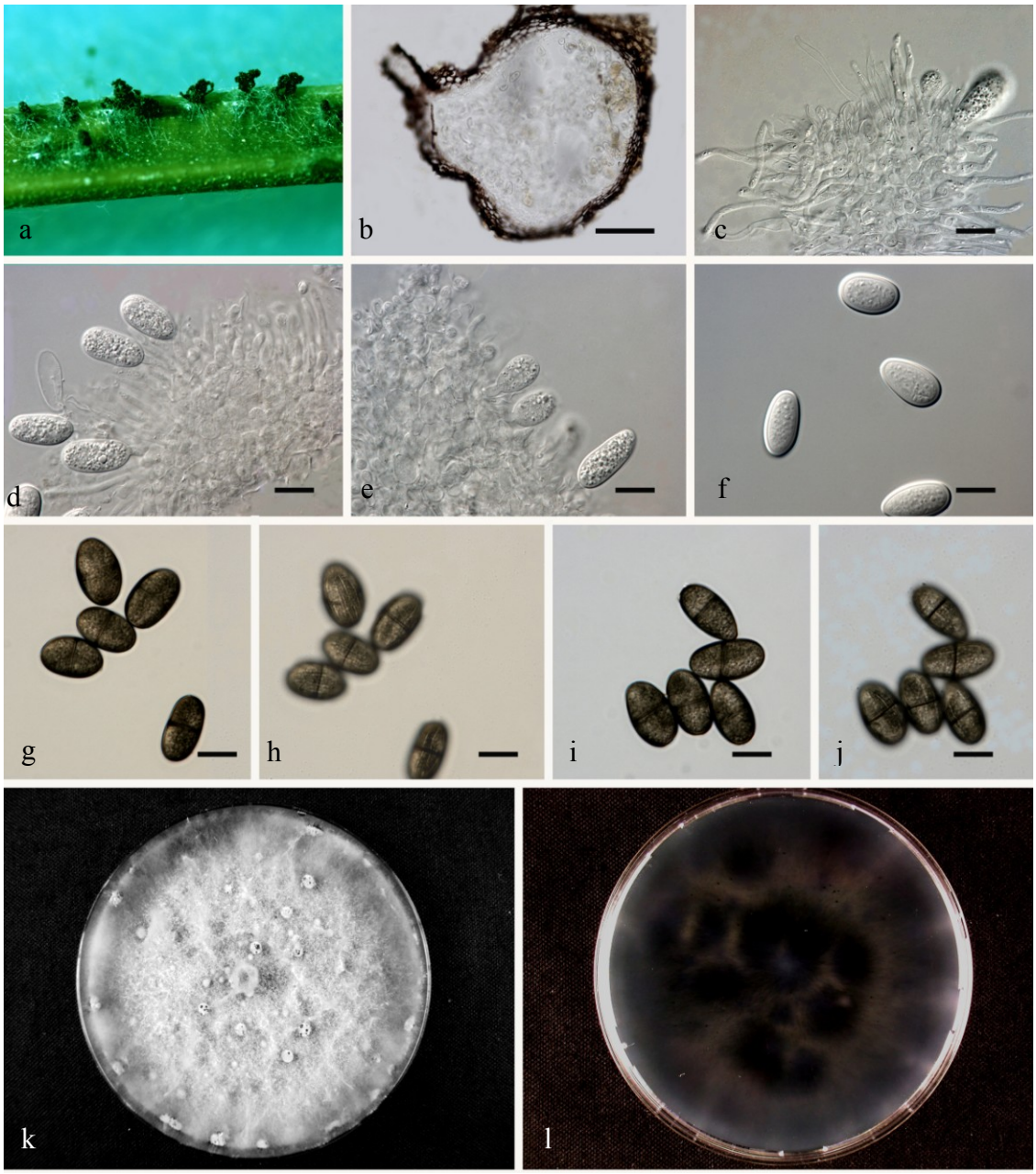


Figure 27 *Lasiodiplodia theobromae* BOT-4 = CBS 130989. (a) pycnidia formed on pine needles on WA; (b) vertical section through papillate pycnidia; (c) hyaline aseptate with rounded apex paraphyses formed between conidiogenous cells; (d, e) conidiogenous cells; (f) hyaline immature thick-walled conidia; (g, h) and (i, j) dark mature conidia at two different focal planes showing the longitudinal striation. Colony morphology: (k) front side; (l) reverse side. Scale bars: (b) = 20 µm; (c, d, e, f, g, h, I, j) = 10 µm.

Lasiodiplodia pseudotheobromae

Conidiomata (Fig. 28a) (stromatic pycnidia produced on WA on pine needles within 10-12 days). Pycnidia were superficial to semi-immersed in the needle tissues, become erumpent when mature, dark brown to black, covered with dense dark grey hairy mycelium, solitary and most often in aggregates, ovoid to ampulliform, mostly uniloculate and sometimes multiloculate, papillate with central ostiole, pycnidial wall composed of brown, thick-walled 3-5 *textura angularis* cell layers (Fig. 28b).

Paraphyses (Fig. 28c) hyaline, cylindrical, apical ends rounded, developed between conidiogenous cells, spetate with 0-1 septum, up to 52.2 µm long, 2-3.2 µm wide. *Conidiogenous cells* (Fig. 28d, e) holoblastic, hyaline, cylindrical, somewhat ellipsoid and sometimes swollen at the base measuring, 4.1-10.8 µm long and 2.5-4.7 µm wide. *Conidia* (Fig. 28f) after release from pycnidia as cirri, initially hyaline, smooth, mostly ellipsoid to sub-ovoid, thick-walled, aseptate, contains granular content, rounded at both apex and base. Conidia become brown to, verruculose, septate with 1-2 septum, with longitudinal striation on the surface due to the melanin deposits (Fig. 28g, h, I, j), measuring (22.8-) 24.8-28.6 (-32.1) × (9.8-) 11.1-13.5 (-14.8), mean of 50 conidia ± SD= 26.7 ± 1.9 µm long, 12.3 ± 1.2 µm wide, L/W ratio = 2.1.

Cultural characteristics (Fig. 28K, l) Colonies with dense, concave with raised margins mycelium, initially white to smoke-grey (21′′′′f) become pale olivaceous grey (21′′′′′d) at the front side and olivaceous grey (21′′′′′i) at the reverse side within 7-days in the dark at 25ºC, the colony colour become iron grey (23′′′′′k) with age. Colonies reached the edge of the Petri plate 85 mm after 3-days in the dark at 25-30°C. Cardinal temperature requirements for growth; minimum 10°C, maximum 35°C, optimum 25-30°C (Fig. 31).

Teleomorph: Unknown *Botryosphaeria* sp. (not seen in this study)

Host: leaf lesions of *Mangiferae indica*

Known distribution: Egypt (Sharkia Province)

Specimen examined Egypt: Sharkia Province, isolated from dark brown lesions on mango leaves 2 Feb 2010, BOT-11= CBS 130990.

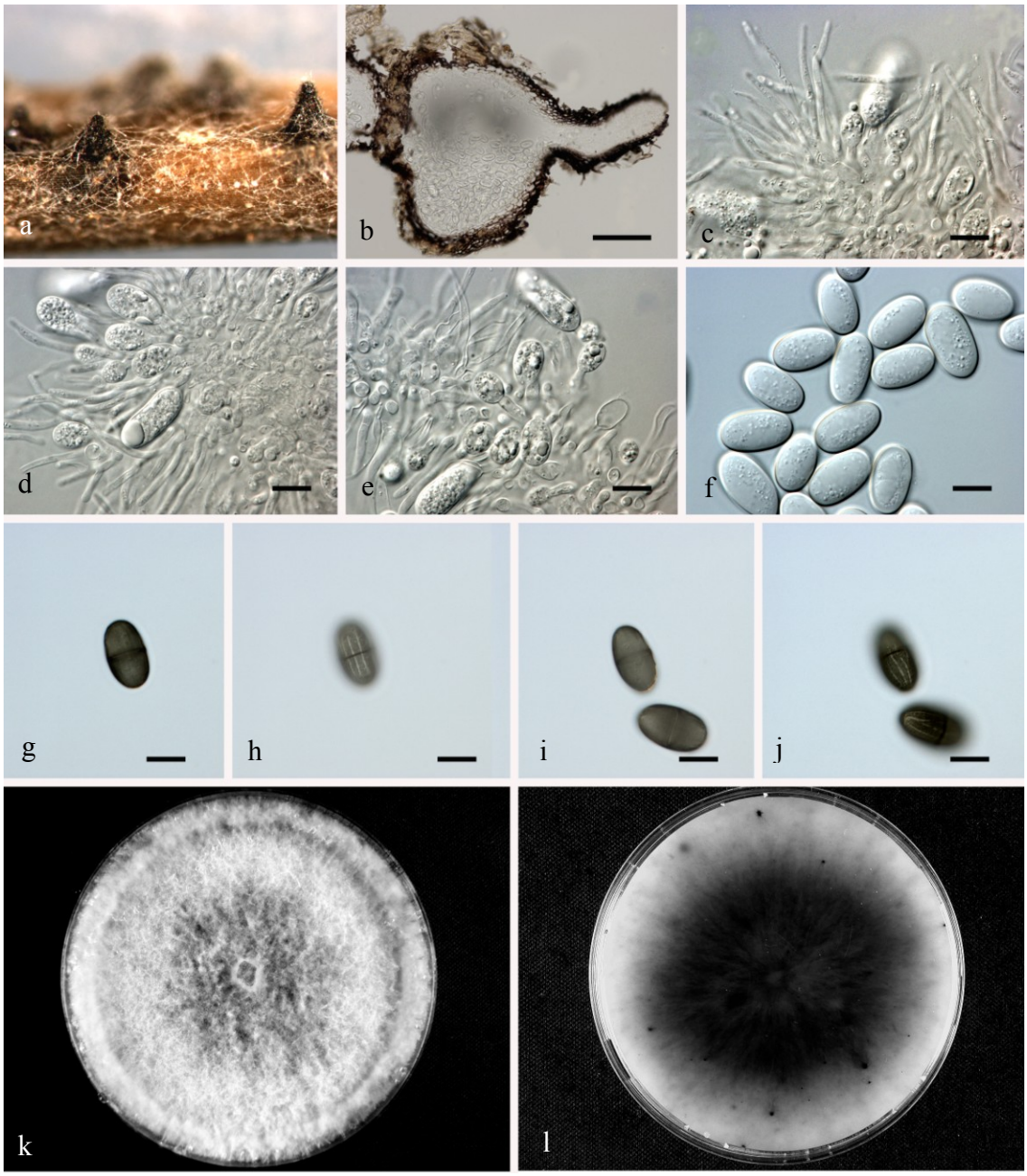


Figure 28 *Lasiodiplodia pseudotheobromae* BOT-11 = CBS 130990. (a) pycnidia formed on pine needles on WA; (b) vertical section through papillate pycnidia; (c) hyaline septate paraphyses formed between conidiogenous cells; (d, e) conidiogenous cells; (f) hyaline immature thick-walled conidia; (g, h) and (i, j) dark mature conidia at two different focal planes showing the longitudinal striation. Colony morphology: (k) front side; (l) reverse side. Scale bars: (b) = 20 µm; (c, d, e, f, g, h, i, j) = 10 µm.

***Lasiodiplodia* sp. 10**

Conidiomata (Fig. 29a) (stromatic pycnidial produced on pine needles on WA withing 10-12 days). Pycnidia were mostly solitary, or in aggregates, dark-grey to black, globose to subglobose, covered with dense hairy mycelium, semi-immersed becomes erumpent when mature, mostly multiloculate to uniloculate, vertical section through pycnidia shows, outer layers of pycnidia composed of 5-7 dark brown thick-walled cells layers of *textura angularis*, followed by hyaline thin-walled cells towards the center (Fig. 29ba).

Paraphyses (Fig. 29c) hyaline, subcylinderical, arising between the conidiogenous cells, aseptate, wider at base and rounded or slightly swollen at apex, up to 57.2 µm long, 1.6-3.4 µm wide. *Conidiogenous cells* (Fig. 29d) holoblastic, thin-walled hyaline, cylindrical, in sometimes swollen slightly at the base, with rounded apex, proliferating precurrently to produce 1-2 minute annelations, 4.6-10.5 µm long, and 3.2-5 µm wide. *Conidia* (Fig. 29e, f) release from pycnidia in chains as cirri, initially hyaline, smooth, thick-walled, aseptate, obovoid to ellipsoid, contain granular contents, mostly tapered at the apex, and rounded at base. Conidia become brown pigmented, septate with 1-septum, with longitudinal striation on the inner surface of the conidia wall due to the melanin deposits (Fig. 29g, h, I, j), measuring (17.2-) 20.2-23.8 (-26.7) × (10.5-) 11- 12.4 (-13.3) µm. av. of 50 conidia ± SD =22 ± 1.8 µm long, 11.7 ± 0.7 µm wide, L/W ratio = 1.8.

Cultural characteristics (Fig. 29k, l) Colonies with moderately dense raised mycelium mat, initially white to smoke-grey (21‴‴f) turning greenish grey (33‴‴i) on the front side and greenish grey (33‴i) on the reverse side of Petri plate, the colour becomes dark slate blue (39‴‴k) with age. Cardinal temperature requirements for growth; minimum 15°C, maximum 35°C, optimum 25-30°C (Fig. 31).

Teleomorph: Unknown *Botryosphaeria* sp. (not seen in this study)
Host: *Mangiferae indica*
Known distribution: Egypt (Sharkia Province)
Specimen examined Egypt: Sharkia Province, isolated from dark brown lesions on mango leaves 2 Feb 2010, holotype, BOT-10 = CBS 130992.

In this study, *Lasiodiplodia* sp. 10 identified as new species based on morphology and DNA sequence data of the ITS and TEF1-α gene regions. The two isolates BOT-10 and BOT-29 obtained from mango in this study were phylogenetically distinct and grouped separately in a well-supported clade with values (BS/BPP: 88%/0.97). Although, *Lasiodiplodia* sp. 10 is phylogenetically closely related to *L. hormozganensis* (Abdollahzadeh et al, 2010), but it can be distinctly differentiated based on its conidia and paraphyses morphology. Conidia of *Lasiodiplodia* sp. 10 are somewhat longer but narrower (22 ± 1.8 ×11.7 ± 0.7 µm) than those of *L. hormozganensis* (21.5 ± 1.9 × 12.5 ± 0.8 µm). Furthermore, conidia of *Lasiodiplodia* sp. 10 are ovoid to sub-ovoid, whereas, those of *L. hormozganensis* are ellipsoid to cylindrical. In addition, the paraphyses of *Lasiodiplodia* sp. 10 are aseptate and shorter up to 57.2 µm long, while, the paraphyses of *L. hormozganensis* are 1-7 septate and longer up to 83 µm long.

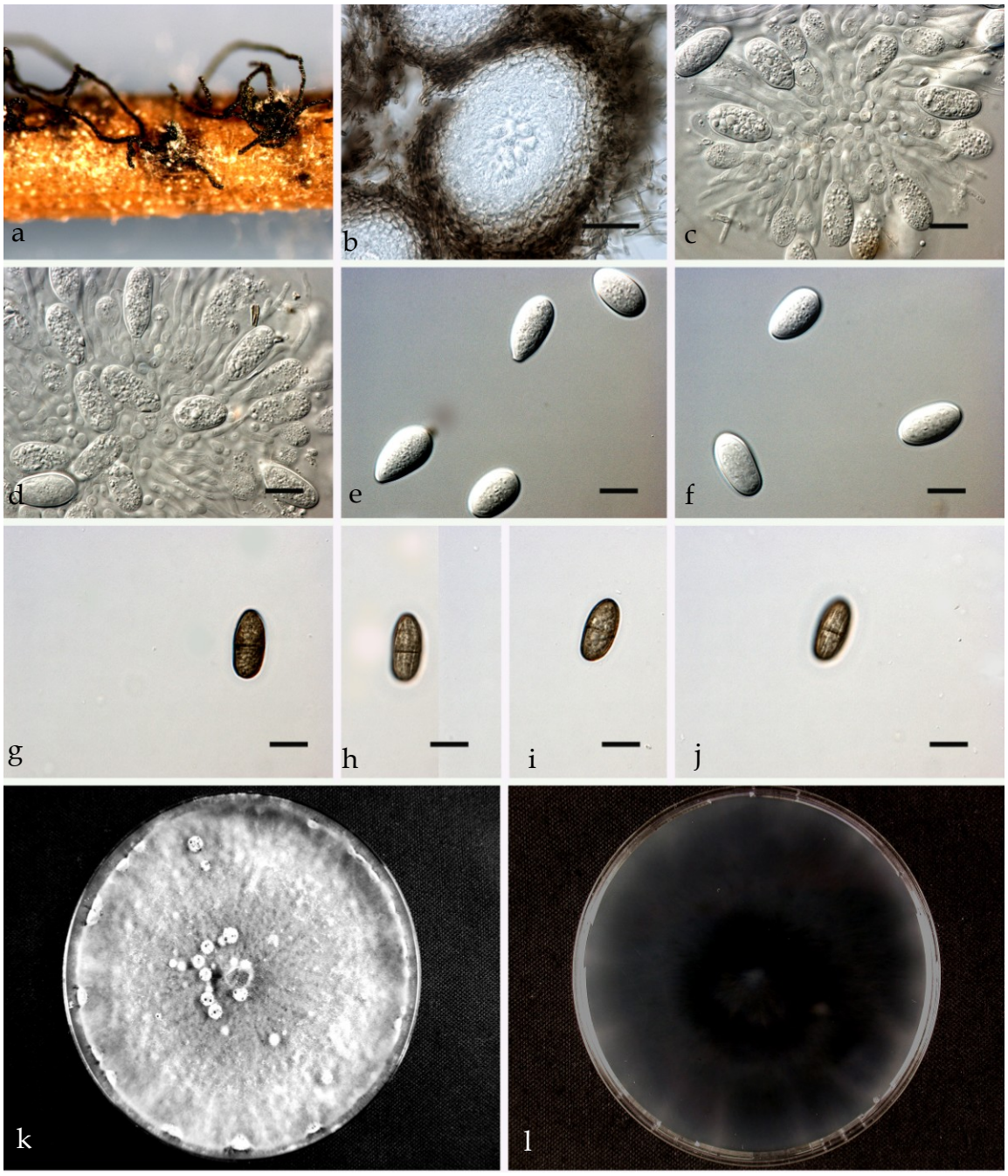


Figure 29 *Lasiodiplodia* sp. 10, holotype BOT-10 = CBS 130992. (a) pycnidia formed on pine needles on WA; (b) vertical section through pycnidia; (c) hyaline aseptate paraphyses formed between conidiogenous cells; (d) conidiogenous cells; (e, f) hyaline immature thick-walled conidia; (g, h) and (i, j) dark mature conidia at two different focal planes to show longitudinal striation. Colony morphology: (k) front side; (l) reverse side. Scale bars: (b) = 20 µm; (c, d, e, f, g, h, i, j) = 10 µm.

***Lasiodiplodia* sp. 8**

Conidiomata (Fig. 30a) (stromatic pycnidial produced on pine needles on WA within 10-12 days). Pycnidia were solitary, ampulliform, dark brown to black, superficial to semi-immersed in needle tissues, unilocular, papillate with central ostiole, vertical section through pycnidia; showing that the outer layers composed of 3-5 dark brown thick-walled cell layers of *textura angularis*, followed by thin-walled hyaline, cell layers beyond the center of pycnidia (Fig. 30b).

Paraphyses (Fig. 30c, d) hyaline, cylindrical, thin-walled, aseptate, developing between the conidiogenous cells, broader at base, rounded at apex, up to 62 µm long, 2.1-3.6 µm wide. *Conidiogenous cells* (Fig. 30e) holoblastic, hyaline, smooth, thin-walled, cylindrical, and discrete, 6.4 - 13.1 µm long, and 3.3-5.1 µm wide. *Conidia* (Fig. 30f) after releasing from pycnidia, initially hyaline, ellipsoidal to cylindrical, thick-walled, aseptate, with granular content, rounded at both ends. Conidia become brown, verruculose, with 1-septum with longitudinal striations (Fig. 30g, h, I, j), measuring (24.3-) 25.9 – 28.1 (-29.7) × (11-) 12.4 – 13.8 (-14.2) µm. av. of 50 conidia ± SD = 27 ± 1.1 µm long, 13.1 ± 0.7 µm wide, L/W ratio = 2.

Cultural characteristics (Fig. 30k, l) Colonies with moderately dense, convex with papillate surface mycelium mat, with sparse aerial mycelium, initially white becomes pale olivacelous grey (21‴‴d) to olivaceous grey (21‴‴i) on the upper surface, olivaceous grey (21‴‴i) on the reverse and becoming iron grey (23‴‴k) with age. Cardinal temperature requirements for growth; minimum 15°C, maximum 35°C, optimum 30°C (Fig. 31).

Teleomorph: Unknown *Botryosphaeria* sp. (not seen in this study)
Host: *Mangiferae indica*
Known distribution: Egypt (Sharkia Province)
Specimen examined Egypt: Sharkia Province, isolated from dark brown lesions on mango leaves 2 Feb 2010, holotype, BOT-8 = CBS 130991.

Lasiodiplodia sp. 8 was the single isolate obtained in this study that grouped separately in a single clade and did not fall with any of *Lasiodiplodia* isolates included in the phylogenetic analysis. *Lasiodiplodia* sp. 8 is identified in this study

as new species emerged from cryptic species of *L. theobromae* basing on the DNA sequence of ITS and TEF1-α and morphology. However, this species is phylogenetically closely related to *L. pseudotheobromae,* and has similar culture morphology, but it can be consistently distinguished based on conidia, conidiogenous cells and paraphyses dimension. Conidia average of *Lasiodiplodia* sp. 8 are longer and wider (27 ± 1.1 ×13.1 ± 0.7 µm) than those of *L. pseudotheobromae* (26.7 ± 1.9 × 12.3 ± 1.2 µm). Furthermore, conidiogenous cells of *Lasiodiplodia* sp. 8 are markedly longer and wider (6.4 - 13.1 × 3.3 -5.1 µm) than those of *L. pseudotheobromae* (4.1-10.8 × 2.5-4.7 µm), and not swollen at base as *L. pseudotheobromae*. Moreover, the paraphyses of *Lasiodiplodia* sp. 8 are aseptate and longer up to 62 µm long, while for *L. pseudotheobromae* are septate and shorter up to 52.2 µm long.

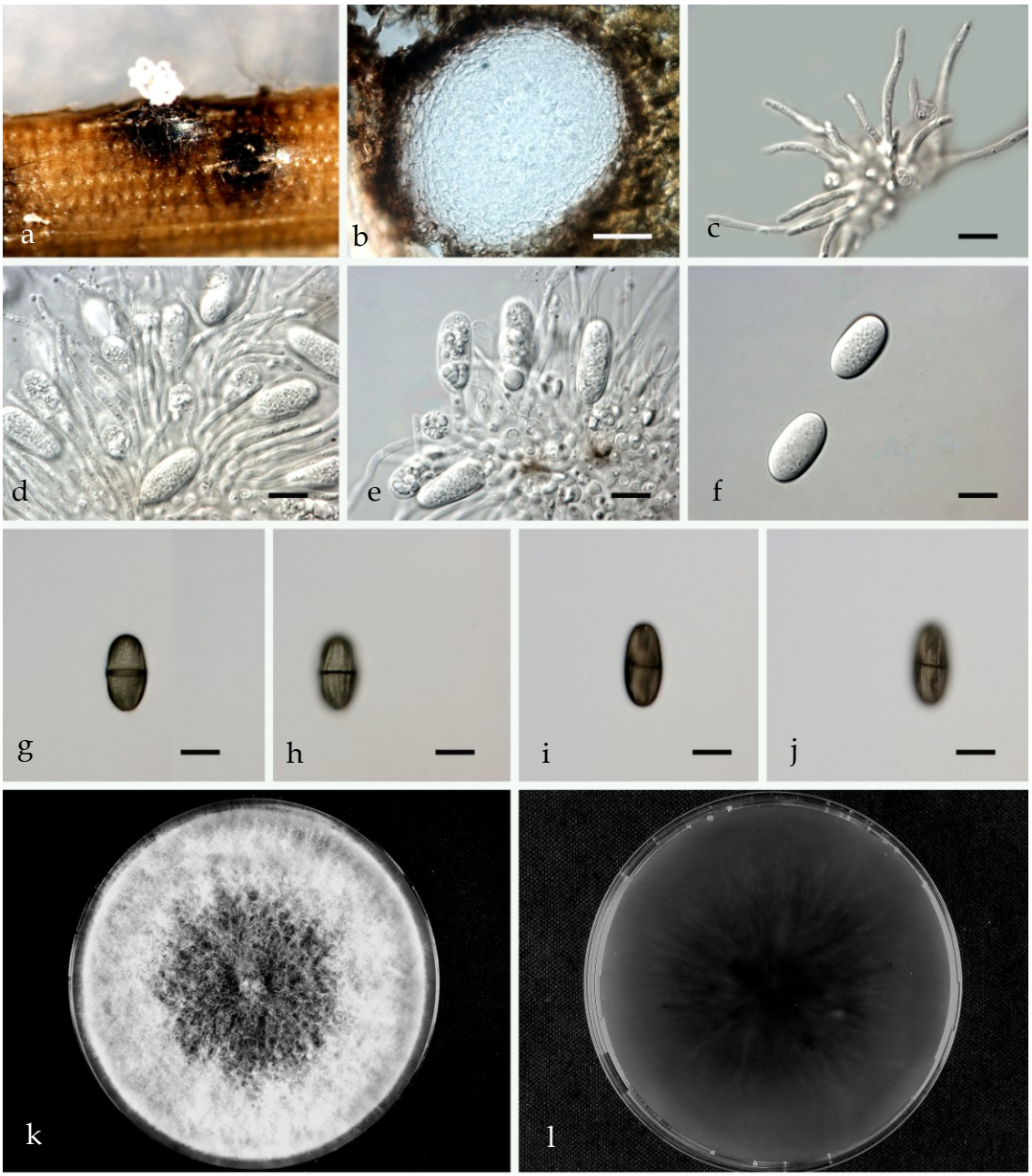


Figure 30 *Lasiodiplodia* sp. 8, holotype BOT-8 = CBS 130991. (a) pycnidia formed on pine needles on WA; (b) vertical section through pycnidia; (c,d) hyaline aseptate paraphyses formed between conidiogenous cells; (e) conidiogenous cells; (f) hyaline immature thick-walled conidia; (g, h) and (i, j) dark mature conidia at two different focal planes to show longitudinal striation. Colony morphology: (k) front side; (l) reverse side. Scale bars: (b) = 20 µm; (c, d, e, f, g, h, i, j) = 10 µm.

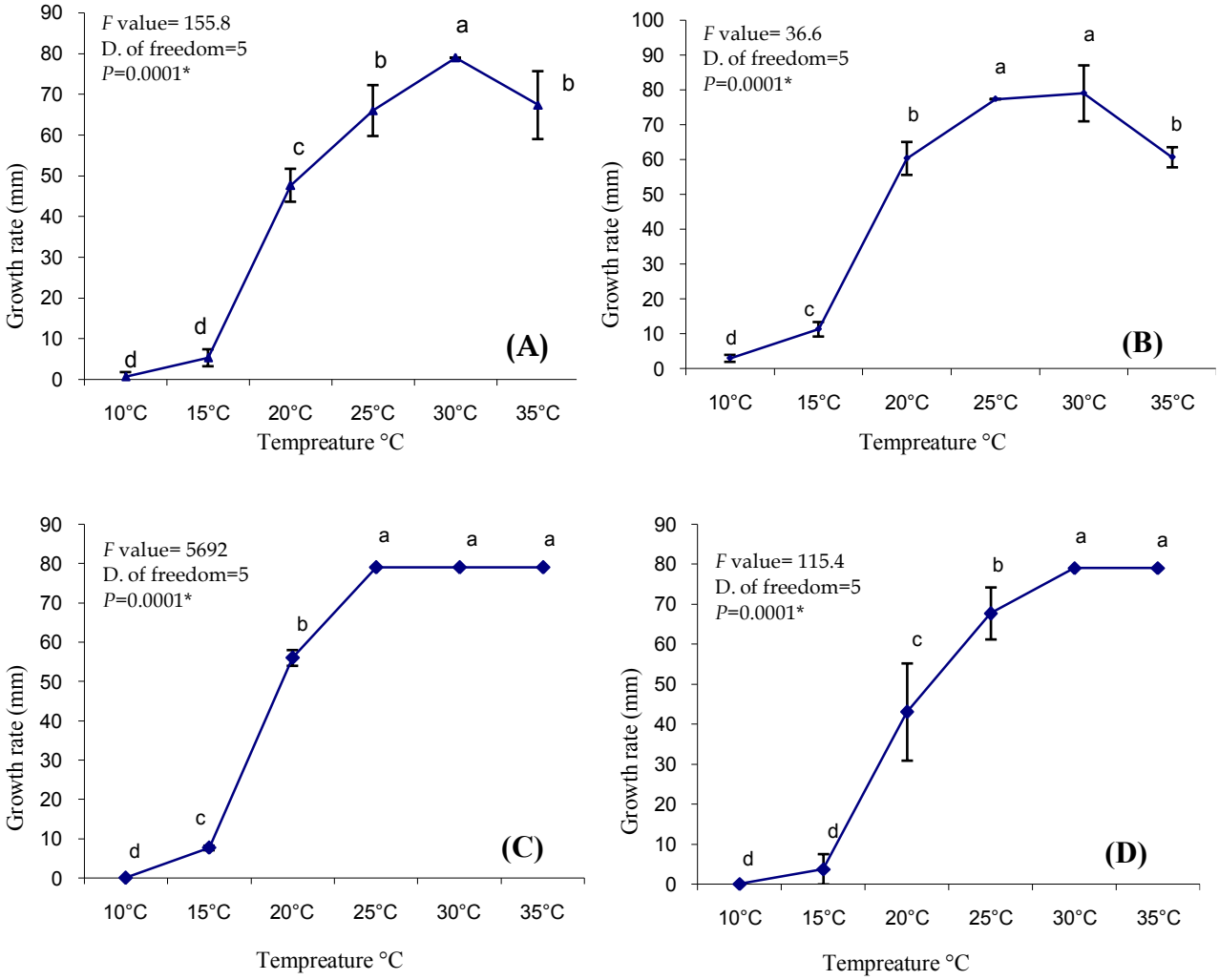


Figure 31 Temperature effect on the growth of representative four species of *Botryosphaeria* isolated from mango in this study. (A) *L. theobromae* (B) *L. pseudotheobromae*, (C) *Lasiodiplodia* sp. 10 and (D) *Lasiodiplodia* sp. 8. The dimension of the fungal plugs (6-mm) was subtracted from the final measurements. Means followed by the same letter are not significantly different according to LSD test (P<0.05).

DISCUSSION

In this study, four species of *Lasiodiplodia* were isolated from mango and associated with dieback disease. These were identified as *L. theobromae*, L. *pseudotheobromae* and two (*Lasiodiplodia* sp. 8 and *Lasiodiplodia* sp. 10) newly recognised species. Both the two new species were distinguished from other taxa in *Lasiodiplodia* based on morphological characters and phylogenetic inference.

The morphological characteristics described here and those previously published together with the combined ITS and TEF1-α sequence data were used to separate *Lasiodiplodia* species. Several studies have relied on morphological characteristics (conidia dimensions, morphology and morphology and size of paraphyses) in association with DNA sequence data to identifying *Lasiodiplodia* species. However, several morphological features can overlap (Abdollahzadeh et al, 2010; Charles, 1970; Kim et al, 2005; Pennycook and Samuels, 1985; Slippers et al, 2004a), but are still complimentary tools when combined with DNA phylogeny to distinguish new species in the Botryosphaeriaceae. However, using morphological features as discriminatory criteria alone should be done with care. Burgess et al, (2006) relied on the septation of paraphyses to discriminate between *Lasiodiplodia* spp. and indicated that *L. gonubiensis*, *L. venezuelensis* and *L. crassispora* have septate paraphyses while other species are aseptate. However, in this study the septate paraphyses were observed for *L. pseudotheobromae* as previously reported by Alves et al, (2008) and Punithalingam (1976). Using a similar approach, Damm et al, (2007) was able to distinguish *L. plurivora* from *L. crassispora* and *L. venezuelensis* based on the morphology of the paraphyses. This was also followed by study of Abdollahzadeh et al, (2010) who distinguished *L. gilanensis* from *L. plurivora* and *L. hormozganensis* from *L. parva* and *L. citricola* using the morphology of the paraphyses. In this study, the shape and length of paraphyses were used to differentiate *Lasiodiplodia* sp. 8 from *L. pseudotheobromae* and *Lasiodiplodia* sp. 10 from *L. hormozganensis.*

Culture characteristics have also played a role in distinguishing *Lasiodiplodia* species. Alves et al, (2008) discriminated *L. parva* and *L. pseudotheobromae* from *L. theobromae* based on the production of pink pigment in culture. In contrast, the findings of Abdollahzadeh et al, (2010) revealed that *L. theobromae* and other *Lasiodiplodia* species, with the exception of *L. hormozganensis,*

produced pink pigment on PDA at 35°C. In the present study, only *L. theobromae* produced a dark pink pigment in PDA after 2 d at 35°C with the colour becoming darker with age. This corresponded with the findings of Úrbez-Torres et al, (2006). Moreover, *L. pseudotheobromae* was the only species that could grow at 10°C, which is in agreement with the observations made by Alves et al, (2008) and in contrast to those of Abdollahzadeh et al, (2010) who found that all studied *Lasiodiplodia* isolates proven to grow at the same temperature. Therefore, culture characteristics, paraphyses and conidia morphology play a limited role in the discrimination of cryptic species unless DNA sequence data is used complementary to morphology.

The extensive phylogenetic inference based on multiple gene sequences has played an important role in delimiting novel species in the genus *Lasiodiplodia.* In this study, the use of combined ITS and TEF-1α sequence data enabled us to identify two cryptic species within *L. theobromae* species complex newly described here as Lasiodiplodia sp. 8 and *Lasiodiplodia* sp. 10. Based on the phylogeny, both novel species were clearly separated from *L. pseudotheobromae* and the morphological characters reinforce this.

Pavlic et al, (2004) relied on a single gene phylogeny using ITS sequence data as well as the original description of *L. theobromae* to distinguish *L. gonubiensis* from *L. theobromae.* Several studies have demonstrated that using a single gene region is insufficient to delimit cryptic species (De Wet et al, 2003; Slippers et al, 2004a, b), and therefore, to resolve species boundaries in the genus *Lasiodiplodia* more than one gene region is required. This approach has revealed the presence of cryptic species in several genera in the family Botryosphaeriaceae (Slippers et al, 2004a, b).

In Egypt, *Lasiodiplodia theobromae* was considered as the main causal agent of fruit rot and dieback of mango (Ragab et al, 1971), and was previously reported as *Botryodiplodia theobromae* (Ragab et al, 1971). This species is cosmopolitan, occurring on a broad range of woody plant hosts worldwide (Burgess et al, 2006; Punithalingam, 1980). This fungus has been reported widely on mango associated with gummosis, twig and branch dieback and decline of mango (Abdollahzadeh et al, 2010; Al Adawi et al, 2003; de Oliveira Costa et al, 2010; Jacobs 2002; Khanzada et al, 2004 a, b). Recently, Sakalidis et al, (2011), reported *L.*

pesudotheobromae for the first time on *Mangifera indica* in Western Australia. In addition, Zhao et al, (2010) also reported *L. pseudotheobromae* on *Mangifera sylvatica* and other tropical and subtropical trees in China. *L. pseudotheobromae* has not been reported on mango in Egypt previously, and therefore, this study represents the first report of this fungus on mango in Egypt associated with sever dieback of branches and twigs, which in some cases led to tree mortality. Reports on various hosts in different geographical areas suggest that *L. pseudotheobromae* has a wide host range and its distribution might extend to other plant hosts as well as new areas (Begoude et al, 2009) *Lasiodiplodia* sp. 8 and *Lasiodiplodia* sp. 10 have been isolated from plant material showing brown to black leaf lesions and branches dieback symptoms. Thus far, there are no information related to their ecology and distribution in mango plantations in Egypt and might have been introduced from alternative hosts in close proximity to the surveyed mango plantations. Therefore, extensive survey and samplings in different geographical areas, in close proximity to mango plantations are needed.

CHAPTER .5

PATHOGENICITY TESTS OF *BOTRYOSPHAERIA* SPP. ASSOCIATED WITH MANGO DIEBACK DISEASE IN ITALY AND IN EGYPT

INTRODUCTION

Species of Botryosphaeriaceae contain important pre and post-harvest pathogens of fruit and woody trees causing several patterns of symptoms such as; leaf spot, dieback, stem-end rot, fruit rot cankers and sometimes tree mortality (Damm et al, 2007; Johnson et al, 1991; Johnson et al, 1993; Ramos et al, 1991; Slippers et al, 2005). Botryosphaeriaceae are not host specific and can affects wide range of hosts and have recognized as successful opportunistic endophytic colonists especially when their plants are subjected to unfavorable conditions or stress due to several factors (Johnson, 1992; Slippers and Wingfield, 2007). Various factors play important role in the predisposition of mango trees to be attacked by *Botryosphaeria* spp.; mechanical injuries, mineral deficiencies (zinc, manganese, iron) and other environmental factors (sunscald and hail damage) (Ploetz et al, 1996a,b; Ramos et al, 1991). The endophytic nature of mango dieback and stem-end rot pathogens as they can systematically colonize vascular tissues without inducing symptoms can explain well their distribution in mango production areas around the world through plant materials (Ploetz et al, 1996a).

These fungi infect mango tissues through wounds (Johnson, 1993; Smith et al, 1994) or directly through natural opening such as stomata, lenticels and other natural openings on healthy plants (Kim et al, 1999). Infection by *Botryosphaeria* fungi in the field (pre-harvest) can express various types of symptoms such as: browning of leaf petioles and midrib leading to dying of terminal leaves of the branches, bud necrosis, discolouration of the xylem vessels, and formation of tyloses, inclusions inside xylems, stem dieback, blight and gummosis (Ploetz et al, 1996a; Ramos et al, 1991). While the most common encountered post-harvest symptoms are, stem-end rot and soft rot (Johnson et al, 1993; Swart et al, 2009). Several *Botryosphaeria* species have reported to cause diseases on mango worldwide (de Oliveira Costa et al, 2010; Jacobs, 2002; Javier-Alva et al, 2009; Khanzana et al, 2004; Ni et al, 2010; Ramos et al, 1991; Ploetz et al, 1996a).

In our study, we have characterised three common species of *Botryosphaeria* occur on mango in Sicily-Italy. They are *N. parvum*, *N. australe* and *N. vitifusiforme*. In addition, two species of *Lasiodiplodia* (*L. theobromae* and *L. pseudotheobromae*) occur on mango in Egypt. To date, no work has done neither to isolate nor to study the pathogenicity of such fungi on mango in Italy. However,

the pathogenicity tests of *L. theobromae* have been done in the past (Ragab et al, 1971), but we incorporated it in a comparison with the newly reported *L. pseudotheobromae*. The objective of this study therefore, was to test the pathogenicity of the five predominant species of *Botryosphaeria* occurring on mango in Italy and Egypt.

MATERIALS AND METHODS

Apple fruit assay

A preliminary test was made to assess the pathogenicity behavior of total 40 representative *Botryosphaeria* spp. isolates (Table .5). Apple fruit assay is a relatively quick, convenient and has been reported to provide an indication of pathogenicity in *Botrysphaeria* spp. (Brown-Rytlewski and McManus, 2000; Jacobs, 2002). The cultivar Granny Smith was chosen for the screening of *Botryosphaeria* isolates. The healthy, ripen and have uniform size fruit were selected for inoculation. Wounds (6 mm wide × 3 mm depth) were made with sterilized cork borer on 2 points on the fruit surface after washing with water and sodium hypochlorite (0.5%NaOCl) and then disinfected with 70% ethanol (EtOH) and left to dry for about 10 min. Inoculation was made by placing 6-mm-diameter PDA plugs from the actively colony margins into wounds. Three fruits were inoculated for each isolate. Nine wounded fruits inoculated with only PDA plugs served as control. All the inoculated fruits were enclosed in plastic bags to maintain high relative humidity and to prevent any external contamination. Fruits then placed on the laboratory bench at room temperature 25 ± 4°C for 4 days and observed daily for the symptoms development. The developed lesions were measured at equatorial and longitudinal axes (Brown-Rytlewski and McManus, 2000). The sizes of the wounded sites (6-mm) weremade by the cork borer were not subtracted from the final measurement.

Table 5 Isolates of *Botryosphaeria* spp. used for apple fruit assay and pathogenicity tests on mango seedlings.

Isolate numbers	Identity	Isolated from (Plant organ)	Sampling locations	Collection date
NF-1	*N. parvum*	branch	Catania	September, 2009
NF-3	*Neofusicoccum* sp. 3	branch	Catania	September, 2009
NF-4	*N. parvum*	twig	Catania	September, 2009
NF-11	*N. parvum*	branch	Catania	September, 2009
NF-14	*N. parvum*	leaf	Messina	September, 2009
NF-17	*N. parvum*	twig	Messina	Sseptember, 2009
NF-20	*N. parvum*	branch	Catania	September, 2009
NF-22	*N. australe*	branch	Catania	September, 2009
NF-24	*N. parvum*	fruit	Catania	September, 2009
NF-26	*N. parvum*	fruit	Catania	September, 2009
NF-28	*N. parvum*	branch	Catania	September, 2009
NF-29	*N. parvum*	fruit	Catania	September, 2009
NF-30	*N. parvum*	twig	Catania	September, 2009
NF-32	*N. parvum*	twig	Catania	September, 2009
NF-34	*N. parvum*	leaf	Catania	September, 2009
NF-36	*N. parvum*	twig	Catania	September, 2009
NF-43	*N. parvum*	leaf	Catania	November, 2009
NF-46	*N. parvum*	leaf	Catania	November, 2009
NF-48	*N. parvum*	leaf	Catania	November, 2009
NF-49	*N. parvum*	leaf	Messina	April, 2010
NF-50	*N. parvum*	twig	Messina	April, 2010
NF-56	*N. parvum*	leaf	Messina	April, 2010
NF-60	*N. parvum*	leaf	Messina	April, 2010
NF-64	*N. parvum*	twig	Messina	April, 2010
NF-69	*N. parvum*	twig	Messina	April, 2010
NF-66	*N. parvum*	branch	Messina	April, 2010
NF-68	*N. parvum*	twig	Palermo	April, 2010
NF-70	*N. australe*	leaf	Palermo	April, 2010
NF-73	*N. australe*	twig	Palermo	April, 2010
NF-74	*N. vitifusiforme*	twig	Palermo	April, 2010
NF-76	*N. australe*	twig	Ragusa	April, 2010
NF-85	*N. parvum*	branch	Catania	September, 2009
BOT-4	*L. theobromae*	leaf	Sharkia	February, 2010
BOT-5	*L. theobromae*	leaf	Sharkia	February, 2010

BOT-7	*L. theobromae*	leaf	Sharkia	February, 2010
BOT-9	*L. theobromae*	leaf	Sharkia	February, 2010
BOT-11	*L. pseudotheobromae*	leaf	Sharkia	February, 2010
BOT-12	*L. pseudotheobromae*	branch	Fayoum	February, 2010
BOT-28	*L. pseudotheobromae*	branch	Giza	February, 2010

Isolates highlighted in the table were selected for pathogenicity test on mango seedlings
Isolates coded with BOT were isolated from local mango varities cultivated in different geographical areas in Egypt

Pathogenicity tests on mango seedlings

Studied isolates

Twenty isolates of *Botryosphaeria* represented the most isolated and identified species were selected in this study: six isolates (NF-4, NF-5, NF-20, NF-50, NF-69 and NF-85) representing *N. parvum*, three isolates (NF-22, NF-70 and NF-76) representing *N. austral*, two isolates (NF-71 and NF-74) representing *N. vitifusiforme*. Four isolates (BOT-4, BOT-5, BOT-7 and BOT-9) representing *L. theobromae*, four isolates (BOT-11, BOT-12, BOT-13 and BOT-28) representing *L. pseudotheobromae* and one isolate (NF-3) of *Neofusicoccum* sp. 3 (Table .5). The selected isolates were plated on PDA for 6-days at 25ºC in the dark prior to inoculation.

Inoculation of mango seedlings

Mango seedlings cv. Kensington Pride 3-4 month-old, with 30-40 cm in height growing in peat moss soil mixture in 15-W x 20-L cm, were selected for pathogenicity. Plants were maintained inside growth chambers under artificial light (10/14h) light-and-dark cycles at 25±2ºC and 70-80% relative humidity (RH). Four plants for each isolate, arranged in a completely randomized design were used. The epidermal tissues of the stem were disinfected with 70% ethanol then washed with sterilized distilled water and left to dry. A 5-mm cut was made with a sterile blade under the epidermis tissues (about 10-12-cm) below the apex of the stem before inoculation. A 5-mm-PDA plug cut from the actively grown margins of cultures was introduced into the stem wounds with the mycelium facing the cambium. The inoculated wounds with fungi were warped with Parafilm®, (Laboratory Film, Chicago, IL, USA) to prevent the dissection of the wounds and inoculum and to avoid the contamination. Control plants were inoculated with

sterile PDA plugs. The inoculated plants were examined periodically for symptoms development. After six weeks, the trial was ended and the bark lesion lengths as well as the length of the vascular discolouration under cambium were measured to assess the pathogenicity of the tested isolates. Re-isolation of the tested isolates was done from the margins of the necrotic lesions, and from the inoculated apple fruits on PDA amended with Streptomycin sulfate ($0.1g/L^{-1}$) and incubated in the dark at 25 ± 2 °C.

Data analysis

Trials were arranged in a completely randomized factorial design and all trials were repeated once. Four replicates (4 plants) per each isolate and one factor (isolates) considered as source of variation. Pathogenicity of the tested isolates was registered as extent of necrotic lesions developed around the inoculation sites for mango seedlings and apple fruit assay in (mm-diam). Data were subjected to the analysis of variance one-way ANOVA and the mean values of the lesions were compared using the Least Significant Difference (LSD) test ($P< 0.05$) (Stat Soft, Inc. 2004).

RESULTS

Apple fruit assay

Most of the inoculated *Botryosphaeria* isolates produced initially sunken rot lesions with light colour margins and showed to be pathogenic to apple fruit with variation (Fig. 32). In general, the analysis of variance revealed significant $P<0.05$ differences in lesion size (mm) between isolates of the same species of *Botryosphaeria* on apple fruit at the end of the trial. Significant differences (F value=348.2, P=0.001) were observed in lesion size within *L. pseudotheobromae* isolates of which, BOT-11 produced significantly larger lesion (85 mm) than the other isolates of the same species (Fig. 34C). Whereas , BOT-28, BOT-12 developed lesions not significantly different among each other (72.3 mm) (73 mm) respectively, but still significantly larger than the control (6 mm). Isolates of *L. theobromae* showed also significant difference (P=0.001) amonge each other. BOT-9 produced the largest lesion (81.6 mm), whereas, the other isolates produced similar lesion diameters and were significantly $P< 0.05$ larger than the control one (6 mm) (Fig. 34B). *L. theobromae* and *L. pseudotheobromae* isolates have shown to be

severly pathogenic to Apple fruits, which developed lesions around the inoculation site faster than the other *Botryosphaeria* isolates and completely rotten the fruit within 4 days of inoculation.

High significant variation (*F* value=72.9, *P*=0.001) was obtained between lesions caused by *N. parvum* isolates (Fig. 33). The majority of isolates produced large lesion sizes of which the largest lesions obtained by isolates NF-68 (89.6 mm), NF-69 (89.6 mm), NF-32 (87.3 mm) and NF-29 (87 mm). While, the smallest lesion sizes were obtained by isolates NF-30 (27.6 mm), NF-4 (16.6 mm) and NF-3 (12.8 mm). The last three isolates (NF-30, NF-4 and NF-3) exhibited very slow expansion of lesions around the inoculation site. Among *N. australe* isolates, a significant variation (*F* value=6.25, *P*=0.009) was observed in lesion sizes according to LSD test. Isolate NF-22 developed the largest lesion (54.3 mm) followed by NF-70 (32 mm) than the other isolates of the same specie as well as the controls (6 mm) (Fig. 34A). The only tested isolate of *N. vitifusiforme* induced lesion size (27.6 mm) similar to NF-30 and NF-56 (Fig. 33).

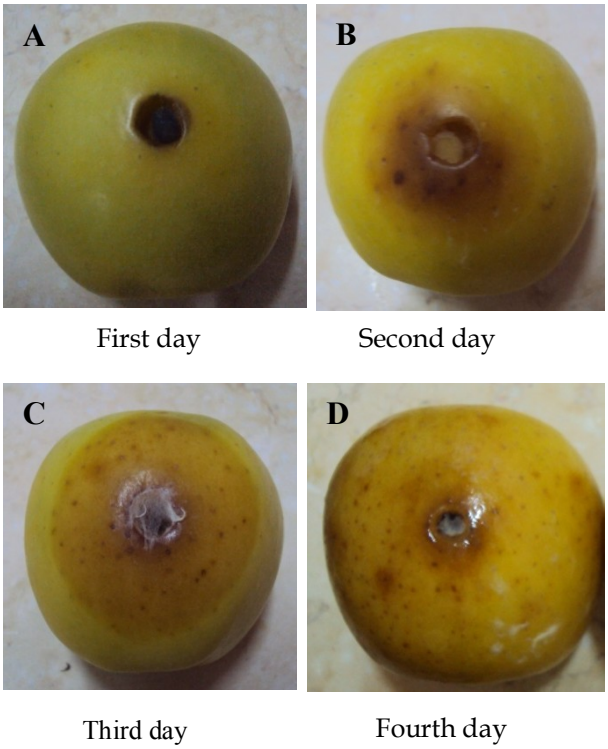
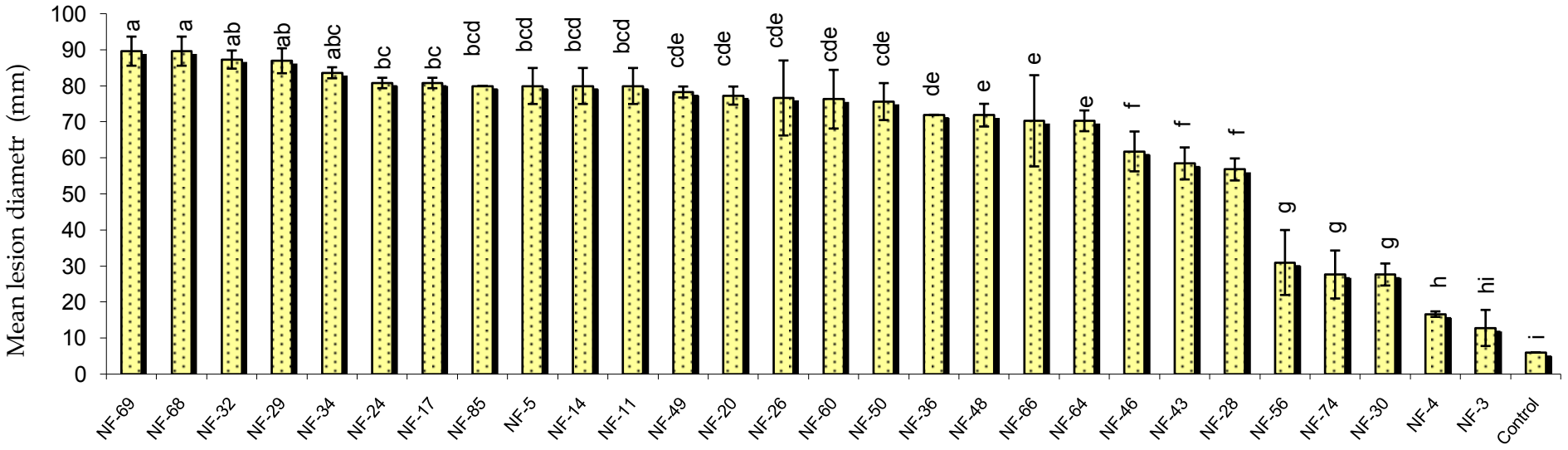


Figure 32 Development of symptoms on apple fruits inoculated with *Botryosphaeria* spp. after incubation at 25°C. A, B, C, D represents the expansion of the lesions around the inoculation sites over time.



N. parvum, *N.vitifusiforme* (NF-74) and *Neofusicoccum* sp.3 (NF-3) isolates

Figure 33 Mean lesion size-mm (y-axis) on cv. Granny Smith inoculated with 27 isolates of *Neofusicoccum parvum*, one isolate of *N. vitifusiforme* and one isolate of *Neofusicoccum* sp.3 (x-axis) after 4-days of incubation at 25±2°C. Data in these columns are the mean of *n*=6 lesions developed on three fruits ± (SD).Values within columns followed by the same letter are not significantly different according to least significant difference LSD test ($P<0.05$).

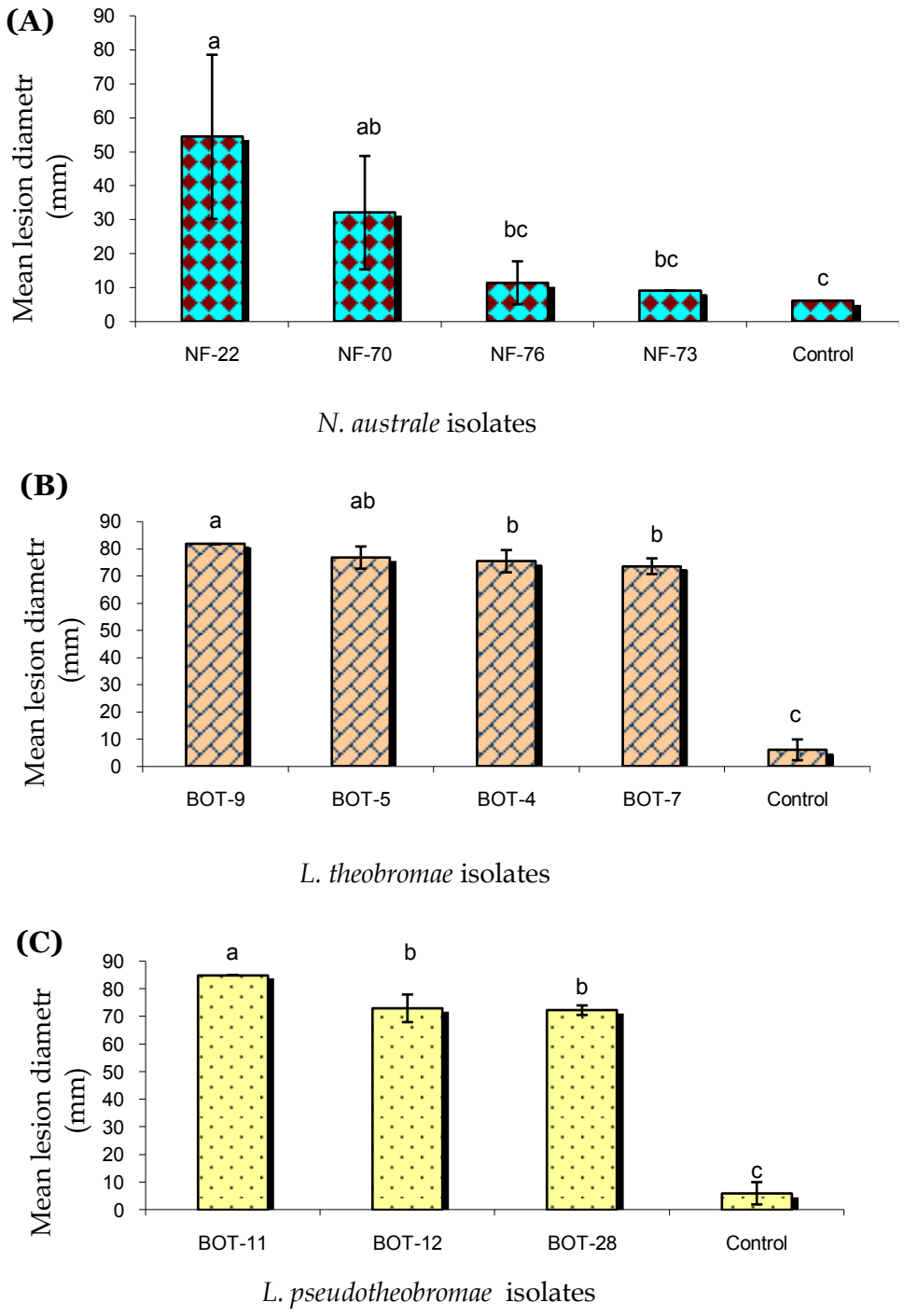


Figure 34 Mean lesion size-mm (y-axis) on cv. Granny Smith apple fruits inoculated with 11 isolates (4 of *N. australe,* 4 of *L. theobromae,* and 3 of *L. pseudotheobromae*) (x-axis) after 4-days of incubation at 25±2ºC. Data in these columns are the mean of *n*=6 lesions developed on three fruits ± (SD). The dimension of the inoculated wounds was not subtracted from final measurements. The real values were used for mean separation and analysis of variance. Means in these columns are not significantly different if followed by the same letter according to least significant difference LSD test ($P<0.05$).

Pathogenicity tests on mango seedlings

One week after inoculation primary symptoms appeared and dark brown to black necrosis developed around inoculated wounds (Fig. 35A). Brown to black vascular discolouration appeared under cambium tissues (Fig. 35B). In the early stage of infection, relatively some isolates (BOT-4, 5, 11, 28) induced typical dieback symptoms (Fig. 35C). There was a progressive in fungus growth upward and downward led to wilting and drying of the apical as well as the terminal leaves giving the scorch appearance. Died leaves remained attached to infected twigs for some time and at the end of the trial all leaves were fall. Fungal structures such as pycnidia and mycelium appeared on the necrotic died tissues with some isolates (Fig. 35D).

Analysis of variance indicated that there were significant variations $P<0.05$ between inoculated plants (lesion length) and *Botryosphaeria* species as well as between plants and isolates of the same species. *N. parvum* isolates showed little variation in lesion lengths ($F=2.89$, $P=0.024$) (Fig. 36A). Very similar lesion lengths were developed by all *N. parvum* isolates of which, the longest lesions were produced by NF-69 (45.66 mm long) and NF-5 (45.03 mm), and shown to be equally more virulent than the other less virulent isolates (NF-4, NF-20, NF-50, NF-85), which produced the smaller lesions significantly longer than the PDA inoculated control (25.84 mm). NF-3 developed significantly very small lesions and proven to be the least pathogenic isolate in both apple assay (Fig. 33A) and mango tree (Fig. 36A) trials comparing to other *N. parvum* isolates. However, the data of this isolate are not reliable because only a single isolate was available and the degree of variability could not evaluate. Althought, *N. australe* isolates (NF-22, NF-70, NF-76) were most, intermediately, least pathogenic respectively, in the apple fruit assay (Fig. 34B). Isolates NF-76 and NF-70 were however, more virulent in mango tree trial and produced lesions not significantly $P<0.05$ different among each other but still significantly ($F=7.42$, $P=0.004$) larger than the control (Fig. 36B). In addition, the two *N. vitifusiforme* isolates NF-71 and NF-74 were equally virulent by producing similar lesion lengths (31.65, 32.41 mm respectively), that significantly $P= 0.008$ larger than the control (Fig. 36C). While the only isolate (NF-74) inoculated on apple fruit was weekly pathogenic that showed slow progress in lesion extension around the inoculation point within 4 days post inoculation (Fig. 33).

Lesion sizes produced by *L. theobromae* isolates didn't differ greatly in both apple fruit and mango tree trials but showed somewhat little variation of which BOT-9 was significantly $P<0.05$ more pathogenic when inoculated on apple fruit than the other isolates. Whereas, the inoculation on mango trees showed that BOT-4 and BOT-5 were highly pathogenic which developed lesions lengths 65.50, 44.59 mm long respectively, significantly $P=0.003$ larger than the other isolates of the same species as well as than PDA plugs inoculated control plants (Fig. 36D). Isolates of *L. pesuotheobromae* developed similar values of lesion sizes in both apple fruit assay and mango tree trials of which BOT-11 was the most pathogenic to apple fruit than the other two isolates. On the opposite, inoculation of mango trees revealed that BOT-11, BOT-28 were significantly ($F=13.54$, $P=0.001$) the most virulent isolates that produced the largest lesions (63.34, 62.60 mm) respectively (Fig. 36E). The other isolates produced smaller lesions but significantly larger than the control.

Only two isolates (NF-5, NF-69) of *N. parvum,* one isolate (BOT-4) of *L. theobromae* and two isolates (BOT-11, BOT-28) of *L. pseudotheobromae* resulted in dieback symptoms at the end of the trials. There were minor differences observed between the length of bark lesions and the internal discolouration of cambium tissues produced by all inoculated *Botryosphaeria* species on mango plants (Fig. 36). However, theses differences are statistically different from control plants according to the Fisher LSD test at $P<0.05$. All *Botryosphaeria* spp. inoculated on mango tree were successfully re-isolated from the respective lesions and therefore, considered potential pathogens of mango.

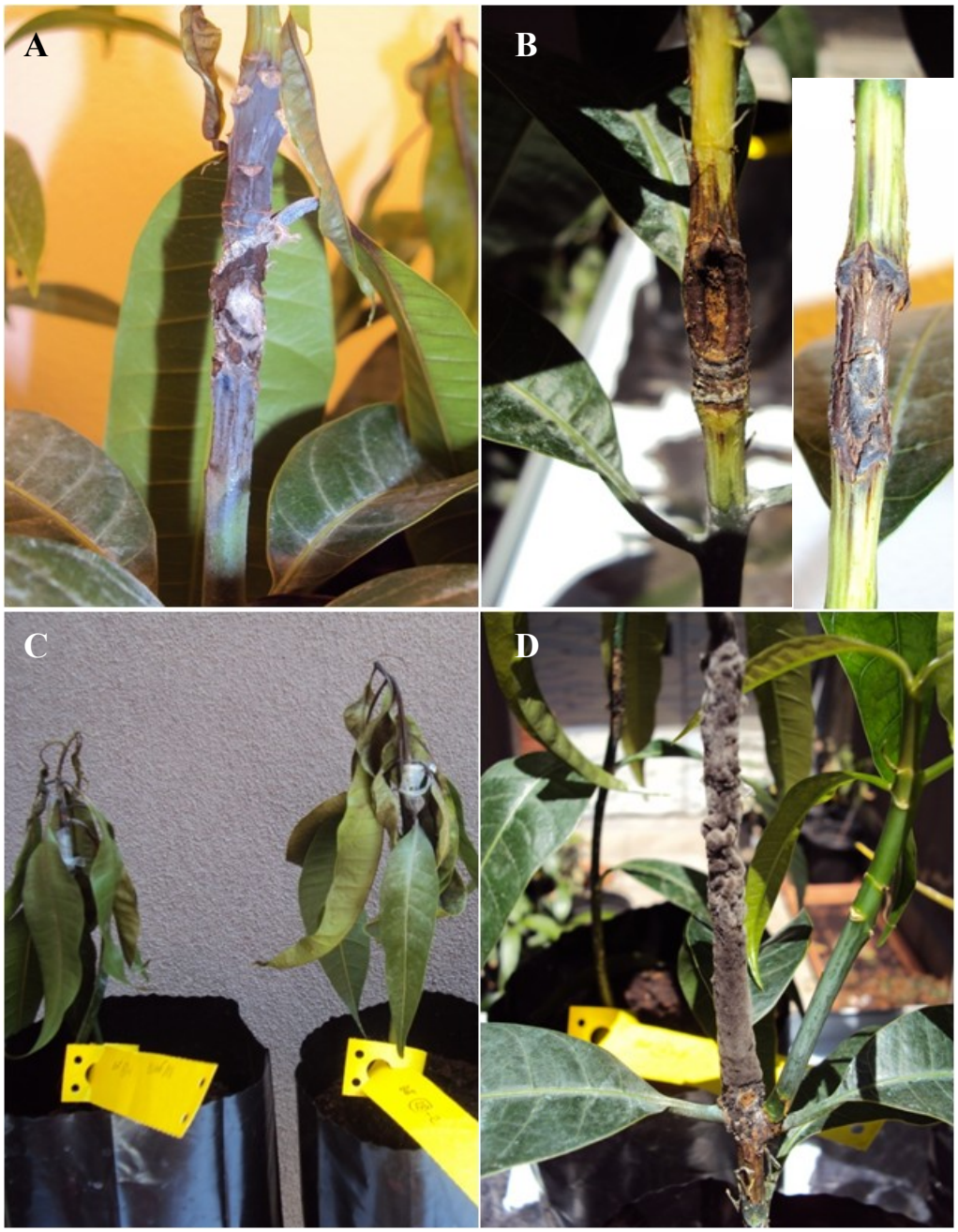
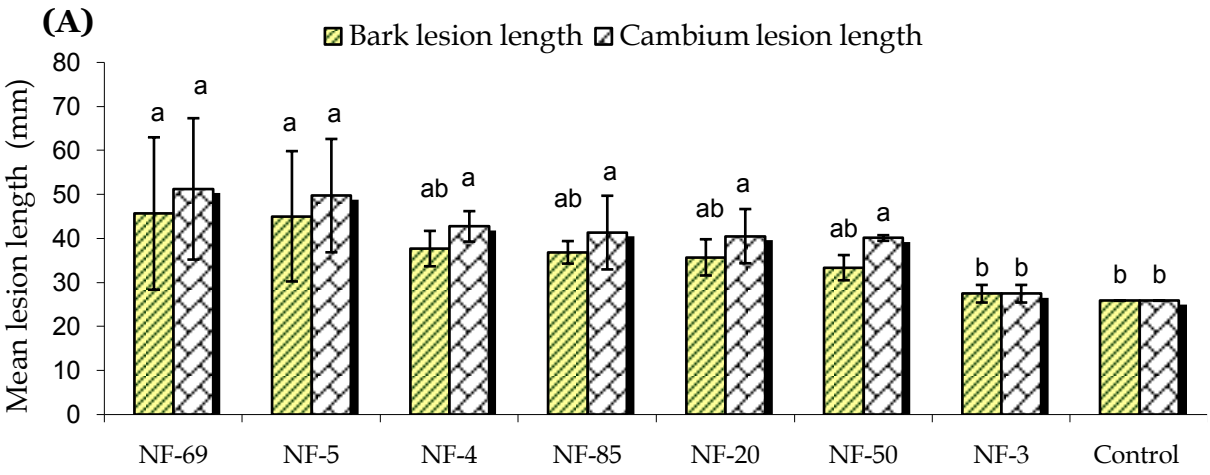
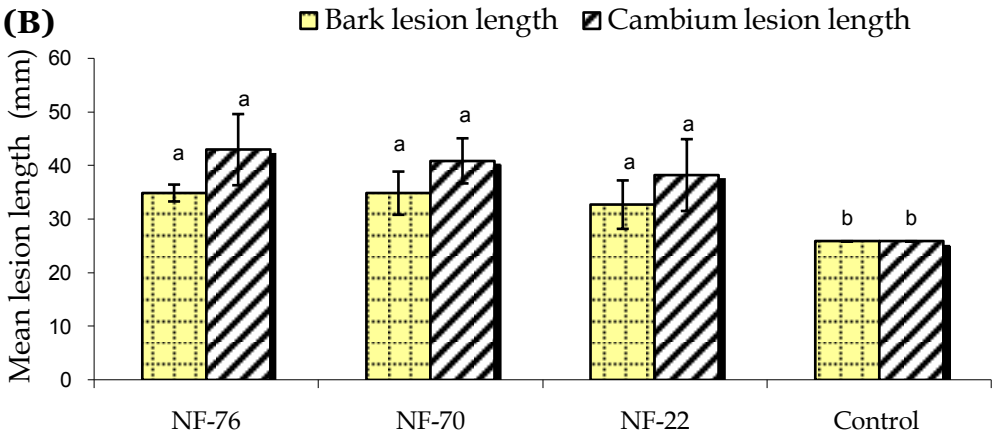


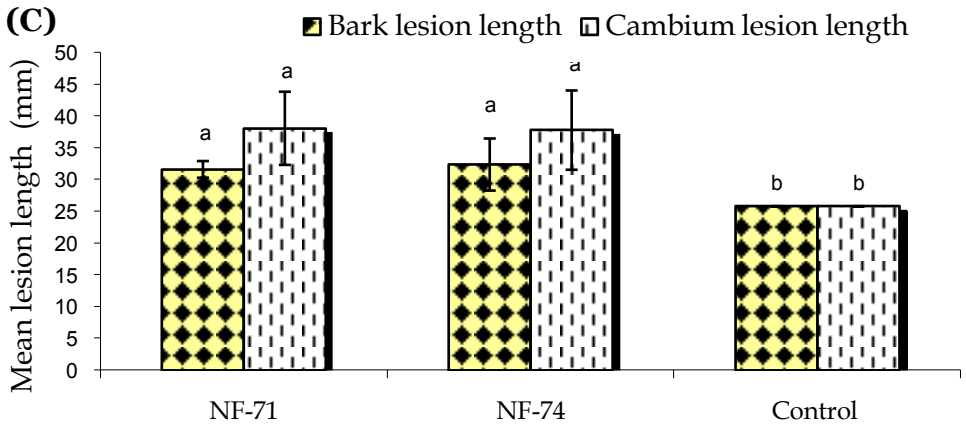
Figure 35 A, Symptoms developed around the inoculation sites as dark black necrosis and craking of the epidermal tissues in all inoculated plants. B, necrosis and brown discolouration of the cambium tissues extended upward and downward of the inoculation point. C, typical dieback symptoms of mango seedling 4-weeks after stem wound inoculation with *Botryosphaeria* spp., wilting and death of the apical shoots giving the scorch appearance appeared after rapid upward and downward progressive of the fungus which led to falling of the died leaves but can remain attached to the died twig for some time. D, abundant mycelial growth appeared on the necrotic tissues of died twigs after complete defoliation of the apical leaves.



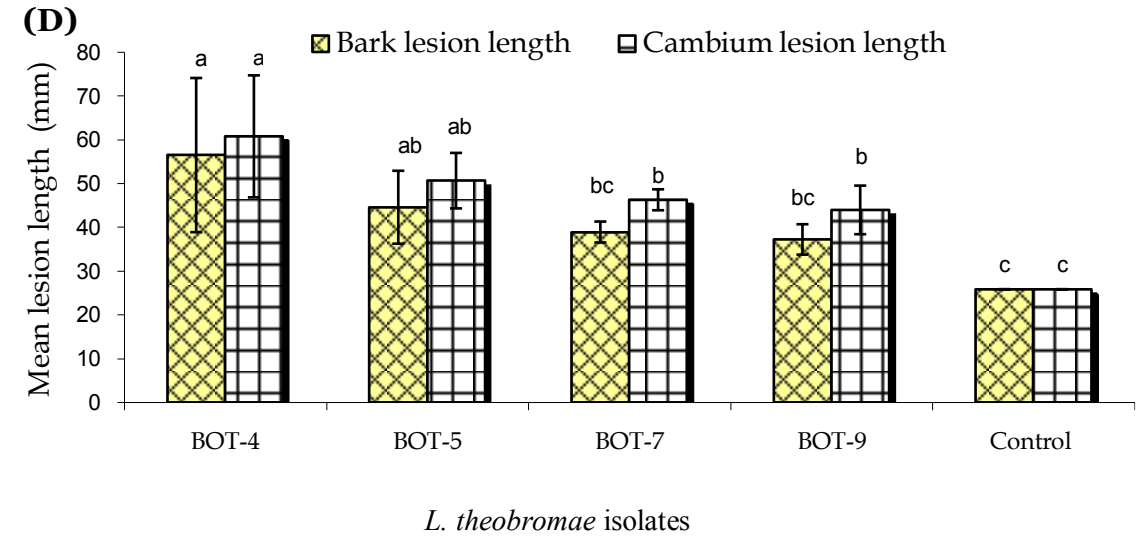
N. parvum isolates



N. australe isolates



N. vitifusiforme isolates



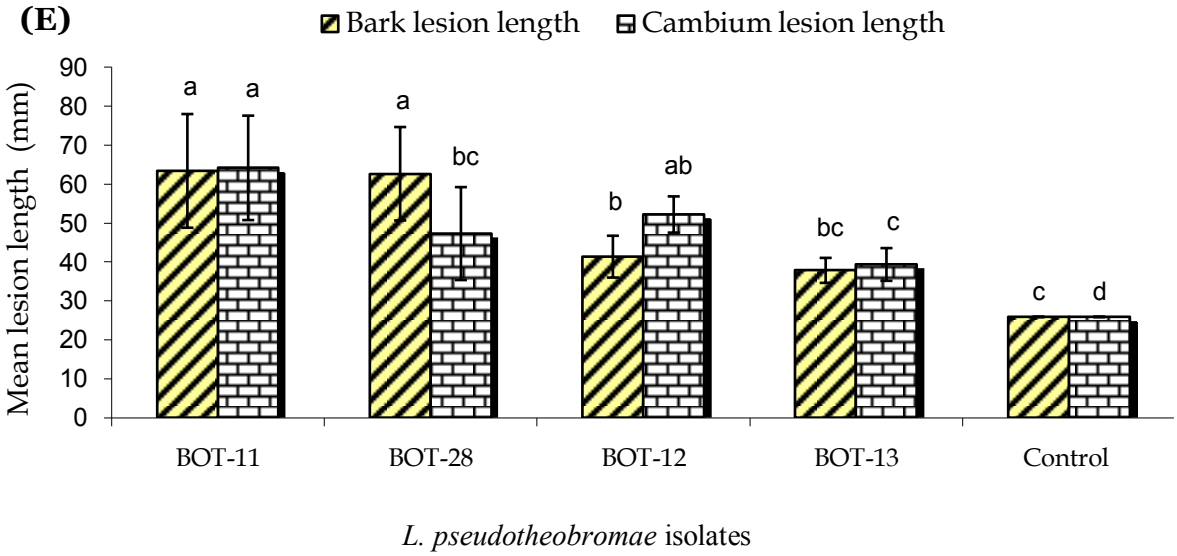


Figure 36 Mean lengths (mm) of bark and cambium lesions 6-weeks after inoculation on mango plants cv. Kensignton Pride with five *Botryosphaeria* species (A) *N. pavum,* (B) *N. australe*, (C) *N. vitifusiforme*, (D) *L. theobromae*, (E) *L. pseudotheobromae*. Data are the mean of total distance of the bark necrotic tissues above and below the point of inoculation ± (SD). Data are the mean of total distance of the internal discolouration above and below the point of inoculation ± (SD). The dimension of the inoculated wounds was not subtracted from final measurements.Values were transformed by Log2 for analysis and separation of means. Means within columns followed by the same letter are not significantly different according to LSD test (P<0.05).

DISCUSSION

Findings of this study revealed that the five-isolated *Botryosphaeria* species (*N. parvum*, *N. australe*, *N. vitifusiforme*, *L. theobromae* and *L. pseudotheobromae*) associated with mango die back symptoms and fruit stem-end rot were proved to be pathohenic on apple fruit as well as mango seedlings cv. Kensignton Pride. On the apple fruit, *N. parvum* produced lesion size larger than *L. pseudotheobromae*, *L. theobromae* and *N. australe* isolates. In contrast, on mango plants, *L. pseudotheobromae*, and *L. theobromae* were the most vireulent species followed by *N. parvum* and *N. australe*. This means that there was no correlation between apple fruit and mango seedlings trials since lesions on apple fruit do not reflect the relative pathogenic behavior of these fungi on mango plants. Our findings are similar to those obtained by Jacobs, (2002) who found also a lack of correlation between apple fruit and mango trials.

In previous studies, *N. parvum* was reported as well-known fruit tree and forest pathogen (Mohali et al, 2007; Slippers et al, 2004a; van Niekerk et al, 2004). In this study, isolates of this species were obtained from leaves, twigs and branches of mango showing dieback symptoms. *N. parvum* and *N. australe* were the most virulent fungi that produced the largest lesion sizes with the fact that they were the most dominant isolated fungi from typical symptoms of dieback, suggest that they might be the primary pathogens that contributed significantly to dieback of mango in Italy. Previous studies supported our suggestions that *N. parvum* and *N. australe* are common damaging pathogens on mango worldwide (de Oliveira Costa et al, 2010; Jacobs, 2002; Johnson et al, 1993; Ploetz et al, 1996; Slippers et al, 2005;). However, all inoculated species showed variable degrees of lesions formation, *N. parvum* and *N. australe* were considered as the most virulent among *Neofusicoccum* species on apple fruit as well as on mango trees.

N. vitifusifome was the least pathogenic specie on both apple fruit and on mango plants. The fungus was isolated from mango leaves and twigs with low frequency from only one location (Palermo). The low incidence together with the fact that the only two isolates of *N. vitifusifome* induced the smallest lesions on mango provide an indication that this species is of a little importance on mango and does not contribute significantly to mango die back disease. Our findings in concord with those of Begoude et al, (2009) who indicated that, *Lasiodiplodia*

mahajagana was not a primary pathogen due to its low incidence as well as low virulence on *Terminalia catappa.* Furthermore, similar results have reported by Jacobs (2002), who considered that *F. bacilliforme* is a weak pathogen to mango plants due to its low isolation frequency and the small lesions produced on mango plants.

The pathogenicity trials showed that both of *L. pseudotheobromae* and *L. theobromae* were the most pathogenic of all tested species on mango plants. Althought, several pathological studies have been conducted on the pathogenecity of *L. theobromae*; little information is available about the pathogenecity of *L. pseudotheobromae* and its geographical distribution. Recently, Sakalidis et al, (2011) reported *L. pesudotheobromae* for the first time on mango in Western Australia. In our study, *L. pesudotheobromae* was isolated for the first time from mango in Egypt and might have been considered as *L. theobromae* in the past. The recent study represents the first attempt to test the pathogenecity of the newly reported *L. pseudotheobromae* in Egypt. The importance of *L. pseudotheobromae* has overlooked in the past years, most likely because it was treated under *L. theobromae.* The results of pathogenecity revealed that *L. pseudotheobromae* was more pathogenic than *L. theobromae* in both apple and mango seedlings trials. The subsequential increase of the host range of this fungus and its importance as pathogen should be taken in consideration when establishing control stratigies.

The low frequency of the newly described species, *Neofusicoccum* sp. 18, *Neofusicoccum* sp. 3, *Lasiodiplodia* sp. 8 and *Lasiodiplodia* sp. 10 may give indication that they are of minor importance on mango since they were isolated in association with other dominant species. Extensive survey and pathological studies are needed in the future to clarify the ecology and to highlight their role to cause diseases on mango.

All tested *Botryosphaeria* species showed that they were able to spread endophytically through the internal tissues above and below points of inoculation causing brown to black discolouration. Similar results were obtained by Ramos et al, (1991), Khanzada et al, (2004a) and Ploetz et al, (1996) who demonstrated that inoculation of mango plants with *Botryosphaeria* species manifested various external and internal symptoms such as, outer necrosis, vascular discolouration, defoliation, apical dieback and gummosis. However, no gummosis symptoms were observed on the inoculated plants in this study. The upward and downward

progress inside the apparently healthy tissues along the mango stem can reflect the well-known endophytic nature of these fungi (Ploetz, 2004; Ploetz et al, 1996; Slippers and Wingfield, 2007). The external and internal symptoms developed after inoculation can evidently reflect the capacity of inoculated fungi to cause diseases and to spread rapidly throughout the vascular tissues even if their hosts are not subjected to stress factors.

The endophytic infections of fruit by Botryopshaeriaceae are common and considered as serious post-harvest diseases of mango (Johnson et al, 1993; Slippers and Wingfield, 2007). Fruit can be infected early in the field but the infection remain quiescent and start to develop with the ripening of the fruit of which small to large scattered depressed rot lesions appear on the fruit surface and lead sometimes to the rotten of the whole fruit. Although mango fruit have been used in pathogenicity studies (de Oliveira Costa et al, 2010; Johnson et al, 1992; Sakalidis et al, 2011), no attempt made to inoculate mango fruits due to the lack of fruits in this period, most of the *Botryosphaeria* species are mostly isolated from mango twigs, leaves and branches, and this may effect on our results.

CHAPTER .6

GENERAL DISCUSSION AND CONCLUSION

Mango as important tropical fruit tree has been adapted to the environmental conditions in the southern Italy (Sicily). It is expected that the commercial and backyard plantings of mango trees will continue to increase. The sanitary status of mango orchards was worthy and needed quick and accurate intervention to understand the unknown causal agents of the phytosanitary problems observed in mango plantations. In the present study, attention was paid for the fungal diseases expressed by several patterns of symptoms that attributed to complex of fungal taxa isolated during this study. Various fungal taxa were associated with different symptoms types and it was quit hard to recognize the causal agents associated with each type of these symptoms since most of them were similar.

The isolated fungi in this study were responsible to one or more of the reported symptoms in the field. *Alternaria* spp. was consistently isolated and recovered from all assessed mango collected samples with high frequency from leaves twigs and branches. The fungus is a well-known mango pathogen, which is common and cosmopolitan on mango in the arid environments (Ploetz, 2003; Ploetz, 2004; Prusky et al, 2002). *Alternaria* spp. was associated with brown and black leaf spot symptoms, which mainly observed on margins of the old leaves and was identified solely based on conidia and colony morphology. *Colletotrichum* spp. was associated with symptoms of anthracnose diseases such as dark brown to black spot and canker on leaves and twigs of mango. *Pestalotiopsis* spp. was associated with distinct symptoms of grey leaf spot on leaves and branches and was isolated from symptomatic and asymptomatic samples. There was a variation in conidia and colony morphology among isolated fungi. These variations were not adequate and unsatisfactory for reliable differentiation between species of the same genus, since the conidia, colony morphology and colour vary due to the influence of environmental factors, composition of media and the existence of intermediate forms. *Alternaria* spp., *Colletotrichum* spp. and *Pestalotiopsis* spp., were proved to be adequately pathogenic to detached mango leaves under laboratory conditions with some variation. These variations in virulence within

fungi as well as within isolates of the same fungus can attributed to several factors not investigated in the present work. However, isolation, traditional identification and pathogenicity tests have been conducted but further studies would be required to overcome the taxonomic confusion existing among these fungi due to the overlapping of morphological criteria and to confirm the incidence of these fungi as limiting factors to mango production.

Investigations of mango plantations revealed the presence of distinct symptoms of tip dieback, cankers, brown leaf lesion, and fruit rot which was mainly attributed to one or more members of Botryosphaeriaceae reported and well described in this study. A diverse array of Botryosphaeriaceae were isolated and identified on the basis of morphology and DNA sequence of ITS and EF1-α. Our study revealed five species of *Neofusicoccum* of which *N. parvum*, *N. australe* and *N. vitifusiforme* are well-known species and other two named *Neofusicoccum* sp. 3 and *Neofusicoccum* sp. 18, were described as novel species. *N. parvum* was previously reported on mango, treated under *D. dominicana* (Johnson, 1992), and considered as a well-known mango pathogen in several countries (de Oliveira Costa et al, 2010; Jacobs, 2002; Javier-Alva et al, 2009; Johnson, 1992; Ramos et al, 1991). The fungus was the most prevalent isolated species from symptomatic mango trees with high incidence. It was isolated from twigs, branches, leaves and fruit of mango cv. Kensington Pride. Therefore, it was evident that the high incidence and frequency of isolation along with the fact that *N. parvum* was the most virulent species among the tested *Neofusicoccum* species on mango, this species can considered as the primary causal agent of mango dieback disease in Italy.

N. asutrale was frequently isolated from mango after *N. parvum*; the fungus was associated mainly with twig and branches dieback symptoms. This fungus was reported as pathogen to several hosts worldwide (Damm et al, 2007; Lazzizera et al, 2008; Taylor et al, 2005; van Niekerk et al, 2004). It has not been reported on mango, thus in this study *N. asutrale* has been reported for the first time on mango in Italy and in the world. The fungus was identified easily from other *Neofusicoccum* species based on morphology and DNA sequence data and showed to be adequately pathogenic to mango seedlings. We suggest that *N. australe* can play significant role along with *N. parvum* in causing dieback disease on mango. *N. vitifusiforme* was found with low frequency from only one site

(Palermo) and was less pathogenic to mango suggesting that it has a minor role in causing disease on mango. It was reported as pathogen to other hosts (Damm et al, 2007; Kong et al, 2010; Lazzizera et al, 2008) and has not been reported on mango; therefore, we consider this is the first report of *N. vitifusiforme* on mango in Italy and the world. Due to their low frequency among the other dominant species, the pathogenicity studies of the newly described species, *Neofusicoccum* sp. 3 and *Neofusicoccum* sp. 18 were not investigated in our study and thus we suggest that they did not associate with mango diseases.

Four species of *Lasiodiplodia* were recovered from mango plantations in Egypt of which *L. theobromae* is considered as the primary causal agent of mango dieback in Egypt for many years (Ragab et al, 1971). This specie has been reported as mango pathogen worldwide associating commonly with cankers and dieback symptoms and appeared to be dominant in warm environments (Abdollahzadeh et al, 2010; de Oliveira Costa et al, 2010; Jacobs, 2002; Khanzada et al, 2004a, b; Sangchote, 1991). It was isolated from leaves, branches and twigs of local mango varieties, showing dieback symptoms. Until the recent study, it was realized that this species was the main causal agent of mango dieback disease in Egypt. However, recent phylogenetic studies basing on the multiple gene sequence demonstrated that *L. theobromae* is a complex species contained other cryptic and unknown species. Therefore, the DNA sequence of ITS and EF1-α along with the morphological data resulted in separation other three *Lasiodiplodia* species that were treated under *L. theobromae* in Egypt. In the present study, DNA sequence and morphology data were able to delimit this species from collection of *Lasiodiplodia*-like isolates previously identified as *L. theobromae*. Recently, Alves et al, (2008) identified and described *L. pesudotheobromae* as new species. Sakalidis et al, (2011) reported this specie on mango (*Mangifera indica* L.) in Western Australia and recently reported on *Mangifera sylvatica* in China by Zhao et al, (2010). *L. pesudotheobromae* proved to be more pathogenic to mango plants than *L. theobromae* that induced the largest lesions and sever dieback symptoms after two weeks of inoculation. Therefore, the high frequency isolation together with the results of pathogenicity led us to consider that *L. pesudotheobromae* is an important fungal pathogen on mango in Egypt. The newly described species *Lasiodiplodia* sp. 8 and *Lasiodiplodia* sp. 10 were phylogeneticaly and morphologically distinct from the other studied *Lasiodiplodia* isolates. These species were found in low incidence and

were isolated in association with the other dominant species. These new species might pose unconstructive effect on mango in the future therefore; additional studies are needed to clarify their role in causing disease to mango.

In conclusion, our aim of this study to identify the causal agents associated with the different symptoms in the field was accomplished. Based on our results, the five species of *Botryosphaeria* occur on mango in Italy and the four species occur on mango in Egypt represent restrictive factors to mango industry in both countries. Therefore, we recommend that the future studies should focus not only on the understading the ecology of such fungi but also to develope control approaches to limit the impact of them on mango production. Some important practices could be implemented in the the integrated management program. These include the agricultural practices: e.g., sanitation of mango orchards by removing plant materials under ground provides a useful approach to reduce the inoculums density and therefore the disease incidence. Pruning of the trees, exposing all tree sides to the sun and maintaining the air cycling inside the canopy could efficiently reduce the humidity and consequently creat unfavorable conditions to the disease to develop, Pruning and removing the died branches and twigs could reduce the primary inoculums required for new infections. Prevention of introducing infected plant materials into new free area should be taken in consideration. In case of high and sever infections, application of different fungicides would be highly essential to implement in the integrated management program in order to reduce the diseases spreed and severity.

CHAPTER .7

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