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Detection of new *Calonectria* spp. and *Calonectria* Diseases and Changes in Fungicide Sensitivity in *Calonectria scoparia* Complex

This thesis is presented for the degree of Doctor of Philosophy by

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CHAPTER 1 - The genus *Calonectria* and the fungicide resistance 1
1.1 Introduction 2
1.1.1 *Calonectria* 2
1.1.2 Importance of *Calonectria* 3
1.1.3 Morphology 6
1.1.4 Pathogenicity 9
1.1.5 Microsclerotia 9
1.1.6 Mating compatibility 10
1.1.7 Phylogeny 12
1.1.7.1 *Calonectria scoparia* species complex 13
1.1.7.2 *Calonectria pauciramosa* 16
1.1.7.3 *Calonectria polizzii* 18
1.1.8 *Calonectria* diseases in Mediterranean environment 19
1.1.9 *Calonectria* diseases control 27
1.2 Fungicide resistance 33
1.2.1 Introduction 33
1.2.2 Fungicide control of crop disease 33
1.2.3 Defining fungicide resistance 34
1.2.4 Occurrence of resistance 36
1.2.5 Resistance mechanism 39
1.2.6 Monitoring 40
1.2.7 Assessing the risk 42
1.2.8 Management strategies 44
1.2.9 Specific management strategies 49
1.2.9.1 Benzimidazoles 50
1.2.9.2 SBIs (sterol biosynthesis inhibitors) 51
1.3 Fungicide resistance in *Calonectria* spp. 53
1.4 Thesis aims 54
CHAPTER 2 - Detection of new disease on *Laurus nobilis* and identification of pathogen species responsible 61
2.1 Introduction 62
2.2 Materials and methods 63
2.3 Results and discussion 67

CHAPTER 3 - *Calonectria* spp. causing leaf spot, crown and root rot of ornamental plants in Tunisia 70
3.1 Introduction 71
3.2 Materials and methods 74
3.2.1 Disease survey and fungal isolates 74
3.2.2 DNA sequence comparisons 74
3.2.3 Taxonomy 77
3.2.4 Pathogenicity 77
3.3 Results 78
3.3.2 Disease survey and fungal isolates 78
3.3.3 DNA sequence comparisons 80
3.3.4 Taxonomy 82
3.3.4.1 *Calonectria pseudomexicana* 82
3.3.4.2 *Calonectria tunisiana* 84
3.3.5 Pathogenicity 86
3.4 Discussion 91
CHAPTER 4 - Changes in sensitivity to prochloraz in *Calonectria scoparia* complex in southern Italy 93
4.1 Introduction 94
4.2 Materials and methods 95
4.2.1 Sampling sites and fungal population 95
4.2.2 Molecular identification by using DNA sequence comparisons 97
4.2.3 Morphological identification 99
4.2.4 Assessment of fungicide sensitivity in vitro 100
4.2.5 Assessment of fungicide sensitivity on model plant-host 102
4.2.6 Assessment of fungicide sensitivity on Feijoa sellowiana 103
4.2.7 Statistical data analysis 104
4.3 Results 106
4.3.1 Molecular identification by using DNA sequence comparisons 106
4.3.2 Morphological identification 109
4.3.3 Assessment of fungicide sensitivity in vitro 109
4.3.4 Assessment of fungicide sensitivity on model plant-host 116
4.3.5 Assessment of fungicide sensitivity on Feijoa sellowiana 120

4.4 Discussion 122
Acknowledgements 126
References 128

CHAPTER 1 - The genus *Calonectria* and the fungicide resistance

1.1 Introduction

1.1.1 *Calonectria*

The genus *Calonectria* (*Ca.*) was erected in 1867 by De Notaris, based on *Ca. daldiniana* collected on leaves of *Magnolia grandiflora* (Magnoliaceae), in Italy (Rossman 1979a). Rossman (1979a) later reduced *Ca. daldiniana* to synonymy under *Ca. pyrochroa*, and defined this nectrioid fungus as having an ascocarp wall structure that is brightly coloured, changing to blood-red in 3 % KOH solution, warty to scaly and with a *Cylindrocladium* (*Cy.*) anamorph (Rossman 1993, Rossman *et al.* 1999). However, due to the restricted morphological characteristics of the teleomorph (Rossman 1979b, 1983), specimens can in many cases only be identified to species level if the anamorph is present (Schoch et al. 2000b, Crous 2002).

The anamorph genus *Cylindrocladium*, which is based on *Cy. scoparium*, was first described by Morgan (1892) in the U.S.A., where it was found as saprobe on a pod of *Gleditsia triacanthos*. Although Morgan (1892) failed to mention the stipe extension terminating in a vesicle of characteristic shape, he defined the genus as having branched conidiophores producing cylindrical conidia. This fungus has a wide distribution in sub-tropical and tropical regions of the world, and species are pathogenic to numerous plants (Crous 2002).

Calonectria resides in the Nectriaceae, one of three families in Hypocreales, an order that has been reviewed extensively (Rogerson 1970, Rossman 1983, Rossman *et al.* 1996, 1999). The Nectriaceae family is circumscribed as having uniloculate ascomata that are orange to purple and not immersed in well-developed stromata (Rossman *et al.* 1999). The family includes approximately 20 genera of socio-economic importance and of these, *Calonectria* are more clearly distinguished from the others by their *Cylindrocladium* anamorphs and relevance as plant pathogens.

The first monograph of *Cylindrocladium*, by Boedjin & Reitsma (1950), introduced seven *Cylindrocladium* species with a *Calonectria* connection to one of these species. Later, in her treatment of *Calonectria*, Rossman (1983) recognized five species including the novel *Ca. ophiospora*. However, this

species description did not include the anamorph state. The circumscribed type, *Ca. pyrochoa*, was also incorrectly reduced to synonymy with several other species based only on the teleomorph morphology. Peerally (1991a) highlighted this in a monograph of *Cylindrocladium*, where he regarded the anamorph morphology as important in distinguishing species of *Calonectria*. He subsequently recognized 10 *Calonectria* species with their *Cylindrocladium* anamorphs, including an additional 16 *Cylindrocladium* species not associated with a teleomorph. However, he mistakenly reduced *Cylindrocladiella*, a genus that accommodates *Cylindrocladium*-like species with small conidia (Boesewinkel 1982), to synonymy with *Cylindrocladium*.

The monograph of *Cylindrocladium* by Crous & Wingfield (1994) entrenched the importance of anamorph characteristics in the taxonomy of *Calonectria* spp. In this monograph, 22 *Cylindrocladium* species and one variety were recognised, associated with 16 *Calonectria* species. Five species were assigned to the genus *Cylindrocladiella* based on morphological characters of the holomorph. The focus on anamorph characteristics is perpetuated in the most recent monograph (Crous 2002), which recognized 28 *Calonectria* species, all associated with *Cylindrocladium* anamorphs and an additional 18 *Cylindrocladium* species for which teleomorph states were not known. Of the latter group, seven taxa were of doubtful authenticity. Actually, 109 *Calonectria* and 96 *Cylindrocladium* species are recognised (Crous 2002, Crous *et al.* 2004b, 2006a, Gadgil & Dick 2004, Lombard *et al.* 2009, 2010d).

A general search on MycoBank (www.mycobank.org; Crous *et al.* 2004a, Robert *et al.* 2005) and Index Fungorum (www. indexfungorum.org) provide a total of 291 and 306 name records respectively for *Calonectria*. A similar search for *Cylindrocladium* species on both electronic databases indicated a total of 98 and 92 names records respectively.

1.1.2 Importance of *Calonectria*

The genus *Calonectria* was initially regarded as a saprobe as no disease symptoms could be induced by inoculating a suspected host (Graves 1915). The first proof of pathogenicity of these fungi was provided by Massey (1917), and

subsequently by Anderson (1919), who showed pathogenicity of *Ca. morganii* (as *Cy. scoparium*). Subsequently, *Calonectria* species have been associated with a wide range of disease symptoms on a large number of hosts worldwide (Crous 2002). In the past, several authors showed that *Calonectria* species cause disease on plants residing in approximately 30 plant families (Booth & Gibson 1973, French & Menge 1978, Peerally 1991a, Wiapara *et al.* 1996, Schoch *et al.* 1999). Upon closer inspection, the number of plant families is actually closer to 100 and approximately 335 plant host species (Crous 2002). The plant hosts include important forestry, agricultural and horticultural crops and the impact of these plant pathogens has likely been underestimated.

The majority of disease reports associated with *Calonectria* species in forestry include hosts in five plant families, of which the most important are associated with Fabaceae (*Acacia* spp.), Myrtaceae (*Eucalyptus* spp.) and Pinaceae (*Pinus* spp.). Disease symptoms include cutting rot (Crous *et al.* 1991, Crous 2002, Lombard *et al.* 2009, 2010d), damping-off (Batista 1951, Cox 1953, Terashita & Itô 1956, Sharma & Mohanan 1982, Sharma *et al.* 1984, Crous *et al.* 1991, Brown & Ferreira 2000, Crous 2002, Taniguchi *et al.* 2008) leaf diseases (Cox 1953, Hodges & May 1972, Barnard 1984, Sharma *et al.* 1984, El-Gholl *et al.* 1986, Peerally 1991b, Crous *et al.* 1993b, Crous & Wingfield 1994, Crous *et al.* 1998b, Schoch & Crous 1999, Schoch *et al.* 1999, Booth *et al.* 2000, Park *et al.* 2000, Crous & Kang 2001, Gadgil & Dick 2004), shoot blight (Sharma *et al.* 1984, Crous *et al.* 1991, 1998b, Crous & Kang 2001), stem cankers (Cox 1953, Sharma *et al.* 1984, 1985, Crous *et al.* 1991, Lombard *et al.* 2009) and root rot (Cox 1953, Hodges & May 1972, Cordell & Skilling 1975, Mohanan & Sharma 1985, Crous *et al.* 1991, Lombard *et al.* 2009). The majority of these diseases are associated with seedling and cutting production in forestry nurseries, but in a few cases *Cylindrocladium* species have also been reported from older, established commercial plantations. In these cases the pathogens have been reported to cause leaf diseases and shoot blight resulting in defoliation of trees leading to loss of vigour (Hodges & May 1972, Sharma *et al.* 1985, Booth *et al.* 2000, Park *et al.* 2000, Crous & Kang 2001, Crous 2002, Old *et al.* 2003, Rodas *et al.* 2005).

In agriculture, *Calonectria* species have been reported to cause diseases on several economically important crops. Several plant families of agricultural

importance are susceptible to *Calonectria* infections, including Fabaceae and Solanaceae. Important diseases in these families are Cylindrocladium black rot of *Arachis hypogea* (peanut) and red crown rot of *Glycine max* (soybean) caused by *Ca. ilicicola* and *Ca. pyrochroa* in the USA (Bell & Sobers 1966, Beute & Rowe 1973, Rowe *et al.* 1973, Sobers & Littrell 1974, Rowe & Beute 1975, Phipps *et al.* 1976, Johnson 1985, Dianese *et al.* 1986, Berner *et al.* 1988, 1991, Culbreath *et al.* 1991, Porter *et al.* 1991, Varon 1991, Hollowell *et al.* 1998, Kim *et al* 1998) and Cylindrocladium tuber rot of *Solanum tuberosum* (potato) (Boedijn & Reitsma 1950, Bolkan *et al.* 1980, 1981) by *Ca. brassicae* (as *Cy. gracile*) in Brazil. Other diseases associated with *Calonectria* species on agricultural crops include root rot and leaf diseases of fruit bearing and spice plants (Jauch 1943, Wormald 1944, Sobers & Seymour 1967, Nishijima & Aragaki 1973, Milholland 1974, Krausz & Caldwell 1987, Hutton & Sanewski 1989, Anandaraj & Sarma 1992, Risède 1994, Jayasinghe & Wijesundera 1996, Risède & Simoneau 2001, Vitale & Polizzi 2008), post-harvest diseases of fruits (Fawcett & Klotz 1937, Boedijn & Reitsma 1950, Sepiah 1990, Fitzell & Peak 1992, Vaidya & Roa 1992, Sivapalan *et al.* 1998), root and crown rot of *Medicago sativa* (alfalfa) (Ooka & Uchida 1982, Hwang & Flores 1987), and sheath net blotch of *Oryza sativa* (rice) (Crous 2002).

On horticultural crops, *Calonectria* species have been reported mostly from the Northern Hemisphere, especially in gardens and ornamental commercial nurseries in Europe and Asia (Polizzi & Crous 1999, Polizzi 2000, Crous 2002, Henricot & Culham 2002, Pérez-Sierra *et al.* 2007, Polizzi *et al.* 2007a, b, Hirooka *et al.* 2008, Polizzi *et al.* 2009a, Vitale *et al.* 2009). Hosts in this sector include ornamental trees, shrubs and cut flowers in several plant families, most commonly in Arecaceae, Asteraceae, Ericaceae and Rosaceae. A wide range of disease symptoms are recorded including crown-, collar- and root rot, leaf spots, and cutting rot (Massey 1917, Anderson 1919, Aragaki *et al.* 1972, 1988, Peerally 1991b, Uchida & Kadooka 1997, Polizzi & Crous 1999, Polizzi 2000, Crous 2002, Henricot & Culham 2002, Henricot & Beales 2003, Poltronieri *et al.* 2004, Lane *et al.* 2006, Pérez-Sierra *et al.* 2006, 2007, Polizzi *et al.* 2006a, b, 2007a, b, Vitale & Polizzi 2007, Aghajani *et al.* 2008, Hirooka *et al.* 2008, Vitale *et al*. 2008, Polizzi *et al.* 2009a, Vitale *et al.* 2009).

1.1.3 Morphology

Morphological or phenotypic characters have played a major role in the description of fungal species (Brasier 1997, Taylor *et al.* 2000) and form the basis of new fungal descriptions as required by the ICBN (McNeill *et al.* 2005). In recent years, the use of morphological characters alone to delimit new species has been set aside because considered not enough, with more focus being placed on biological and phylogenetic characters (Rossman 1996, Brasier 1997, Taylor *et al.* 2000). This trend is also evident in recent studies on *Calonectria* species (Crous *et al.* 2004b, 2006a).

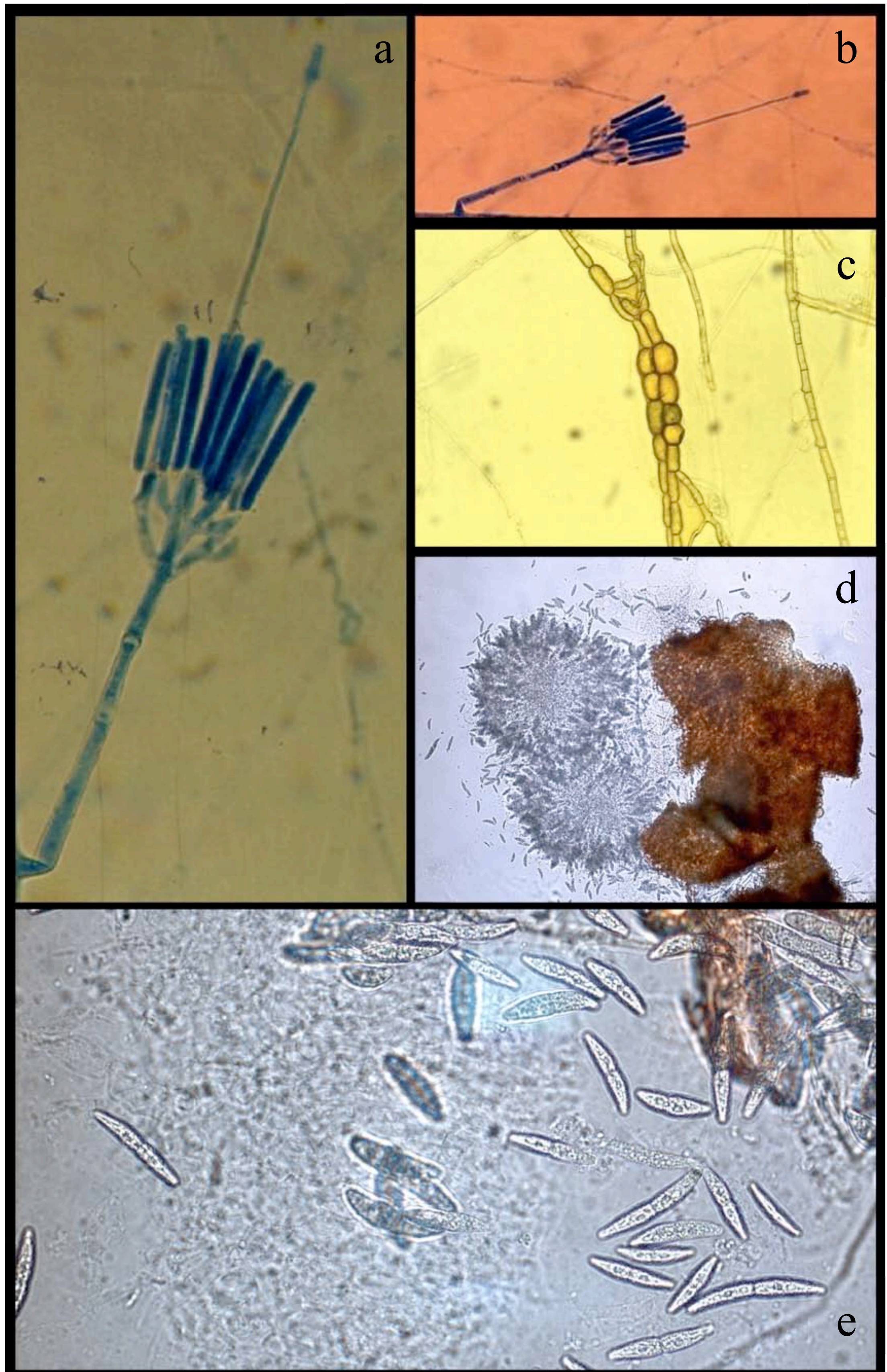
The morphology of *Calonectria* and to a greater extent its anamorph, *Cylindrocladium*, has been important in the taxonomic history of these fungi. Prior to the 1990s, identification of species was based on morphological characteristics and to a lesser extent on sexual compatibility using standardised media (Boedijn & Reitsma 1950, Peerally 1991a, Crous *et al.* 1992, Crous & Wingfield 1994, Crous 2002). This resulted in the establishment of several species complexes, as many *Cylindrocladium* species are morphologically very similar. These include the *Ca. scoparia* complex (Schoch *et al.* 1999), *Ca. brassicae* (as *Cy. gracile*) complex (Crous *et al.* 2004b) and *Ca. kyotensis* complex (Crous *et al.* 2006a). Characteristics of the anamorphs that are extensively employed in identifications include vesicle shape, stipe extension length and macroconidial septation and dimensions (Boesewinkel 1982, Peerally 1991a, Crous & Wingfield 1994, Crous 2002). The morphological characteristics of the teleomorph those are important for identifications are ascospore septation and dimensions, ascospore number within the asci and perithecial colour. Perithecia of *Calonectria* species are morphologically very similar and these are not typically useful in identifications (Crous & Wingfield 1994, Crous 2002).

Recently, for morphological identification of the anamorphs and teleomorph, single conidial cultures were prepared on synthetic nutrient-poor agar (SNA; Nirenburg 1981, Lombard *et al.* 2009, 2010). Inoculated plates were incubated at room temperature and examined after 7d. Gross morphological characteristics were determined by microscopic observations. The measurements of conidia, optimal growth temperatures were determined generally on MEA at 5–35 °C in

5 °C intervals in the dark. Colony colours were determined after 7 d on MEA at 25 °C in the dark. Descriptions, nomenclature, and illustrations were deposited in MycoBank (Crous *et al.* 2004a). This kind of morphological characterization was applied in last years (Lombard et al. 2010b,c,d Lombard et al.2011, Chen et al. 2011), but any difficulties experienced in morphological identification due to the high level of similarity among the species observed, have led to several molecular approaches being employed to identify *Calonectria* spp.

The use of biochemical techniques can also be used in phenotypic characterisation. These include substrate utilisation and cell wall polysaccharide analysis. The use of aminopeptidase specificity (Stevens *et al*. 1990) and utilisation of nitrogen and carbon (Hunter & Barnett 1978, Sharma *et al*. 1992) have been used successfully to separate several *Cylindrocladium* species. The use of polysaccharides obtained from cell walls of *Cylindrocladium* positively identified linkages between asexual species and their respective *Calonectria* teleomorphs (Ahrazem *et al*. 1997). However, this method has been found to limit value as some species in complexes could not be distinguished (Crous 2002).

Figure 1. a-b. Conidiophores with terminal vesicles of *Calonectria* spp. c. Cluster of chlamydospores. d. Perithecium with asci and ascospores. e. Ascospores.



1.1.4 Pathogenicity

Representatives strains of *Cylindrocladium* were initially considered to be saprophytic (Graves, 1915). This is not totally surprising, as most species are readily retrieved from soil samples, and are also found to sporulate a lot on debris collected from damp areas. The first disease reports of *Cy. scoparium* by Massey (1917) and Anderson (1919) were on plants of rose. Subsequent to these reports, numerous others have been made, documenting a wide range of symptoms such as damping-off, root rot, crown canker, fruit rot, stem lesions, tuber rot, etc. (Crous *et al*. 1991). The germination of conidia, microsclerotia or ascospores, is the first step of infections, which requires free water, infact the role of rain, dew and irrigation practices is very important. High humidity and free water increase diseases caused by species of *Cylindrocladium* and related genera. Conidia are easily splash-disperded, which underlines the importance or seedling spacing and general nursery hygiene. The disease also appears to be more severe if seedlings are exposed to nutrient stress (Arentz 1991).

Conidia of *Cy. reteaudii* (as *Cy. quinqueseptatum*) were observed to germinate faster *in vivo* than *in vitro* (Sharma & Mohanan 1990). Germination occurred after 3 h on leaves of 2 month old *Eucalyptus grandis* seedlings. After germination, germ tubes originating from the same or different conidia were observed to anastomose. Earlier reports (Bolland *et al*. 1985) stated that *Cy. reteaudii* could only infect eucalypts through stomata. This was refuted by Sharma & Mohanan (1990), who observed that isolates of *Cy. reteaudii* favoured direct penetration, and rarely formed appressoria over stomata for stomatal penetration. Direct penetration on eucalypt leaves has on several occasions also been observed for *Cy. pauciramosum* and is probably the more common mode of penetration for most species.

1.1.5 Microsclerotia

In proximity of infection, chlamydospores and microsclerotia (chain of chlamydospores) have been observed to develop in substomatal chambers in pine needles or in the inner cortex cells (Bugbee & Anderson 1963). When the

infected plant material disintegrates or the plants are harvested, the leaves and the other plant materials fall to the ground, releasing microsclerotia into the soil. Microsclerotia can survive in the absence of the host for periods of 15 years or more (Thies & Patton 1970, Sobers & Littrell 1974), and are the primary survival structure in soil (Phipps *et al*. 1976). A large number of fungicides have been reported as effective in inhibiting conidial germination or mycelial growth, but not so for microsclerotia (Sharma & Mohanan 1991a). Microsclerotia din not survive in soil with a low water content (Sung *et al*. 1980), but they survived for longer periods when buried, as the cooler conditions favoured a higher moisture content (Pataky & Beute 1983). A temperature of 25 °C was optimal for microsclerotial survival, irrespective of soil moisture (Almeida & Bolkan 1981). Microsclerotia have been reported to be present at depths of up to 66 cm below the soil surface (Anderson 1919). Dumas *et al*. (1998) found that an Egedal® bed steamer produced sufficient heat to kill microsclerotia of *Cy. floridanum* at 5-10 cm soil depths in a bare root forest seedlings nursery, but did not affect the microsclerotia at or below 15 cm.
Very low soil temperatures and severe drought were also found to affect the number of viable microsclerotia recovered (Phipps & Beute 1979, Roth *et al*. 1979, Taylor *et al*. 1981). Moreover, preliminary study, in which solarization effectively suppressed *C. pauciramosa* microsclerotia (Polizzi *et al.* 2003), were confirmed by recent research showing that different tested solarizing materials, had potential in eradicating *Calonectria* inocula from soil acting on microsclerotia (Vitale *et al.* 2012b).

1.1.6 Mating compatibility

Mating strategies have been employed in the taxonomy of *Calonectria* and have played an important role in identifying new species of the genus (Schoch *et al.* 1999, Crous 2002). Based on these studies, there were approximately 18 species of *Calonectria* considered homothallic and 34 heterothallic (Crous 2002, Crous *et al.* 2004b, Gadgil & Dick 2004, Crous *et al.* 2006a). Studies in the female fertility of *Cylindrocladium* by Schoch *et al.* (1999, 2000a, 2001a) have also shown that several species are self-sterile hermaphrodites requiring fertilisation

from an opposite mating type. This is typical of heterothallic ascomycetes (Leslie & Klein 1996).

Several difficulties associated with applying the BSC have been highlighted (Brasier 1997, Taylor *et al.* 1999, 2000, Kohn 2005). The most relevant problem occurs where genetically isolated fungal strains retain the ancestral ability to recombine to produce viable progeny (Brasier 1997). This phenomenon has also been found with several phylogenetic species that are closely related in *Calonectria*. *Cy. hawksworthii*, *Ca. insularis* and *Ca. morganii* were capable of recombining, but that the progeny had low levels of fertility (Crous 2002). Other mating studies done by Overmeyer *et al.* (1996) and Neubauer & Zinkernagel (1995) have found that induction of fertile perithecia requires the presence of an additional isolate that, however, does not contribute to the genetic make-up of the progeny. Isolates of *Ca. polizzii* (species very closed to *Ca. pauciramosa*) were not capable of mating with the *Ca. pauciramosa* mating-tester strains or other *Ca. pauciramosa* isolates from different geographic regions, while *Ca. colombiana* and *Ca. zuluensis* have a homothallic mating system, showing that the presence of homothallic and heterothallic mating strategies in closely related fungi is interesting and could well provide another opportunity to analyse the genetics of mating systems in ascomycetes (Lombard *et al*. 2010b).

All this data clearly highlights the need for further studies regarding the mechanism of perithecial formation and recombination in *Calonectria*.

Figure 2. Production of perithecia on CLA medium.

1.1.7 Phylogeny

During last years phylogenetic studies on *Calonectria* and its *Cylindrocladium* anamorphs have substantially influenced the taxonomy of these genera. Application of new molecular techniques and particularly DNA sequence comparisons to distinguish between species, showed news in the recognition of numerous cryptic species. Several molecular approaches have been employed that include total protein electrophoresis (Crous *et al.* 1993a, El-Gholl *et al.* 1993a), isozyme electrophoresis (El-Gholl *et al.* 1992, 1997, Crous *et al.* 1998a), random amplification of polymorphic DNA (RAPD) (Overmeyer *et al.* 1996, Victor *et al.* 1997, Schoch *et al.* 2000a, Risède & Simoneau 2004) restriction fragment length polymorphisms (RFLP) (Crous *et al.* 1993b, 1995, 1997b, Jeng *et al.* 1997, Victor *et al.* 1997; Risède & Simoneau 2001) and DNA hybridisation (Crous *et al.* 1993b, 1995, 1997a, Victor *et al.* 1997). All these mentioned techniques have been useful, but DNA sequence comparisons and associated phylogenetic inference had the most important influence on the taxonomy of *Calonectria* and are most widely applied today.

In the first study using 5.8S ribosomal RNA gene and flanking internally transcribed spacers (ITS) sequences Jeng *et al.* (1997) were able to distinguish between *Cy. scoparium* and *Cy. floridanum* isolates. Subsequently, it was found that this gene region contains few informative characters (Crous *et al.* 1999, Schoch *et al.* 1999, Risède & Simoneau 2001, Schoch *et al.* 2001b). Therefore, the β-tubulin (Schoch *et al.* 2001b) and histone H3 (Kang *et al.* 2001a) gene regions have been applied in order to allow for improved resolution in separating species.

The first complete DNA sequence-based phylogenetic study using partial β-tubulin gene sequences (Schoch *et al.* 2001b) compared phenotypic, biological and phylogenetic concepts used in the taxonomy of *Cylindrocladium*. This also highlighted the fact that *Calonectria* represents a monophyletic lineage (Schoch *et al*. 2000b, 2001b). Subsequently, combined DNA sequence data for the ITS, β-tubulin and histone H3 gene regions have been widely used in studies relating to taxonomic issues surrounding *Cylindrocladium* and *Calonectria* (Crous *et al.* 1999, Schoch *et al.* 2000a, 2000b, Crous & Kang 2001, Kang *et al.* 2001a, 2001b, Henricot & Culham 2002, Crous *et al.* 2004b, 2006a, Lombard *et al.* 2009, 2010d). Other partial gene sequences recently used include translation elongation 1-alpha (TEF-1α) and calmodulin (Crous *et al.* 2004b, Lombard *et al.* 2010d). For *Cylindrocladium* and *Calonectria*, there are only six studies (Kang *et al.* 2001a 2001b, Crous *et al.* 2004b, 2006a, Lombard *et al.* 2009, 2010d) that provide files on TreeBase (www.treebase.org).

1.1.7.1 *Calonectria scoparia* species complex

Several past studies have focused on the taxonomy of *Calonectria* spp. with small, 1-septate macroconidia and ellipsoidal to obpyriform vesicles (Crous *et al.* 1993, Overmeyer *et al.* 1996, Schoch *et al.* 1999, 2000a). These *Calonectria* spp. were initially regarded as either *Ca. morganii* (= *Cylindrocladium scoparium*) or *Ca. scoparia* (= *Cy. candelabrum*) based on their morphological similarities. However, the anamorph state of *Ca. morganii* was circumscribed as having ellipsoidal to pyriform vesicles and *Ca. scoparia* having ellipsoidal to obpyriform vesicles by Crous *et al.* (1993a). Later studies, incorporating DNA

sequence data, have shown that *Ca. morganii* is restricted to the Northern Hemisphere and Brazil (Crous *et al.* 1993a, Overmeyer *et al.* 1996, Schoch *et al.* 2000a). In contrast, *Ca. scoparia* is found worldwide and forms part of a species complex consisting of four mating groups, each representing a different *Calonectria* species that includes *Ca. pauciramosa* (anamorph: *Cy. pauciramosum*), *Ca. scoparia*, *Ca. mexicana* (anamorph: *Cy. mexicanum*) and *Ca. insularis* (anamorph: *Cy. insulare*) (Schoch *et al.* 1999).

Calonectria pauciramosa has been reported worldwide on numerous plant hosts (Schoch *et al.* 1999, Koike *et al.* 1999, Koike & Crous 2001, Polizzi & Crous 1999, Polizzi 2000, Polizzi & Catara 2001, Polizzi & Vitale 2001, Crous 2002, Polizzi *et al.* 2006a, 2007b, 2009a, Vitale *et al.* 2009), where it causes different symptoms such as cutting rot, damping-off, root rot and leaf blight. In South Africa and Australia, *Ca. pauciramosa* is regarded as the most important pathogen in commercial forest nurseries (Crous 2002) and it is also found on various horticultural crops in commercial nurseries in Italy and the U.S.A. (Schoch *et al.* 2001, Crous 2002, Polizzi *et al.* 2006a, 2007b, 2009a,c, Vitale *et al.* 2009).

Schoch *et al.* (2001a) considered female fertility in populations of *Ca. pauciramosa* from various geographical regions to determine the ratio of mating types present, and based on these data suggested that *Ca. pauciramosa* was endemic to South America given that the ratio of both mating types approached 1:1. Furthermore, the study also indicated that *Ca. pauciramosa* isolates from California were represented by only one mating type, supporting the view that this represented an introduced pathogen. Isolates from Italy showed higher ratios of hermaphrodites and some variation was observed in the β-tubulin sequences. In contrast, South African isolates had close to a 1:1 mating type ratio and showed variation in β-tubulin sequence data (Schoch *et al.* 1999, 2001a), indicating that this was either a native pathogen or that there had been multiple introductions into the country.

Initial investigations using DNA sequence comparisons and mating studies on *Ca. pauciramosa* isolates from South Africa and Colombia showed some variation amongst isolates. These findings and those of Schoch *et al.* (2001a) suggested that *Ca. pauciramosa* might accommodate a number of cryptic species. A recent study, considering a variation observed amongst isolates of

Ca. pauciramosa from different geographical localities, had the aim to consider the phylogenetic relationships, morphological characters and mating compatibility of available isolates of *Ca. pauciramosa* and to determine whether this species represented an assemblage of cryptic taxa. This study revealed the presence of three cryptic species accommodated in cultures that have collectively been treated as *Ca. pauciramosa* (Lombard *et al*. 2010d). This result is homogeneous with the results of previous studies (Schoch *et al.* 1999, 2001a), which noted variation within *Ca. pauciramosa,* although at that time the sample size was inordinately small to consider the matter further. The descriptions of *Ca. colombiana*, *Ca. zuluensis* and *Ca. polizzii* add three new species to the *Ca. scoparia* species complex. This complex is characterised by species having ellipsoidal to obpyriform vesicles and producing 1-septate macroconidia (Schoch *et al.* 1999, Crous 2002). The complex was previously regarded as having a biallelic, heterothallic mating system (Schoch *et al*. 1999, 2001a). However, both the newly described *Ca. colombiana* and *Ca. zuluensis* are homothallic. Schoch *et al.* (2001a) considered female fertility of *Ca. pauciramosa*, and found variation in BT sequence data for isolates from Italy. This new species has thus been shown as unique based on morphological, phylogenetic inference and biological characteristics, separating it from *Ca. pauciramosa*. Morphologically, *Ca. polizzii* can be distinguished from *Ca. pauciramosa* by its smaller 1-septate macroconidia. Isolates of *Ca. polizzii* were also not capable of mating with the *Ca. pauciramosa* mating-tester strains or other *Ca. pauciramosa* isolates from different geographic regions (Lombard *et al*. 2010b).

Figure 3. Morphological variability among different strains belonging to *Calonectria scoparia* species complex.

1.1.7.2 *Calonectria pauciramosa*

Ca. pauciramosa C.L. Schoch & Crous, anamorph *Cy. pauciramosum* C.L. Schoch & Crous, has been described as a member of the species complex *Cy. candelabrum* (Schoch *et al.* 1999). It is a polyphagous fungal species widely reported in Australia, New Zealand, Brazil, Colombia, Mexico, South Africa, USA, and Europe, probably introduced from Australia or South Africa by the trade of plant-propagation material, new plant-species and cultivars.

The first reports of this species concerned *Medicago truncatula* (Lamprecht 1986), *Acacia longifolia* (Hagemann & Rose 1988), *Rododendron* spp., *Azalea* spp., *Eucalyptus* spp. and *Protea* spp. (Botha & Crous 1992).

Subsequently, this pathogen has been reported for the first time in North America on *Erica capensis* (Koike *et al.* 1999), while Polizzi & Crous (1999) have confirmed the discovery in Europe on plants of *Polygala myrtifolia*.

In Italy *Ca. pauciramosa* is a species widely present in the nurseries of eastern Sicily and other parts of southern Italy, responsible for significant losses on

several ornamental plants, especially on young seedlings, where it finds the optimal conditions for its development. This pathogen was found in several plants such as *Fejioa sellowiana*, *Arbutus unedo*, *Acacia retinodes* and *Dodonaea viscosa* (Polizzi & Catara 2001). *Ca. pauciramosa* also causes leaf spots, defoliation, stem blight on different species belonging to the family of Mirtaceae which bottlebrushes, blue eucalyptus, red eucalyptus, melaleuca, myrtle, and *Metrosideros* spp. (Polizzi 1996).

Until some years ago, for the identification of this species the application of the keys of Crous & Wingfield (1994) and Schoch *et al*. were considered necessary and enough (1999).

Difficulties experienced in morphological identification, have led to several molecular approaches being employed to identify *Calonectria* spp. These include total protein electrophoresis (Crous *et al.* 1993a, El-Gholl *et al.* 1993), isozyme electrophoresis (El- Gholl *et al.* 1992, El-Gholl *et al.* 1997, Crous *et al.* 1998a), random amplification of polymorphic DNA (RAPD) (Overmeyer *et al.* 1996, Victor *et al.* 1997, Schoch *et al.* 2000a, Risède & Simoneau 2004), restriction fragment length polymorphisms (RFLP) (Crous *et al.* 1993b, Crous *et al.* 1995, Crous *et al.* 1997, Jeng *et al.* 1997, Victor *et al.* 1997, Risède & Simoneau 2001) and DNA hybridisation (Crous *et al.* 1993a, 1995, 1997, Victor *et al.* 1997). However, DNA sequence comparisons and associated phylogenetic inference has the most significant impact on the taxonomy of the group. It is also most widely applied in contemporary species descriptions. The 5.8S ribosomal RNA gene and flanking internally transcribed spacer (ITS) sequences made it possible for Jeng *et al.* (1997) to distinguish between *Cy. scoparium* and *Cy. floridanum* isolates. Subsequently, it was found that this gene region contains few informative characters for members of the genus (Crous *et al.* 1999, Schoch *et al.* 1999, Risède & Simoneau 2001, Schoch *et al.* 2001b). As a consequence, this resulted in the β-tubulin (BT) (Schoch *et al.* 2001b) and histone H3 (HIS3) (Kang *et al.* 2001b) gene regions being widely employed to improve the resolution of phylogenetic trees for species of *Calonectria*.

In addition, DNA sequence comparisons and mating studies on *Ca. pauciramosa* isolates from South Africa and Colombia showed some variation amongst isolates. These findings and those of Schoch *et al.* (2001) suggested that *Ca. pauciramosa* might accommodate a number of cryptic species.

The morphological measures of perithecia, conidia, terminal vesicle, conidiophores and other morphological characters, still are fundamental for the distinction among different species (Peerally 1991, Crous *et al.* 1992). In *Ca. pauciramosa,* macroconidiophores form a stipe. This represents a sterile extension from which the bundle of branches fertile started. The stipe is septate, hyaline, 120-230 µm in length, terminating in a vesicle of variable shape from ellipsoidal to pyriform and with diameter of (5-) 7-9 (-11) µm. The primary branches are aseptate or with only one septum (12-45 x 5-6 µm), the secondary branches are aseptate (15-20 x 5 µm), and also the tertiary (12-15 x 5 µm). The terminal branches produce 2-6 phialides, with shape variable from doliiform to reniform, hyaline, without septum, (10-13 x 2.5-4 µm), with a small enlargement at the apex.

The conidia are cylindrical, hyaline, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. The microconidiophores have never been observed. Chlamydospores extensive throughout the medium, represent the preservation organs; dark brown in colour, aggregated to form microsclerotia, while the teleomorph produces perithecia subglobose to ovoid with height of 250-400 µm and width of 170-300 µm, with a variable colour from orange to red to brown. Asci 8-spored, clavate, 70-140 µm, with filiform stipe, contain 8 ascospores, hyaline, fusoid, septate, guttulate and slightly curved with rounded tip.

Colonies fast growing (35–40 mm diameter after 7 d) with optimal growth temperature at 25 ^{o}C (growth at 10– 30 ^{o}C) on MEA, reverse amber to sepia-brown after 7 d; abundant white aerial mycelium with sparse sporulation.

1.1.7.3 *Calonectria polizzii*

Calonectria polizzii L. Lombard, Crous & M.J. Wingf. is a cryptic species belonging to *Calonectria scoparia* complex**.** The teleomorph is unknown.

Conidiophores with a stipe bearing a penicillate suite of fertile branches, stipe extensions, and terminal vesicles. Stipe are septate, hyaline, smooth, 58–108 × 5–7 µm; stipe extensions septate, straight to flexuous, 111–167 µm long, 5–6 µm wide at the apical septum, terminating in an obpyriform to ellipsoid vesicle, 6–9 µm diam. The conidiogenous apparatus 27–57 µm long, and 28–51 µm

wide; primary branches aseptate or 1-septate, 15–35 × 4–6 µm; secondary branches aseptate, 12–26 × 3–5 µm; tertiary branches aseptate, 10–15 × 4–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8–13 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia are cylindrical, rounded at both ends, straight, (31–)32–42(–49) × 3–5 µm (av. = 37 × 4 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Megaconidia and microconidia absent.

Colonies are fast growing (35-40 mm diameter after 7 d) with optimal growth temperature at 25°C (growth at 10-30 ^{o}C) on MEA, reverse amber to sepia-brown after 7 d; abundant white aerial mycelium with sparse sporulation; chlamydospores extensive throughout the medium, forming microsclerotia.

Ca. polizzii is morphologically similar to *Ca. pauciramosa* and *Ca. zuluensis.* The macroconidia of *Ca. polizzii* (av. 37 × 4 µm) are smaller to those of *Ca. pauciramosa* (av. 50 × 4.5 µm).

1.1.8 *Calonectria* diseases in Mediterranean environment

Several studies of last twenty years reported a broad plant-host group susceptible to *Calonectria* diseases. Different symptoms such as cutting rot, damping-off, crown rot, root rot, leaf blight and petiole rot are caused by *Calonectria* spp. in different countries of Mediterranean basin. In Italy, the most widespread species is *Ca. pauciramosa* reported as agent causal of leaf spots on *Arbutus unedo*, *Acacia retinodes*, *Feijoa sellowiana*, *Dodonaea viscosa*, *Pistacia lentiscus*, *Brahea armata*, *Agonis flexuosa* (Polizzi & Catara 2001, Vitale & Polizzi 2007, Polizzi *et al*. 2007a, Polizzi *et al*. 2010), crown rot and stem blight on plants of *Pistacia lentiscus* (Vitale & Polizzi 2007), defoliation and stem blight on plants of *Eugenia myrtifolia* (Polizzi *et al.* 2009a), crown and root rot on *Ceanothus thyrsiflorus, Arbutus unedo, Eugenia myrtifolia* and *Feijoa sellowiana* (Polizzi *et al.* 2006b, Vitale *et al.* 2008, Vitale *et al.* 2009, Polizzi *et al.* 2009a).

Ca. morganii was also widely reported as responsible of crown rot, leaf spot, stem blight and defoliation on *Pistacia lentiscus* (Polizzi *et al*. 2006a),

damping-off, crown, root rot and leaf spot on *Callistemon* spp. (Polizzi *et al*. 2007b), leaf spot and shoot blight on *Melaleuca acuminata* (Polizzi *et al*. 2009b).

Moreover, Carrai & Garibaldi (1990) observed during 1987 in several Italian nurseries initial symptoms such as leaf spot, petiole rot, stem blight and subsequently the death of plants of *Spathiphyllum.* The causal agent of these symptoms was identified as *Cylindrocladium spathiphylli* (Schoultles & El-Gholl 1980) reported for the first time as the agent of crown and root rot of *Spathiphyllum* plants cv. Clevelandii in 1977 in Florida (Schoulties & El-Gholl, 1980).

In Italy, a further species was reported on plants of *Buxus sempervirens* in which defoliation and stem blight were observed; the fungal species involved in this disease was identified as *Cylindrocladium buxicola* (Saracchi *et al*. 2008)*,* due to the recent outbreaks in northern Europe, in 2007 *Cy. buxicola* was placed in a list established by the European Parliament (www.eppo.org/quarantine/quarantine.htm), as regards quarantine pathogens.

In Spain *Ca. pauciramosa* was reported as responsible of leaf spot on *Callistemon citrinus* (Perez-Sierra *et al.* 2007), while in Portugal was observed on plants of *Myrtus communis* causing crown and root rot (Henricot & Beales 2003).

Recently three isolates collected in Italy from symptomatic plants of *Arbutus unedo* and *Callistemon citrinus* were identified as *Ca. polizzii*, one of the three cryptic species recognized within *Ca. pauciramosa*; the pathogenicity of these isolates was not still established.

Table 1. Ornamental-plants host of *Calonectria* spp. in Mediterranean basin.

HOST	SYMPTOMS	FUNGAL SPECIES	REFERENCE
Acacia retinodes	Leaf spot, stem blight	*Cy. pauciramosum*	Polizzi & Catara 2001
Agonis flexuosa	Leaf spot	*Cy. pauciramosum*	Polizzi *et al*. 2010
Arbutus unedo	Leaf spot, stem blight	*Cy. pauciramosum*	Polizzi & Catara 2001
Brahea armata	Leaf spot	*Cy. pauciramosum*	Polizzi *et al*. 2007
Buxus sempervirens	Leaf spot, stem blight, defoliation	*Cy. buxicola*	Saracchi 2008
Callistemon spp.	Leaf spot, defoliation	*Cy. scoparium*	Polizzi *et al*. 2007
Callistemon spp.	Leaf spot, stem blight	*Cy. pauciramosum*	Polizzi 1996
Ceanothus thyrsiflorus	Crown and root rot	*Cy. pauciramosum*	Polizzi *et al*. 2006
Chamaerops humilis	Leaf spot	*Cy. pauciramosum*	Polizzi *et al*. 2007
Dodonaea viscosa	Leaf spot	*Cy. pauciramosum*	Polizzi & Catara 2001
Eugenia myrtifolia	Crown and root rot	*Cy. pauciramosum*	Polizzi *et al.* 2009a
Eucalyptus globulus	Leaf spot	*Cy. pauciramosum*	Polizzi 1996
Eucalyptus rostrata	Leaf spot	*Cy. pauciramosum*	Polizzi 1996
Eucalyptus spp.	Leaf spot	*Cy. pauciramosum*	Polizzi 1996
Feijoa sellowiana	Leaf spot	*Cy. pauciramosum*	Polizzi & Catara 2001
Melaleuca acuminata	Leaf spot, shoot blight	*Cy. scoparium*	Polizzi 2009b
Melaleuca fulgens	Crown and root rot	*Cy. pauciramosum*	Polizzi 2009c
Melaleuca hypericifolia	Leaf spot	*Cy. pauciramosum*	Polizzi 1996
Metrosideros robustus	Leaf spot, stem blight	*Cy. pauciramosum*	Polizzi 1996
Myrtus communis	Leaf spot, stem blight, crown and root rot	*Cy. pauciramosum*	Polizzi 1996
Pistacia lentiscus	Leaf spot, stem blight, crown and root rot, defoliation	*Cy. scoparium*	Polizzi *et al*. 2006
Pistacia lentiscus	Leaf spot, stem blight,	*Cy. pauciramosum*	Vitale & Polizzi 2008
Polygala myrtifolia	Crown and root rot	*Cy. pauciramosum*	Polizzi & Crous 1999
Spathiphyllum spp.	Leaf spot, petiole rot, stem blight	*Cy. spathiphylli*	Carrai & Garibaldi 1990

Figure 4. Symptomatic plants of *Myrtus communis*. a. wilting . b. crown-rot with presence of mycelia. c-d. leaf spot.

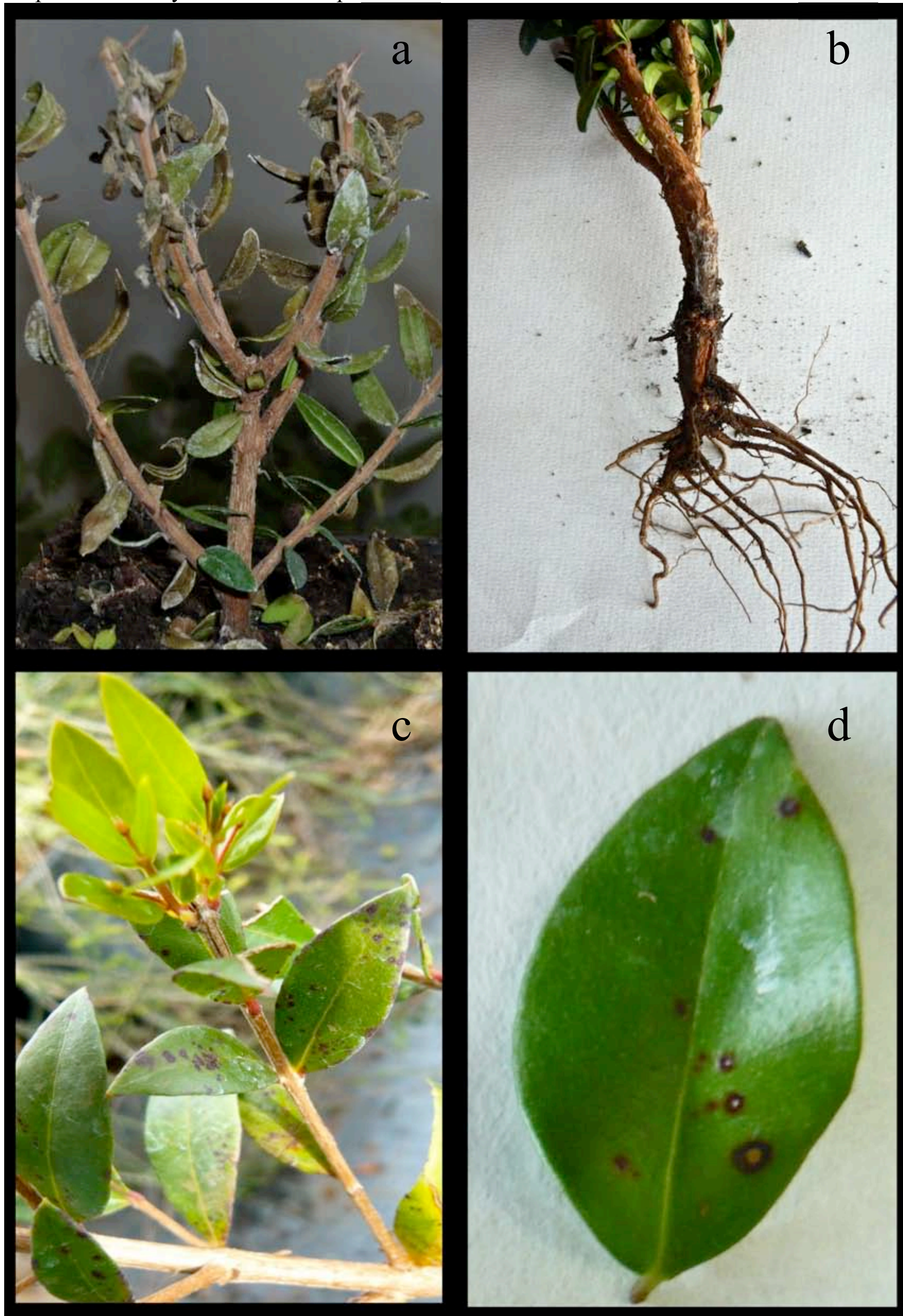


Figure 5. a. Leaf spot and b. severe defoliation on *Metrosideros* spp. plants. c,e. Leaf spots and d,f. damping-off on plants of *Callistemon* spp.

Figure 6. a-b. Different severity of disease on plants of *Eugenia myrtifolia* c-d. Leaf spots on *Agonis flexuosa.*

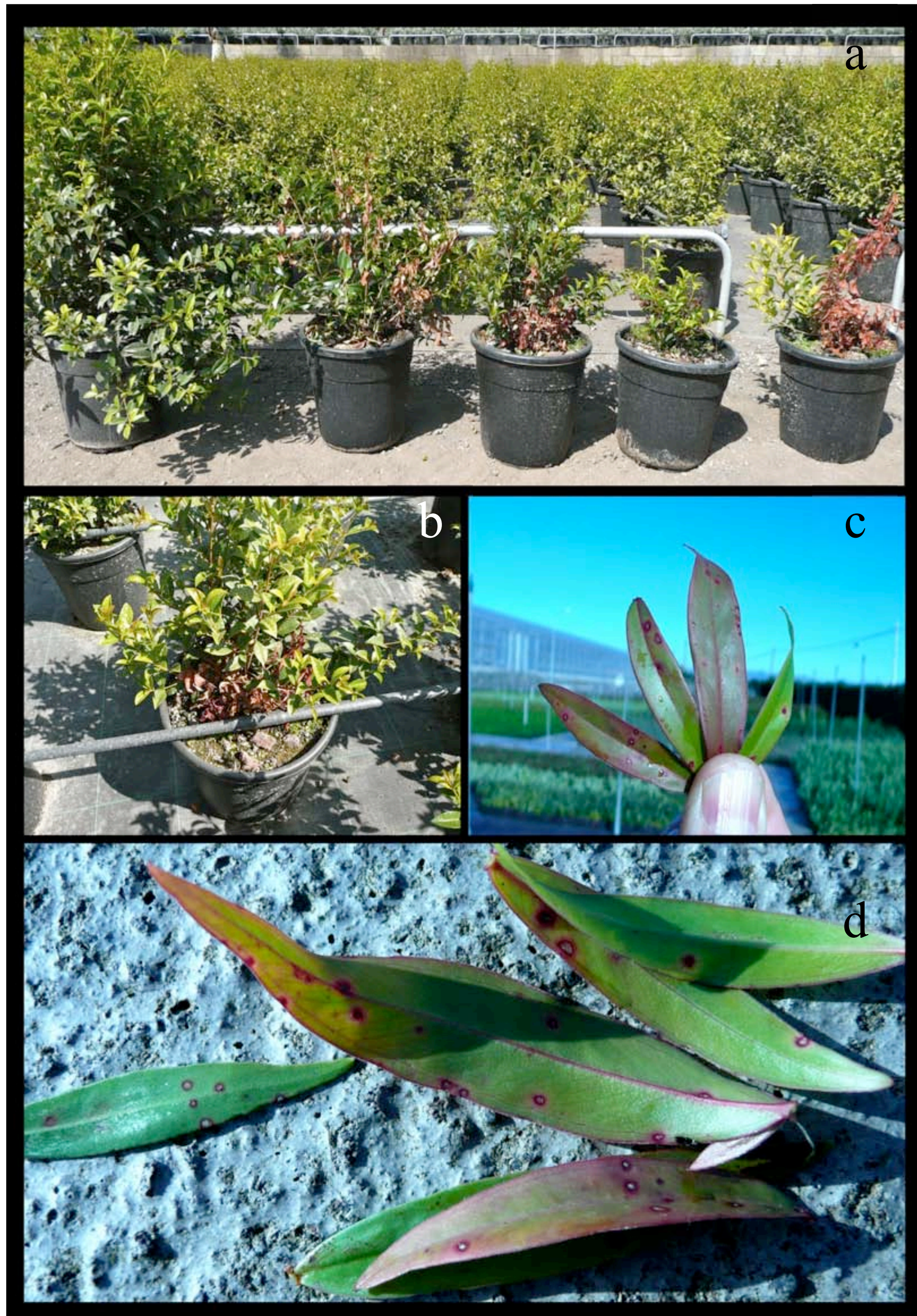


Figure 7. a-c. Wilting and crown and root rot on *Polygala myrtifolia* cuttings.

Figure 8. a. Defoliation and b. leaf spots on *Melaleuca fulgens*. c. Leaf spot on *Melaleuca hypericifolia*. Leaf spots on *Dodonaea viscosa*. e. Death of *Arbutus unedo* plants as a consequence to crown and root rot.

1.1.9 *Calonectria* diseases control

Various fungicides and different methods of application have been proposed to control diseases caused by *Calonectria* spp.

Soil fumigation or sterilization has also been reported as effective in reducing soil populations of *Calonectria* (Jauch 1943). Soil temperature, potting medium, compaction and pH were found to influence Cylindrocladium root and petiole root of *Spathiphyllum*, with the fungus favored by warm, moist conditions with lower pH and higher soil compaction. However higher pH only appeared to delay the onset of disease development (Chase & Poole 1987). At least, soil pH could play a positive role in disease control when temperatures were sub-optimal. Diseases increased from 25-30 °C, but decreased at 32 °C (Chase & Poole 1987). Only preventative measures were found effective, while no curative effects could be obtained for this disease. In Florida (U.S.A.), *Calonectria* diseases were controlled by use of fungicide in association with good nursery practices, including immediate removal of diseased plants, use of new, uninfected potting medium, and minimal watering.

To control *Calonectria* diseases in cuttings during rooting, effort must be made to reduce primary inoculum by using healthy shoots and adopting management practices such as selective and continuous shoot harvesting and the use of inoculum-free trays (Silveira 1996). Good control of Calonectria rot was obtained on azaleas when mother plants were treated with benomyl 9-12 days before cutting were taken (Roos 1980, 1981). Control was further improved if cuttings were dipped in a benomyl solution. Care should be taken, however, as Alfenas *et al.* (1988) observed that constant use of benomyl to control *Calonectria* in nursery cuttings led to the selection of benomyl-resistant strains. This led to recommendation that fungicides with different modes of action be used in rotation.

Since chemical control of Cylindrocladium Leaf Blight (CLB) is expensive (Sharma & Mohanan 1992), the long-term solution should be to control CLB by selecting resistant provenances or species. Sharma & Mohanan (1991b, 1992) found, however, that the susceptibility rating of a provenance to different species of *Cylindrocladium* was related to the eucalypt species or subgenus. These results also provided the first evidence for the existence of physiological

strains in *Cy. reteaudii* (Sharma & Mohanan 1991b). In an experiment screening 22 fungicides against several species of *Cylindrocladium* and *Cylindrocladiella* Sharma & Mohanan (1991a) also demonstrated a differential effect of many fungicides depending on the species of *Cylindrocladium* involved, and that the ED_{100} also varied with species.

In Florida the disease was contained by benomyl, triflumizole and prochloraz treatments associated with good agronomic practices such as using uninfected potting medium (Chase 1987).

For some fungicide, the activity to prevent and eradicate the disease and the activity to inhibit the sporulation of *Cy. candelabrum* were valuated. Tebuconazole, eposiconazole and eposiconazole + pyraclostrobin showed an high ability to inhibit conidial germination and mycelial growth of *Cy. candelabrum.* In *in vivo* experiments these active ingredients reduced the foliar infection severity to 96, 98 and 95% respectively (Ferreira *et al*. 2006).

Grigoletti & Auer (2003) tested several fungicides to manage foliar infections on *Ilex paraguariensis* caused by *Cy. spathulatum.* Benomyl and captan resulted the most effective.

Experiments with fungicides were carried out on *Polygala myrtifolia* showing a good efficacy on different strains of *Cy. pauciramosum* (Polizzi 2000). In the first trial the preventive activity of prochloraz and tebuconazole was valuated to control root rot and both reduced the symptoms; moreover, the fresh and the dry weight of roots not treated were lower than those of the treated plants. Tebuconazole provide symptoms of stunting with marked reduction of leaf edges on treated plants. The second trial, again on potted plants of *P. myrtifolia*, showed good effectiveness of repeated applications of prochloraz every 10-12 days.

One more assay with fungicide to manage natural infections caused by *Cy. pauciramosum* was carried-out on young plants of *Callistemon citrinus* var. 'Captain Cook'. The results showed stunting symptoms, reduced average length of plants and a reduced total number of shoot on plants treated with tebuconazole, the Copper hydroxide caused necrotic, dark red dot on young leaves and delay of blooming. Foliar treatments were applied also on two cultivars ('*baetica*' *e* '*lusitanica*') of *Myrtus communis* for infection caused by *Cy. pauciramosum* on leaves. A lower susceptibility of cultivar '*lusitanica*' and

a good effectiveness of copper hydroxide were observed; no effectiveness of prochloraz on plants of *M. communis* was observed, underlining the variability of activity of this fungicide (Vitale *et al*. 2003).

Brand (2006) tested the activity of 9 active ingredient for the management of *Cy. buxicola.* The conidial germination on amended medium was inhibited for 16 hours at low rates of tolylfluanide, mancozeb, chlorothalonil and fludioxonil + cyprodinil. The mycelial growth was totally inhibited by prochloraz, propiconazole, thiophanate-methyl and carbendazim + flusilazole for 2 weeks.

Chemical control experiments carried out on plants of myrtle showed the effectiveness of regular applications with copper oxychloride for management of foliar infections caused by *Cy. pauciramosum* while, benomyl, chlorothalonil, prochloraz and dithianon were ineffective (Polizzi & Azzaro 1996).

Experimental trials were carried out with the aim to evaluate the efficacy of the antagonist *Trichoderma harzianum* T22 and prochloraz in management of crown and root rot of *P. myrtifolia* caused by *Cy. pauciramosum.* The biological antagonist provided an excellent control compared to the untreated control and to the chemicals treatments. In addition, plants treated with the strain of *T. harzianum*, were more vigorous and larger than plants of others treatments, showing a higher commercial value (Polizzi & Vitale 2002).

Recently, Aiello *et al.* (2013) showed the effectiveness of preventive applications of tebuconazole, fosetyl-Al, Cu hydroxide, thiophanate-methyl, prochloraz + cyproconazole, trifloxystrobin, azoxystrobin and prochloraz in controlling leaf spots caused by both *Ca.morganii* and *Ca. pauciramosa* on bottlebrush plants, while cyproconazole, propamocarb + fosetyl-Al and K phosphite were discouraged. Moreover, K phosphite, fosetyl-Al, prochloraz + cyproconazole, cyproconazole, Cu hydroxide, thiophanate-methyl, trifloxystrobin and azoxystrobin provided a significant reduction of crown and root rot caused by *Ca. pauciramosa* in feijoa plants, detecting variable results for prochloraz applications and suggesting further investigations about the efficacy of this compound.

Soil fumigation (metham-sodium and 1, 3-dichloropropene + methyl isothiocyanate) combined with resistant cultivars significantly reduced the build-up of Cylindrocladium black rot (CBR) in groundnuts (Phipps 1990).

Good control of CBR was obtained using a combination of metham sodium fumigation and resistant genotypes (Cline & Beute 1986). Genetic breeding proved promising in obtaining durable resistance to *Sclerotinia minor* and *Cy. parasiticum* (Coffelt *et al*. 1994).

Practices for controlling CBR and red collar rot (RCR) include the use of resistant varieties (Berner *et al.* 1988) and cultural practices such as reducing the inoculum and delayed planting (Sidebottom & Beute 1989). A direct correlation was observed between weed-free and disease-free areas in soybean fields (Berner *et al.* 1991). This led to the investigation of the possible fungicidal activity of herbicides that contain glyphosate. Other recommendations for CBR control include crop rotation with non-legumes, control of nematodes, use of resistant cultivars, and soil fumigation with products containing methyl isothiocyanate (Phipps 1990).

CBR of groundnut was slowed when soil temperatures exceeded 25 °C and stopped when temperatures exceeded 35 °C. Sidebottom & Beute (1989) proposed cultural practices to modify soil temperature, and hence control the disease. In experiments inoculating resistant and susceptible groundnut cultivars, Hadley *et al.* (1979) concluded that races of *Cy. parasiticum* could occur, even though host resistance appeared to be quantitatively inherited.

The use of antagonistic microorganism is a viable alternative for suppression of plant pathogens in cutting nurseries, since the rooting substrate and the humidity and temperature conditions required for the development of roots favor biocontrol agents (Kunieda-de-Alonso 1997). The incorporation and pre-incubation of *Trichoderma* isolates in *Eucalyptus* bark compost resulted in 100% suppression of *Rhizoctonia* and 90% of *Cylindrocladium* (A.C. Alfenas).

Most species of *Cylindrocladium* are soil-borne, and many have been associated with root disease problems. In Canada, isolates of *Cy. floridanum* species complex have also been isolated from asymptomatic seedlings (Juzwik *et al.* 1988). To date these pathogens have primarily been controlled by means of soil fumigation with either methyl bromide or chloropicrin. The present mandate, however, is that these synthetic pest control agents should be reduced, and that environmentally safe alternatives be explored for disease control. Species of *Trichoderma* are well-known agents of biocontrol. Dumas *et al.* (1996) therefore evaluated isolates of *T. harzianum*, *T. viride*, *T. koningii*, *T.*

polysporum, *T. hamatum* and *T. virense* for their ability to inhibit *Cy. floridanum in vitro.* Isolates that inhibited mycelial growth and microsclerotia production were found to produce 6-n-pentyl-2H-pyran-2-one in culture. However, *Cy. floridanum* isolates were also found to produce a substance that inhibited the mycelial growth of *Trichoderma* species. Isolates of *Trichoderma* or fluorescent *Pseudomonas* reduced root disease caused by *Cy. scoparium* and *Cy. gracile* (Blum & Lin 1991).

Some *Bacillus* isolates obtained from *Eucalyptus grandis* leaves were antagonistic to *Cy. scoparium* (Bettiol *et al*. 1988). By applying a bacterial suspension from a 10-d-old culture to detached leaves 1, 24 and 48 h before inoculation with the pathogen, disease control similar to that obtained with benomyl (0,5g/l) was obtained. A *Bacillus* sp. was also observed to provide good control of *Cy. scoparium* on eucalypt seedlings at the pre-emergence stage (Santos *et al.* 1993) Under greenhouse conditions *Bacillus* and *Streptomyces* spp. were the most effective in controlling the disease.

The effect of various ectomycorrhizal fungi to control common root pathogens were screened by Natarajan & Govindasamy (1990), who reported *Suillus brevipes* to control most pathogens, including *Cy. scoparium*. Ectomycorrhizal fungi such as *Paxillus involutus* and *Hebeloma cylindrosporum* also inhibited *Cy. canadense in vitro* (Morin *et al.* 1999).

Soils treated with *Phaeotheca dimorphospora* led to an increase in the number of propagules of *T. harzianum,* whereas the population of *Cy. scoparium* decreased rapidly and was not detectable 30 days after treatment (Yang *et al.* 1995). An extract of the bark of *Barringtonia ceylanica* was found to inhibit growth of *Cy. reteaudii* by up to 50% (Palanakumbura *et al.* 1996). Field applications of compost and dazomet controlled *Cy. scoparium* (Lyons *et al.* 1997): while field application of sewage and spent mushroom compost also suppressed *Cy. scoparium* (Hunter *et al.* 1995).

Cell-wall homogenates of *Cy. spathiphylli* markedly stimulated the accumulation of tolytoxin, an antifungal secondary metabolite, in cultures of the cyanobacterium *Scytonema ocellatum* (Patterson & Bolis 1997). These results suggest that tolytoxin is an inducible chemical defense agent (phytoalexin) capable of protecting *S. ocellatum* against fungal invasion.

Activities and effective dose of different types of *Ginkgo biloba* have been tested *in vitro* against *Cy. colhouni*. The greatest effect was obtained from extracts diluted in ethanol with an efficiency of 37.4% followed by extracts diluted in ether-petroleum (23.7%), and from fresh extracts (18.4%). When the concentration of the extracts has reached high values it is obtained an efficacy of 100% (Feng *et al*. 2007). The C184 strain of *T. harzianum* isolated from banana was tested *in vitro* against *Cy. pteridis*, the causal agent of necrosis of the roots on *Musa acuminata*. Culture filtrates of the antagonist reduced the mycelial growth of the pathogen from 52 to 87% (Ngueko & Tong 2002).

T. harzianum, T. viride and *Pseudomonas fluorescens* were tested to evaluate their effectiveness against damping-off of seedlings of *Eucalyptus tereticornis* and *E. grandis* caused by *Rhizoctonia solani* and *Cy. quinqueseptatum*. Potential antagonists were inoculated alone or in combination with each other, in the soil and on the seeds by dipping. The treatment to the soil with *T. harzianum* was the most effective in limiting disease (Mohanan 2007).

Other studies showed a good activity of *T. harzianum* T-22 (Rootshield) for the management of roots infections caused by *Cy. pauciramosum* on *P. myrtifolia*. The treatment with the antagonist showed a higher activity than prochloraz and than the association between the same active ingredient and antagonist. In addition, the inoculation of the antagonist determined an increase of vegetative growth and commercial value of plants (Polizzi & Vitale 2002). *T. harzianum* T-22 was also tested on plants of *M. communis* subspecies 'tarentina' for the control of natural infections caused by *Cy. pauciramosum* and *Phytophthora palmivora*. Contrary to the results obtained previously, the treatments to the collar and soil, showed a reduced activity of the antagonist in the control of mixed infections (Vitale *et al*. 2003).

T. harzianum strain T22 showed the potential ability to reduce microsclerotia production on carnation leaf tissue used as cultural-like debris and in controlling collar and root rot on selected hosts. However, antagonist activity was not always complete and variable effects were detected among the tested isolates. As regards its effect on microsclerotia, reduction of the primary resting structures of *Ca. pauciramosa* was dependent on the application timings of the antagonist and on the tested isolate. Even T22 effectiveness in controlling *Ca. pauciramosa* infections on red clover was related to treatment timing and

pathogen isolate. Based on our results, the T22 efficacy in controlling *Ca. pauciramosa* varied among the isolates tested. However, greatest T22 effectiveness in reducing the collar and root rot of selected hosts was related to the lowest virulence of the pathogen (Vitale *et al.* 2012a).

1.2 Fungicide resistance

1.2.1 Introduction

Since the first cases of widespread fungicide resistance arose, agrochemical manufacturers, academic and government scientists, and crop advisers, have put a great work into analysing the phenomenon and establishing countermeasures.

1.2.2 Fungicide control of crop disease

Fungicides have been used for over 200 years to protect plants against disease attack by fungi. From the beginnings of using, mainly to protect cereal seeds and grape-vines, the number of crops and crop diseases treated, the range of chemicals available, the area and frequency of their use, and the effectiveness of treatments, have increased enormously, especially since the second world war.
Two very old-established remedies, copper-based formulations and sulphur, are still used widely and effectively. A broad number of effective fungicides, of novel structure and mostly with systemic activity not found in the earlier products, were introduced in the late 1960s and 1970s. These included 2-amino-pyrimidines, benzimidazoles, carboxanilides, phosphorothiolates, morpholines, dicarboximides, phenylamides, and sterol demethylation inhibitors (DMIs). Introductions in the 1980s mainly were analogues of existing fungicides, particularly DMIs, with generally similar though sometimes improved properties. Over the past decade, however, a number of novel compounds have been introduced commercially or have reached an advanced stage of development – these include phenylpyrroles, anilinopyrimidines, quinone outside inhibitors (QoIs, including strobilurin analogues), benzamides, carboxylic acid amides, azanapthalene (AZN, quinoxyfen and proquinazid) and

succinate-dehydrogenase (SDHI). Spraying has always been the principal method of fungicide application, and the conventional hydraulic sprayer still predominates. Reduction in spray volume, and more stable and safer formulation, are probably the most significant advances that have been made in application technology. Systems of integrated crop management involving minimum necessary chemical and energy inputs, and use of complementary non-chemical protection measures wherever possible, have been widely adopted and to some extent have led to a reduction in spray number and dose in some situations.

At present some 150 different fungicidal compounds, formulated and sold, are used in world agriculture. The total value of fungicide sales to end-users is approximately 7.4 billion US dollars. Nearly half of the usage is in Europe, where fungal diseases cause the most economic damage to crops. Most of the recommended treatments generally provide 90% or greater control. Although many fungicides are marketed, any one major crop disease typically is well controlled by only three or four different types of fungicide, so that any fall in effectiveness caused by resistance development can be a very serious matter for the grower.

1.2.3 Defining fungicide resistance

A potential new fungicide is identified in laboratory and glasshouse tests on different types of fungal pathogen, and is then tested in field trials against an appropriate range of crop diseases in different regions and countries. Only if it works uniformly well against pathogens involved in important crop and in numerous trials over several seasons is it considered effective and good for marketing. The pathogens it works against are considered to be ‘sensitive’, and those that it does not affect or hardly affects are regarded as ‘naturally’ or ‘inherently resistant’. Reasons for natural resistance are seldom investigated, although sometimes they can be comprised from studies of the mode of action.

The fungicide resistance is a different phenomenon, sometimes called ‘acquired resistance’. Sooner or later during the years of commercial use of a fungicide, populations of the target pathogen can arise that are no longer sufficiently

sensitive to be controlled adequately. They generally appear as a response to repeated use of the fungicide, or to repeated use of another fungicide which is related to it chemically and/or biochemically through a common mechanism of antifungal action. This emergence of resistant populations of target organisms, which were formerly well controlled, has been widely known for antibacterial drugs (e.g. sulphonamides, penicillin, streptomycin) and for agricultural and public health insecticides (e.g. DDT) for almost sixty years.

Some people prefer to call this phenomenon ‘insensitivity’ or ‘tolerance’. The former term is preferred by some plant pathologists, because they believe that fungicide resistance is easily confused with host-plant resistance to certain species or pathotypes of fungi. Some agrochemical companies have also tended to use ‘insensitivity’, ‘loss of sensitivity’ or ‘tolerance’, because these sound less alarming than ‘resistance’. On the other hand, two studies on terminology recommended that ‘resistance’ should be the preferred term (Anon 1979; Delp & Dekker 1985). Also ‘resistance’ has been in use for many years to describe precisely the same phenomenon in bacteriology and entomology, and it is now very widely used with reference to fungicides also.

Workers within the agrochemical industry have objected from time to time to the use of ‘resistance’ to describe shifts in fungicide sensitivity occurring either in non-crop situations such as the laboratory or experimental glasshouse, or in the field but to a degree which is too small to affect disease control. They recommend that ‘resistance’ should denote only situations where failure or diminution of crop disease control is known to have resulted from a change in sensitivity. ‘Field resistance’ (in contrast to ‘laboratory resistance’) has been used sometimes to denote specifically a crop disease control problem caused by resistance. However, detection of some signs of resistance in the field can still be a far cry from having a control failure. It seems preferable to use ‘field resistance’ to indicate the presence of resistant variants in field populations (at whatever frequency or severity), and ‘practical resistance’ to indicate consequent, observable loss of disease control, whenever such precise terminology is necessary. ‘Laboratory resistance’ or ‘artificially induced resistance’ also are useful and precise terms.

1.2.4 Occurrence of resistance

Table 2. Occurrence of practical fungicide resistance in crops (Source: Brent & Hollomon, 2007a, modified).

Date first observed	Fungicide or fungicide class	Years of commercial use before resistance observed	Main crop disease and pathogen affected	References
1960	Aromatic hydrocarbons	20	Citrus storage rots, *Penicillium* spp.	1
1964	Organo-mercurials	40	Cereal leaf spot and stripe, *Pyrenophora spp.*	2
1969	Dodine	10	Apple scab, *Venturia inaequalis*	3
1970	Benzimidazoles	2	Many target pathogens	4
1971	2-Amino-pyrimidines	2	Cucumber and barley, powdery mildews, *Sphaerotheca fuliginea & Blumeria graminis*	5
1971	Kasugamycin	6	Rice blast, *Magnaporthe grisea*	6
1976	Phosphorothiolates	9	Rice blast, *Magnaporthe grisea*	6
1977	Triphenyltins	13	Sugar beet leaf spot, *Cercospora betae*	7
1980	Phenylamides	2	Potato blight and grape downy mildew, *Phytophthora infestans & Plasmopara viticola*	8
1982	Dicarboximides	5	Grape grey mould, *Botrytis cinerea*	9
1982	DMIs	7	Cucurbit and barley powdery mildews. *S. fuliginea & B. graminis*	10
1985	Carboxanilides	15	Barley loose smut, *Ustilago nuda*	11
1998	QoI	2	Many target pathogens	12
2002	Melanin biosynthesis inhibitors	2	Rice blast, *Magnaporthe grisea*	13

(References: 1.Eckert,1982; 2. Noble *et al.*, 1966; 3. Gilpatrick, 1982; 4. Smith, 1988; 5. Brent, 1982; 6. Kato, 1988; 7. Giannopolitis, 1978; 8. Staub, 1994; 9. Lorenz, 1988; 10. De Waard, 1994; 11. Locke, 1986; 12. Heaney *et al.*, 2000; 13. Kaku *et al.*, 2003.)

Table 2 gives a summarized history of the occurrence of practical fungicide resistance world-wide, and lists major fungicide groups for which resistance is well documented. Leading examples are given of the more important diseases affected, and a few key literature references are cited. Up to 1970 there were a few sporadic cases of fungicide resistance, which had occurred many years after the fungicide concerned was introduced. With the introduction of the systemic fungicides, the incidence of resistance increased greatly, and the time taken for resistance to emerge was often relatively short, sometimes within two years of first commercial introduction. Many of the fungicides introduced since the late

1960s have been seriously affected, with the notable exceptions of the amine fungicides ('morpholines'), fosetyl-aluminium, anilinopyrimidines, phenylpyrroles and some of the fungicides used to control rice blast disease (e.g. probenazole, isoprothiolane and tricyclazole). Some recently introduced fungicides such as benzamides and carboxylic acid amides have not yet encountered serious resistance problems. Most of the older materials such as copper fungicides, sulphur, dithiocarbamates (e.g. mancozeb), phthalimides (e.g. captan) and chlorothalonil, have retained their full effectiveness in all their uses, despite their extensive and sometimes exclusive use over many years.

Often the occurrence of resistance has been associated with total, or almost total, failure of disease control. Indeed the observations carried-out by the growers of obvious and sudden loss of effect that generally gave the first indication of resistance. It was necessary to show that resistance really was the cause, by checking for abnormally low sensitivity of the pathogen in tests under controlled conditions. There are several possible reasons for failures in disease control, such as poor application, deteriorated product, misidentification of the pathogen, unusually heavy disease pressure. However, there remained many examples where no other explanation was found, and where serious loss of control was clearly correlated with greatly decreased sensitivity of the pathogen population as revealed in laboratory tests on representative samples.

Resistance of the kind just described, characterised by a sudden and marked loss of effectiveness, is variously referred to as 'qualitative', 'single-step', 'discrete', 'disruptive' or 'discontinuous' resistance. Once developed, it tends to be stable. If the fungicide concerned is withdrawn or used much less, pathogen populations can remain resistant for many years; a well documented example is the sustained resistance of *Cercospora betae*, the cause of sugar-beet leafspot, to benzimidazole fungicides in Greece (Dovas *et al*. 1976). A gradual recovery of sensitivity can sometimes occur, as in the resistance of *Phytophthora infestans*, the potato late blight pathogen, to phenylamide fungicides (Cooke *et al*. 2006). In such cases, resistance tends to return quickly if unrestricted use of the fungicide is resumed, but re-entry involving also a partner fungicide has proved useful in some instances.

Sometimes, as in the case of the DMI fungicides, and of the 2-amino-pyrimidine fungicide ethirimol, resistance has developed less suddenly. In such cases, both

a decline in disease control and a decrease in sensitivity of pathogen populations as revealed by monitoring tests, manifest themselves gradually, and are partial and variable in degree. This type of resistance is referred to as ‘quantitative’, ‘multi-step’, ‘continuous’, ‘directional’ or ‘progressive’.

The first appearance of resistance in a particular fungicide-pathogen combination in one region has almost always been accompanied, or soon followed, by parallel behaviour in other regions where the fungicide is applied at a similar intensity. Whether the fungicide also meets resistance in other of its target pathogens depends on the individual case. Generally it does occur in other target pathogens that have a comparable rate of multiplication, provided that the fungicide is used in the same way. It is notable that rust fungi, despite their abundant sporulation and rapid spread, appear to be low-risk, seldom producing resistance problems (Grasso *et al*. 2006).

Pathogen populations that develop resistance to one fungicide automatically and simultaneously become resistant to those other fungicides that are affected by the same gene mutation and the same resistance mechanism. Generally these have proved to be fungicides that have an obvious chemical relationship to the first fungicide, or which have a similar mechanism of fungitoxicity. This is the phenomenon known as ‘cross-resistance’. For example, pathogen strains that resist benomyl are almost always highly resistant to other benzimidazole fungicides such as carbendazim, thiophanate-methyl or thiabendazole. Sometimes cross-resistance is partial, even when allowance is made for the greater inherent activity of different members of a fungicide group.

There is a converse phenomenon, ‘negative cross-resistance’, in which a change to resistance to one fungicide automatically confers a change to sensitivity to another.

Some pathogen strains are found to have developed separate mechanisms of resistance to two or more unrelated fungicides. These arise from independent mutations that are selected by exposure to each of the fungicides concerned. This phenomenon is totally different from cross-resistance in its origin and mechanism, and is usually termed ‘multiple resistance’.

1.2.5 Resistance mechanism

A lot of experimental studies has focussed on this subject, particularly in laboratories. A broad outline of current information is provided by Table 3. Some of the information is derived from resistant strains generated in the laboratory (e.g. for quinoxyfen) and not from field isolates. There is extensive information concerning the DMI fungicides, identifying four major resistance mechanisms that may operate. However, there are still many gaps in our knowledge, not only for established fungicide groups (e.g. anilinopyrimidines), but also for new fungicide groups defined by cross-resistance (e.g. carboxylic acid amides, CAAs).

Many types of resistance mechanism are known. These include:

- alteration of the biochemical target site so that it is no longer sensitive;
- increased production of the target protein;
- developing an alternative metabolic pathway that bypasses the target site;
- metabolic breakdown of the fungicide;
- exclusion or expulsion of the fungicide through ATP-ase dependent transporter proteins.

By far the commonest mechanism appears to be an alteration to the biochemical target site of the fungicide. This could explain why many of the older products have not encountered resistance problems. Once they have penetrated the fungal cell, the older fungicides act as general enzyme inhibitors, affecting many target sites (hence they are sometimes called 'multi-site' inhibitors). They act selectively on fungi because they penetrate and accumulate much more readily in fungi. Many sites in the fungus would have to change simultaneously in order to stop the fungicide from working.

In contrast, modern fungicides work primarily at single target sites, and are often referred to as 'single-site' or 'site-specific' fungicides. Thus just a single gene mutation can cause the target site to alter, so as to become much less vulnerable to the fungicide. The rapid development over the past 10 years of PCR-based diagnostic methods for detection of point mutations causing resistance has aided the identification of resistance mechanisms, especially

those involving target site changes. Several major resistance genes have now been isolated and characterised.

Table 3. Mechanism of fungicide resistance.

Fungicide or fungicide class	Mechanism of resistance
Aromatic hydrocarbons	Unknown
Organo-mercurials	Detoxification by binding substances
Dodine	Unknown
Benzimidazoles	Altered target site (β-tubulin)
2-Amino-pyrimidines	Unknown
Kasugamycin	Altered target site (ribosomes)
Phosphorothiolates	Metabolic detoxification
Phenylamides	Possibly altered target site (RNA polymerase)
Dicarboximides and phenylpyrroles	Altered target site (protein kinase involved in osmoregulation)
DMIs	Increased efflux; altered target site; decreased demand for target-site product; target-site over-production
Carboxanilides	Altered target site (succinate-ubiquinone oxidoreductase)
QoIs (strobilurins)	Altered target site (ubiquinol-cytochrome c reductase)
Melanin Biosynthesis Inhibitors (Dehydratase) MBI-D	Altered target site (scytalone dehydratase)

(Source: Brent & Hollomon, 2007a, modified)

1.2.6 Monitoring

By 'monitoring for fungicide resistance' we mean testing samples of field populations of target pathogens for their decrease of sensitivity to one or more fungicides. This is a crucial area of resistance research, because virtually all our knowledge of the distribution, evolution and impact of resistance in the field has depended on monitoring. It was originally done in the early 1960s to investigate possible resistance in seed-borne diseases of wheat and oats, and in storage mould on citrus fruit. A much larger amount of monitoring is now routinely done world-wide.

Monitoring can be done to gain early warning of an impending resistance situation. However, as discussed above, single-step resistance only becomes immediately detectable in field samples when a relatively high frequency of the

resistant variants (>1%) is reached. The next or next-but-one treatment would fail to give normal control. Therefore useful early warning is unlikely to be obtained, unless impractically large numbers of samples are tested (300 samples are needed to give a 95% chance of detecting resistance at 1% frequency).With multi-step resistance, partially resistant strains can exist at high frequency before practical loss of disease control occurs. Another important reason for monitoring is to check that management strategies are working. This involves monitoring regularly over large areas of use, an expensive operation but one which has been justified by situations of high commercial risk. Molecular diagnostics have been successfully used to monitor the degree of success of anti-resistance strategies aimed at combating QoI resistance in powdery mildew and septoria diseases of wheat (Fraaije *et al*. 2002, 2005). Monitoring is also done at specific sites in order to investigate complaints from growers of an apparent loss of performance of the fungicide, and/or to give guidance on the selection of future fungicide treatments at the site or in the district. Many otherwise competent monitoring operations have, in the past, given inconclusive results because one or both of two extremely important steps have been omitted. The first of these is to develop monitoring methods early, and then to use them to obtain base-line data on typical pathogen populations before they are exposed to any widespread use of a new fungicide. This initial assessment of the 'natural' range of sensitivity, which can be considerable, is an enormous help to the interpretation of any later monitoring data in terms of possible shifts in sensitivity. It also ensures that suitable sampling and assay methods have been worked out and tested.

A second crucial activity to complement resistance monitoring, is to monitor practical performance. Knowledge of the continued degree of effectiveness of field performance is often surprisingly vague and badly recorded, and yet it is a critical indicator of the occurrence of practical resistance. Systematic observations, over time, must be made on amounts of disease in commercial crops treated and untreated with the at-risk fungicide, and also in any replicated plot trials that are done. In order to confirm that practical resistance has appeared, it is essential to establish a clear correlation, both in time and geographically, between the incidence of resistant biotypes and the deterioration of field performance of the fungicide. Much experience has now been gained

with regard to the reliability, logistics, costs and necessity of monitoring. Timely and representative sampling is important. It has been found very revealing to obtain some samples of the pathogen early in the season before treatment starts, if sufficient infection exists. The observation of a high resistance level after treatment can be a sign of very successful control, the resistant forms being concentrated in the small surviving population. Of course practical problems would follow if the resistant population persisted and formed the inoculum for the following year, but this is not necessarily the case. Experience has also shown that the risk of resistance can vary greatly between regions where disease pressures and fungicide use are high, and neighbouring areas where there is less disease or where yields are too low to support widespread fungicide use. For example, in Northern Europe several key cereal pathogens have developed resistance to a number of fungicide groups, whereas in southern Europe the same pathogens have remained sensitive, and the requirement for monitoring is less important (Kuck 2005).

1.2.7 Assessing the risk

This is a matter of great importance to the chemical manufacturer who is about to develop a new product. Knowledge of the risk of resistance will help to determine whether the product should be developed and marketed, and, if so, of what nature and how stringent should be the resistance management strategies and how much further monitoring should be done.

The possibility that strains resistant to existing fungicides may be cross-resistant to the candidate product is readily determined. The chemical structure of the potential product, or its mode of action if known, may resemble those of existing fungicides, and thus indicate a likelihood of cross-resistance. More direct guidance can be obtained by testing the candidate against field isolates of the target pathogen that are known to resist other fungicides, and this is now done as a matter of routine.

Knowledge of the mechanism of action of a fungicide can be informative. For example, a mechanism involving inhibition of tubulin assembly would, by

analogy with the benzimidazole fungicides, be considered a high risk indicator, whereas a multi-site action would indicate relatively low risk.

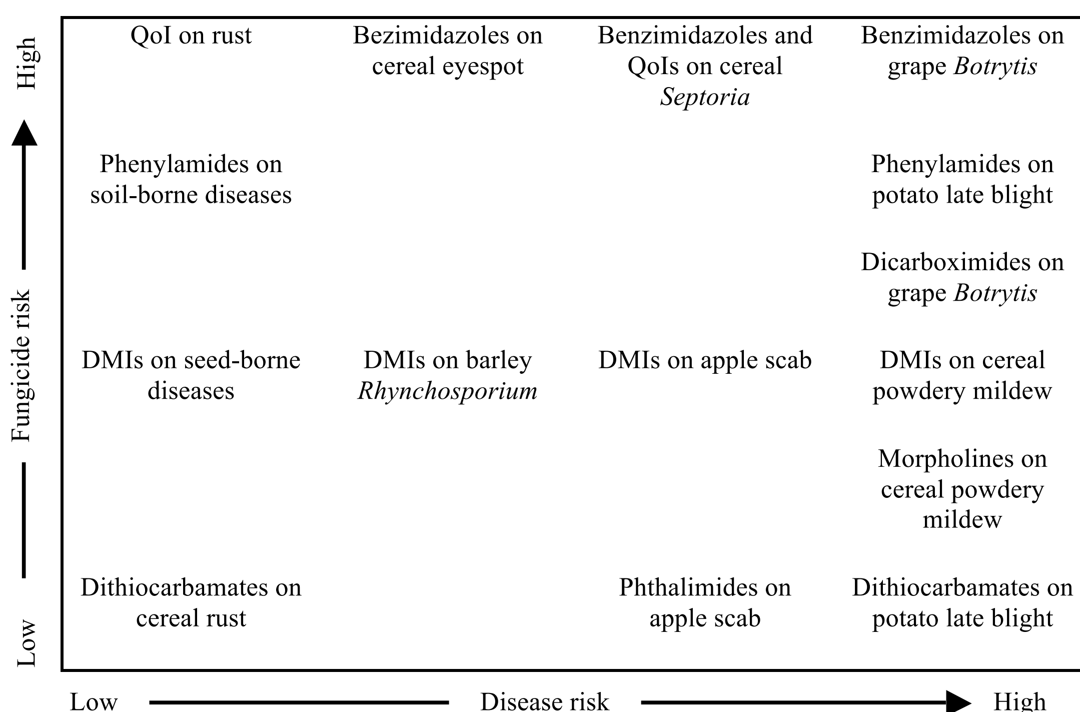
As discussed earlier, classes of fungicide differ greatly in their basic vulnerability to resistance arising in target pathogens. Indications of the degree of this intrinsic fungicide risk, whether low, medium or high level, can emerge from mutagen treatments or training experiments, or more reliably (although only after first commercial introduction) from performance-checking and monitoring during early years of commercial use, and from cross-resistance studies.

Different classes of pathogen also vary in their ability to become resistant to fungicides. Higher pathogen risk is associated with a shorter life cycle, more abundant sporulation of the pathogen, and rapid, long-distance dispersal of spores. For example, resistance to the benzimidazole fungicides was much slower to develop in cereal eyespot disease, where the pathogen (*Oculimacula* spp.) generally has only one generation per year, with limited spore production and dispersal, and only one fungicide application is made per year, than in cucurbit powdery mildew (*Sphaerotheca fuliginea*) which has many short generations, abundant sporulation and widespread dispersal, and requires repeated fungicide treatments. The way in which 'fungicide risk' and 'pathogen risk' combine to determine the overall intrinsic risk of resistance problems is illustrated in Fig. 9. Overall risks of resistance development in crop disease situations depend not only on these intrinsic or inherent risks attached to particular types of fungicide or pathogen, but also on the conditions of fungicide use. Unlike the intrinsic risks, the conditions of use can vary much between regions and from farm to farm. They comprise environmental factors, especially climatic and topographic conditions that affect the severity and spread of crop disease, and a range of farmer-determined agronomic factors. The latter include fungicide selection, application frequency and dose, use of glass-houses or polythene tunnels (these tend to isolate pathogen populations and prevent ingress of sensitive strains), pattern of crop rotation, choice of cultivar and its degree of susceptibility to infection, and the extent of use of hygienic practices.

Assessment of degree of risk of resistance development for a particular location must take into account and integrate as far as possible all influential factors including the intrinsic risk for each fungicide-pathogen combination, the

environmental conditions and their likely effects on disease incidence, and relevant agronomic practices which should incorporate any specific fungicide use strategies recommended by the fungicide manufacturer. Inevitably, such risk assessment can only be an approximate estimate, at best indicating low, medium or high level, because many factors are involved, and with our present state of knowledge their effects cannot be measured precisely or given accurate weightings for relative importance.

Figure 9. Graphical representation of the combined risk-resistance for some combinations fungicide-pathogen. (Source: Brent & Hollomon, 2007a, modified).



1.2.8 Management strategies

In practice, however, resistance management strategies must combine the long-term conservation of fungicide effectiveness with an amount and pattern of use that are sufficient both to satisfy the needs of the farmer and to provide a reasonable pay-back to the manufacturer.

Strategies must be applied uniformly over large areas in order to obtain their full biological benefit, and also to ensure that any short-term commercial disadvantage and long-term advantage are shared amongst all manufacturers of the same group of fungicides. Thus to have a chance of success any strategy

must be reached by agreement and depend upon a commitment to implementation from all supply companies involved. It must also be understandable and acceptable to the farmer. The integrated use of combinations of different strategies is feasible, beneficial, and often implemented.

A. Do not use the product exclusively

Apply it as a mixture with one or more fungicides of a different type, or as one component in a rotation or alternation of different fungicide treatments.

The ‘companion’ or ‘partner’ compounds applied in either of these ways will dilute the selection pressure exerted by the at-risk fungicide and inhibit the growth of any resistant biotypes that arise. The partner compound can be a multi-site compound known to have a low risk of inducing resistance. Alternatively, it can be a single-site fungicide that is known not to be related to its partner by cross-resistance or (in the absence of known resistance) by a similar mode of action. Use of a mixture of two single-site fungicides must carry some element of risk of selecting dual-resistant strains. This type of strategy is widely recommended by industry and also by advisory bodies. The use of formulated (‘pre-packed’) mixtures of two different fungicides has often been favoured by manufacturers. Also the control of many pathogens only requires one or two treatments per annum so that the rotational approach is not appropriate. Questions of what application rate is appropriate for each mixture component are difficult and have been debated many times. Some reduction relative to the full recommended separate rates has often been made, to keep down costs.

The models all indicate that use of both mixtures and rotations can delay, but not prevent, the build-up of resistant variants. They favour one or other of these two approaches to different degrees depending on the various assumptions that are incorporated. Experimental data relating to the effectiveness of mixture and rotation strategies are limited. Studies on *Phytophthora infestans*, showed that applications of mixtures of a phenylamide fungicide with mancozeb or mancozeb plus cymoxanil decreased the build-up of phenylamide resistance, compared with phenylamide alone (Staub & Sozzi 1984; Samoucha & Gisi 1987). Selection for QoI resistance in *Plasmopara viticola* was delayed by a mixture with folpet, fosetyl- aluminium or mancozeb (Genet *et al*. 2006).

A recent modelling study (Parnell *et al*. 2006) has predicted that the regional spread of single gene resistance over large distances will depend on the proportion of fields of a particular crop that are sprayed, and not only on within-field use strategies. The extent of any loss in fitness caused by the resistant mutation, and the effectiveness of the fungicide against the wild-type sensitive pathogen, also influence the speed that resistance will spread. It is suggested that some fields should be left untreated, or treated with different, non-cross-resistant fungicides. Field experimentation on resistance management strategies is always a difficult task, requiring large, replicated plots, and sustained cropping, treatments and assessments for several successive years. Variation in infection conditions and disease pressure from year to year, irregular availability of adequate samples of the pathogen, movement of inoculum between plots, ingress of external inoculum into the experimental area, and other difficulties often render such work inconclusive. An early field experiment on *Cercospora beticola* showed that alternation of benomyl and a tin fungicide delayed the development of benomyl resistance (Dovas *et al*. 1976). In several studies on cereal powdery mildews (*Blumeria graminis* f. sp. *tritici* and *hordei*), field application of mixtures of triazoles with morpholine or aminopyrimidine fungicides was found to hinder the development of resistance to one or to both of the fungicides applied, which did occur after sequential applications of each fungicide alone (Heaney *et al*. 1988, Brent *et al*. 1989, Lorenz *et al*. 1992). Effects of fungicide alternation were less regular, giving either a similar or a smaller benefit according to the particular study.

Development of resistance of *Botrytis cinerea* on tunnel-grown strawberries to dicarboximides, and of *Polyscytalum pustulans* and *Helminthosporium solani* on potatoes to thiabendazole, was shown to be delayed by the application of certain fungicide mixtures (Hunter *et al*. 1987, Carnegie *et al*. 1994). In experiments on grape powdery mildew (*Uncinula necator*) a mixture of triadimenol with sulphur or dinocap at roughly half normal rates did not slow down the evolution of triadimenol resistance; however, alternations, at full rates, did decrease resistance development (Steva 1994). Build-up of QoI resistance in *Mycosphaerella graminicola* in field plots of wheat was much reduced by application of an azoxystrobin/epoxiconazole mixture, compared with a solo azoxystrobin treatment (Gisi *et al*. 2005). Overall, field experimentation does

appear to support the adoption of mixture and rotation strategies, but since there are some inconsistencies and the range of diseases and fungicides worked on is rather limited, further work should be encouraged.

Practical experience also suggests that both mixture and rotation strategies have delayed resistance development, and examples are discussed later.

B. Restrict the number of treatments applied per season, and apply only when strictly necessary. Use other fungicides both beforehand and subsequently

This approach, like rotation, reduces the total number of applications of the at-risk fungicide and therefore must slow down selection to some extent. It can also favour decline of resistant strains that have a fitness deficit.

C. Maintain manufacturers' recommended dose

For many years farmers have often used reduced rates of application of fungicides, mainly to reduce costs, especially in conditions where disease pressures are usually low, or where the risk of financial loss from reduced performance was not great. Also, advisory services in pursuing lower-input approaches for economic and environmental reasons, have recommended use of smaller doses for certain situations. On the other hand it is the view of FRAC that recommended doses must be maintained, because it is possible that reducing the dose could enhance the development of resistance.

However, relationships of fungicide dose to risks of resistance are not yet fully established, and it seems likely that they may vary according to the fungicide in question. With regard to multi-step resistance, it has been argued that lowering dose can enhance resistance development by favouring the survival of low-level resistant forms which would be inhibited by the full dose.

Experimental data regarding effects of different doses are still rather limited and confusing. In a growth chamber experiment, selection for resistance to triazoles in barley powdery mildew was slowed down by lowering fungicide concentrations (Porras *et al*. 1990). Again the work is more difficult to do in the field, partly because degrees of effectiveness, which must be critical, vary greatly between and within growing seasons. Decreasing application rates appeared to slow down development of resistance of triadimefon to barley powdery mildew (Hunter *et al*. 1984), but in other experiments on strawberry *Botrytis* and wheat eyespot altering fungicide doses made little difference to

resistance build-up (Hunter *et al*. 1987, 1993). When a benomyl-mancozeb mixture was applied to control apple scab, build-up of benomyl resistance was delayed by reducing the benomyl concentration and increasing the mancozeb concentration (Lalancette *et al*. 1987). Decreasing the dose of DMI fungicides from one-quarter to one-eighth of the full recommended dose was found to reduce resistance development in *Mycosphaerella graminicola* (Metcalfe *et al*. 2000, Mavroeidi & Shaw 2006).

It is now widely accepted, on theoretical grounds, limited experimental data and practical experience, that risks of major-gene (single-step) resistance are unlikely to increase, and may well decline as dose is lowered. The situation with regard to polygenic resistance is still not at all clear, and more experimental work is justified in order to obtain a sounder base for recommendations. Some of the published data refer specifically to 'split' schedules, in which dose is lowered but frequency of application is correspondingly increased, to give the same total mount applied each season. It is important to distinguish these from reduced-dose applications made on normally timed schedules so that the total dose per season is decreased. The use of more frequent 'split' applications could increase resistance risk and should be avoided.

D. Avoid eradicant use

One of the advantages of systemic fungicides is that they can eradicate or cure existing infections. This property greatly assists their use on a 'threshold' basis, where application is made only when a certain, economically acceptable, amount of disease has already appeared, in order to prevent further spread. However, avoidance of the use of systemic fungicides in this way has been recommended in two different situations as an anti-resistance strategy.

FRAC has recommended that eradicant use of phenylamides should be avoided. This is because they are now always applied for control of foliage diseases as a mixture with a multi-site companion fungicide. The latter does not work as an eradicant, so that the phenylamide is acting alone when the mixture is applied to existing infections. To the authors' knowledge there is no experimental evidence comparing the resistance risks of prophylactic versus threshold-based schedules, and research on this would be useful.

E. Integrated disease management

The integrated use of all types of countermeasures against crop disease is not only highly desirable on economic and environmental grounds, but is also a major strategy for avoiding or delaying fungicide resistance. The use of disease resistant crop varieties, biological control agents, and appropriate hygienic practices, such as crop rotation and removal of diseased parts of perennial crop plants, reduces disease incidence and permits the more sparing use of fungicides, and in both these ways decreases selection of fungicide-resistant forms (Brent & Hollomon 2007).

F. Chemical diversity

The availability of a number of different types of fungicide for the control of each major crop disease is highly beneficial both environmentally and in order to overcome resistance problems. The continued use of one or a very few types of compound over many years presents a much greater risk of side-effects and favours resistance in the target organisms. A new fungicide does not necessarily have to be superior to existing ones in order to be of value. It has to be effective, and, in the resistance context, it should work against strains that are resistant to existing fungicides. This latter property is usually associated with a new mode of action, and ideally there should be more than one site of action to decrease the risk of evolution of resistance to the new fungicide.

1.2.9 Specific management strategies

FRAC (Fungicide Resistance Action Committee) decided to operate through Working Groups, one for each major class of fungicides to which resistance is known, and which has more than one manufacturer, or potential manufacturer with an announced development product. Currently there are seven Working Groups, dealing with SBI (sterol biosynthesis inhibitor) fungicides, AP (anilinopyrimidines), QoI (quinone outside inhibitor) fungicides, CAA (carboxylic acid amide) fungicides, azanapthalene (AZN, quinoxyfen and proquinazid) fungicide, succinate-dehydrogenase (SDHI) and Banana group. These Groups collect and publish data on resistance status in different crops, pathogens and countries, and issue and review annually resistance management guidelines. Three former Working Groups, concerned with benzimidazoles,

dicarboximides and phenylamides, have now converted to Expert Fora, giving relevant information and advice on request. The latest information and guidelines from each Working Group are available on the FRAC website (www.frac.info).

1.2.9.1 Benzimidazoles

Many pathogens adapted very quickly to benzimidazoles, for example *Botrytis* spp. Others took about 10 years before being detected e.g. *Oculimacula* spp., cause of cereal eyespot disease (Locke 1986) or even 15 years (e.g. *Rhynchosporium secalis*, cause of barley leaf-scald (Kendall *et al*. 1993).

Over the years the use of mixtures or alternations with non-benzimidazole fungicides has been encouraged with varying degrees of vigour by the individual companies concerned and by advisory services. Often this was done too late. When benzimidazole resistance has already become established, it usually persists.

An example of the successful early use of a mixture strategy is the application of benzimidazoles to control *Cercospora* leaf-spots of peanut in the USA. In the southeastern states, where there was sole use of benomyl, practical resistance soon appeared. In Texas, where benzimidazole-mancozeb mixtures were used from the start, no resistance developed over many years except in trial plots where a benzimidazole alone was applied repeatedly (Smith 1988). The FRAC Working Group supported the use of mixtures or alternation in a general way, and the avoidance of eradicant use unless absolutely necessary, but did not make specific recommendations or initiate major monitoring projects.

Use of benzimidazole fungicides worldwide is still substantial, despite the widespread incidence of resistance since the early 1970s. In the absence of data it is hard to say to what extent benzimidazole fungicides are now still effective, and whether use on the present scale is fully justified. Monitoring in 1997-2003 in France revealed the common occurrence at high frequency of benzimidazole-resistant strains of *Mycosphaerella graminicola* and *Oculimacula* spp in wheat (Leroux *et al*. 2003, 2005). One special and interesting approach to overcoming benzimidazole resistance has been the application of a mixture of the

benzimidazole fungicide carbendazim with diethofencarb, to control *Botrytis* in grapes and other crops. Diethofencarb shows negative cross-resistance with respect to benzimidazoles. Remarkably, it inhibits only benzimidazole-resistant strains of the target pathogens and does not affect benzimidazole-sensitive strains. In practice a formulated carbendazim-diethofencarb mixture, introduced in 1987 initially gave good control of *Botrytis*, irrespective of whether pathogen populations were benzimidazole-resistant or not. However, the appearance and spread of strains resistant to both fungicides caused problems (Elad *et al*. 1992, Leroux & Moncomble 1994) and the product is no longer used.

1.2.9.2 SBIs (sterol biosynthesis inhibitors)

This large class of fungicides comprises three distinct groups: the sterol C14-demethylation inhibitors (DMIs, e.g. triazoles, imidazoles, fenarimol, triforine); amines (morpholines e.g. tridemorph, fenpropimorph, piperidines e.g. fenpropidin, spiroketalamines e.g. spiroxamine); hydroxyanilides (e.g. fenhexamid).

DMIs were first used in the 1970s, triforine, triadimefon and imazalil being early representatives. Since then at least 30 more DMIs have been used in agriculture. At the time the FRAC Working Group formed, in 1982, there were very few reports of DMI resistance.

Several mechanisms have been described leading to DMI resistance; these mechanisms include alteration in sterol biosynthesis pathways, amino acid changes in CYP51 target sites, increased active efflux by membrane transporters, increased level of CYP51 gene expression, changes in cell-wall composition and sequestration of the antifungal agents in cell membranes and reduced positive influx (Ma & Michailides 2005, Zhan *et al.* 2006).

However, such mutants had reduced pathogenicity and other fitness attributes, so that development of practical resistance was deemed unlikely (Fuchs & Drandarevski 1976). Practical resistance did in fact develop in several pathogens during the 1980s (e.g. powdery mildews, *Venturia inaequalis*, *Mycosphaerella fijiensis* var *difformis*), but relatively slowly and with fluctuating severity, as is considered to be characteristic of polygenic resistance.

Although amine fungicides have been used extensively for many years, they continue to perform well. Considering the amount of use, their potency, the high multiplication rates of the main target pathogens (e.g. powdery mildews and *Mycosphaerella fijiensis*), and the ease of generating resistant mutants in the laboratory, the stability of their performance has been remarkable. Some reports of decreased sensitivity have appeared from time to time. The slightly resistant field isolates were not cross-resistant to the DMI fungicides, which act at a different stage of sterol biosynthesis.

Interestingly, several studies have revealed cross-resistance between isolates of barley and wheat powdery mildews with respect to fenpropimorph and fenpropidin, but little cross-resistance to tridemorph appears to occur (Readshaw & Heaney 1994). This pattern correlates well with information on mechanisms of action, since fenpropimorph and fenpropidin are considered mainly to inhibit the Δ14 -15 reduction step, and tridemorph mainly the Δ8 -7 isomerisation step, in sterol biosynthesis (Hollomon 1994). However, there is evidence for additional sites of action, and a multi-site action, coupled with the flexible, multi-configurational nature of the carbon chain, could account for the durability of action of the morpholine fungicides.

Hydroxyanilide fungicides inhibit yet another step in sterol biosynthesis, catalysed by C3-keto-reductase. Fenhexamid, the sole hydroxyanilide in commercial use is applied specifically for control of *Botrytis* spp. and related pathogens, which does not show cross-resistance to other classes of anti-Botrytis fungicides.

FRAC has made the following general recommendations regarding use of SBI fungicides:

- Do not use repeated applications of SBIs alone on the same crop in one season against a high-risk pathogen in areas of high disease pressure for that pathogen.
- For crop/pathogen situations requiring multiple spray applications, e.g. orchard crops/powdery mildews, use mixtures or alternate (in block sprays or in sequence) with effective non-cross-resistant fungicides.
- If mixture or alternation is not possible, reserve SBI use for the critical part of the season or critical crop growth stage.

- If DMI or amine performance declines and less sensitive forms of the pathogen are detected, SBIs should only be used in mixture or alternation with effective non-cross-resistant fungicides.
- Complementary use of other fungicide classes with different modes of action should be maximised.
- Use as recommended on the label. Do not use reduced doses.
- Use other measures such as resistant varieties, good agronomic practice, plant hygiene.

These recommendations have been widely implemented, and in general the SBI fungicides are continuing to give good control of most target pathogens some 30 years after their introduction. The warning against reduced rates could be open to debate since, as discussed earlier, the relevant experimental data are limited and conflicting. This is clearly an important area for further research. However, it is of course always necessary to use DMIs in amounts sufficient to ensure cost-effective disease control under the particular conditions of use.

1.3 Fungicide resistance in *Calonectria* spp.

Several authors have reported the effectiveness of the benzimidazoles for the management of different species belonging to *Calonectria* (Barnard 1984, Chase 1987, Nan *et al*. 1992, Kucharek & Atkins 1993), but the continuous use of these fungicides in nurseries may determine the selection of resistant strains with a consequent decrease in the activity of the fungicide (French & Menge 1978, Alfenas *et al*. 1988).

The constant use of benomyl induced the selection of resistant strains of *Cy. scoparium* at concentrations close to 1000 µg/ml (Alfenas *et al*. 1988).

An experiment was carried out in greenhouse on cuttings of rhododendron to assess the presence of sensitive and resistant strains to carbendazim in a population of *Cy. scoparium*. In conditions of low pressure of the disease, a treatment to the soil with prochloraz showed a good control of both the sensitive strain and the resistant. Captan, chlorothalonil and diclofuanide were ineffective. None of the compounds tested was effective in the case of high pressure of the disease (De Prest & Poppe, 1988).

In vitro trials have shown that the mycelial growth of six strains of *Cy. pauciramosum* were completely inhibited at concentrations of 1 µg/ml of carbendazim while 500 µg/ml did not allow the complete inhibition of other four strains (Polizzi 2000).

In several experiments, resistance to benomyl was observed in the 58% of 200 strains of *Cy. pauciramosum* collected in different nurseries located in southern Italy. The largest number of resistant strains have been found in plants frequently treated with benzimidazoles during an year. Many resistant strains grew at concentrations of the fungicide higher than 500 mg/l, while no growth was observed for those sensitive at the concentration of 1 mg/l. The benomyl-resistant isolates showed cross-resistance to carbendazim. In addition, the higher resistant strains showed a slow-growing phenotype when compared to the sensitive fast-growing isolates (Polizzi & Vitale 2001).

Sensitivity to benzimidazole was determined also in an Italian *Cy. scoparium* population; 14 isolates were tested *in vitro* at different rates of benomyl and 13 were resistant to the active ingredient. The benomyl-resistant isolates were also resistant to carbendazim. Among the resistant isolates, five grew in the presence of 10 µg a.i./ml and one isolate grew at 100 µg a.i./ml (Vitale *et al.* 2009).

Considering the risks arising from the continuous use of benzimidazoles, a correct anti-resistance strategy should be expected to limit their use, especially where the percentage of resistant isolates is high, or to use them in mixture or alternation with fungicides with a different mode of action or low risk of resistance.

1.4 Thesis aims

In southern Italy, the ornamental-plant nurseries occupies of course an important role. Significant development has seen the production of ornamental plants with particular reference to those designed for the outdoors design. The general interest in this commercial sector induced the need of introduction of more new products and, therefore, an intensive exchange of planting material among different countries around the world. These activities have sometimes favored the introduction of new pathogens.

The species belonging to the genus *Calonectria* are pathogens of a large number of crops interesting in agriculture and forestry worldwide (Peerally 1991a, Crous & Wingfield 1993, Crous & Wingfield 1994). It has also been reported that two or more species belonging to this genus may be associated giving origin to a "complex disease" (Crous *et al.* 1993a, Schoch *et al*. 1999). *C. pauciramosum, C. morganii* and *C. polizzii* have recently been reported in Europe (Polizzi & Crous, 1999, Polizzi *et al*. 2006a, Polizzi *et al*. 2007b, Polizzi *et al*. 2009a,b,c Lombard *et al*. 2010b).

In southern Italy *Calonectria* spp. cause serious symptoms in ornamental plants, especially seedlings and cuttings in the nursery. In relation to its geographical distribution, the large number of host plants, the severity of the diseases and the difficulty of the management in the nursery, *Calonectria* disease represent a serious threat for ornamental nurseries of southern Italy and it is one of the fungal pathogens "key" for some crops such as *Myrtus communis*, *Polygala myrtifolia* and *Callistemon* spp. The large economic losses that may occur, justify the use of chemical means of control (Polizzi 2000, Polizzi & Crous 1999, Aiello *et al.* 2013).

Considering the large expansion of ornamental-plants sector in agriculture and considering the spatial distribution of the pathogen complex, the large number of host plants, the severity of the diseases and the difficulties of their management in the nursery, it is possible to establish that *Calonectria* spp. represent a serious phytopathological threat of great importance in the agricultural industry. To complicate the management of these diseases should be added any epidemiological characteristics of these pathogens, such as their ability to perform more cycles during a growing season and their phenotypic and genetic variability.

For all these reasons, the disease complex in Mediterranean environment should be examined monitoring important areas of ornamental plants production.

Indeed, the first goal of this study was to detect new *Calonectria* diseases. For the achieving of this goal, several surveys in ornamental-plants nurseries were carried out to monitor symptomatic plants, to identify the fungal species associated to the disease and to confirm the pathogenicity of isolates collected. The scientific activity was concerning in particular: visual analysis and the selection of samples with symptoms similar to infection caused by *Calonectria*,

the isolation in culture of *Calonectria* spp. from infected tissues; obtaining *Calonectria* single-spore colonies, the morphological and molecular identification of isolates and, at least, to carry out pathogenicity tests of isolates collected on healthy plants. During this study, several survey were localized in eastern Sicily, an ornamental plant-growing areas with an important role by several decades, and in Carthage, Tunisia, where this agriculture sector, is now expanding.

Chemical control of *Calonectria* diseases is necessary for reducing damage to young plants in the nursery. Only preventative measures were found effective, while no curative effects could be obtained for controlling these diseases (Crous 2002). Various fungicides and different methods of application have been proposed to control diseases caused by *Calonectria* species (Crous 2002, Henricot *et al*. 2008).

Several authors report the effectiveness of different chemicals such as benomyl, chlorothalonil and prochloraz in numerous tests against fungal pathogen belonging to the genus *Calonectria* although it has been shown that the effectiveness was variable depending on the host species, the mode of application, the localization of the pathogen in the organs of the plant and the pressure of the disease (Engelhard 1971, Alfieri & Graham 1972, Miller & McRitchie 1973, Bertus 1976, Barnard 1984, Jamart & Kamoen 1986, Lutz *et al.* 1988). On the basis of this knowledge, the use of benzimidazoles and prochloraz have achieved a primary role in nurseries-practices, becoming the most used chemical for the management of *Calonectria* infections in last decades.

In literature, several works report the effectiveness of benzimidazoles fungicides for containment of cuttings rot (Horst & Hoitink 1968) and foliar infections (Bertus 1976) caused by *Cy. scoparium* or others different species of *Cylindrocladium* (Barnard 1984, Chase 1987, Nan *et al.* 1991, Kucharek & Atkins, 1993). Grigoletti & Auer (2003) tested several fungicides to manage foliar infections on *Ilex paraguariensis* caused by *Cy. spathulatum,* observing that benomyl and captan resulted the most effective, but the continuous exposure of these fungicides in nurseries may determine the selection of resistant strains with a consequent decrease in the fungicide activity (French & Menge 1978, Alfenas *et al.* 1988).

The intensive use (sometimes exclusive use) of some of the above chemicals such as benomyl led to the occurrence of resistance to fungicides in ornamental plants cultivated in nurseries. Resistance to benomyl had already been reported several decades ago for *C. floridanum* (French & Menge 1978) and *C. scoparium* (Alfenas *et al.* 1988).

The resistance to benzimidazoles has been observed for all location of southern Italy examined. Of the 200 isolates tested, 58% were resistant to benomyl, while the remaining 42% did not grow at concentrations of 1 ppm of the fungicide (Polizzi & Vitale 2001). Analyzing the results obtained, it is clear that the use of benzimidazoles for the control of this pathogen, despite the known efficacy, should be carefully considered, especially in nurseries and in those areas with a high percentage of resistant isolates in the population. These data should be considered for a correct management of anti-resistance strategies in the nursery.

Considering the risks arising by the continuous use of benzimidazoles, a proper strategy to combat resistance should be expected to limit its use or use them in combination or alternation with fungicides with different mode of action or low risk of resistance.

However, recent research revealed a good fungicidal activity by some chemicals, as well as the antagonistic activity of microorganisms and fungi typically used in biological control.

Regular applications of copper oxychloride that have ensured a good containment of the pathogen, as well as confirmed by some tests carried out on plants of myrtle while benomyl and prochloraz were ineffective for management of foliar infections caused by *Cy. pauciramosum* (Polizzi & Azzaro 1996).

The most encouraging results have been obtained using prochloraz + cyproconazole and a cultural filtrate of *Trichoderma* spp., and have been obtained in experiments in which prochloraz was applied on *Polygala myrtifolia,* showing a good efficacy on different strains of *Cy. pauciramosum* (Polizzi 2000).

Application with only prochloraz on plants of *Myrtus communis*, contrary to the previous work, has shown inefficacy in containing the disease, underlining the variability in efficacy of this active ingredient (Vitale *et al*. 2003).

Experimental trials for evaluation of the efficacy of the antagonist *T. harzianum* T22 and prochloraz in management of crown and root rot of *P. myrtifolia* caused by *Cy. pauciramosum* provided an excellent control of the biological antagonist compared to the untreated control and to the fungicide treatments that was no effective (Polizzi & Vitale 2002).

Recently, Aiello *et al.* (2013) suggested the effectiveness of preventive applications of several chemicals, such as prochloraz, in controlling leaf spots caused by both *Ca. morganii* and *Ca. pauciramosa* on bottlebrush plants. Moreover these authors detected with the use of prochloraz, no significant reduction of crown and root rot caused by *Ca. pauciramosa* in feijoa plants, suggesting further investigations about the efficacy of this compound.

Demethylase inhibitors fungicides (DMIs) represent one of the largest groups of organic fungicides used extensively to control agriculturally important fungal pathogens by several decades. This group of single-site mode of action fungicides shows a broad spectrum of antifungal activity against most yeasts and filamentous fungi, and includes triazole, imidazole, piperazine, pyridine and pyrimidine fungicides.

The risk of developing resistance to DMI fungicides in field conditions was thought to be low. Unlike many other fungicides, such as phenylamides and benzimidazoles, resistance to DMIs is polygenic (Gisi *et al*. 2007, Blatter *et al*. 1998, Dyer *et al*. 2000) and mediated by several mechanisms. Because of the polygenic inheritance of resistance, it was expected that the emergence of phenotypes resistant to DMI would be less easily detected than would those of other fungicides with single target sites (De Waard 1994). Furthermore, it was noticed that laboratory-generated DMI-resistant phenotypes had a reduced fitness with respect to spore germination, mycelial growth rate and virulence. Despite the apparent fitness costs, resistance to DMI fungicides has been reported in several pathogens such as powdery mildews, *Venturia inaequalis*, *Mycosphaerella fijiensis* (Hildebrand *et al*. 1988, Kendall *et al*. 1989,).

Prochloraz, an imidazole, member of DMIs, was introduced in Italy in the early of 1990s. Prochloraz is a protectant, translaminar fungicide, effective on several plant pathogens (Nan *et al*. 1991, Henricot *et al*. 2008, Gea *et al.* 1996). In last decades any pathogens such as *Verticillium fungicola, V. dahliae, Mycosphaerella graminicola, Pseudocercosporella herpotrichoides*, showed

reduced sensitivity to this active ingredient (Gea *et al* 2005, Kurt *et al*. 2003, Mavroeidi & Shaw 2006, King *et al.* 1986).
In conclusion, the rising concern about the widespread resistance to benzimidazole fungicide within different *Calonectria* spp. (Alfenas *et al*. 1988, Polizzi & Vitale 2001, Vitale *et al.* 2009) and the occurrence of resistance to prochloraz of others pathogens, have been established. In addition, a variability in prochloraz efficacy for the management of infections caused by *Calonectria* spp. has been demonstrated in last decade and recently by Aiello *et al*. (2013).
On the basis of this knowledge, a study about sensitivity to prochloraz of isolates belonging to *Calonectria* was considered necessary.
The second purpose of this was, therefore, to determine if reduced sensitivity to prochloraz has occurred within an Italian population of *Calonectria* spp. The specific objectives of the second part of this experimental thesis were: to identify the different isolates belonging the Italian population studied, to assess the *in vitro* sensitivity to prochloraz and to assess if a shift in sensitivity to prochloraz has occurred among pathogen strains collected in different year periods in the same areas. In addition, the *in vitro* sensitivity to prochloraz was compared with the *in vivo* sensitivity, using both seedlings of *Trifolium pratense* L. as a model system (Vitale *et al.* 2012a) and *Feijoa sellowiana,* as an ornamental species cultivated in commercial nurseries (Vitale *et al*. 2008).
Thus, the aims of this thesis were:

Objective 1: Detection of new *Calonectria* diseases in eastern Sicily.

Approaches:

A) Field survey, symptoms observations and collection of plant materials.
B) Isolation and morphological and molecular characterisation of the causal agents of symptoms associated with already known *Calonectria* diseases.
C) Testing of the isolated fungi for pathogenicity.

Objective 2: Detection of new *Calonectria* diseases in eastern Tunisia.

Approaches:

A) Field survey, symptoms observations and collection of plant materials.

B) Isolation and morphological and molecular characterisation of the causal agents of symptoms associated with already known *Calonectria* diseases.

C) Testing of the isolated fungi for pathogenicity.

Objective 3: To study and monitor shift in prochloraz sensitivity within *Calonectria* spp.

Approaches:

A) to identify the different isolates belonging to the Italian population studied.

B) to assess the *in vitro* sensitivity to prochloraz

C) to assess if a shift in sensitivity to prochloraz has occurred among pathogen strains collected in different year periods

D) to compare the *in vivo* sensitivity to the *in vitro* sensitivity to prochloraz using seedlings of *Trifolium pratense* L.

E) to compare the *in vivo* sensitivity to the *in vitro* sensitivity to prochloraz using *Feijoa sellowiana* seedlings.

CHAPTER 2 - Detection of new disease on *Laurus nobilis* and identification of pathogen species responsible

2.1 Introduction

During 2009 and 2010, a new disease was observed on approximately 10% of 10 000 2-year-old potted bay laurel (*Laurus nobilis* L., Lauraceae family) plants in a nursery located in Mascali (Giarre, Catania province, eastern Sicily, Italy). Infected plants showed internal large brown areas with necrotic tissues at the crown level, the basal stem and root rots. Death of bay laurel potted plants as a consequence of the symptoms above mentioned was observed. Orange to red perithecia referable to *Calonectria* spp. were frequently detected in groups on the crowns and basal stems of symptomatic plants (Fig._). Brayford & Chapman (1987) reported a wilting disease of *L. nobilis* in nurseries on the Isles of Scilly (UK). The causal agent was identified as *Cylindrocladium ilicicola* (Hawley) Boedijn & Reitsma, but incorrectly linked to the teleomorph name *Ca. ilicicola* Boedijn & Reitsma. Based on a molecular comparison of ex-type strain, Crous *et al*. (1993c) showed *Ca. ilicicola* is the teleomorph of *C. parasiticum* Crous, M.J. Wingf. & Alfenas. More recently, Lechat *et al.* (2010) proposed *Ca. laurii* (Vanderw.) Lechat & Crous as the teleomorph name of *C. ilicicola*. The aim of the present study was to identify the *Calonectria* species involved on bay laurel in Italy with both morphological and molecular characterisation and to verify the pathogenicity of this fungus.

Figure 10. a-b. Death of *Laurus nobilis* potted plants as a consequence of crown rot and root rot caused by *Calonectria ilicicola*. c. Brown discolouration of the basal stem. d. Perithecia of *Ca. ilicicola*. e. Mycelia of *Ca. ilicicola*

2.2 Materials and methods

Isolations were performed transferring fragments of symptomatic crown, stem and root tissues of *L. nobilis*, surface-sterilized with 1% NaClO for 1 min, on potato dextrose agar (PDA). Petri dishes were incubated at 25 °C in the dark. For the morphological identification of the anamorph stage, single hyphal tip or single viable ascospore were transferred on malt extract agar (MEA), carnation leaf agar (CLA) and synthetic low-nutrient agar (SNA). Because we were unable to induce the formation of the anamorph stage on different media, ascospore suspension ($1.0 \cdot 10^4$ ascopores⁄ml) obtained from 30-day-old cultures grown on CLA was used for the inoculation of red clover (*Trifolium pratense* L.) seedlings. Morphological characteristics of the anamorph and teleomorph stages of twenty isolates were examined. Total genomic DNA was

extracted from five representative 7-day-old single- conidial isolates using the UltraCleanÔ Microbial DNA isolation kits (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) as described by the manufacturer. Partial gene sequences were determined for calmodulin (CAL), histone H3 (HIS3) and translation elongation factor 1-α (TEF-1α), as described by Lombard *et al*. (2010c), and the amplicons were sequenced in both directions using the same PCR primers. Sequence data from Lombard *et al*. (2010c) were used as reference data, and subsequent alignments were generated as described in Lombard *et al*. (2010c) (Table_). Nucleotide substitution models were determined for each locus using the Akaike information criterion in MrModeltest v. 2.2 (Nylander 2004). A phylogenetic tree with Bayesian probabilities for the combined data set was generated using the Markov Chain Monte Carlo (MCMC) algorithm of MrBayes v. 3.1.1 (Ronquist & Heulsenbeck 2003). The four chains of the MCMC analysis were run simultaneously for one million generation from random trees. The posterior probabilities were determined after the first 1000 trees were discarded as the burn-in phase. A maximum parsimony genealogy was also done using PAUP v.4.0b10 (Swofford 2002). The heuristic search was based on 1000 random addition sequences and tree bisection–reconnection, with the branch swapping option set on 'best trees' only. Alignment gaps were treated as missing data, and all characters were weighted equally. Branch support was determined using bootstrap analysis based on a 1000 replications. Pathogenicity of the five representative isolates used in the molecular analysis was confirmed by applying 5 ml of a ascospore suspension (1.0 x 10^4 ascospores⁄ml), obtained on CLA, to the crowns of potted 1-month-old seedlings of bay laurel that were subsequently covered with plastic bags and maintained in a growth chamber (23–25°C). Twenty plants for each isolate were used. The same number of control plants were treated with water.

Table 4.

Sequences of *Calonectria* spp. used in the study

Species	Accession number[a]	GenBank number		
		Calmodulin	Histone H3	TEF-1α
Calonectria asiatica	CBS 112711; CPC 3898; SFE 744	AY725738	AY725655	AY725702
	CBS 114073[b]; CMW 23782; CPC 3900; SFE 726	AY725741	AY725658	AY725705
Calonectria chinensis	CBS 112744; CMW 30986; CPC 4104	AY725746	AY725660	AY725709
	CBS 114827[b]; CMW 23674; CPC 4101	AY725747	AY725661	AY725710
Calonectria colombiensis	CBS 112220[b]; CMW 23676; CPC 723	AY725748	AY725662	AY725711
	CBS 112221; CMW 30985; CPC 724	AY725749	AY725663	AY725712
Calonectria curvispora	CBS 116159[b]; CMW 2369; CPC 765	GQ267374	AY725664	GQ267302
Calonectria hongkongensis	CBS 114711; CMW 30995	AY725754	AY725666	AY725716
	CBS 114828[b]	AY725755	AY725667	AY725717
Calonectria ilicicola	CBS 190.50[b]; IMI 299389; CMW 30998; CPC 2482	AY725764	AY725676	AY725726
	CBS 115897	GQ267403	GQ267256	GQ267321
	DISTEF-LN1; CBS 129185	JF714949	JF714954	JF714959
	DISTEF-LN3; CBS 129186	JF714950	JF714955	JF714960
	DISTEF-LN5; CBS 129187	JF714951	JF714956	JF714961
	DISTEF-LN6; CBS 129188	JF714952	JF714957	JF714962
	DISTEF-LN11; CBS 129189	JF714948	JF714953	JF714958
Calonectria indonesiae	CBS 112823[b]; CMW 23683; CPC 4508	AY725756	AY725668	AY725718
	CBS 112840; CPC 4547	AY725758	AY725670	AY725720
Calonectria kyotensis	CBS 170.77; IMI 299388; CMW 23679	GQ267380	GQ267249	GQ267308
	CBS 413.67; CMW 23678; CPC 2391	GQ267379	GQ267248	GQ267307
Calonectria malesiana	CBS 112710; CPC 3899	AY725759	AY725671	AY725721
	CBS 112572[b]; CMW 23687; CPC 4223	AY725760	AY725672	AY725722
Calonectria multiphialidica[c]	CBS 112678; CMW 23688	AY725761	AY725673	AY725723
Calonectria naviculata[c]	CBS 101121[b]; CMW 30974	GQ267399	GQ267252	GQ267317
Calonectria pacifica	CBS 109063[b]; IMI 35428; CMW 16726; CPC 2541	AY725762	GQ267255	AY725724
	CBS 114038; CMW 30988	GQ267402	AY725675	GQ267320
Calonectria sumatrensis	CBS 112829[b]; CMW 23698; CPC 4518	AY725771	AY725696	AY725733
	CBS 112934; CMW 30987; CPC 4516	AY725773	AY725698	AY725735

[a]CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; CMW: Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CPC: Pedro Crous Working collection housed at CBS.
[b]Ex-type culture.
[c]*Calonectria* spp. used as outgroup in the phylogenetic analysis.
Bold values indicate Ca. ilicicola isolates, principal subject of this paper.

Figure 11. Health plant compared to artificially inoculated plant.

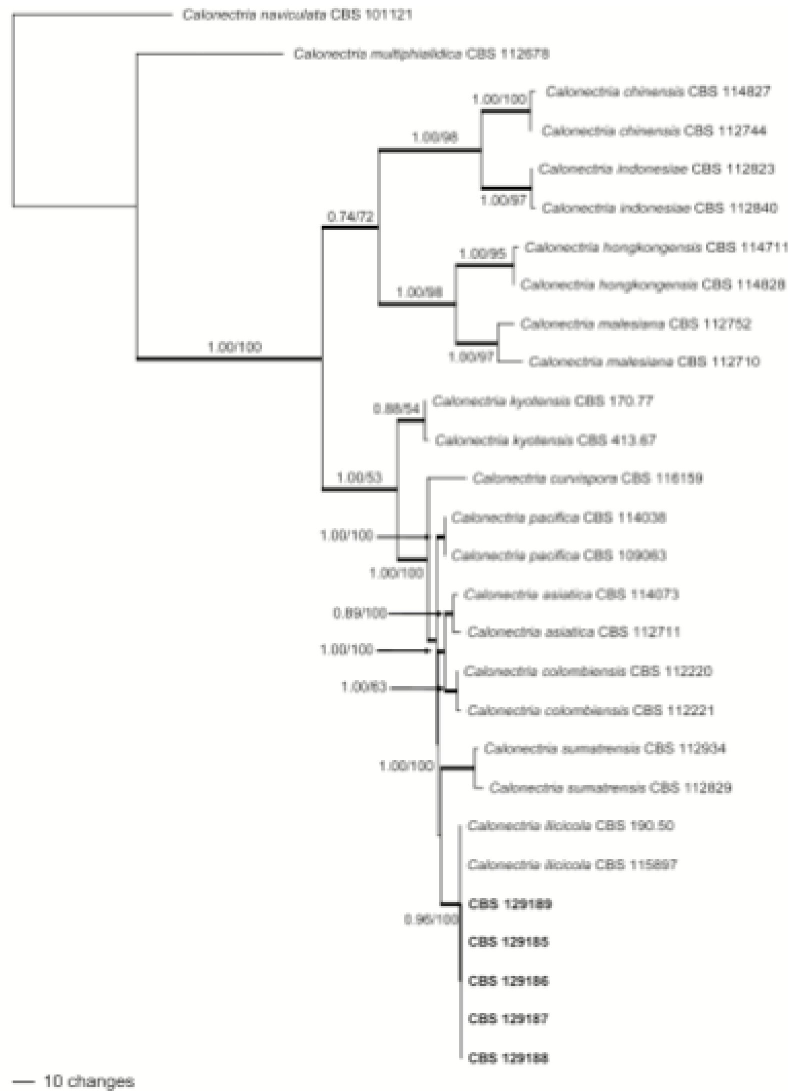
Figure 12. *Ca. ilicicola* reisolated from symptomatic plants artificially inoculated.

2.3 Results and discussion

A *Calonectria* sp. was consistently obtained from symptomatic tissues of bay laurel. On PDA, colonies were fast growing with abundant white, floccose, aerial mycelia and reverse salmon-buff colour. Conidia were cylindrical, rounded at both ends, straight, ranged from 40 to 50 µm long x 4 to 5 µm wide (mean= 44.8 x 4.6 µm), 1(-3) septate and were produced only on red clover seedlings. Stipe extension was terminating in sphaeropedunculate vesicles. Perithecia were produced after 20–30 days in all media and occurred solitary or in groups, orange to red–brown, subglobose to ovoid and ranged from 330 to 460 µm long x 330 to 400 µm in diameter (mean = 402 x 355 µm). Ascospores were hyaline, fusoid with rounded ends, straight to slightly curved, 1(-3) septate, not or slightly constricted at the septum and ranged from 31 to 45 µm long·4 to 7 µm wide (mean = 37 x 5.2 µm). Amplicons of approximately 450 bases for HIS3 and 500 bases each for CAL and TEF-1α were determined. The adjusted alignment for each gene region consisted of 28 taxa including *Ca. naviculata* (CBS 101121) and *Ca. multiphialidica* (CBS 112678) as outgroup taxa. The outgroup taxa were selected based on their phylogenetic position as determined by Lombard *et al*. (2010c). A HKY+I+G model for CAL and TEF-1α and a GTR+I+G model for HIS3 was selected for Bayesian analysis. The Bayesian consensus tree obtained confirmed the tree topology obtained with maximum-parsimony including bootstrap support. For the maximum-parsimony analysis, the combined sequence data set of 1431 bases, including alignment gaps, consisted of 1035 constant characters, 121 uninformative characters and 275 parsimony-informative characters. Parsimony analysis yielded one most parsimonious tree (Fig. 13). In the tree, all the isolates isolated from *L. nobilis* grouped with *Ca. ilicicola* in the Sphaero-naviculate group (Lombard *et al.* 2010c) with good bootstrap and posterior probability support. Symptoms identical to those observed in the nurseries developed 10–14 days after the inoculation, and most part of the inoculated plants were killed within 2 months. No variation on virulence degree was observed among the isolates tested. This pathogen was reisolated from the infected tissues and identified as previously described.

Morphological features of the fungus associated with disease symptoms on bay laurel including conidia and perithecial morphology, as well as partial gene sequences for CAL, HIS3 and TEF-1α, identify the isolates studied as *Ca. ilicicola*. In addition, Koch's postulates demonstrated the pathogenicity of this fungus and its role as causal agent of the new disease detected in Italy. *Ca. ilicicola* induces peg, pod and root necrosis of peanuts, but also leaf spot, damping-off, crown root and blight of several hosts (Crous 2002). This species has a large distribution on different countries (Crous 2002). In Europe, *Ca. ilicicola* was previously reported only on *Nerium oleander* in United Kingdom (Crous 2002). To our knowledge, this is the first report of the occurrence of a disease caused by *Ca. ilicicola* on *Laurus nobilis* and it is the first detection with molecular data of this fungal pathogen in Europe.

Figure 13. The most parsimonious tree obtained from a heuristic search with a 1000 random additions sequences for the combined calmodulin (CAL), HIS3 and TEF sequences alignments. Scale bar shows 10 changes, and posterior probabilities⁄bootstrap supports are indicated at the nodes. Thickened lines indicate branches also present in Bayesian consensus tree



CHAPTER 3 - *Calonectria* spp. causing leaf spot, crown and root rot of ornamental plants in Tunisia

3.1 Introduction

Species of *Calonectria* are common pathogens of a wide range of plant hosts in nurseries cultivated through seedings or vegetative propagation (Crous 2002, Lombard *et al.* 2010a). Nursery disease symptoms associated with these fungi include crown, collar and root rot, leaf spots and cutting rot (Crous 2002, Vitale & Polizzi 2008, Polizzi *et al*. 2009a, Lombard *et al*. 2010a). *Calonectria* spp. have been reported worldwide from agricultural and forestry nurseries (Crous *et al*. 1991, Crous 2002, Lombard *et al*. 2009, 2010a,b,d), whereas in Europe, they have only been reported from commercial ornamental nurseries (Polizzi & Crous 1999, Polizzi 2000, Crous 2002, Henricot & Culham 2002, Polizzi *et al.* 2007a, b, Vitale & Polizzi 2008, Polizzi *et al*. 2009, Lombard *et al*. 2010a).

Past reports have shown that *C. morganii* and *C. pauciramosa* are the most common *Calonectria* spp. found in ornamental nurseries in the Northern Hemisphere (Polizzi & Crous 1999, Polizzi 2000, Polizzi & Catara 2001, Polizzi *et al*. 2006a, b, 2007a, b). Based on phylogenetic studies, *C. morganii* appears to be restricted to Brazil, Europe and the USA (Crous *et al*. 1993, Overmeyer *et al.* 1996, Schoch *et al*. 2000a), whereas *C. pauciramosa* has a more global distribution and has been shown to better adapt to different environmental conditions (Crous 2002, Lombard *et al*. 2010b, Chen *et al.* 2011). *Calonectria pauciramosa* was also regarded as the dominant pathogen in nurseries in Australia and South Africa (Crous 2002, Schoch *et al*. 2001, Lombard *et al*. 2010b).

In November 2010, a survey was conducted in an ornamental nursery in Carthage, Tunis, Tunisia. Various plant species were collected showing symptoms of leaf spots, crown and root rot. Isolations consistently yielded *Calonectria* spp. and the aim of this study was to identified these species and their pathogenicity.

Figure 14. Symptoms of leaf spot caused by *Calonectria* spp. on different ornamental plants. a. *Callistemon* spp. b. *Myrtus communis*.

Figure 15. a. Crown and root rot on *M. communis.* b,c. Leaf spot on *Metrosideros excelsa.*

3.2 Materials and methods

3.2.1 Disease survey and fungal isolates

During November 2010, an ornamental nursery located in Carthage, Tunis, Tunisia was surveyed for diseased plants. Several samples of *Callistemon* spp., *Dodonaea viscosa*, *Myrtus communis* and *Metrosideros* spp. showing leaf spots, crown and root rot were randomly collected for analysis (Table 5). Infected tissues collected from symptomatic plants were superficially disinfected with 1,0% sodium hypoclorite for 2 min, rinsed with sterile water, placed on potato-dextrose agar (PDA, Oxoid) and incubated in the dark at 24 °C. Representative isolates of *Calonectria* from each ornamental species were obtained from single-spore colonies made from 14 d old cultures grown on PDA. Representative isolates have been deposited at the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands (Table 5.

3.2.2 DNA sequence comparisons

Total genomic DNA was extracted from single-conidial isolates grown on 2% malt extract agar (MEA) for 7d, using the UltraClean^{TM} Microbial DNA isolation kits (Mo Bio Laboratories, Inc., California, USA) according to the manufacturer's protocol. Partial gene sequences were determined for β-tubulin (BT), histone H3 (HIS3) and translation elongation factor-1α (TEF-1α) using the primers and protocols described by Lombard *et al*. (2010c).

To ensure the integrity of the sequences, the amplicons were sequenced in both directions using the same primer pairs used for amplification. Sequence data from Lombard *et al.* (2010b,d) were used as reference data and subsequent alignments were generated using MAFFT v.6 (Katoh & Toh 2010) and manually corrected where necessary.

Congruency of the sequence datasets for the separate loci was determined using tree topologies of 70% reciprocal Neighbour-Joining bootstrap trees with Maximum Likelihood distances that were compared visually to identify conflicts between partitions (Gueidan *et al.* 2007). Molecular evolution models

for the separate gene regions were determined in Modeltest v. 3.7 (Posada & Crandall 1998) and bootstrap analyses were run for 10 000 replicates.

PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10, Swofford 2002) was used to analyse the DNA sequence data-sets. Phylogenetic relationships were estimated by heuristic searches with 1 000 random addition sequences and tree bisec- tion-reconnection was used, with the branch swapping option set on 'best trees' only. All characters were weighted equally alignment gaps were treated as missing data. Measures and calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). Bootstrap analysis (Hillis & Bull 1993) was based on 1 000 replications.

A second phylogenetic analysis using a Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003). Nucleotide substitution models were determined using MrModeltest (Nylander 2004) for each gene region and included in the analyses. Two analyses of four MCMC chains were run from random trees for one million generations and sampled every 100 generations. All runs converged on the same likelihood score and tree topology and therefore the first 800 trees were discarded as the burn-in phase of each analysis and posterior probabilities determined from the remaining trees.

The phylogenetic analyses included 46 partial gene sequences for each gene region, representing 20 *Calonectria* spp. (Table 5). *Calonectria colombiensis* (CBS 112221) and *C. chinensis* (CBS 112744) were used as outgroup taxa in both analyses (Lombard *et al.* 2009). All novel sequences were deposited in GenBank and the alignments in TreeBASE (http://www.treebase.org).

Table 5. *Calonectria* isolates used in the phylogenetic analyses and pathogenicity trials.

Species	Isolate number[1]	β-tubulin[2]	Histone H3[2]	TEF-1α[2]	Host/substrate
Calonectria brasiliensis	CBS 230.30[4]	GQ267241	GQ267259	GQ267328	*Eucalyptus* sp.
	CBS 114257	GQ267242	GQ267260	GQ267329	Leaf litter
C. cerciana	CBS 123693[4]	FJ918510	FJ918528	FJ918559	*E. grandis × urophylla*
	CBS 123695	FJ918511	FJ918529	FJ918560	*E. grandis × urophylla*
C. chinensis	CBS 112744	AY725618	AY725660	AY725709	Soil
C. colombiana	CBS 115127[4]	FJ972423	FJ972442	FJ972492	Soil
	CBS 115638	FJ972422	FJ972441	FJ972491	Soil
C. colombiensis	CBS 112220[4]	GQ267207	AY725662	AY725711	Soil
C. hawksworthii	CBS 111870[4]	AF333407	DQ190649	FJ918558	*Nelumbo nucifera*
C. insularis	CBS 114558[4]	AF210861	FJ918526	FJ918556	Soil
	CBS 114559	AF210862	FJ918525	FJ918555	Soil
C. leucothoës	CBS 109166	FJ918508	FJ918523	FJ918553	*Leucothoë axillaris*
C. mexicana	CBS 110918[4]	AF210863	FJ972460	FJ972526	Soil
	CBS 130353[3]	JN607280	JN607265	JN607295	*Dodonaea viscosa*
C. morganii	CBS 110666	FJ918509	FJ918527	FJ918557	*Ilex vomitoria*
	CBS 119669	DQ521599	DQ521601	GQ421796	*Pistacia lentiscus*
C. pauciramosa	CPC 971	FJ918514	FJ918531	FJ918565	*E. grandis*
	CPC 416	FJ918515	FJ918532	FJ918566	*E. grandis*
C. polizzii	CBS 123402[4]	FJ972419	FJ972438	FJ972488	*Arbutus unedo*
	CBS 125270	FJ972417	FJ972436	FJ972486	*Callistemon citrinus*
	CBS 130351[3]	JN607270	JN607255	JN607285	*Myrtus communis*
	CBS 130352[3]	JN607275	JN607260	JN607290	*Metrosideros thomasii*
	DISTEF-TMC2	JN607269	JN607254	JN607284	*Myrtus communis*
	DISTEF-TMEA1	JN607272	JN607257	JN607287	*Metrosideros excelsa* cv. Aurea
	DISTEF-TMN3	JN607274	JN607259	JN607289	*Metrosideros* sp.
C. pseudomexicana	**CBS 130354[3,4]**	JN607281	JN607266	JN607496	*Callistemon* sp. (rouge)
	CBS 130355[3]	JN607282	JN607267	JN607497	*Callistemon* sp. (rouge)
	DISTEF-TCROU4	JN607283	JN607268	JN607498	*Callistemon* sp. (rouge)
C. pseudoscoparia	CBS 125256	GQ267228	GQ267277	GQ267348	*E. grandis*
	CBS 125257[4]	GQ267229	GQ267278	GQ267349	*E. grandis*
C. scoparia	CPC 1675	FJ972426	FJ972476	FJ972525	*Eucalyptus* sp.
	CPC 1679	FJ972427	GQ267246	GQ267298	*Eucalyptus* sp.
C. spathulata	CBS 112689	AF308463	FJ918524	FJ918554	*E. viminalis*
	CBS 555.92[4]	GQ267215	GQ267261	GQ267331	*Araucaria angustifolia*
C. sulawesiensis	CBS 125248	GQ267223	GQ267272	GQ267343	*Eucalyptus* sp.
	CBS 125253	GQ267220	GQ267269	GQ267340	*Eucalyptus* sp.
C. tunisiana	**CBS 130356[3]**	JN607277	JN607262	JN607292	*Callistemon* sp. (rouge)
	CBS 130357[3,4]	JN607276	JN607261	JN607291	*Callistemon laevis*
	DISTEF-TCV1	JN607278	JN607263	JN607293	*Callistemon viminalis*
	DISTEF-TCROS4	JN607279	JN607264	JN607294	*Callistemon* sp. (rosè)
	DISTEF-TME1	JN607271	JN607256	JN607286	*Metrosideros excelsa*
	DISTEF-TMN1	JN607273	JN607258	JN607288	*Metrosideros* sp.
C. variabilis	CBS 112691	GQ267240	GQ267264	GQ267335	*Eucalyptus* sp.
	CBS 114677	AF333424	GQ267263	GQ267334	*Eucalyptus* sp.
C. zuluensis	CBS 125268[4]	FJ972414	FJ972433	FJ972483	*Eucalyptus* sp.
	CBS 125272	FJ972415	FJ972434	FJ972484	*Eucalyptus* sp.

1 CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Pedro Crous working collection housed at CBS; DISTEF: Dipartimento di Scienze e Tecnologie Fitosanitarie, Catania, Italy.
2 GenBank accession numbers.
3 Isolates used for the pathogenicity trials.
4 Ex-type cultures; Isolates in **bold** obtained during survey.

3.2.3 Taxonomy

Morphological characterisation of the *Calonectria* isolates was done using single conidial cultures prepared on MEA and synthetic nutrient-poor agar (SNA; Nirenburg 1981, Lombard *et al*. 2009). Inoculated plates were incubated at room temperature and examined after 7d. Gross morphological characteristics of the anamorph state were determined by mounting fungal structures in clear lactic acid and 30 measurements at X 1000 magnification were made for each isolate using a Zeiss Axioscope 2 microscope with interference contrast (DIC) illumination. The 95% confidence levels were determined and extremes of conidial measurements are given in parentheses. For other structures, only extremes are presented. Colony characteristics were noted after 7 d of growth on MEA at 24°C and colony colours determined using the colour charts of Rayner (1970). Descriptions, nomenclature and illustrations were deposited in MycoBank (Crous *et al.* 2004).

3.2.4 Pathogenicity

In order to test the pathogenicity of the *Calonectria* spp. collected in this study, seven isolates representing different *Calonectria* species identified by morphology and DNA sequence comparisons were selected for inoculation trials (Table 5). A conidial suspension (1.0 X 10^5 conidia/mL) was prepared for each isolate by adding sterile water to plates of carnation leaf agar (CLA; Fisher *et al*. 1982) 7 d after inoculation and dislodging the conidia. The conidial suspension was sprayed onto the canopy (until run-off) of potted 2-6 months old plants of *Callistemon citrinus* cv. Splendens, *C. laevis*, *C. viminalis*, *Dodonaea viscosa*, *Metrosideros excelsa*, *M. excelsa* cv. Aurea, *M. thomasii*, *Myrtus communis*, *M. communis* subsp. *tarentina*. The conidial suspension of the isolate CBS 130351 was also applied to the crown of *M. communis* plants (10 ml/plant). All plants were subsequently covered with plastic bags for 48 h and maintained in a growth chamber at 25 ± 1 °C for 14 days. Five plants for each isolate and host were used and the same number of control plants were treated

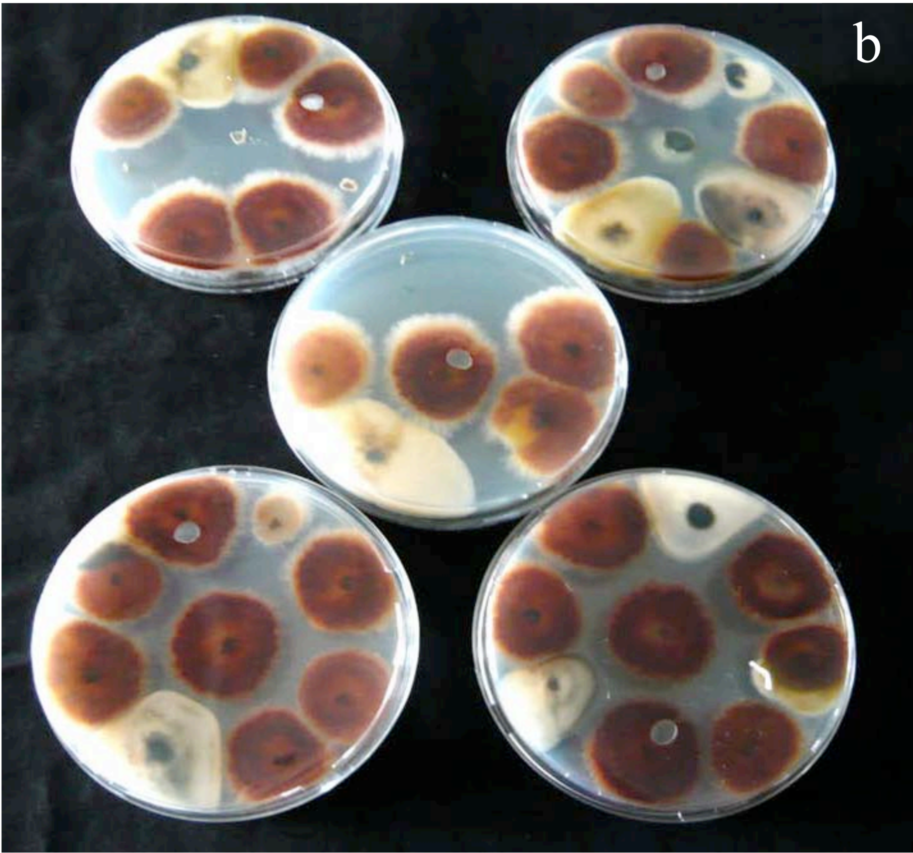
using sterile water. Pathogenicity tests were evaluated 5, 10 and 25 d after inoculation.

3.3 Results

3.3.2 Disease survey and fungal isolates

During the survey, a total of 46 *Calonectria* isolates were collected from ornamental hosts sampled. Majority of the isolates (41) were associated with leaf spots or leaf blight of *Callistemon* spp. (18), *D. viscosa* (1), *Metrosideros* spp. (17) and *Myrtus communis* (5), and the remaining (5) with crown and root rot of *M. communis*. Leaves showed minute brown spots, which often enlarged, forming a necrotic centre surrounded by a dark purple halo. Young, non-lignified terminal shoots often exhibited dieback or lesions similar to those on the leaves. Severe defoliation was observed on *M. communis* and *M. excelsa* cv. Aurea. Several seedlings of *M. communis* had crown and root rot, and fungal sporulation occurred on the lower part of the crown. Initial symptoms were brown lesions that expanded rapidly to girdle the stem at the seedling crown, above and below the soil line, resulting in plant death.

Figure 16. Colonies growing from infected tissues at 24 ^{o}C on PDA a. on surface and b. on reverse.

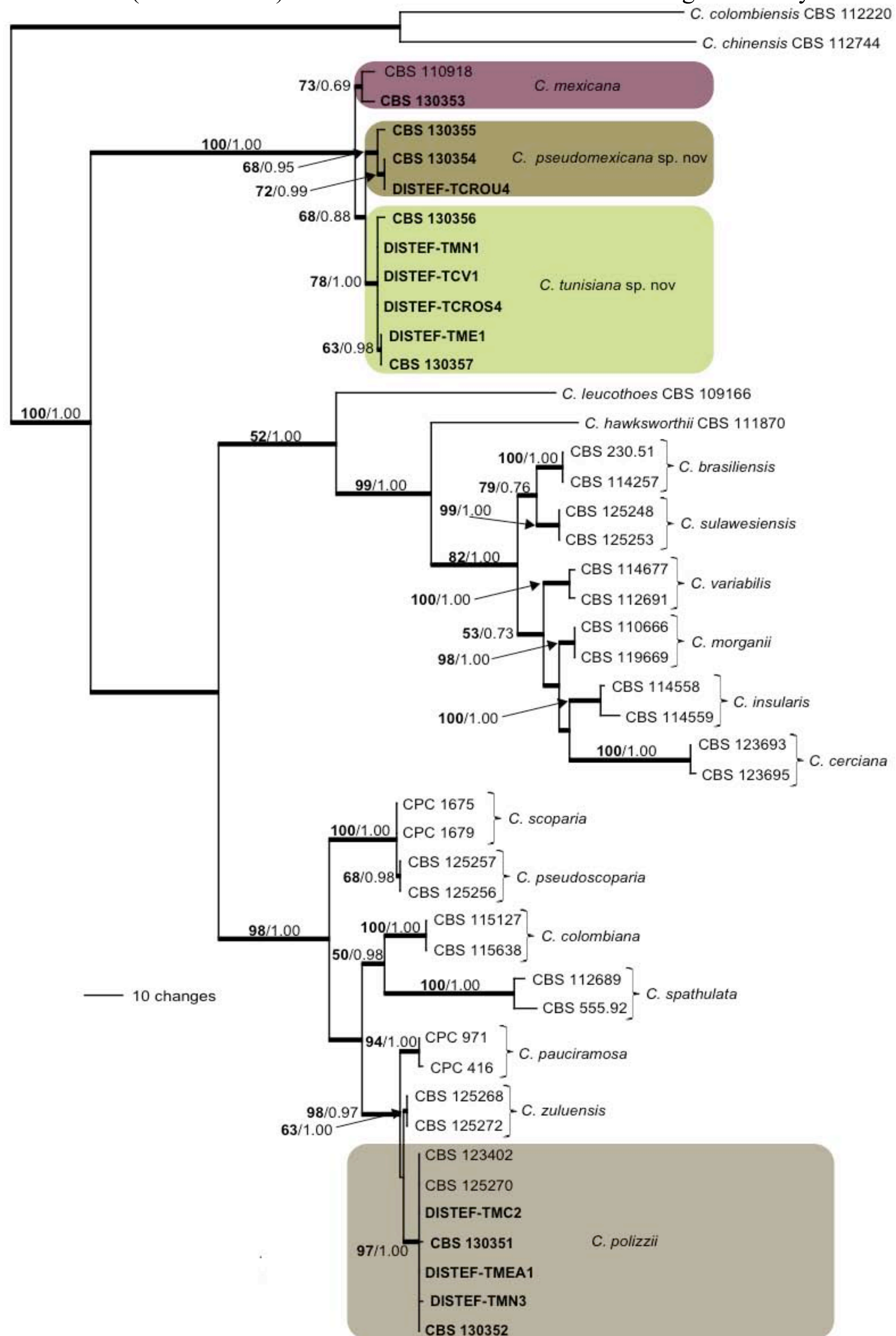


3.3.3 DNA sequence comparisons

Amplicons of approximately 450 bases for HIS3 and 500 bases each for BT and TEF-1α were generated. The 70% reciprocal bootstrap trees showed no conflict in tree topologies for the three gene regions and therefore they were combined in a data- set consisting of 1532 characters including gaps. Of these characters, 1187 were constant and parsimony uninformative. Analysis of the 345 parsimony informative characters yielded 16 equally most parsimonious trees (TL = 814, CI = 0.721, RI = 0.923, RC = 0.666), of which the first tree is presented (Fig. 2). A HKY+I model for BT, a GTR+I+G model for HIS3 and a GTR+G model for TEF-1α was selected for Bayesian analysis. The Bayesian consensus tree confirmed the tree topology obtained with maximum parsimony including bootstrap support.

The phylogenetic tree illustrates a number of well-supported clades containing the *Calonectria* isolates obtained during the survey. Some of the isolates clustered in a clade representing *C. polizzii* with a bootstrap value (BP) of 97 and a Bayesian posterior probability (PP) value of 1.00. Several isolates also grouped with and close to *C. mexicana* in two separate well- supported clades (BP = 68, PP = 0.95 and BP = 78, PP = 0.98, respectively), which could represent novel phylogenetic species.

Figure 17. One of 16 most parsimonious trees obtained from a heuristic search with 1000 random addition sequences of the combined sequences of β-tubulin, histone H3 and translation elongation factor 1α sequence alignments of the *Calonectria* isolates obtained during the survey and other closely related species. Scale bar shows 10 changes. Bootstrap support values (in **bold**) and Bayesian posterior probability values are shown at the nodes.
Thickened lines indicate branches in the strict consensus tree and the consensus tree of the Bayesian analyses. The tree was rooted to *C. chinensis* (CBS 112744) and *C. colombiensis* (CBS 112220). Isolates in **bold** were obtained during the survey.



3.3.4 Taxonomy

DNA sequence and morphological comparisons of the *Calonectria* isolates obtained during the survey show that these isolates belong to *C. mexicana* and *C. polizzii* and also constitute two previously undescribed taxa. Based on morphological comparisons, isolate CBS 130353 agrees with *C. mexicana* (Schoch *et al.* 1999) and isolates DISTEF-TMC2, CBS 130351, DISTEF-TMEA1, DISTEF-TMN3 and CBS 130352 represent *C. polizzii* (Lombard *et al.* 2010b). The remaining isolates are newly described as follows:

3.3.4.1 *Calonectria pseudomexicana*

L. Lombard, G. Polizzi & Crous, *sp. nov*. MycoBank MB563138; Fig.__

Teleomorph unknown.

Etymology. Name reflects the fact that this species closely resembles *C. mexicana*.

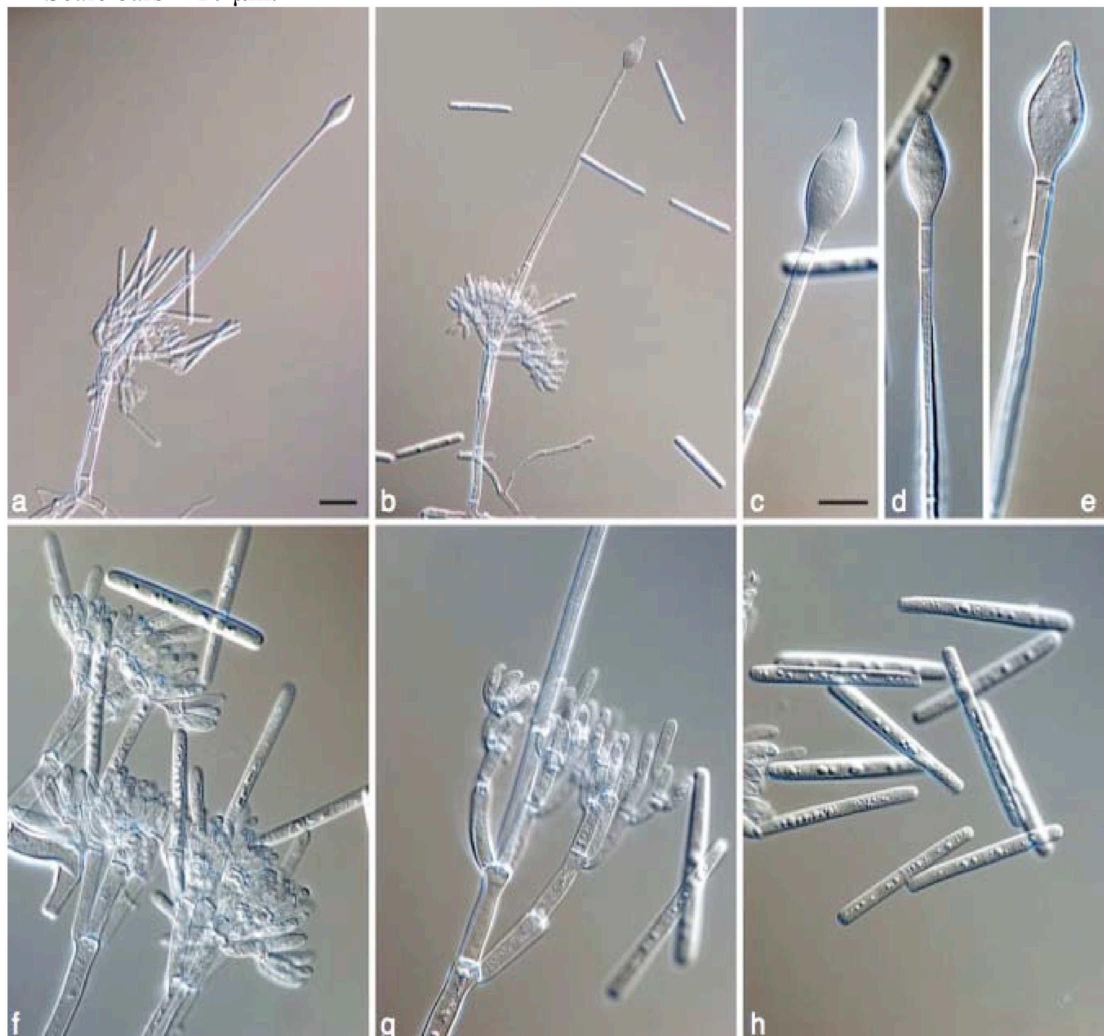
Conidiophores with a stipe bearing penicillate suites of fertile branches, stipe extensions, and terminal vesicles; stipe septate, hyaline, smooth, 38-69 x 5-9µm; stipe extension septate, straight to flexuous, 175-251 µm long, 3-6 µm wide at the apical septum, terminating in a fusiform to broadly ellipsoidal vesicle 9-14 µm diam with papillate apex. *Conidiogenous apparatus* 38-68 µm long, 32-64 µm wide; primary branches aseptate or 1-septate, 21-43 x 4-7 µm; secondary branches aseptate, 13-26 x 4-7 µm; tertiary branches and additional branches (-4), aseptate, 10-18 x 2–6 µm, each terminal branch producing 2-6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6-14 x 2-6 µm; apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (40-)43-48(-49) x (4-)5-6 µm (av. = 45 x 5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Megaconidia and microconidia not seen.

Culture characteristics. Colonies fast growing at 24 ^{o}C on MEA, sienna to bay on surface, reverse sienna after 7 d; moderate white aerial mycelium with sparse to moderate sporulation; chlamydospores extensive throughout medium.

Specimens examined. TUNISIA, Carthage, Tunis, from *Callistemon* sp., Nov. 2010, *G. Polizzi*, (CBS H-20685, holotype of *C. pseudomexicana*, culture ex-type CBS 130354 = DISTEF-TCROU1); Carthage, Tunis, from *Callistemon* sp., Nov. 2010, *G. Polizzi*, culture CBS 130355 = DISTEF-TCROU3; Carthage, Tunis, from *Callistemon* spp., Nov. 2010, *G. Polizzi*, culture DISTEF-TCROU4.

Notes. *Calonectria pseudomexicana* is morphologically similar to *C. mexicana*. *Calonectria pseudomexicana* has four or less conidiophore branches while *C. mexicana* or less conidiophore branches while *C. mexicana* has five as reported by Schoch *et al*. (1999).

Figure 18. *Calonectria pseudomexicana*. a,b. Macroconidiophores; c-e. fusiform to broadly ellipsoidal vesicles with papillate apices; f,g. conidiogenus apparatus with conidiophore branches and doliiform to reniform phialides; h. 1-septate macroconidia. — Scale bars = 10 µm.



3.3.4.2 *Calonectria tunisiana*

L. Lombard, G. Polizzi & Crous, *sp. nov*. MycoBank MB563139; Fig.__

Teleomorph unknown.

Etymology. Name refers to the country Tunisia, where the fungus was collected.

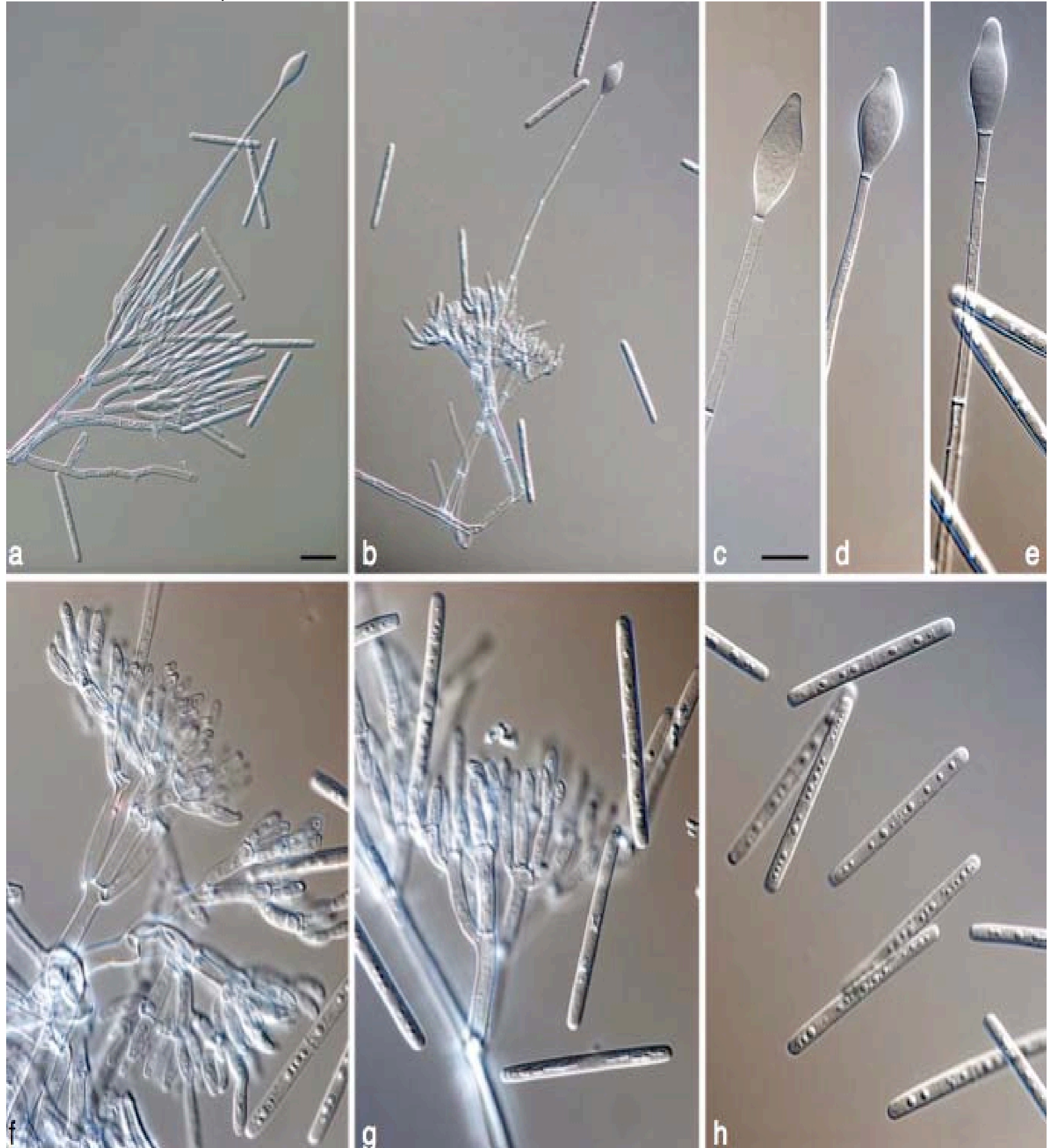
Conidiophores with a stipe bearing penicillate suites of fertile branches, stipe extensions, and terminal vesicles; stipe septate, hyaline, smooth, 42-95 x 7-11 µm; stipe extensions septate, straight to flexuous, 147-199 µm long, 4-5 µm wide at the apical septum, terminating in a fusiform to broadly ellipsoidal vesicle 8-14 µm diam with papillate apex. *Conidiogenous apparatus* 40-68 µm long, 30-66 µm wide; primary branches aseptate or 1-septate, 17-41 x 5-7 µm; secondary branches aseptate, 10-22 x 4-7 µm; tertiary branches aseptate, 9-18 x 4-5 µm, each terminal branch producing 2-6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8-13 x 3-5 µm; apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (43-)47-51(-53) x 4-6 µm (av. = 49 x 5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Megaconidia and microconidia not seen.

Culture characteristics. Colonies fast growing at 24 °C on MEA, sienna to bay on surface, and reverse sienna after 7 d; sparse white aerial mycelium with sparse sporulation; chlamydo- spores extensive throughout the medium.

Specimens examined. Tunisia, Carthage, Tunis, from *Callistemon laevis*, Nov. 2010, *G. Polizzi*, (CBS H-20684, holotype of *C. tunisiana*, culture ex-type CBS 130357 = DISTEF-TCL1); Carthage, Tunis, from *Callistemon* sp., Nov. 2010, *G. Polizzi*, culture CBS 130356 = DISTEF-TCROU2; Carthage, Tunis, from *Metrosideros excelsus*, Nov. 2010, *G. Polizzi*, culture DISTEF-TME1.

Notes. Morphologically, *C. tunisiana* is similar to *C. mexicana* and *C. pseudomexicana*, but can be distinguished from both taxa by its shorter stipe extensions. The conidiophores of *C. tunisiana* (-3) also form fewer fertile branches than *C. mexicana* (-5) and *C. pseudomexicana* (-4) (Schoch *et al*. 1999).

Figure 19. *Calonectria tunisiana*. a,b. Macroconidiophores; c-e. fusiform to broadly ellipsoidal vesicles with papillate apices; f,g. conidiogenus apparatus with conidiophore branches and doliiform to reniform phialides; h. 1-septate macroconidia.
— Scale bars = 10 µm.



3.3.5 Pathogenicity

All plants inoculated with the *Calonectria* spp. in this study developed leaf spot, leaf blight or crown and root rot symptoms. The first symptoms of leaf spot and leaf blight were observed 5 d after inoculation on all test plants inoculated with the *Calonectria* spp., resembling the symptoms observed during the survey. Isolates of *C. pseudomexicana* (CBS 130354, 130355), *C. tunisiana* (CBS 130356, 130357) as well as the single isolate of *C. mexicana* (CBS 130353) produced the most severe symptoms. Isolates of *C. polizzii* (CBS 130351, 130352) also caused leaf spot and leaf blight on all inoculated plants, but less severe than the other three *Calonectria* spp. tested. Ten days after inoculation, severe or moderate defoliation of *M. communis* and *M. excelsa* cv. Aurea plants was observed.

All inoculated plants of *M. communis* developed crown rot, basal stem rot and root rot 25 d after inoculation with the isolate representing *C. polizzii* (CBS 130351). All un-inoculated control plants remained healthy and re-isolations from the test plants consistently yielded the test fungi.

Figure 20. Leaf spots on artificially-inoculated plants of: a. *Dodonaea viscosa* and b. *Callistemon citrinus* cv. Splendens.

Figure 21. Leaf spots on artificially-inoculated plants of: a. *C. laevis*, and b. *C. viminalis*.

Figure 22. Leaf spots on artificially-inoculated plants of: a. *M. thomasii* and b. *M. excelsa*.

Figure 23. Leaf spots on artificially-inoculated plants of: a. *Metrosideros* sp. and b. *M.excelsa* cv. Aurea.

3.4 Discussion

During a survey of diseased plants at an ornamental nursery in Tunis, Tunisia, a number of *Calonectria* spp. were isolated from plants exhibiting crown, root rot and leaf spots. DNA sequence and morphological comparison allowed the identification of two of these isolates as *C. mexicana* and *C. polizzii* as well as the description of two new species, *C. pseudomexicana* and *C. tunisiana*, both in the *C. scoparia* complex (Schoch *et al*. 1999).

Calonectria mexicana resides in the *C. scoparia* complex (Schoch *et al*. 1999) and can be distinguished from the other seven *Calonectria* spp. in the complex based on their unique papillate vesicles (Schoch *et al*. 1999, Lombard *et al*. 2010b, c, Chen *et al*. 2011). Until now, *C. mexicana* has only been reported from soil samples collected in Mexico, and its pathogenicity was unknown (Schoch *et al*. 1999, Crous 2002). This study represents the first report of this fungus outside Mexico and also demonstrates its pathogenicity on some plant hosts.

Calonectria polizzii has previously been reported from ornamental plants collected at a nursery in Sicily, Italy (Schoch *et al*. 2001, Lombard *et al*. 2010b), although its pathogenicity was not confirmed. This study represents the first confirmation of the pathogenicity of *C. polizzii* and widens its distribution to Tunisia. *Calonectria polizzii* is a member of the *C. scoparia* complex and can be distinguished from the other members by its smaller macroconidial dimensions (Lombard *et al*. 2010b).

The description of *C. pseudomexicana* and *C. tunisiana* adds two more species to the *C. scoparia* complex. This complex is characterised by 1-septate macroconidia and the formation of ellipsoidal to obpyriform terminal vesicles on the stipe extensions (Schoch *et al.* 1999, Crous 2002, Lombard *et al*. 2010b). Based on phylogenetic inference, both these newly described species are closely related to *C. mexicana*, which they also resemble in morphology. They can be distinguished from *C. mexicana* and each other by the number of fertile branches produced on the conidiophores. *Calonectria tunisiana* (av. = 49 x 5 µm) has slightly larger macroconidia than both *C. mexicana* (av. = 45 x 4 µm; Schoch *et al*. 1999) and *C. pseudomexicana* (av. = 45 x 5 µm).

The pathogenicity tests with isolates of *C. mexicana*, *C. polizzii*, *C.*

pseudomexicana and *C. tunisiana* clearly showed that they are able to cause symptoms similar to those observed during the survey. *Calonectria polizzii* was less virulent than the other three species, but should still be regarded as an important nursery pathogen. This supports the view that most *Calonectria* spp. can induce leaf spots if the environmental conditions are favourable (Crous 2002). All four species caused similar disease symptoms on the nine inoculated plant species, suggesting that little is known about the host specificity and mechanisms of infection of this group of plant pathogens.

This study stresses the importance of *Calonectria* spp. as nursery pathogens. Their soil-borne nature has contributed to their ease of movement globally and little is known about their origins. Furthermore, it is not known if these fungal pathogens originated from Tunisia or were introduced, and more isolates are needed for a study of their population dynamics.

CHAPTER 4 - Changes in sensitivity to prochloraz in *Calonectria scoparia* complex in southern Italy

4.1 Introduction

Calonectria spp. represent fungal species with a worldwide distribution that causes cutting rot, damping-off, root rot, crown rot and leaf blight on numerous ornamental plants (Schoch *et al.* 1999, Koike *et al.* 1999, Polizzi & Crous 1999, Polizzi & Catara 2001, Crous 2002, Lombard *et al.* 2010a,b,c,d). In South Africa and Australia *Calonectria* spp. causes extensive losses on commercial forest nurseries (Crous 2002), in U.S.A., Mediterranean basin and UK, are the causal agent of diseases in several ornamental plants (Schoch *et al*. 2001, Henricot & Beales 2003, Lane *et al*. 2006, Pérez-Sierra *et al*. 2006, Pérez-Sierra *et al*. 2007). In Italy *Calonectria pauciramosa* C.L. Schoch & Crous, present on anamorph stage and occasionally under perfect stage, have been associated on broad plant host range such as Myrtaceae, Polygalaceae, and Sapindaceae, causing numerous disease symptoms on many ornamental plants, especially on young seedlings (Polizzi *et al*. 2006a, 2006b, 2007a, 2009a, 2009b, 2010; Vitale & Polizzi 2007; Vitale *et al*. 2008, 2009). Recent studies revealed that *Ca. pauciramosa* included cryptic species belonging to *Ca. scoparia* complex and multigene phylogeny analysis recognised *Ca. polizzii* sp. nov. L. Lombard, Crous & M.J. Wingf. in Italy (Lombard *et al.* 2010) and in Tunisia as pathogen on several hosts (Lombard *et al.* 2011). Both species are very similar, but basing on observations of three *Ca. polizzii* strains*,* is known that this species can be distinguished from *Ca. pauciramosa* by its smaller 1-septate macroconidia (Lombard *et al.* 2010b), but further studies incorporating more isolates will be required to confirm this, and in the present work this morphological character has been studied. Chemical control is the most effective approach to manage diseases caused by *Calonectria*. Good results were obtained with benzimidazoles (MBCs) applications to control diseases caused by *Calonectria* species (Polizzi & Azzaro 1996; Barnard 1984; Kucharek & Atkins 1993). However resistant MBCs-strains were reported within an Italian population of *Ca. pauciramosa* and *Ca. morganii* (Polizzi & Vitale 2001; Vitale *et al*. 2009). Sterol Demethylase Inhibitors (DMIs) represents the most common alternative to MBCs fungicides (Nan *et al*. 1991). Prochloraz, an imidazole, member of

DMI, was introduced in Italy in the early of 1990s. Prochloraz is a protectant, translaminar fungicide, effective on several pathogens of ornamental plants (Nan *et al*. 1991, Henricot *et al*. 2008). In Sicily and Calabria the effectiveness of prochloraz to control *C. pauciramosa* was established in 2000 (Polizzi), but recent monitoring activities in nurseries of the same areas showed variability of efficacy and, recently, unstable treatments results were observed in *Calonectria* spp. diseases management (Aiello *et al*. 2013). The observed variability of effectiveness has raised concern regarding the presence of resistant pathogen strains to this active ingredient (a.i.). Moreover, continuous exposure to the a.i. could provide selection pressure for more resistant isolates. Thus, the aim of this paper were to determine if reduced sensitivity to prochloraz has occurred within an Italian population of *Calonectria* spp. The specific objectives of this study were: (I) to identify the different species belonging to this population on molecular and morphological basis (II), to assess the *in vitro* sensitivity to prochloraz and (III) if a shift in sensitivity to prochloraz has occurred among pathogen strains collected in different year periods in the same areas. In addition (IV) the *in vitro* sensitivity to prochloraz was compared with *in vivo* sensitivity, using both seedlings of *Trifolium pratense* L. as a model system (Vitale *et al.* 2012a) and *Feijoa sellowiana* Berg*,* as a ornamental species cultivated in commercial nurseries (Vitale *et al*. 2008). *Trifolium pratense* and *Feijoa sellowiana* were reported as highly susceptible hosts to *Calonectria* diseases (Vitale *et al*. 2008, Waipara *et al*. 1996).

4.2 Materials and methods

4.2.1 Sampling sites and fungal population

A total of 105 single-spore *Calonectria* isolates, previously identified on the morphological basis, collected during the period 1993 - 2009 from several symptomatic hosts cultivated in 6 nurseries located in eastern Sicily and Calabria (Southern Italy), were used. In nurseries 1, 3 and 4 (Tab___) prochloraz has been used extensively, in average, with four applications per

season, since 2000. This fungal population included a group (group A) of isolates collected during the period 1993 - 1996 and a second group (group B) belonging only isolates collected during 2005 - 2009. All isolates were grown on malt extract agar (Oxoid®) at 25 ± 1°C in darkness for 8 days to obtain actively growing colonies for the molecular identification and for the production of conidial suspension and mycelia.

Table 6. Provenience of all isolates used in this work.

Nursery 1	Praiola (CT)
Nursery 2	Milazzo (ME)
Nursery 3	Carrubba (CT)
Nursery 4	Grotte (CT)
Nursery 5	Barcellona (ME)
Nursery 6	Lamezia (CZ)

Table 7. *Calonectria* spp. isolates belonging to group A collected during the period 1993-1996.

Isolates	Location	Hosts	Symptoms
1, 14868*	Nursery 1	*Polygala myrtifolia*	crown rot
180, 181, 182	Nursery 1	*Callistemon citrinus*	leaf spot
187	Nursery 1	*Melaleuca hypericifolia*	leaf spot
14887*, 200	Nursery 1	*Myrtus communis*	leaf spot
6, 14869*	Nursery 2	*Callistemon citrinus*	leaf spot
65, 14876*	Nursery 2	*Callistemon leavis*	leaf spot
12, 19, 20, 31	Nursery 3	*Callistemon* 'Mauve Mist'	leaf spot
17, 30	Nursery 3	*Myrtus communis*	leaf spot
44	Nursery 3	*Callistemon leavis*	leaf spot
117, 122	Nursery 3	*Arbutus unedo*	leaf spot
14874*, 52	Nursery 3	*Myrtus communis*	leaf spot
177	Nursery 3	*Polygala myrtifolia*	crown rot
24	Nursery 4	*Myrtus communis*	leaf spot
183, 184	Nursery 4	*Eucalyptus rostrata*	leaf spot
188	Nursery 4	*Metrosideros robustus*	leaf spot
241, 14877*, 242,1831, 1832, 1833, 14879*, 1841, 1842	Nursery 4	*Acacia retinodes*	leaf spot
139, 140, 147, 148, 149, 155, 156, 164, 168, 171	Nursery 5	*Callistemon viminalis*	leaf spot
75	Nursery 5	*Callistemon viminalis*	leaf spot
128	Nursery 6	*Callistemon citrinus*	leaf spot
193, 196	Nursery 6	*Polygala myrtifolia*	crown rot

* This number is referred to strains deposited in ITEM Culture Collection (www.ispa.cnr.it/Collection)

Table 8. *Calonectria* spp. isolates belonging to group B collected during the period 2005-2009.

Isolates	Location	Hosts	Symptoms
14890*, My2, My3, My4	Nursery 1	*Myrtus communis*	leaf spot
CaMa2	Nursery 1	*Callistemon* 'Masotti'	crown and root rot
AS1, AS2, AS3, AS4, AS5, AS11, 14891*	Nursery 1	*Feijoa sellowiana*	crown and root rot
ER1, ER2	Nursery 1	*Escallonia rubra*	crown and root rot
AU2, 14892*, AU4	Nursery 1	*Arbutus unedo*	crown and root rot
CA, CB, C2, C3, C4, C5, C6, C7, C8, CT9, C10, C11	Nursery 3	*Ceanothus thyrsiflorus*	crown and root rot
14895*, Met2, Met3, Met4, Met5, Met7, Met8	Nursery 3	*Metrosideros robustus*	crown and root rot
GPP1, 14894*, GP4, GP5, GP6, GP7	Nursery 4	*Pistacia lentiscus*	crown and root rot
AS7	Nursery 4	*Feijoa sellowiana*	leaf spot
BA1, BA2, BA3	Nursery 4	*Brahea armata*	leaf spot
14889*, AF2	Nursery 4	*Agonis flexuosa*	leaf spot
MFR1, MFR2	Nursery 4	*Melaleuca fulgens*	crown rot
EN1, EN2, EN3	Nursery 4	*Eugenia myrtifolia*	crown and root rot
CAL2	Nursery 4	*Callistemon laevis*	leaf spot

* This number is referred to strains deposited in ITEM Culture Collection (www.ispa.cnr.it/Collection)

4.2.2 Molecular identification by using DNA sequence comparisons

The species level identification was obtained by DNA sequencing and phylogenetic analyses of β-tubulin (*benA*), histone H3 (*HIS3*) and translation elongation factor-1α (*TEF-1a*) gene sequences of the 105 strains tested in this study. Each fungal strain was grown in shake culture (150 rpm, 25°C, 2 days) on Wickerham medium, containing 40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and water up to 1 l; then the mycelium was filtered and lyophilized for total DNA isolation. The fungal DNA extraction was done with the Wizard Magnetic DNA purification Kit for food (Promega) with some modifications by halving the volume of the reaction, starting from 10 mg of lyophilized mycelium. The quality of genomic DNA

was determined by agarose gel electrophoresis and the quantification using a ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Amplification of part of the β-tubulin gene (*benA*) was performed using the primers T1 (O'Donnell & Cigelnik 1997) and CYLTUB1R (Crous *et al.* 2004), for the Histone 3 region (*his3*) primers CYLH3F and CYLH3R (Crous *et al*. 2004) were used and for the translation elongation factor-1α (*tef-1α*) the primers EF1-728F (Carbone & Kohn 1999) and CylEF-R2 (Crous *et al.* 2004) were used. Primers and relevant temperature used in the PCR analysis are listed in the following table (Table 9).

Table 9. Primers and relevant temperatures used in the PCR analysis.

		T °C
T1	5-AACATGCGTGAGATTGTAAGT-3	58
CYLTUB1R	5-AGT TGT CGG GAC GGA AGA G-3	
CYLH3F	5-AGG TCC ACT GGT GGC AAG-3	58
CYLH3R:	5-AGC TGG ATG TCC TTG GAC TG-3	
EF1-728F	5-CATCGAGAAGTTCGAGAAGG-3	58
CylEF-R2	5-CAT GTT CTT GAT GAA (A/G)TC ACG-3	

The preliminary alignment of the three sequenced loci (*benA*, *his3*, *tef-1α*) was performed using the software package BioNumerics version 5.1 (Applied Maths) and manual adjustment for improvement was made by eye where necessary. The phylogenetic analysis was conducted firstly on the three single locus alignments and successively the combined alignment of the three loci was analyzed for inferring the organismal phylogeny. The multilocus alignment was conducted using the Clustal W algorithm in MEGA version 5 (Tamura *et al*. 2011). Phylogenetic and molecular evolutionary analyses were inferred by using the Maximum Likelihood method based on the Tamura-Nei model (1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma

distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 2.1893). Evolutionary analyses were conducted in MEGA5. Maximum parsimony analysis was also performed for all data sets, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The MP trees were obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The trees are drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence (Nei & Kumar 2000). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option),

In addition, also a Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v3.2 (Ronquist *et al.* 2012) for the combined sequences datasets. The analysis was run in duplicate with four MCMC chains and setting random trees for 10 000 000 generations sampled every 100 generations. A total of 7582 trees were read in the two runs, 3791 for each, therefore the first 947 trees (25%) were discarded in each run, as belong to the burn-in phase of the analysis, so posterior probabilities were determined from the remaining trees (2844 in each run).

4.2.3 Morphological identification

The morphological identification of the isolates was achieved on the basis of macroconidia length, single conidial cultures were prepared on synthetic nutrient-poor agar (SNA; Nirenburg 1981, Lombard *et al.* 2009, 2010b) for seven different isolates previously identified as *C. polizzii* on molecular basis. Inoculated plates were incubated at room temperature and examined after 7d. Gross morphological characteristics were determined by mounting fungal structures in lactic acid and 30 measurements at ×100 magnification were made for each isolate. For conidia length the 95% confidence intervals

was determined from 30 observations/isolate and the minimum and maximum ranges given in parentheses.

4.2.4 Assessment of fungicide sensitivity *in vitro*

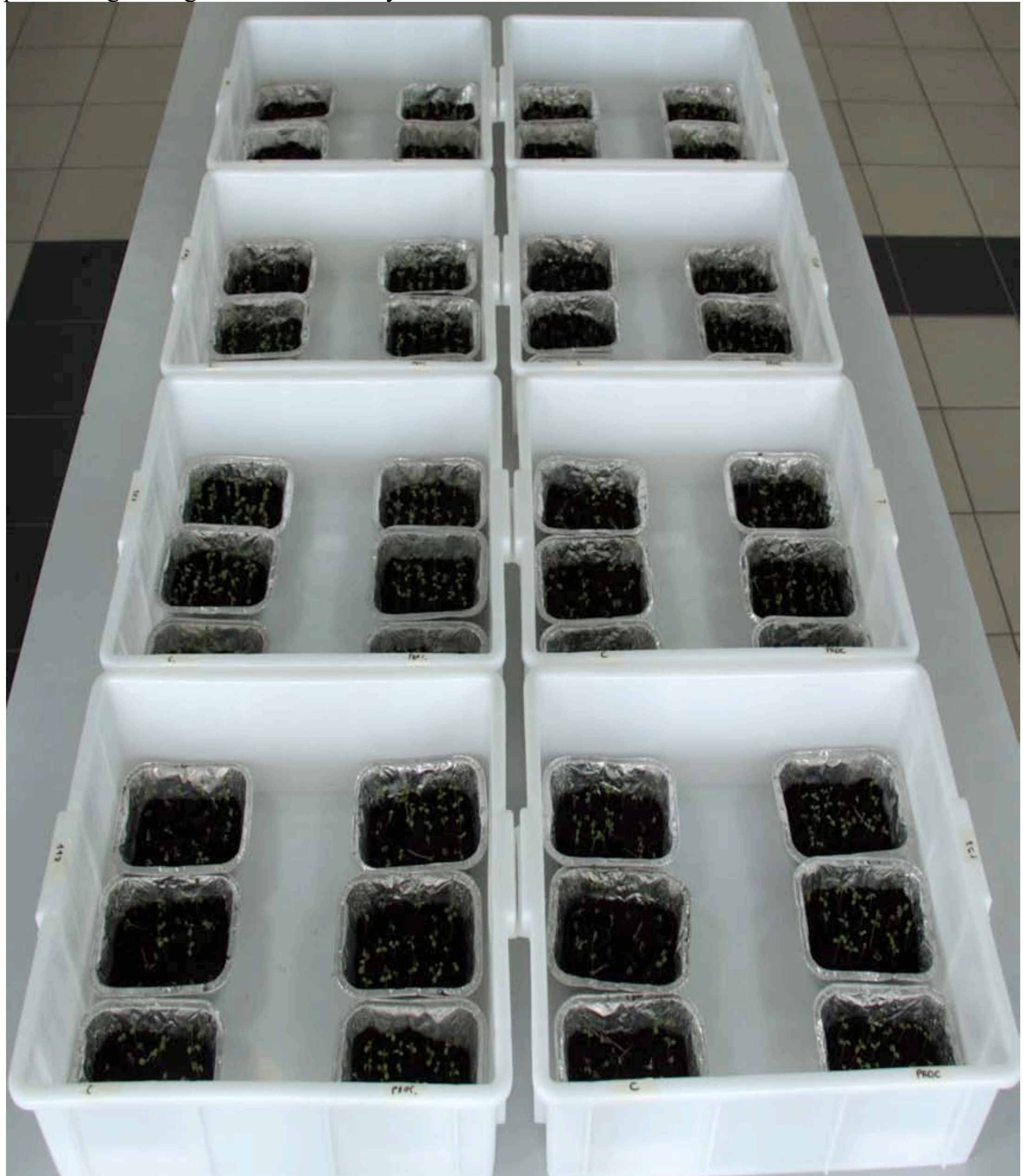
Sensitivity measurements to prochloraz was based on mycelial growth inhibition. A commercial formulation of prochloraz 46,1% WP (Octave®, Basf-Agro) was used as a.i. The fungicide formulation was dissolved in sterilised distilled water (SDW) to make a 100 mg L^{-1} stock solution. Aliquots of the stock solution were added to potato dextrose agar (PDA) after autoclaving to yield final concentrations of 10, 20, 50, 100 mg L^{-1}. Then the amended medium was dispensed into 9 cm plastic Petri plates; control plate did not contain fungicide. Mycelial plugs of *Calonectria* isolates were removed from the edge of actively growing 5 days old colonies on MEA and placed upside down on the centre of Petri dishes containing approximately 20 ml of amended or unamended PDA. Cultures were incubated at 25° C in the dark for 5 days. Then, the mean colony diameter was measured for calculating the growth-reduction (%) by the fungicide, comparing with the mycelial growth on unamended PDA. ED_{50} (effective dose to reduce growth by 50%) values were determined and all the isolates were assigned into different ED_{50} sensitivity-ranges, established as follows: $0 < ED_{50} \leq 10$; $10 < ED_{50} \leq 20$; $20 < ED_{50} \leq 50$; $50 < ED_{50} \leq 100$.

Figure 24. Reduction of mycelial growth in *Calonectria* spp. isolates exposed to different rates of prochloraz.

4.2.5 Assessment of fungicide sensitivity on model plant-host

Sensitivity of *Calonectria* spp. isolates to prochloraz was assayed *in vivo* using seedlings of red clover (*T. pratense*). Eight isolates sensitive to the a.i. and eight exhibiting reduced sensitivity were used. These isolates were selected basing on the ED_{50} values obtained in the *in vitro* sensitivity tests. Red clover seeds were selected, surface disinfected for 2 min with NaOCl (1.2% v/v), rinsed three times in SDW and then left to germinate on aluminium trays (9 × 11.5cm) containing sterile peat. 6.5ml of pathogen conidial suspension was then sprayed onto each tray 7 days after seeding. A conidial suspension (ca 1×10^5 conidia mL^{-1}) was prepared for each isolate by adding SDW to plates of carnation leaf agar (CLA; Fisher *et al*. 1982) 7 d after inoculation and dislodging the conidia. The CLA plates were inoculated with mycelial plugs of *Calonectria* isolates in actively growing from 5 d on MEA. Prochloraz was applied 3 hours before pathogen inoculation by spraying 7 ml of solution/tray using the label rate. Control seedlings were sprayed with SDW. All plants were transferred and kept in a growth chamber at 25°C under near-UV light with a 16:8 light/dark regimen and at about 95% relative humidity (RH). After 7 days, all young plants were removed from peat and rinsed to observe dead plants and the symptoms such as crown-rot, stem-rot and leaf-spot. The percentage of infected seedlings within the total examined (disease incidence, DI) was evaluated. The sensitivity to prochloraz among isolates was established by the reduction of disease spread in plants treated. The experiment was conducted twice, under similar conditions.

Figure 25. Different isolates of *Calonectria* spp. inoculated on seedlings of *T. pratense* growing in aluminium trays.



4.2.6 Assessment of fungicide sensitivity on *Feijoa sellowiana*

Two sensitive and two resistant isolates were used for artificial inoculation on seedlings of *F. sellowiana* (Myrtaceae). The efficacy evaluation of prochloraz was performed on four month-old feijoa plants. Feijoa seeds were surface sterilized and grown in 8 cm pots containing sterile peat. In all, 90 healthy seedlings were used for the inoculation in a randomized block design

with three replicates for each isolate and treatment. The fungicide solution and the conidia suspension were made and applied as the previous experiment, although the fungicide applications were repeated every ten days and the inoculation was on collar and root portions. This experiment was done twice. Control trays were inoculated with conidia suspension and sprayed with SDW. DI and symptoms severity (SS) were calculated 40 days after inoculation. SS of *Calonectria* infections on the crown and root was assayed by the mean disease rating (MDR) that was calculated using an empirical 1-to-5 rating scale that takes into account the percentage of infected hypocotyls surface, where 1 = no symptoms, 2 = 1 to 10%; 3 = 11 to 25%; 4 = 25 to 50%; 5 = more than 50% of the surface infected. The experiment was conducted twice, under similar conditions.

Figure 26. Example of empirical 1-to-5 rating scale used in this work.

4.2.7 Statistical data analysis

Data from all experiments regarding mycelial growth *in vitro*, mortality index on red clover assays, disease incidence (DI) and severity symptoms (SS) index on feijoa trials for each treatment were calculated averaging corresponding values determined for each replicate. Analysis of variance (ANOVA; Statistica

10, Statsoft Inc., Analytical Software for Windows) was performed to examine the fungicide treatment in reducing above pathogen and disease parameters, including treatment × assay and treatment × trial. Percentage data concerning mortality index and DI were transformed using arcsine (sin–1 square root *x*) prior to statistical analysis. The corresponding mean values of mycelial growth (mm), mortality (%) and DI were compared and separated by Fisher's least significant difference test ($P < 0.05$ or 0.01) for adopted randomized complete block design. Untransformed arithmetic means of red clover mortality (%), mycelial growth (± standard error of the mean = SEM), and DI are presented in the tables. In addition, for *Calonectria* mortality values on red clover assays was calculated coefficient of variation (CV) for both sensitive and reduced-sensitive group of pathogen isolates. Otherwise, *Calonectria* severity symptoms (SS) data were analyzed according to non-parametric approach because an ordinal scale was adopted to evaluate disease symptoms on feijoa plants. In detail, two-ways analysis according to Friedman and one-way analysis according to Mann-Whitney test were respectively used to evaluate treatment × trial interactions and prochloraz effects on SS reduction on feijoa plants. Furthermore, Kendall's coefficient of concordance (*W*) was calculated to assess if the rankings of the SS data are similar within each trial. For one resistant isolate W was < 0.75 with an associated low $\chi 2$ (chi-square) value, and therefore the single trials (1st and 2nd) were presented, calculating z according to Mann Whitney test and *P*-level associated.

4.3 Results

4.3.1 Molecular identification by using DNA sequence comparisons

We obtained amplicons of about 600 bases for *benA*, 450 for *HIS3* and 500 for *TEF-1α* for all isolates, no conflict in tree topology was observed for the three loci, however only *benA* is able to clearly differentiate with high bootstrap between the closely related species *C. polizzii* and *C. pauciramosa.* The combined sequence dataset for *benA*, *HIS3* and *TEF-1α* consisted of 1508 characters, of which 1140 were constant, 367 are variable of which 361 were parsimony informative. The evolutionary history was inferred by using the Maximum Likelihood method that resulted in a tree with the highest log likelihood (-3952.5559). The percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrap analysis, Fig. 27a). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 2.1893)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 117 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1432 positions in the final dataset. In addition the consensus tree obtained Maximum Parsimony analysis confirmed the tree topology obtained with the ML methods as well as bootstrap support. In particular the evolutionary history was inferred using the Maximum Parsimony method. One tree out of 1243 most parsimonious trees (length = 428) was obtained. The consistency index is (0.929078), the retention index is (0.971884), and the composite index is 0.903761 (0.902956) for all sites and parsimony-informative sites (in parentheses). The three loci dataset phylogenetic analysis showed a monophyletic group for *C. polizzii* strains with a bootstrap (BP) value of 97 (Fig.27b) and less of 70% for *C. pauciramosa*, while the cluster of these two sister species had high BP values in both the analysis (MP and ML) respect to the other closely related species of the group.

Fig. 27a. Multilocus (*benA*, *TEF-1α* , *HIS-3*) phylogenetic tree by Maximum Likelihood method. The tree with the highest likelihood (-3952.3559) is shown. Numbers above branches are bootstrap values**.** Only values above 70% are indicated.

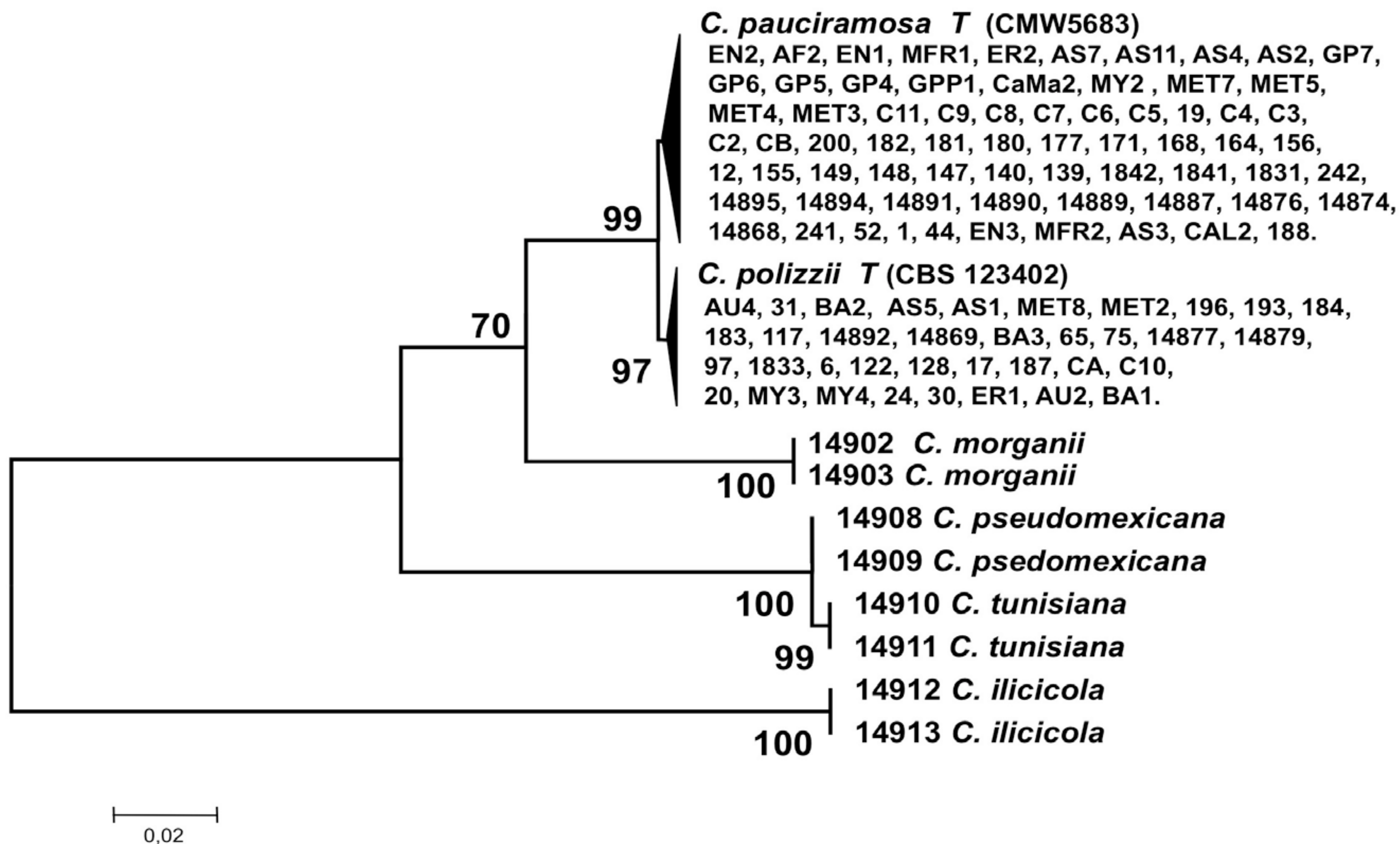
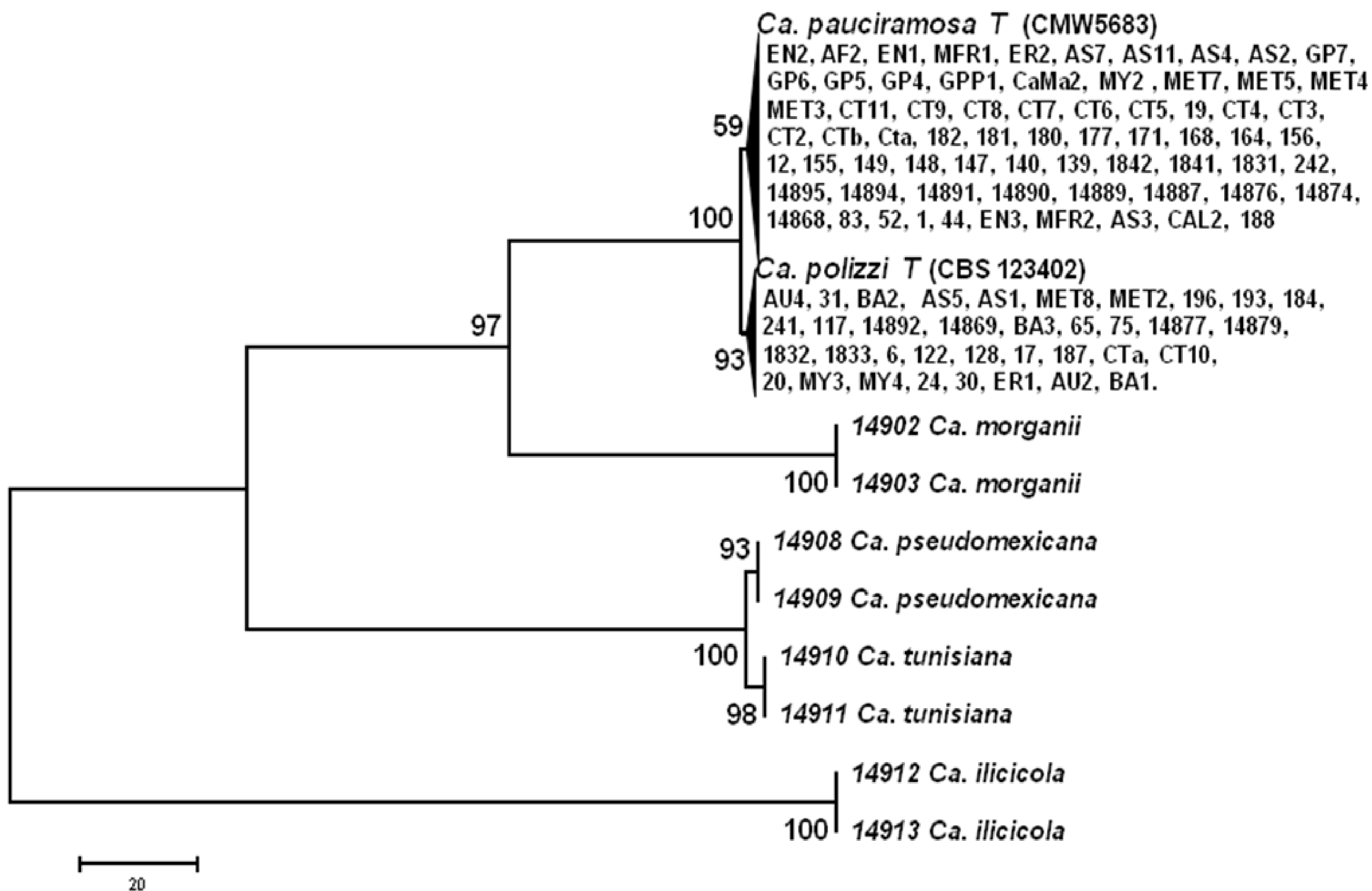


Fig. 27b. Multilocus (*benA*, *TEF-1α* , *HIS-3*) phylogenetic tree by Maximum Parsimony method. One tree out of 1243 most parsimonious (lenght = 428) is shown. Numbers above branches are bootstrap values**.** Only values above 70% are indicated.



4.3.2 Morphological identification

The microscopic observations provide to describe the macroconidia characters of seven isolates of *C. polizzii*. Macroconidia observed were cylindrical, rounded at both ends, straight, (31–)40–43(–52) × 3–5 µm (av. = 41 × 4 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime.

4.3.3 Assessment of fungicide sensitivity *in vitro*

With regard to sensitivity within group A, no one isolate was totally inhibited by the minimum concentration of the fungicide used (10 mg L^{-1}). However, for all isolates tested, a reduction in growth compared to the unamended medium between 10,60% and 88,79%, was recorded at that concentration.

Even at a concentration of 20 mg L^{-1}, all isolates were increased although in this case, reductions in growth were slightly more pronounced: from 10,47% to 89,38%.

At the concentration of 50 mg L^{-1} of the fungicide, only one isolate was completely inhibited by the action of the fungicide. As regards the other isolates, the reductions of growth fall within a range between the values of 13,95% and 100%.

At the highest concentration of fungicide, 100 mg L^{-1}, there was a good growth inhibition. 21 isolates were inhibited completely by the fungicide. The values of reductions in growth of the remaining isolates varied between 67.44% and 100%.

Also all the isolates belonging to group B had increased at the lowest concentration of the fungicide (10 mg L^{-1}). At this concentration, there were reductions in growth, compared to the growth in unamended medium, between 15.58% and 79.08%.

At the second concentration evaluated (20 mg L^{-1}) all isolates have also increased despite reductions in growth was between 28.49% and 87.15%.

The isolates increased at the concentration of 50 mg L^{-1} showed a reduction compared to the control comprised between 43,78% and 97,66%. At the

highest concentrations of prochloraz (100 mg L^{-1}), 4 isolates were totally inhibited in growth. The reductions in growth measured for isolates having increased varied between 52,15% and 100%.

The mean reduction growth of isolates belonging to group B is lower compared to the mean reduction growth of isolates of group A at each dose tested.

Table 11. Mean mycelial growth (mm) on PDA media supplemented with prochloraz of 51 isolates belonging to *Calonectria scoparia* complex collected within 1993-1996 year period in this study.

Prochloraz concentration (mg L^{-1})	Growth (mm)	Growth reduction (%)
0	32.07 (± 0.64) a	-
10	14.60 (± 0.67) b	54.47
20	11.72 (± 0.73) c	63.45
50	7.11 (± 0.66) d	77.83
100	2.12 (± 0.64) e	93.42

[a] Colony diameter was determined 5 days after treatment.
[b] Numbers are means ± standard error of the mean (SEM) of 3 replicates each formed by 51 values.
[c] Means followed by the same letter are not significantly different according to Fisher's least significance difference ($P = 0.01$).

Table 12. Mean mycelial growth (mm) on PDA media supplemented with prochloraz of 54 isolates belonging to *Calonectria scoparia* complex collected within 2005-2009 year period in this study.

Prochloraz concentration (mg L^{-1})	Growth (mm)	Growth reduction (%)
0	27.82 (± 0.39) a	-
10	14.80 (± 0.79) b	46.80
20	11.17 (± 0.71) c	59.85
50	6.36 (± 0.61) d	77.13
100	3.72 (± 0.50) e	86.62

[a] Colony diameter was determined 5 days after treatment.
[b] Numbers are means ± standard error of the mean (SEM) of 3 replicates each formed by 54 values.
[c] Means followed by the same letter are not significantly different according to Fisher's least significance difference ($P = 0.01$).

Among the isolates of *C. pauciramosa* belonging to group A, the mean growth reduction was lower than the isolates belonging to group B at the concentration of 10, 20 and 100 mg L^{-1}, while the growth reduction was slightly higher at the concentration of 50 mg L^{-1}.

Table 13. Mean mycelial growth (mm) on PDA media supplemented with prochloraz of 30 *Calonectria pauciramosa* isolates collected within 1993-1996 year period in this study.

Prochloraz concentration (mg L^{-1})	Growth (mm)	Growth reduction (%)
0	32.55 (± 0.90) a	-
10	15.31 (± 0.90) b	52.95
20	12.18 (± 0.99) c	62.57
50	7.91 (± 0.95) d	75.66
100	2.13 (± 0.55) e	93.45

[a] Colony diameter was determined 5 days after treatment.
[b] Numbers are means ± standard error of the mean (SEM) of 3 replicates each formed by 30 values.
[c] Means followed by the same letter are not significantly different according to Fisher's least significance difference ($P = 0.01$).

Table 14. Mean mycelial growth (mm) on PDA media supplemented with prochloraz of 39 *Calonectria pauciramosa* isolates collected within 2005-2009 year period in this study.

Prochloraz concentration (mg L^{-1})	Growth (mm)	Growth reduction (%)
0	27.60 (± 0.49) a	-
10	14.43 (± 0.89) b	47.83
20	10.91 (± 0.78) c	60.51
50	6.18 (± 0.69) d	77.61
100	3.19 (± 0.53) e	88.44

[a] Colony diameter was determined 5 days after treatment.
[b] Numbers are means ± standard error of the mean (SEM) of 3 replicates each formed by 39 values.
[c] Means followed by the same letter are not significantly different according to Fisher's least significance difference ($P = 0.01$).

Similarly, among the isolates of *C. polizzii*, the growth reduction within the group B at each concentration tested, was lower compared to that of group A, showing a fungicide response in accordance to that observed in *C. pauciramosa* isolates.

Table 15. Mean mycelial growth (mm) on PDA media supplemented with prochloraz of 21 *Calonectria polizzii* isolates collected within 1993-1996 year period in this study.

Prochloraz concentration (mg L^{-1})	Growth (mm)	Growth reduction (%)
0	31.39 (± 0.88) a	-
10	13.58 (± 1.02) b	56.74
20	11.07 (± 1.11) c	64.73
50	5.96 (± 0.87) d	81.01
100	2.09 (± 0.62) e	93.35

[a] Colony diameter was determined 5 days after treatment.
[b] Numbers are means ± standard error of the mean (SEM) of 3 replicates each formed by 21 values.
[c] Means followed by the same letter are not significantly different according to Fisher's least significance difference ($P = 0.01$).

Table 16. Mean mycelial growth (mm) on PDA media supplemented with prochloraz of 15 *Calonectria polizzii* isolates collected within 2005-2009 year period in this study.

Prochloraz concentration (mg L^{-1})	Growth (mm)	Growth reduction (%)
0	28.10 (± 0.53) a	-
10	15.80 (± 1.49) b	43.77
20	12.01 (± 1.47) c	57.26
50	6.62 (± 1.27) d	76.44
100	4.73 (± 1.08) e	83.17

[a] Colony diameter was determined 5 days after treatment.
[b] Numbers are means ± standard error of the mean (SEM) of 3 replicates each formed by 15 values.
[c] Means followed by the same letter are not significantly different according to Fisher's least significance difference ($P = 0.01$).

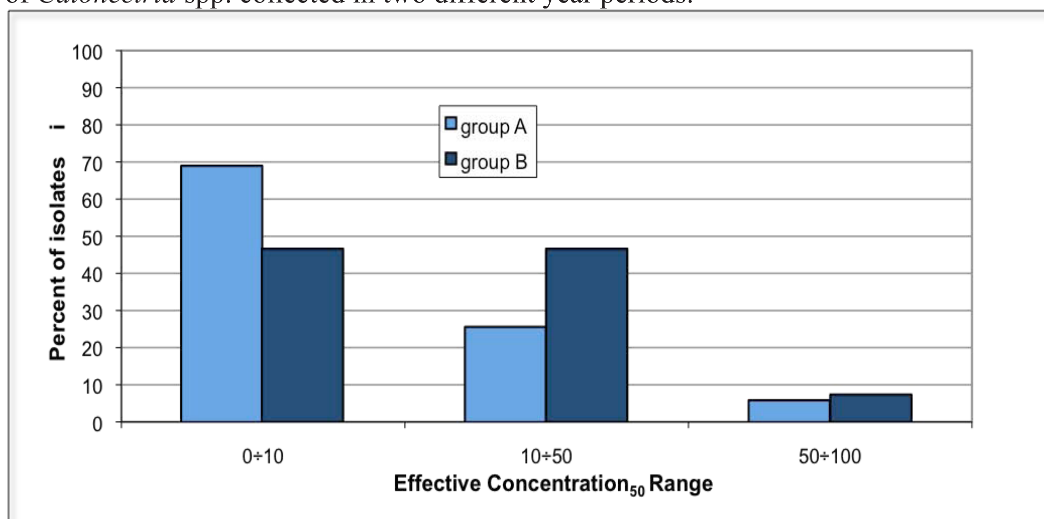
On the basis of the results obtained in the group A, all isolates tested were grouped in "sensitivity ranges", using the DE_{50} values as a parameter for comparing. Since such processing is shown that out of a total of 51 isolates, 35 (68.62%) have a DE_{50} between 0 and 10 mg L^{-1}.

Within the range between 10 and 50 mg L^{-1}, 13 (25.49%) isolates were

included, while the interval between 50 and 100 mg L^{-1} was composed of a total of 3 isolates that represent the 5,9% of the total number of isolates tested.

Also all isolates belonging to group B, were grouped in "sensitivity ranges", through the DE_{50} values. The first interval, that with DE_{50} values comprised between 0 and 10 mg L^{-1}, was composed by 25 (46,29%) isolates. The second interval (DE_{50} between 10 and 50 mg L^{-1}) had 25 (46,29%) isolates. In the range between 50 and 100 mg L^{-1}, there were 4 (7,42%) isolates.

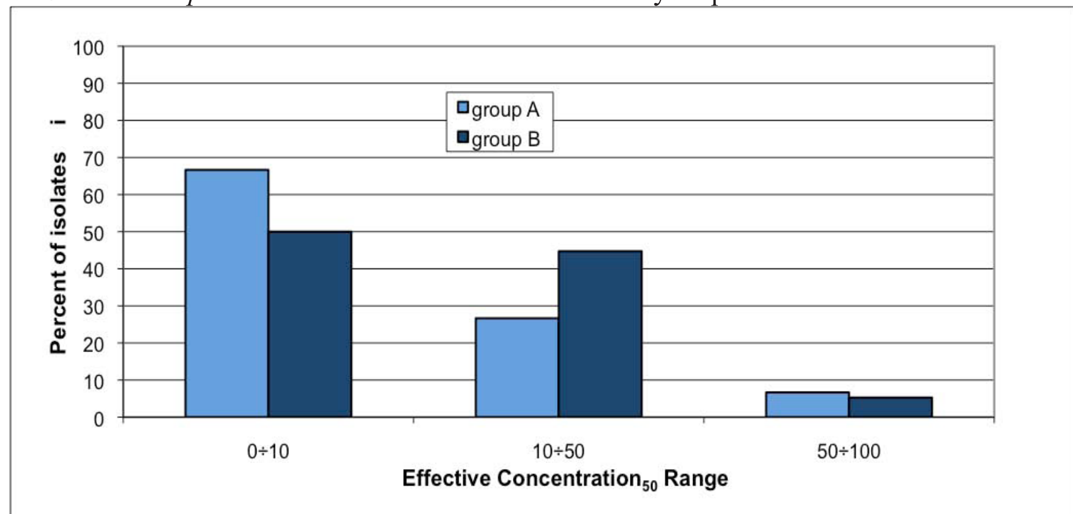
Figure 28. Changes of sensitivity (EC_{50} values) to prochloraz in an Italian population of *Calonectria* spp. collected in two different year periods.



Among the isolates of *C. pauciramosa* belonging to group A, the 66,66% of those were in the first range, the 26,66% of isolates were included in the interval comprised between 10 and 50 mg L^{-1}, while the 6.66% had a DE_{50} in the third range (50 - 100 mg L^{-1}).

In accordance with the temporal shift observed in group B, all isolates of *C. pauciramosa* of this group showed a shift in sensitivity distribution: in the range comprised between 0 and 10 mg L^{-1} there were the 50% of the 38 total isolates, in the second range the 44.73% of isolates were included, and, at least, 5.73% of isolates were grouped in the interval comprised between 50 and 100 mg L^{-1}.

Figure 29. Changes of sensitivity (EC_{50} values) to prochloraz in an Italian population of *Calonectria pauciramosa* collected in two different year periods.



Similarly a shift in sensitivity distribution was observed among isolates of *C. polizzii* of group A that were grouped within the three ranges respectively with percentages of 71,24%, 23,80%, 4,96% and isolates of *C. polizzii* of group B with percentages 37,50%, 50% and 12,50%.

Figure 30. Changes of sensitivity (EC_{50} values) to prochloraz in an Italian population of *Calonectria polizzii* collected in two different year periods.

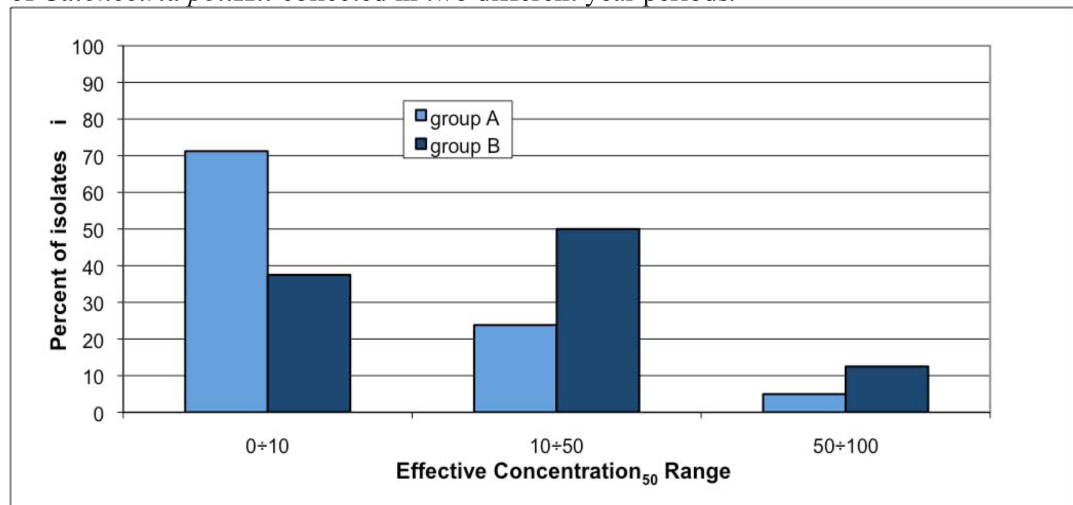


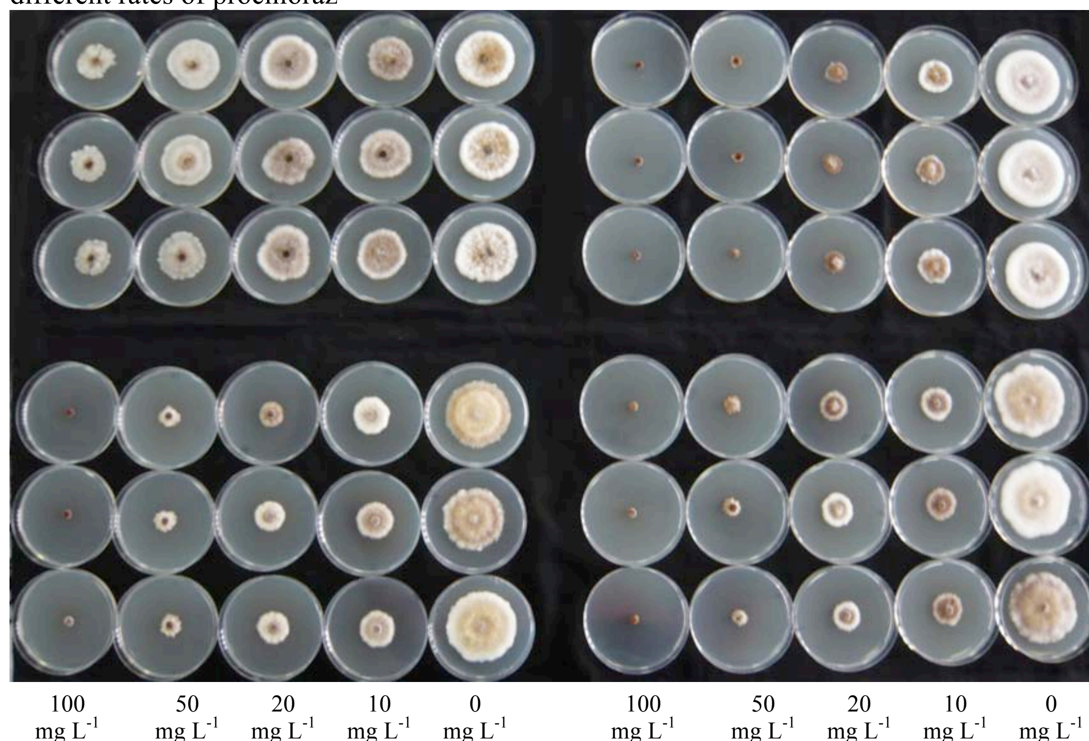
Table 17. Distribution in sensitivity ranges of isolates belonging to group A.

Isolates	ED_{50} Range	Nursery
14868, 180, 181, 182, 200	$0<DE_{50}<10$	1
6, 65, 14876		2
12, 19, 31, 17, 30, 117, 122, 52		3
24, 183, 188, 14877, 242, 1831, 1832, 14879, 1841, 1842		4
75, 139, 148, 149, 156, 168, 171		5
193, 196		6
20, 44, 14874, 177	$10<DE_{50}<20$	3
184		4
155, 164		5
128		6
1	$20<DE_{50}<50$	1
14869		2
241, 1833		4
140		5
187, 14887	$50<DE_{50}<100$	1
147		5

Table 18. Distribution in sensitivity ranges of isolates belonging to group B.

Isolates	ED_{50} Range	Nursery
MY2, MY3, AS5, ER2	$0<DE_{50}<10$	1
CB, C3, C4, C5, C6, C7, C8, C9, C10, C11, 14895, MET2		3
14894, GP4, GP5, BA1, BA3, AF2, EN2, EN3, CAL2		4
AS1, AS2, AS4, AS11, ER1, AU4	$10<DE_{50}<20$	1
CA		3
AS7, GPP1, GP6, MFR1, EN1		4
14890, MY4, CaMa2, AS3, AU2	$20<DE_{50}<50$	1
C2, MET3, MET4, MET5, MET8		3
GP7, BA2, MFR2		4
14891, 14892	$50<DE_{50}<100$	1
MET7		3
14889		4

Figure 31. Variability of mycelial growth in four isolates of *Calonectria* spp. at different rates of prochloraz



4.3.4 Assessment of fungicide sensitivity on model plant-host

With regard to this trial, all isolates caused symptoms on plants of red clover at the rate used (100 g/hl), although with variable reductions of the disease incidence compared to the untreated seedlings inoculated. However, for all isolates, a reduction of disease comprised between 10,67% and 83,56% was recorded at that concentration.

The spread of symptomatic plants inoculated in the untreated thesis were high for each isolate, showing values included between 51,77% and 100%.

While, observing the DI in treated thesis, the values were reduced. In details, regarding the 8 isolates considered highly sensitive (basing on the results of previous *in vitro* tests) the values of the DI varied from 8,51% to 37.39%; while in the case of the 8 isolates considered with low sensitivity those values were between 43.80% and 89.23%.

The commercial product containing prochloraz showed activity in containment of infections caused by *Calonectria* spp. with a DI reduction variable, in

relation to the molecule sensitivity by each isolate. This activity was high in the case of the 8 isolates considered highly sensitive in which the reduction was observed between 37,83% and 83.56%, while lower values are observed in the case of the 8 resistant isolates, where the reduction varied from 10,67% to 38,52%, thus demonstrating a lower activity in containment of the disease.

Within the group of isolates with high sensitivity to prochloraz, statistical differences between the fungicide untreated and the treated thesis for all isolates tested and for the mean value, were established; meanwhile in the group of isolates with reduced sensitivity, four isolates and the mean value of growth reduction did not present statistical differences, while the others four isolates were statistically different.

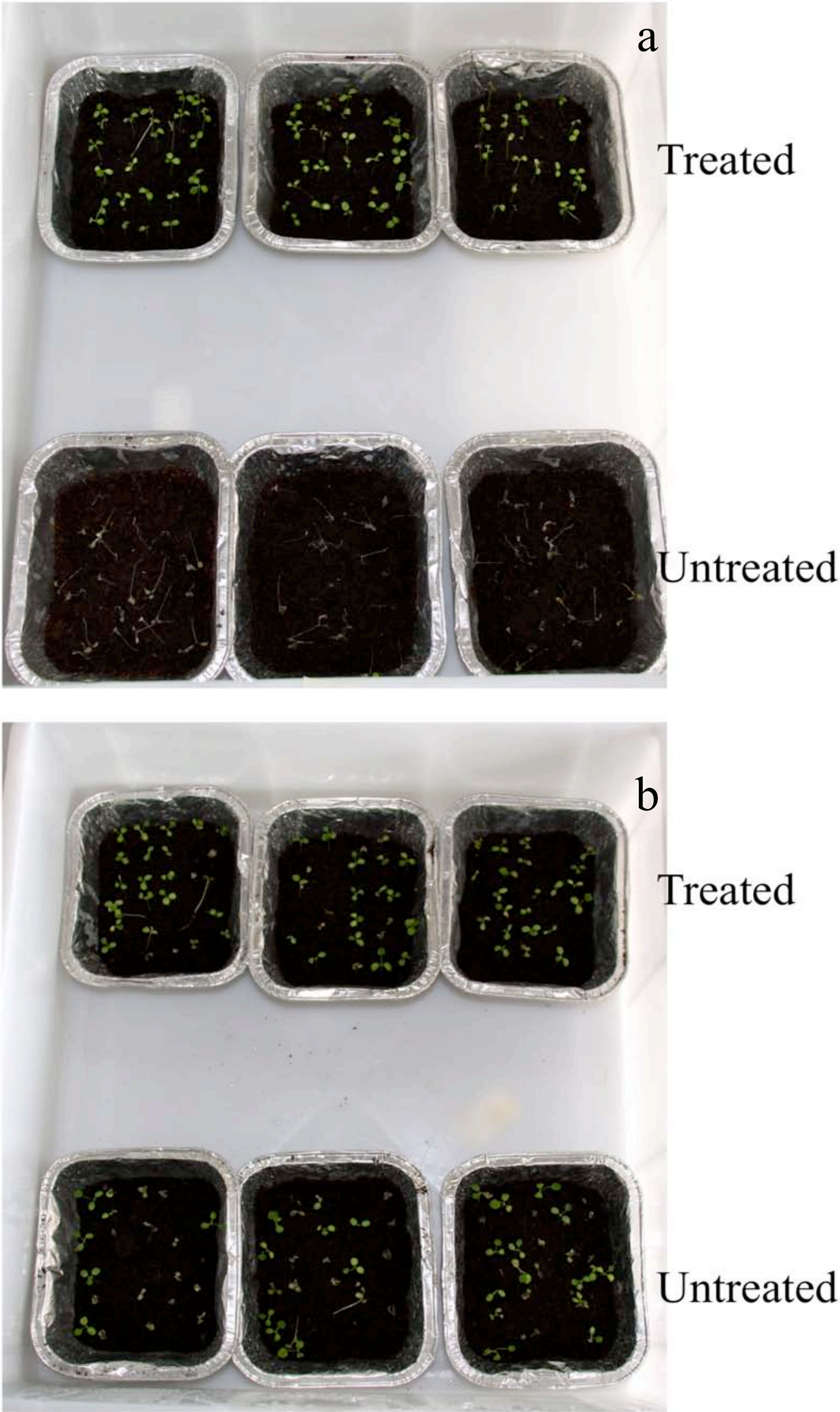
Table 19. Effects of prochloraz on DI (%) of crown and root rot caused by *Calonectria* spp. on feijoa under nursery conditions.

Isolates[a]	Untreated[b]	Treated[b]	Reduction (%)	*F-test (P) (Trtm)*	*F-test (P) (Trtm×assay)*
R.S.					
C. polizzii 31	92.21±1.72	29.42±6.59	68.09	155.72 **	3.77 ns
C. polizzii 14879	71.21±4.17	23.72±3.35	66.69	118.52**	< 1 ns
C. pauciramosa 188	53.88±7.78	13.46±2.95	75.02	29.95 **	1.87 ns
C. polizzii 193	61.59±3.14	19.23±4.21	68.78	79.72 **	1.77 ns
C. pauciramosa EN3	60.14±3.82	37.39±3.85	37.83	34.02 **	1.47 ns
C. pauciramosa Cal2	79.15±3.65	21.42±3.39	72.94	162.79 **	1.27 ns
C. pauciramosa 14894	68.30±8.35	18.26±4.15	73.26	44.50 **	< 1 ns
C. pauciramosa 14895	51.77±11.73	8.51±1.61	83.56	31.75 **	1.01 ns
Mean	*67.28±4.79*	*21.43±3.19*	*68.15*	*52.26* **	-
CV (%)	20.12	42.08			
S.					
C. pauciramosa 1	57.14±3.61	50.49±3.00	11.64	3.49 ns	< 1 ns
C. polizzii 75	78.96±8.40	57.92±6.12	26.61	4.96 ns	< 1 ns
C. polizzii 104	70.48±5.68	53.97±5.63	23.42	7.32 ns	1.59 ns
C. polizzii 187	100.0±0.00	89.23±6.20	10.67	7.06 ns	< 1 ns
C. pauciramosa 14887	70.68±2.85	43.80±4.36	38.52	49.21 **	5.74 ns
C. pauciramosa 14889	82.53±5.79	61.94±3.85	24.95	17.36 *	3.36 ns
C. pauciramosa 14891	73.70±2.50	55.64±4.48	24.50	19.51 *	3.51 ns
C. polizzii 14892	94.04±1.19	69.62±2.96	25.97	76.59 **	< 1 ns
Mean	*78.44±4.86*	*60.33±4.93*	*23.09*	*5.63* ns	-
CV (%)	17.54	23.13			

[a] For all *Calonectria* isolates disease incidence was determined 4 days after treatment.
[b] Data are means ± SEM of 3 replicates each formed by 30-to-50 red clover seedlings
*, ** denote significant differences at P ≤ 0.01 and 0.001, respectively. ns: not significant.
R.S.: Isolates with reduced sensitivity
S.: Isolates sensitives

Figure 32. Symptoms on plants of *Trifolium pratense.*

Figure 33. Significant difference between treated and untreated in plants inoculated with sensitive isolate and no difference in plants inoculated with reduced-sensitivity isolates.



4.3.5 Assessment of fungicide sensitivity on *Feijoa sellowiana*

As regards the evaluation of sensitivity *in vivo* to prochloraz of 4 isolates of *Calonectria* spp., on seedlings of *Feijoa sellowiana*, a variable efficacy of prochloraz was observed.

Both sensitive isolates tested, in the untreated thesis, had DI values of 73.33% and 72.19% in the first trial and 77.78%, 75.56% in the second trial; however those values, in the prochloraz treated thesis, became respectively 21.10%, 25.53% in the first trial and 27.78%, 31.11% in the second trial. As regarding both resistant isolates, in the untreated thesis, the DI values were 78.88% and 80% in the first trial, while in the second trial they were 78.89% and 75.56, contrary to the treated thesis where the DI were 62.22% and 55.51 in the first trial and 71.11% and 58.89% in the second.

In the case of both isolates having showed high sensitivity in the *in vitro* tests, in trial 1 and 2, the reduction of DI observed in treated thesis was variable by 58.83% to 71.23%, while this reduction values of DI of both isolates with low sensitivity were comprised between 9.86% and 30.61%. Comparing those data with the percentage of reduction of SS in sensitive isolates was variable by 81.60% and 86.46%, while in resistant isolates was comprised between 9.36% and 48.10%.

Table 20. Effects of fungicide on DI (%) and SS of crown and root rot caused by *Calonectria pauciramosa* on feijoa under nursery conditions.

Isolate	Treatment	1st trial		2nd trial	
		DI (%)[y]	**SS**[y,z]	**DI (%)**[y]	**SS**[y,z]
R.S.					
	Control [x]	78.88	2.03	78.89 a	2.37 a
14889	Prochloraz	62.22	1.84	71.11 b	1.44 b
	Reduction(%)	*21.12*	*9.36*	*9.86*	*39.24*
		$F = 5.79^{ns}$	$Z = 0.91^{ns}$	*F = 24.29***	*Z = 5.29****
	Control [x]	80.00 a	2.37 a	75.56 a	2.31 a
14887	Prochloraz	55.51 b	1.23 b	58.89 b	1.67 b
	Reduction(%)	*30.61*	*48.10*	*22.06*	*27.71*
		*F = 13.29**	*Z = 4.95****	*F = 25.31***	*Z = 2.59***
S.					
	Control [x]	73.33 a	1.94 a	77.78 a	2.29 a
14894	Prochloraz	21.10 b	0.27 b	27.78 b	0.31 b
	Reduction(%)	*71.23*	*86.08*	*64.28*	*86.46*
		*F = 147.57****	*Z = 7.04****	*F = 827.48****	*Z = 7.98****
	Control [x]	72.19 a	1.97 a	75.56 a	2.24 a
14879	Prochloraz	25.53 b	0.34 b	31.11 b	0.41 b
	Reduction(%)	*64.63*	*82.74*	*58.83*	*81.70*
		*F = 61.77****	*Z = 6.92****	*F = 99.69****	*Z = 7.43****

[x] Control = untreated, inoculated seedlings.
[y] Data are the mean of 3 replicates each formed by 32 young feijoa plants. Values followed by the same letters within a column are not significantly different according to the Fisher's least significant difference test (*P = 0.01*) for disease incidence (DI). Arcsine square root transformation was applied on percentage prior to data analysis.
*,**,*** denote significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively according to Mann Whitney non parametric rank test. ns: not significant.
R.S.: Isolates with reduced sensitivity
S.: Isolates sensitives

4.4 Discussion

The agriculture in southern Italy, includes a commercially largely developed sector: the production of ornamental plant. During last twenty years a broad ornamental-plant group was reported as susceptible to *Calonectria* diseases. Different symptoms caused by *Calonectria* spp., became a serious threat for the production of many marketable plants. Considering the importance of this industry, the chemical management in ornamental plants nurseries plays a primary role.

MBCs have proved their efficacy against *Calonectria* spp. (Bertus 1976, Rattan & Dhanda 1985, Horst & Hoitink 1968, Barnard 1984, Chase 1987, Nan *et al.* 1991, Kucharek & Atkins 1993), but the intensive use of some chemicals belonging to MBCs such as benomyl led to the occurrence of resistance phenomena. The resistance to MBCs has been observed also in Italy within a population of *C. pauciramosa* (Polizzi & Vitale 2001) and of *C. morganii* (Vitale *et al.* 2009).

Prochloraz, member of DMI fungicides, is considered as an important fungicide worldwide for the effectiveness on several plant pathogens (Nan *et al*. 1991, Henricot *et al*. 2008, Gea *et al.* 1996, Fletcher *et al.* 1983). The prochloraz activity on *Calonectria* spp., has been showed in several studies (Nan *et al.* 1991, Brand 2006, Henricot *et al.* 2008).

In Mediterranean basin, prochloraz showed a good efficacy in management of *Calonectria* disease (Polizzi 2000, Polizzi & Vitale 2002), moreover, in others cases this a.i. provided changing results (Polizzi & Azzaro 1996, Vitale *et al*. 2003, Aiello *et al.* 2013).

On the basis of this variability of prochloraz efficacy, the rising concern about the widespread resistance to MBCs of *Calonectria* spp. and the occurrence of resistance to prochloraz of others pathogens, a study about sensitivity to prochloraz of isolates belonging to *Calonectria* was considered necessary.

Recent studies have indicated that *C. pauciramosa* included cryptic species and multigene phylogeny analysis recognised *C. polizzii* sp. nov. L. Lombard, Crous & M.J. Wingf. in Italy (Lombard *et al.* 2010b). Phylogenetic analyses conducted recently in another study (Vitale *et al.* 2012a), showed that *C. polizzii* is widespread in Sicily. The widespread presence of *C. polizzii* was

confirmed by this study. Infact, the analyses of combined sequence dataset for *benA*, *HIS3* and *TEF-1α* show the presence of this species at the rate of 34.3% and the presence of *C. pauciramosa* at the 65.7%, within the population tested. Among the total of isolates identified as *C. polizzii* in this work, the 58,33% of those belongs to the group composed by isolates collected during 1993-1996, showing, for the first time, the presence of this species in southern Italy since the early of 1990s.

Regarding morphological identification, Lombard *et al.* (2010b) showed that the macroconidia of *C. polizzii* (av. 37 × 4 μm) are smaller to those of *C. pauciramosa* (av. 50 × 4.5 μm). Microscopical observation carried out in this work provide a different average in macroconidia measures of *C. polizzii* (41 × 4 μm), showing that there are not significant differences in macroconidia length between *C. polizzii* and *C. pauciramosa.*

This study was initiated to investigate a problem of ineffective treatments of prochloraz to manage infections caused by *Calonectria pauciramosa* in several nurseries located in southern Italy where the a.i. has been extensively used.

The fungal population studied consists of 'group A' belonging isolates collected during the period 1993-1996 and a 'group B' composed by isolates collected during 2005-2009 in the nurseries where prochloraz was used for many years. All 105 isolates tested were sensitive to the fungicide action of prochloraz, despite within both populations the sensitivity showed some variability. Reductions of mycelial growth recorded for all isolates were variables between 10.47% and 100% compared to the control. Comparing the ED_{50} values of the two groups belonging isolates collected in two different time periods, the distribution in sensitivity-ranges was different between the two groups. In group A the range with greater frequency was that comprised between 0 and 10 mg L^{-1} with the 68.62% of the isolates, while the same range in group B the 46.29% of isolates contained. In group B the majority of the isolates, precisely the 53.71% (in opposite to the 31.39% of the group A) grew at higher rates, grouping into intervals between 10 and 100 mg L^{-1}.

No significant differences were recorded between the responses to prochloraz by *C. pauciramosa* and *C. polizzii* isolates, establishing the accordance within each of both species with the entire population.

These data confirm the gradual decrease of sensitivity of *C. pauciramosa* and *C. polizzii* to prochloraz, assuming that, over the years, has been a shift and that this shift was presumably due to long period of exposure to fungicide. The distribution of isolates within ranges of sensitivity represented graphically, shows the shift in sensitivity between the two populations (Fig 28, 29, 30).
Evaluating the results obtained, the resistance occurrence to prochloraz in the population of *C. pauciramosa* and *C. polizzii* has been established. Moreover, we can assume that this variation in sensitivity to the molecule was at the base of the variability of effectiveness observed in nurseries and in microcosms, as reported in some recent studies (Polizzi & Azzaro 1996, Aiello *et al.* 2013).
Inoculating red clover seedlings, treated and not treated in advance, was a reliable method to confirm *in vivo* the presence of isolates with reduced sensitivity. Among the high sensitive isolates tested, the DI was highly significantly different, for each isolate, compared to the control; as long as, among the isolates that showed reduced sensitivity in experiment *in vitro*, only the 25% of isolates showed a DI highly significantly different, a further 25% were slightly significantly different and the 50% of isolates presented no significant difference in DI between prochloraz treated and not treated.
In accordance with experiment on model plant, the results of trials on a susceptible marketable plant, showed an high significant difference about DI and SS between prochloraz treated and not treated for both sensitive isolates artificially inoculated, while the isolates with reduced sensitivity showed a low significant difference for both DI and SS. In detail, for one of those isolates tested in the first trial, no significant difference for both DI and SS, was observed.
In conclusion, the *in vivo* tests conducted with artificially inoculating model plant and marketable plant hosts, revealed that prochloraz gave only partial disease control when used at label rates, confirming the recorded presence of isolates with low reduced sensitivity by *in vivo* tests.
The findings of the present study showing the selection of *C. pauciramosa* and *C. polizzii* strains with reduced sensitivity to prochloraz, and that there may soon be a major problem concerning the successful control of this pathogen. However, from the data reported, the shift in sensitivity detected did not result in total loss of disease control, from this reason the preference to use the term

"reduced-sensitivity" to describe the change in several isolate, hence in the entire population of *Calonectria* , in response to prochloraz fungicide.

Although the nature of the disease and the biology of the fungus play important roles in the development of fungicide resistance, this work provide to establish the threat due to the continuous applications over time of the same a.i., to decrease his activity.

Avoidance of disease control failure due to reduced-sensitivity fungicide development is an important aim for disease control strategies in ornamental-plant crops.

However, further research is required to determine the precise mechanisms about the acquisition and stability of tolerance within a pathogen population, fitness parameter and competitive ability among different isolates.

Overall, management of *Calonectria* diseases in the nursery cannot rely only on chemical control and the steps to developing an integrated program for efficient management of these diseases is complex. Since only preventative measures were found effective for *Calonectria* disease control, chemical control would always be adopted in association with good nursery practices, including reduction of primary inoculum, removal of infected plants and utilization of uncontaminated potting medium. Moreover, the use of other sustainable strategies as well, such as soil solarization or biological control agents could improve disease control.

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