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# Sustainable approaches to control Calonectria diseases in ornamental nursery

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# Contents

## 1. Introduction: Calonectria disease in the Mediterranean basin

In last decades, ornamental plant nurseries have occupied an increasingly important role in the agricultural economy, in particular in southern Italy. For this reason, trade of plants between countries is becoming more frequent. These activities could favour the introduction of new pathogens. Among these, *Calonectria* spp. represent a serious threat for the nursery production in several countries, among which also Italy (Koike *et al*., 1999; Polizzi and Crous, 1999; Schoch *et al*., 2001b; Henricot and Beales, 2003; Lane *et al*., 2006; Pérez-Sierra *et al*., 2007; Chen *et al*., 2011).

The species belonging to the genus *Calonectria* cause several symptoms, such as damping-off, cutting rot, crown and root rot, stem canker, leaf blight and petiole rot on a wide range of plant hosts.

Although in the Mediterranean basin *Calonectria* species were little known until few years ago, today they are a serious threat for crop. The first disease caused by *Calonectria* has been described by Booth and Murray in 1960. He identified a leaf spot of *Hedera helix* caused by *Calonectria hederae* G. Arnaud ex C. Booth. et D. Murray. Later, in 1964, a damping-off of rose cutting caused by *Ca*. *morganii* Crous, Alfenas et M.J. Wingf (as *Cy*. *scoparium*) was detected by Storey.

In Italy, symptoms of root and petiole rot caused by *Cy*. *spathiphylli* Schoult., El-Gholl et Alfieri were detected, for the first time, on *Spathiphyllum* cv. Mauna Loa by Carrai and Garibaldi in 1990.

In South Italy *Ca*. *pauciramosa* is the most important species among these nectroid fungi. This species, found more frequently, is responsible for different symptoms on several plant-host, such as leaf spots on *Arbutus unedo*, *Acacia retinodes*, *Feijoa sellowiana*, *Dodonaea viscosa*, *Pistacia lentiscus*, *Brahea armata*, *Agonis flexuosa* (Polizzi and Catara, 2001; Vitale and Polizzi, 2007; Polizzi *et al*., 2007a; Polizzi *et al*., 2010), crown rot and stem blight on plants of *Pistacia lentiscus* (Vitale and 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*., 2009a; Polizzi *et al*., 2009a).

In 1999, Polizzi and Crous published the first report of a new disease of *Polygala myrtifolia* caused by *Ca*. *pauciramosa* C.L. Schoch et Crous (= *Cy*. *pauciramosum* C.L. Schoch et Crous) in Italy and Europe. Initially this fungus had been identified as *Ca*. *morganii* after isolations from myrtle plants (Polizzi and Azzaro, 1996).

*Ca*. *pauciramosa* has a centre of origin in Central and South America and is well established in Australia, Europe, South Africa and the USA. Variability in the DNA sequence data from a series of *Ca*. *pauciramosa* isolates from Australia, California, Italy, South Africa and South America illustrated the possibility that more than one introduction of this species could have occurred into Italy from South Africa or other countries (Schotch *et al*., 2001a).

Since the early 1990s, *Ca*. *pauciramosa* was reported as agent causal of several diseases in most ornamental nurseries in the areas of southern Italy (Polizzi and Catara, 2001; Polizzi *et al*., 2006a; Vitale *et al*., 2008; Polizzi *et al*., 2009a, 2010), where it causes considerable losses to ornamental plants.

More recently, Polizzi *et al*. (2006b) have identified *Ca*. *morganii* (as *Cy*. *scoparium*) as causal agent of leaf spot, blight and crown rot on mastic trees (*Pistacia lentiscus*). Then, damping-off and leaf spot caused by *Ca*. *morganii* on different cultivars of bottlebrush cuttings were described in Italy (Polizzi *et al*., 2007b). More recently, Polizzi *et al*. (2009b) have linked *Ca*. *morganii* to leaf spot and twig blight on mallee honey myrtle in Italy.

Furthermore, subsequent studies conducted by Vitale and Polizzi in 2008 have shown the co-existence of *Ca*. *pauciramosa* and *Ca*. *morganii* in the same host.

In 1994, in Hampshire *Buxus sempervirens* was identified as host of a new species of the genus *Calonectria*. Symptoms of blight were observed on this plant and the causal agent was identified in *Ca*. *pseudonaviculata* L. Lombard, M.J. Wingf et Crous (as *Cy*. *pseudonaviculatum* Crous, J.Z. Groenew et C. F. Hill) based on DNA sequence and morphological data (Henricot *et al*., 2000; Crous *et al*., 2002).

More recently, in December 2002 Henricot and Culham (2002) described this species as *Cy*. *buxicola* Henricot. More recently, it has been proposed to retain the name of *Cy*. *buxicola* against *Ca*. *pseudonaviculata* (Henricot *et al*., 2012).

Although the origin of this new species is unknown, it was hypothesized that it was first introduced into Europe, where it is widespread in many countries including Austria, Belgium, Croatia, Czech Republic, France, Germany, Italy, the Netherlands, Slovenia, Spain and Switzerland (Crepel and Inghelbrecht, 2003; Brand, 2005; CABI, 2007; Henricot *et al*., 2008; Saracchi *et al*., 2008; Benko Beloglavec *et al*., 2009; Varela *et al*., 2009; Cech *et al*., 2010; Šafránková *et al*., 2012) and then to New Zealand (EPPO, 2004). *Cy*. *Buxicola* represents the main causal agent of blight of different species of *Buxus*, such as *B*. *macrophylla*, *B*. *sempervirens*, *B*. *sinica* and *B*. *colchica* (Henricot *et al*., 2008; Gorgiladze *et al*., 2011).

Symptoms of peg, pod, and root necrosis of peanuts, but also leaf spot, damping-off, blight, crown and root rot of several plant hosts are caused by another species of the genus *Calonectria*, such as *Ca. ilicicola* Boedijn et Reitsma (= *Cy*. *parasiticum* Crous, M.J. Wingf. et Alfenas) (Crous, 2002).

Based on molecular data, *Ca*. *ilicicola* has been identified for the first time as causal agent of crown and root rot on potted of *Laurus nobilis* plants (Polizzi *et al*., 2012). In the Isles of Scilly (UK) a wilting disease on *L*. *nobilis* was first identified caused by *Cy*. *ilicicola*, known as *Ca*. *lauri*, but incorrectly linked to the sexual morph *Ca*. *ilicicola*. Later, based on molecular comparison of the ex type strain, Crous *et al*. (1993b) proved the linkage between *Ca*. *ilicicola* and the asexual morph *Cy*. *parasiticum*. Furthermore, *Ca*. *lauri* was isolated from leaves of *Ilex quaquifolium* in France and Netherlands and from root of *B*. *sempervirens* in Belgium (Lechat *et al*., 2010).

More recently, three new cryptic species, such as *Ca. polizzii*, *Ca. pseudomexicana* and *Ca. tunisiana*, have been described based on multigen phylogenetic analyses, morphological characters and mating compatibility (Lombard *et al*., 2010b, 2011).

*Ca*. *polizzii* L. Lombard, Crous et M.J. Wingf was identified for the first time on *Arbutus unedo* and *Callistemon citrinus* plants showing leaf spot symptoms, but

its pathogenicty was confirmed in 2011 by Lombard *et al*. Currently, it is present as plant pathogen in the Mediterranean basin, in particular in Tunisia and in Italy (Lombard *et al*., 2011; Aiello *et al*., 2013; Vitale *et al*., 2013b).

Furthermore, two new *Calonectria* species, such as *Ca*. *pseudomexicana* L. Lombard, G. Polizzi, et Crous and *Ca*. *tunisiana* L. Lombard, G. Polizzi, et Crous were reported for the first time in Tunisia in an ornamental nursery (Lombard *et al*., 2011). These species reside in *Calonectria scoparia* complex and are closely to *Ca*. *mexicana*. The presence of *Ca*. *mexicana* in the Mediterranean basin, and in particular in Africa, was reported for the first time by Lombard *et al*. in 2011.

These three cryptic species, such as *Ca*. *polizzii*, *Ca*. *tunisiana* and *Ca*. *pseudomexicana*, are responsible for crown and root rot and leaf spot on seedlings of *Callistemon* spp., *Dodonaea viscosa*, *Metrosideros* spp. and *Myrtus communis* (Vitale *et al*., 2013b).

## 2. The genus *Calonectria*: history and taxonomy

The genus *Calonectria* (Ca.) was first identified in 1867 by De Notaris, with *Ca*. *daldiniana* as the type (Rossman, 1979a). Subsequently, this species was incorrectly confronted with *Ca*. *pyrochroa* because it was identified based only on the teleomorph morphology (Rossman, 1979a). The fungi of this genus are characterized by having an ascocarp wall structure that is brightly colored, changing to blood-red in 3 % KOH solution, warty to scaly (Rossman, 1993; Rossman *et al*., 1999). In addition, it is important to stress that *Cylindrocladium* (*Cy*.) is the anamorph of this genus (Rossman, 1993; Rossman *et al*., 1999). The presence of the anamorph allows the identification of some specimens to species level (Schoch *et al*., 2000b; Crous, 2002), considering to the restricted morphological characteristics of the teleomorph (Rossman, 1979b, 1983).

Morgan in 1892 identified for the first time the anamorph genus *Cylindrocladium*, based on *Cy*. *scoparium*, found as a saprobe in a pod of *Gleditsia triacanthos*. This genus is characterized by branched conidiophores producing cylindrical conidia (Morgan, 1892).

More lately, Crous (2002) mentioned that some species belonging to this genus are pathogenic to numerous plants with a wide distribution in sub-tropical and tropical regions.

Taxonomically, the genus *Calonectria* belongs to the family of *Nectriaceae*, one of three family of the order *Hypocreales* (Rogerson, 1970; Rossman, 1983; Rossman, 1996; Rossman *et al*., 1999; Hirooka *et al*., 2012). The *Nectriaceae* is characterized by having uniloculate ascomata that are orange to purple and not immersed in well-developed stromata (Rossman *et al*., 1999).

*Calonectria* is distinguished from the others 20 genera of this family by its *Cylindrocladium* anamorph and its importance as plant pathogens (Lombard *et al*., 2010a).

After the first description of seven *Cylindrocladium* species and their teleomorph (Boedijn and Reitsma, 1950), Rossman in 1983 identified five species, in which it was described the novel *Ca*. *ophiospora* but not its anamorph state.

As a matter of fact, the anamorph morphology is an important characteristic in order to distinguish species of *Calonectria* (Peerally, 1991a). More lately, 10 *Calonectria* species and corresponding *Cylindrocladium* anamorphs were identified. In addition, 16 *Cylindrocladium* species without any associated teleomorph were reported (Lombard *et al*., 2010b).

The genus *Cylindrocladiella* was mistakenly identified as having *Cylindrocladium*-like species with small conidia (Boesewinkel, 1982) and *Nectricladiella* teleomorphs, to synonymy with *Cylindrocladium* (Schoch *et al*., 2000b).

According to Crous and Wingfield (1994), the anamorph characteristics are most important in the taxonomy of *Calonectria* spp. Accordingly, in their monograph on *Cylindrocladium* they detected 22 *Cylindrocladium* species, by associating with them 16 *Calonectria* species, of which five species were assigned to the genus *Cylindrocladiella* based on morphological characters of the holomorph.

In one of the most recent monograph, 28 *Calonectria* species were identified, all associated with *Cylindrocladium* anamorphs (Crous, 2002). Moreover, 18

*Cylindrocladium* species were recognized, although no information was found on their teleomorph states. In detail, seven taxa, belonging to this latter group, were of doubtful authenticity. Until a few years ago, several authors reported a total of 109 *Calonectria* and 96 *Cylindrocladium* species (Crous, 2002; Crous *et al*., 2004b, 2006; Gadgil and Dick, 2004; Lombard *et al*., 2009, 2010c).

At present, by searching on Index Fungorum (www.indexfungorum.org) it is possible found a total of 317 and 92 names records respectively for *Calonectria* and *Cylindrocladium*. A similar search on Mycobank shows a total of 325 and 115 names records respectively for *Calonectria* and *Cylindrocladium* (www.mycobank.org; Crous *et al*., 2004a; Robert *et al*., 2005).

*Calonectria* spp. are characterized by their yellow to dark red perithecia, with scaly to warty ascocarp walls giving rise to long-stalked, clavate asci with 1–multi-septate ascospores and *Cylindrocladium* (*Cy*.) anamorphs (Rossman 1993; Crous, 2002; Lombard *et al*., 2010a).

The genus *Cylindrocladium* is characterized by branched conidiophores with stipe extension terminating in characteristics vesicles and producing cylindrical, 1-multispetate conidia (Crous and Wingfield, 1994; Crous, 2002).

The anamorph, that is the state most common in nature, showed different types of morphological characters for distinguishing *Calonectria*, such as vesicle shape, stipe extension length, conidial septation, and dimensions on a standardized medium under defined growth conditions (Boesewinkel, 1982; Peerally, 1991a; Crous and Wingfield, 1994; Schoch *et al*., 2001a; Crous, 2002). Moreover, it is important to stress that some intraspecific variation in vesicle shape and conidial dimension can be common, resulting in confusion in the taxonomic level (Crous and Peerally, 1996; Crous *et al*., 1998a).

*Calonectria* spp. differ three different morphological forms of conidia, of which the macroconidia are present in all but *Ca*. *multiseptata* (Peerally, 1991a; Crous and Wingfield, 1994; Crous *et al*., 1998b; Crous, 2002). Mega- and microcondia are less frequent, so they are not considered as important characters to identify different species (Sobers, 1971; Crous and Wingfield, 1994; Crous and Seifert, 1998a; Crous, 2002).

*Calonectria* species have homothallic and heterothallic mating systems (Alfieri *et al*., 1982; Schubert *et al*., 1989; Crous and Wingfield, 1994; Crous, 2002). Heterothallic *Calonectria* spp. have a biallelic heterothallic mating system with the female structures (protoperithecia) spermatised by conidia or hyphae of an opposite mating type strain (Schoch *et al*., 1999, 2000a, 2001a). Crous (2002) points out that some *Calonectria* spp. has retained the ability to recombine with other closely related *Calonectria* spp., although the progeny from these crosses have low levels of fertility. This has complicated the application of the biological species concept for *Calonectria*, although it has been useful for some species (Schoch *et al*., 1999; Lombard *et al*., 2010a, b).

### 2.1. Importance of *Calonectria*

After the first identification as a saprobe (Graves, 1915), Massey in 1917 detected the pathogenicity of *Calonectria* species, and in particular Anderson in 1919 proved the pathogenicity of *Ca*. *morganii* (as *Cy*. *scoparium*).

Initially, *Calonectria* species were recognized as causal agent of disease on 30 plant families (Booth and Gibson, 1973; French and Menge, 1978; Peerally, 1991a; Wiapara *et al*., 1996; Schoch *et al*., 1999). More recently, Crous has reported *Calonectria* species as causal agents of disease symptoms on about 100 plant families and 335 plant host species, among which important forestry, agricultural and horticultural crops (Crous, 2002).

In Europe and in Asia, *Calonectria* species are capable to cause diseases on horticulture crops, especially in garden and ornamental commercial nurseries (Polizzi and Crous, 1999; Polizzi, 2000; Crous, 2002; Henricot and Culham, 2002; Pérez-Sierra *et al*., 2007; Polizzi *et al*., 2007a, b; Hirooka *et al*., 2008; Polizzi *et al*., 2009a, b, c; Vitale *et al*., 2009a). In detail, plants belonging to the families of *Anacardiaceae*, *Aquifoliaceae*, *Araceae*, *Araliaceae*, *Arecaceae*, *Asteraceae*, *Buxaceae*, *Ericaceae*, *Myrtaceae*, *Polygaceae*, *Rhamnaceae* and *Rosaceae* are attacked by these pathogens, with several disease symptoms such as crown rot, collar rot and root rot, leaf spots, and cutting rot (Massey, 1917; Anderson, 1919; Storey, 1964; Aragaki *et al*., 1972, 1988; Peerally, 1974; de Prest and Poppe, 1988; Carrai and Garibaldi, 1990; Uchida and Kadooka, 1997;

Litterick and McQuilken, 1998; Polizzi and Crous, 1999; Polizzi 2000; Crous, 2002; Polizzi and Catara, 2001; Henricot *et al*., 2000; Henricot and Culham, 2002; Henricot and Beales, 2003; Poltronieri *et al*., 2004; Lane *et al*., 2006; Polizzi *et al*., 2006a, b; Pérez-Sierra *et al*., 2006, 2007; Polizzi *et al*., 2007a, b; Vitale and Polizzi, 2007; Aghajani *et al*., 2008; Henricot *et al*., 2008; Hirooka *et al*., 2008; Vitale *et al*., 2008; Polizzi *et al*., 2009c; Vitale *et al*., 2009a; Lechat *et al*., 2010; Alfenas *et al*., 2013b).

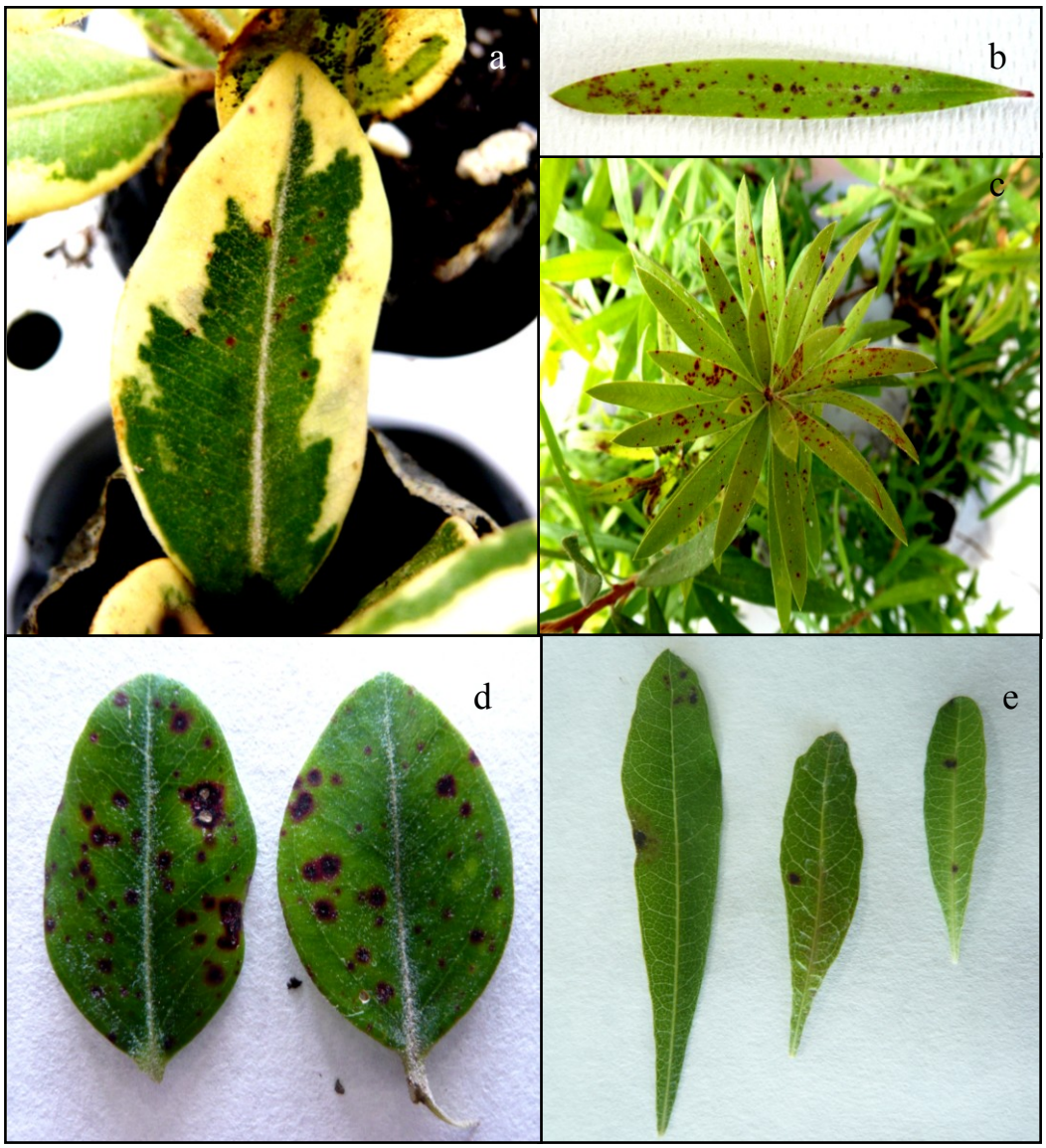


**Figure 1 - Leaf spots on *Metrosideros excelsa* "aurea" (a), *Callistemon* spp. (b,c), *Metrosideros excelsa* (d), *Dodonaea viscosa* (e)**

In agriculture, several economically important crops have been reported as susceptible plants to infections of *Calonectria* species. In particular, *Calonectria* infections are very common in the family of *Fabaceae* and *Solanaceae*. *Ca*. *ilicicola*, *Ca*. *pyrochroa*, *Ca*. *brassicae* (as *Cy*. *gracile*) were reported as responsible for Cylindrocladium black rot on *Arachis hypogeal* (peanut), red crown rot of *Glycine max* (both in USA) and *Cylindrocladium* tuber rot of *Solanum tuberosum* (Brazil), respectively (Bell and Sobers, 1966; Beute and Rowe, 1973; Rowe *et al*., 1973; Sobers and Littrell, 1974; Rowe and 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 AF de, 1991; Hollowell *et al*., 1998; Kim *et al*., 1998; Boedijn and Reitsma, 1950; Bolkan *et al*., 1980, 1981). Other diseases associated to *Calonectria* species on agricultural crops include root rot and leaf diseases of fruit bearing and spice plants (Jauch, 1943; Wormald, 1944; Sobers and Seymour, 1967; Nishijima and Aragaki, 1973; Milholland, 1974; Krausz and Caldwell, 1987; Hutton and Sanewski, 1989; Anandaraj and Sarma, 1992; Risède, 1994; Jayasinghe and Wijesundera, 1996; Risède and Simoneau, 2001; Vitale and Polizzi, 2008), post-harvest diseases of fruits (Fawcett and Klotz, 1937; Boedijn and Reitsma, 1950; Sepiah, 1990; Fitzell and Peak, 1992; Vaidya and Rao, 1992; Sivapalan *et al*., 1998), root and crown rot of *Medicago sativa* (alfalfa) (Ooka and Uchida, 1982; Hwang and Flores, 1987) and sheath net blotch of *Oryza sativa* (Crous, 2002).

These plant pathogens also cause diseases on some forestry plant families, such as *Fabaceae*, *Meliaceae*, *Myrtaceae* and *Pinaceae*. In particular, they are responsible for symptoms of cutting rot (Crous *et al*., 1991; Crous, 2002; Lombard *et al*., 2009, 2010d; Alfenas *et al*., 2013a), damping-off (Batista, 1951; Cox, 1953; Terashita and Itô, 1956; Sharma and Mohanan, 1982; Sharma *et al*., 1984; Crous *et al*., 1991; Brown and Ferreira, 2000; Crous, 2002; Taniguchi *et al*., 2008) leaf diseases (Cox, 1953; Hodges and May, 1972; Barnard, 1984; Sharma *et al*., 1984; El-Gholl *et al*., 1986; Peerally, 1991a,b; Crous *et al*., 1993b; Crous and Wingfield, 1994; Crous *et al*., 1998b; Schoch and Crous 1999; Schoch *et al*., 1999; Booth *et al*., 2000; Park *et al*., 2000; Crous and Kang, 2001; Gadgil and Dick, 2004; Lombard *et al*., 2010b, 2011; Alfenas *et al*.,

2013a), shoot blight (Sharma *et al*., 1984; Crous *et al*., 1991, 1998b; Crous and 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 and May, 1972; Cordell and Skilling, 1975; Mohanan and Sharma, 1985; Crous *et al*., 1991; Lombard *et al*., 2009; Alfenas *et al*., 2013a, b).

Whereas, in many cases, *Calonectria* species cause disease in seedling and cutting production, on the other in few cases *Cylindrocladium* species cause leaf disease e shoot blight resulting in defoliation of trees leading to loss of vigour (Hodges and May, 1972; Sharma *et al*., 1985; Booth *et al*., 2000; Park *et al*., 2000; Crous and Kang, 2001; Crous, 2002; Old *et al*., 2003; Rodas *et al*., 2005).

## 2.2. Epidemiology

As mentioned before, species of *Calonectria* (*Ca*.) are commonly reported as responsible for several disease symptoms, including crown, root and stem rot, stem or crown canker, blight, root and pod rot (Crous, 2002).

At first, several species of this genus were reported as saprobe (Graves, 1915). Moreover, after its first identifications as causal agent of disease on rose, *Cy. scoparium* was reported as responsible for several disease symptoms, such as damping-off, root rot, crown canker, fruit rot, stem lesions, tuber rot, etc. (Massey, 1917; Anderson, 1919; Crous *et al*., 1991).

*Calonectria* species are polycyclic fungi. They are able to differentiate chlamydospores, which form microsclerotia, in soil and senescing plant tissue. Microsclerotia represent the primary inoculum of *Calonectria* spp. in the soil (Phipps *et al*., 1976), where they are present in the infected plant material, such as leaves or stems, fall to the ground and release them in the soil.

Bugbee and Anderson in 1963 (a) showed that the pathogen formed microsclerotia in infected needles. *Cy. scoparium* produced microsclerotia in substomatal chambers in leaf spot on azalea 10 days after inoculation (Reis and Chaves, 1967).

Although microsclerotia aren't capable to survive well in the soil if the water content is low (Sung *et al*., 1980), they have vitality than about 15 years even

without host plant (Thies and Patton, 1970; Sobers and Littrell, 1974), as long as there is an adequate soil moisture level (Pataky and Beute, 1983).

Microsclerotia are able to survive in the soil with an optimal temperature of 25 °C, even without soil moisture (Almeida and Bolkan, 1981). Anderson in 1919 reported the presence of microsclerotia at depth of up to 66 cm below the soil surface.

Some authors performed experiments to evaluate the effect of heat on the vitality of microsclerotia. Certain levels of heat produced by an Egedal bed steamer are able to reduce microsclerotia of *Cy*. *floridanum* present at 5-10 cm soil depths. Moreover, at the same time, microsclerotia present at or below 15 cm have maintained their vitality (Dumas *et al*., 1998). In fact, moisture, soil temperature and depth of soil are three important factors that can determine the level of survival of microsclerotia (Phipps and Beute, 1977, 1979; Roth *et al*., 1979; Taylor *et al*., 1981; Pataky and Beute, 1983; Crous, 2002; Kuruppu *et al*., 2004). Moreover, it was demonstrated that solarization effectively suppressed *Ca*. *pauciramosa* microsclerotia (Polizzi *et al*., 2003). Subsequently, Vitale *et al*. (2013a) confirmed that short-term soil salarization is capable to suppress *Calonectria* microsclerotia in nurseries and demonstrated that different solarizing materials, such as ETFE, could improve this technique.

The infection can also start with the germination of conidia or ascospores. The presence of water or humidity, represented by rain and irrigation practices, plays a key rule during this process, creating an environment favorable to the development of infection. Moreover, the onset of disease caused by these pathogens is favoured by conditions of nutrient stress of seedlings (Arentz, 1991).

In ornamental nurseries, the movement of water is one of the main factors in the spread of conidia originating from microsclerotia, onto potting mixes and plants (Vitale *et al*., 2013b).

On host plant, conidia are able to germinate rapidly, as followed reported. Indeed, Sharma and Mohanan 1990 asserted that conidia of *Cy*. *quinqueseptatum* germinate faster *in vivo* than *in vitro*. It was demonstrated that it isn't true that *Cy*. *reteaudii* could only infect eucalypts through stomata

(Bolland *et al*., 1985), because this pathogen penetrates directly, without need to appressoria over stomata for stomatal penetration (Sharma and Mohanan, 1990). This type of penetration is spread in many *Calonectria* species, as observed for *Cy*. *pauciramosum*. Some *Calonectria* species are able to produce phytotoxin that influence the virulence of pathogen. It was reported that *Cy*. *scoparium* and and *Cy*. *pteridiidis* were able to produce the phytotoxin Cyl-2 (Hirota *et al*., 1973; Nikolskaya *et al*., 1995).

**Figure 2 - Wilting and crown rot caused by *Calonectria pauciramosa* on *Polygala myrtifolia***

## 2.3. Morphology

For many years, the description of fungal species has been performed taking into consideration morphological and phenotypic characters (Brasier, 1997; Taylor *et. al*., 2000; McNeill *et al*., 2005). However, nowadays this is an old method that cannot allow to distinguish different *Calonectria* species due to their morphological similarity (Lombard *et al*., 2010c; Lombard *et al*., 2011; Chen *et al*., 2011). Thus, biological and phylogenetic characters are the basis for the identification of new *Calonectria* species (Rossman, 1996; Brasier, 1997; Taylor *et al*., 2000; Crous *et al*., 2004b, 2006).

Also, the characterization of the genus *Calonectria* and of its anamorph *Cylindrocladium* has been based on morphology of its species (Lombard *et al*., 2010a).

Until the 1990s, sexual compatibility and mostly morphological characteristics were used to identify different species (Boedijn and Reitsma, 1950; Peerally 1991a; Crous *et al*., 1992; Crous and Wingfield, 1994; Crous, 2002), thanks to which several complexes, such as *Ca*. *scoparia* complex (Schoch *et al*., 1999), *Ca*. *brassicae* (as *Cy*. *gracile*) complex (Crous *et al*., 2004b) and *Ca*. *kyotensis* complex (Crous *et al*., 2006) were identified.

Vesicle shape, stipe extension length and macroconidial septation and dimensions are characteristics of the anamorphs, widely used for identifications (Boesewinkel, 1982; Peerally, 1991a; Crous and Wingfield, 1994; Crous, 2002). In particular, on the one hand some authors stated that vesicle morphology was one of the most important taxonomic criteria to distinguish species of *Cylindrocladium* (Sobers, 1968; Peerally, 1973; EI-Gholl *et al*., 1986; EI-Gholl *et al*., 1989; Peerally, 1991a), but on the other, some authors asserted that this characteristic was highly variable (Hunter and Barnett, 1978; Rossman, 1983). More lately, some studies demonstrated that this criterion is reliable when examined on carnation-leaf agar (CLA), under pre-determined conditions of incubation (Fisher *et al*., 1982; Crous *et al*., 1992). Besides, ascospore septation and dimensions, ascospore number within the asci and perithecial colour are the most important morphological characteristics of the teleomorph used for identifications. In detail, it is important to emphasize that perithecia of

*Calonectria* species shouldn't be used in identifications because they are morphologically very similar (Crous and Wingfield, 1994; Crous, 2002).

*Calonectria* spp. has widely been studied by culturing colonies on carnation leaf agar medium (CLA) (Fisher *et al*., 1982; Crous, 2002). More recently, the diagnosis of the genus *Calonectria* is based on the use of synthetic nutrient-poor agar (SNA; Nirenburg, 1981; Lombard *et al*., 2009, 2010) and minimal salt agar (MSA; Guerber and Correll, 2001; Halleen *et al*., 2006; Lombard *et al*., 2010b) with sterile toothpicks, which have been used to induce the morphological characters (Lombard *et al*., 2010b).

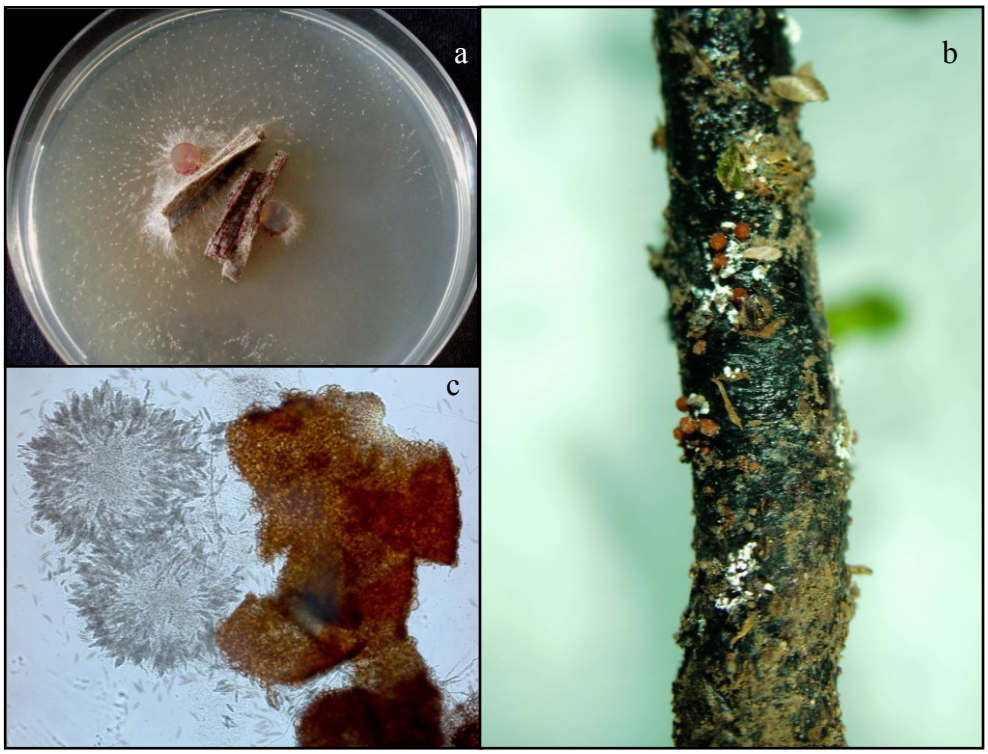


**Figure 3 - Perithecia, asci and ascospore of *Calonectria pauciramosa* on PDA medium (a,c); perithecia of *Calonectria ilicicola* on *Laurus nobilis* (b)**

The use of malt extract agar (MEA) as growth medium is an effective method to determine the size of conidia and other features. In addition, optimal growth temperatures are generally determined on MEA at 5-35 °C in 5°C intervals in the dark, while colony colours were determine after 7 day on MEA at 25 °C in the dark (Vitale *et al*., 2013b).

In addition, substrate utilization and cell wall polysaccharide are biochemical techniques, which could be used in phenotypic characterization. Different

*Cylindrocladium* species have been identified by using aminopeptidase specificity (Stevens *et al*., 1990) and utilisation of nitrogen and carbon (Hunter and Barnett, 1978; Sharma *et al*., 1992). The identification of *Cylindrocladium* species and of its teleomorph is possible by using polysaccharides extracted from cell walls of *Cylindrocladium* (Ahrazem *et al*., 1997). However, some species belonging to the same complex cannot be separate based solely on the use of this method (Crous, 2002).

Relatively to the optimum growth temperature, most of the *Calonectria* species, such as *Ca*. *morganii*, *Ca*. *pauciramosa* and *Ca polizzii*, are considered eurithermal with a temperature range of less than 10°C and 30°C, while others, such as *Ca*. *mexicana*, *Ca*. *spathiphylli* and *Ca*. *ilicicola* are high-temperature species, growing well both at 10°C or above and 30°C or above. Instead, *Ca*. *pseudonaviculata* has a thermal optimum between 5°C and 35°C (Crous *et al*., 2002). This classification has been drawn up taking into account different growth temperatures between *Calonectria* species (Crous, 2002).

### 2.4. Mating compatibility and strategies

In the genus *Calonectria* the identification of new species required the use of mating strategies (Schoch *et al*., 1999; Crous, 2002), which has allowed the identification of about 18 homothallic and 34 heterothallic *Calonectria* species (Crous, 2002; Crous *et al*., 2004b; Gadgil and Dick, 2004; Crous *et al*., 2006). Moreover, it was found that the heterothallic species showed a biallelic mating system (Schoch *et al*., 1999).

In the genus *Calonectria* some species are self-sterile hermaphrodites, which required fertilization from an opposite mating type. This feature has been demonstrated by studies on female fertility of *Cylindrocladium*, as described for other heterothallic ascomycetes (Schoch *et al*., 1999, 2000a,b, 2001a; Leslie and Klein, 1996).

The application of biological species concept (BCS) has shown several issues, in particular when genetically isolated fungal strains retain the ancestral ability to recombine to produce viable progeny (Brasier, 1997; Taylor *et al*., 1999, 2000; Kohn, 2005). More narrowly, the formation of fertile perithecia requires the

presence of a further isolate that does not contribute to the genetic make-up of the progeny (Overmeyer, *et al*., 1996; Neubauer and Zinkernagel, 1995). *Ca*. *colombiana* and *Ca*. *zuluensis*, two new species of *Ca*. *scoparia* complex, have a homotallic mating system, which are distinct from *Ca*. *pauciramosa* with a biallelic, heterothallic mating system (Schoch *et al*., 2001a; Lombard *et al*., 2010a). Although *Ca*. *polizzii* is a species closely related to *Ca*. *pauciramosa*, its isolates were not able to mate with either of the tester strains of *Ca*. *pauciramosa* or other *Ca*. *pauciramosa* isolates from different geographic regions (Lombard *et al*., 2010a).

### 2.5. Multigene phylogeny

Although the morphological and phenotypic characters have been played an important role in the description of *Calonectria* species, nowadays it has been set aside with more focus on biological and phylogenetic characters (Rossman, 1996; Brasier, 1997; Taylor *et al*., 2000).

The identification of species, based on the morphological characters and sexual compatibility using standardized media (Boedijn and Reitsma, 1950; Peerally, 1991a; Crous *et al*., 1992; Crous and Wingfield, 1994; Crous, 2002), resulted in the identification of several species complexes, as many *Cylindrocladium* species are morphologically very similar.

The identification of the anamorph is based on the vesicle shape, stipe extension length and macroconidial septation and dimensions (Boesewinkel, 1982; Peerally, 1991a; Crous and Wingfield, 1994; Crous, 2002). Ascospore septation and dimensions, ascospore number within the asci and perithecial colour are the characteristics used to identify the teleomorph. As mentioned previously, perithecia of *Calonectria* species are morphologically very similar and these are not typically useful in identifications (Crous and Wingfield, 1994; Crous, 2002).

One of the techniques, that can be used to separate the different *Calonectria* species, is the biochemical one, which can also be employed in the phenotypic characterization (Lombard *et al*., 2010c).

This technique allowed to distinguish different *Cylindrocladium* species, based on the use of aminopeptidase specificity (Stevens *et al*., 1990) and utilization of

nitrogen and carbon (Hunter and Barnett, 1978; Sharma *et al*., 1992). However, this method has been allowed the identification of some species within complex not easily discernible (Crous, 2002).

The taxonomy of this genus has been influenced by phylogenetic studies, which allowed to distinguish the anamorph from teleomorph. The first phylogeny of the genus *Cylindrocladium* was published by using β-tubulin sequence (Schoch *et al*., 2001b). Thanks to application of molecular techniques and particularly DNA sequence, it was possible the recognition of several cryptic species (Lombard *et al*., 2010c). The multi-gene approach is very important for studying the phylogenetic relationships of phenotypic closely related *Calonectria* spp. (Lombard *et al*., 2010b).

Although a serious of molecular approaches has been useful, such as total protein electrophoresis (Crous *et al*., 1993a; El-Gholl *et al*., 1993), isozyme electrophoresis (El-Gholl *et al*., 1992, 1997; Crous *et al*., 1998b), random amplification of polymorphic DNA (RAPD) (Overmeyer *et al*., 1996; Victor *et al*., 1997; Schoch *et al*., 2000a; Risède and Simoneau, 2004) restriction fragment length polymorphisms (RFLP) (Crous *et al*., 1993b, 1995, 1997b; Jeng *et al*., 1997; Victor *et al*., 1997; Risède and Simoneau, 2001) and DNA hybridization (Crous *et al*., 1993b, 1995, 1997a; Victor *et al*., 1997), important results in the taxonomy of *Calonectria* have been obtained by applying DNA sequence comparisons and associated phylogenetic inference (Lombard *et al*., 2010c).

The first distinction between two different species such as *Cy. scoparium* and *Cy. floridanum* was obtained by employing 5.8S ribosomal RNA gene and flanking internally transcribed spacers (ITS) sequences (Jeng *et al*., 1997).

Although past studies have shown that the ITS gene region provides limited information to distinguish *Calonectria* spp. (Schoch *et al*., 1999, 2001b; Crous, 2002; Henricot and Culham, 2002; Crous *et al*., 2004b, 2006), it is still a very importance diagnostic tool (Vitale *et al*., 2013b). However, the β-tubulin (Schoch *et al*., 2001b) and histone H3 (Kang *et al*., 2001a) gene regions have been applied for the identification of cryptic species within *Calonectria* species complexes (Lombard *et al*., 2010a).

Although, at first, β-tubulin (BTUB) sequence data was the major phylogenetic study used in the taxonomy of *Calonectria* (Schoch *et al*., 2000b), subsequently taxonomic studies were developed by using multigene DNA sequence data relative to the nuclear ribosomal internal transcribed spacer (ITS), BTUB, Histone H3 (HIS3) and translation elongation factor 1-α (TEF-1α) (Crous *et al*., 1999; Schoch *et al*., 2000a,b; Crous and Kang, 2001; Kang *et al*., 2001a,b; Henricot and Culham, 2002; Crous *et al*., 2004b, 2006; Lombard *et al*., 2009, 2010). The first comprehensive multigene data set of *Calonectria* has been provided by Lombard *et al*. (2010d). He obtained these results by applying seven gene regions such as 28S large subunit (LSU), actin (ACT), β-tubulin, calmodulin (CAL), HIS3, ITS and TEF-1α, and was able to identify 11 species complexes.

More lately, translation elongation 1-alpha (TEF-1α) and calmodulin are the most common gene sequences used for the identification of the species (Crous *et al*., 2004b; Lombard *et al*., 2010a).

### 2.6. *Calonectria morganii* complex

Species belonging to *Calonectria morganii* complex are characterised by having uniseptate macroconidia and vesicles varying from pyriform to obpyriform or ovoid to ellipsoidal. This complex includes *Ca*. *cerciana* L. Lombard, M.J. Wingf. and Crous, *Ca*. *insularis* C.L. Schoch and Crous, *Ca*. *morganii*, *Ca*. *sulawesiensis*, *Ca*. *hawksworthii* (Peerally) L. Lombard, M.J. Wingf. and Crous, *Ca*. *leucothöes* (El-Gholl, Leahy and T.S. Schub.) L. Lombard, M.J. Wingf. and Crous, *Ca*. *variabilis* Crous, B.J.H. Janse, D. Victor, G.F. Marias and Alfenas and *Ca*. *brasiliensis* (Peerally) L. Lombard, M.J. Wingf. and Crous and *Ca*. *hodgesii* (Schoch *et al*., 2001a; Crous, 2002; Lombard *et al*., 2010d; Alfenas *et al*., 2013a).

#### 2.6.1. *Cylindrocladium scoparium*

*Cylindrocladium scoparium* Morgan (teleomorph *Calonectria morganii* Crous, *Al*fenas and M. J. Wingf) is the type species of the anamorph genus *Cylindrocladium* Morgan. A polyphagous fungal species, widely reported as

causal agent of disease on over 30 plant families belonging to *Gymnosperms* and *Angiosperms* (Both and Gibson, 1973; Bertus, 1976; Frence and Menge, 1978; Peerally, 1991a; Wiapara *et al*., 1996). In addition, it was reported as plant pathogen both in forest and ornamental nurseries (Bugbee and Anderson, 1963a, b). It was isolated for the first time from a dead pod of honey locust in Ohio (Morgan, 1892).

This pathogen is able to cause different symptoms such as crown and root rot, leaf spots, damping-off, stem canker, cutting rot, needle blight, epicormic growth, death of trees seedling and shoot blight (Batista 1951; Cordell and Rowan, 1975; Cordell and Skilling, 1975; Ferreira, 1989; Neubauer and Zinkernagel, 1996). In addition, this species was reported as causal agent of wilt disease in Rhododendron and Azalea (Backhaus, 1994) and of fruit rot in post-harvest (Sivapalan *et al*., 1998).

Although *Cy*. *scoparium* has been reported from Africa (Doidge, 1950; Darvas *et al*., 1978; Botha and Crous, 1992), South America (Palmucci *et al*., 1996; Tozzetto and Ribeiro, 1996), Europe (Overmayer *et al*., 1996; Polizzi and Azzaro, 1996), Asia (Mohanan and Sharma, 1985; Srinivasan and Gunasekaran, 1995) and New Zeeland (Wiapara *et al*., 1996), its presence has only been confirmed from North and South America (Crous *et al*., 1993a) and, recently, in Europe (Polizzi *et al*., 2006b). Furthermore, recently, several studies have shown that many of these reports were incorrectly ascribed to *Cy*. *scoparium* (Schoch *et al*., 1999), because some isolates have proven to be *Cy*. *pauciramosum* C.L. Schoch and Crous, which forms part of the *Cylindrocladium candelabrum* species complex (Schoch *et al*., 1999).

In Sicily, *Cy*. *scoparium* has been initially reported as causal agent of leaf spot, blight and crown rot on mastic tree seedlings (*Pistacia lentiscus*) (Polizzi *et al*., 2006b). The next year, Polizzi *et al*. (2007b) reported this pathogen as causal agent of damping-off and leaf spot on different *Callistemon* cultivars. More recently, in Sicily, it has been observed that *Cy*. *scoparium* caused leaf spot and shoot blight on malle honeymyrtle (*Melaleuca acuminate* F. Muell) (Polizzi *et al*., 2009b). All these first reports confirm the spread of this pathogen in Sicilian ornamental nurseries.

*Calonectria morganii* is newly described as the teleomorph of *Cylindrocladium scoparium*.

*Ca*. *morganii* Crous, Alfenas and M.J. Wingfield is characterized by having perithecia solitary or in groups, yellow to orange, which become red-brown with age. In section, perithecia show apex orange to dark orange, body yellow to orange, base red-brown, globose to subglobose, 280-520 µm high, 280-400 µm diameter, apex turning pale red to red, body turning red, and base red-brown to dark red-brown (KOH+). The perithecia wall consists of an outside layer of textura globulosa, 30-60 µm wide, an inner layer of textura angularis of 15-25 µm wide and a center one that is composed of a thin-walled and hyaline. Asci are clavate, 75-100x 8-15 µm, tapering to a long thin stalk, and contain ascospores aggregated in the upper third of the ascus, hyaline, guttulate, fusoid with rounded ends, straight to slightly curved, 1-septate. Macroconidiophores consist of a stipe septate, hyaline, smooth, 60-110 x 6-7 µm, terminating in an ellipsoidal to pyriform or clavate vesicle, with a diameter of 6-8 µm. Conidiogenous apparatus is 60-110 µm long and 60-100 µm wide. Its primary branches are aseptate or 1-septate with dimensions of 11-40 x 4-5 µm, while the secondary and tertiary are aseptate with dimensions of 11-20 x 4-5 µm, 10-15 x 4-5 µm, respectively. Also, additional branches are 6-aseptate with dimensions of 10-15 x 3-4 µm. Each terminal branche produce 2-6 phialides, that are doliiform to reniform, hyaline, aseptate, with dimension of 9-15 x 3-4 µm. Conidia are cylindrical, rounded at both ends, straight, (40-)42-50(-66) x 3-4(-5) µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Megaconidia and microconidia are unknown (Crous, 2002).

*Cy*. *scoparium*, on cultural medium such as Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA), presents colonies amber brown to buckthorn brown to sayal brown. Chlamydospores extensive, dense, distributed throughout medium, form microsclerotia. Its optimum growth temperature is 25-30 °C, with minimum above 5°C and maximum above 35°C (Crous, 2002).

### 2.7. *Calonectria scoparia* complex

The *Calonectria scoparia* species complex is characterized by species having small, 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). The complex was previously considered as having a biallelic, heterothallic mating system (Schoch *et al*., 1999, 2001a). The morphological similarities between *Ca*. *morganii* (= *Cylindrocladium scoparium*) and *Ca*. *scoparia* (= *Cy*. *candelabrum*) didn't allow their distinction. Indead, these two *Calonectria* species are different: the anamorph state of *Ca*. *morganii* is characterized by having ellipsoidal to pyriform vesicles and *Ca*. *scoparia* by having ellipsoidal to obpyriform vesicles (Crous *et al*., 1993a).

If on the one hand, incorporating DNA sequence data proved that *Ca*. *morganii* is limited to the Northern Hemisphere and Brazil (Crous *et al*., 1993a; Overmeyer *et al*., 1996; Schoch *et al*., 2000a), on the other *Ca*. *scoparia* constitutes 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*. *pseudoscoparia*, *Ca*. *mexicana* (anamorph: *Cy*. *mexicanum*) and *Ca*. *spathulata* (Schoch *et al*., 1999). *Ca*. *colombiana*, *Ca*. *zuluensis* and *Ca*. *polizzii* increase the number of species of *Ca*. *scoparia* complex. Among these species, *Ca*. *polizzii* is morphologically closely related to *Ca*. *pauciramosa*, from which it is considered different for its smaller 1-septate macroconidia (Lombard *et al*., 2010a).

More recently, two new species belonging to the genus *Calonectria*, such as *Ca*. *pseudomexicana* L. Lombard, G. Polizzi and Crous and *Ca*. *tunisiana* L. Lombard, G. Polizzi and Crous, were added to this complex (Lombard *et al*., 2011).

In 2013, a new species of *Calonectria* has been isolated from infected plants of *Metrosideros polymorpha*. The isolated fungal pathogen has not previously described, so that it has been identified as *Calonectria metrosideri* R.F. Alfenas, O.L. Pereira, P.W Crous and A.C. Alfenas, sp. nov (Alfenas *et al*., 2013b). Through phylogenetic analysis, this species was found closely related to other species of the *Calonectria scoparia* complex (Schoch *et al*., 1999; 2001b).

### 2.7.1. *Calonectria mexicana*

*Calonectria mexicana* has only been reported from soil samples collected in Mexico (Schoch *et al*. 1999; Crous, 2002), until it has been observed for the first time its pathogenicity and reported as causal agent of leaf spot, crown rot and root rot on young plants of *Dodonaea viscosa*, *Callistemon* spp., *Metrosideros* spp. and *Myrtus communis* (Lombard *et al*., 2011).

*Ca*. *mexicana* C.L. Schoch and Crous (anamorph: *Cylindrocladium mexicanum* C.L. Schoch and Crous) belong to the *Ca*. *scoparia* complex (Schoch *et al*., 1999). The distinction of this species from the other seven *Calonectria* spp. in the complex is based on their unique, widely ellipsoidal and papillate vesicles (Schoch *et al*., 1999, Lombard *et al*., 2010a; Chen *et al*., 2011).

*Ca*. *mexicana* is characterized by having perithecia solitary or in groups, orange to red. In section its perithecia show apex and body orange to red, base red-brown, subglobose to ovoid, 400-500 µm high, 350-450 µm diameter, body turning dark red-brown, and base dark red-brown (KOH+). Perithecial walls consist of two thick-walled layers: outside layer of *textura globulosa*, 35-90 µm wide, becoming more compressed towards inner layer of *textura angularis*, 5-15 µm wide, becoming thin-walled and hyaline towards the center. Outer cells are 20-35 x 20-30 µm, while inner cells are 5-15 x 4-6 µm (Crous, 2002).

Asci 8-spored are calvate, 70-120 x 10-20 µm, tapering to a long thin stalk, and contain ascospores aggregated in the upper third of the ascus, hyaline, guttulate, fusoid with rounded ends, straight to slightly curved, 1-septate, (35-)40-55(-65) x 5-6(-7) µm (Crous, 2002).

Macroconidiophores consist of a stipe, a penicillate arrangement of fertile branches, a stipe extension and terminating in a broadly ellipsoidal vesicle with papillate apex, (7-)8-10(-12) µm of diameter. Conidiogenous apparatus is 40-70 µm long and 25-60 µm wide. Its primary branches are aseptate or 1-septate with dimensions of 17-45 x 4-6 µm, while the secondary, tertiary and quaternary branches are aseptate with dimensions of 15-25 x 4-5 µm, 11-17 x 3-5 µm and 10-15 x 2.5-4 µm, respectively. Each terminal branche produce 2-6 phialides, that are doliiform to reniform, hyaline, aseptate, with dimension of 7-16 x 3-4 µm. Conidia are cylindrical, rounded at both ends, straight, (35-)40-48(-52) x 3-

4(-4.5) µm, 1- septate, lacking a visible abscission scar. Megaconidia and microconidia of *Ca*. *mexicana* are unknown.

The colonies of this fungus are orange to sienna, present irregular margin with extensive chlamydospores and sparse sporulation on aerial mycelium. Its optimum temperature is 25-30°C, with minimum above 10 °C and maximum above 35 °C (Crous, 2002).

### 2.7.2. *Calonectria pauciramosa*

*Calonectria pauciramosa* C.L. Schoch and Crous, with its anamorph *Cy*. *pauciramosum* C.L. Schoch and Crous, is one of the species most frequently encountered of *Calonectria scoparia* complex (Schoch *et al*., 1999).

*Ca*. *pauciramosa* is an important plant pathogen, responsible for several diseases on numerous host plants, with different symptoms such as damping-off, cutting rot, crown and root rot (Schoch *et al*., 1999; Koike *et al*., 1999; Koike and Crous, 2001; Polizzi and Crous, 1999; Polizzi, 2000; Polizzi and Catara, 2001; Polizzi and Vitale, 2001; Crous, 2002; Polizzi *et al*., 2006a, 2007a, 2009a,c; Vitale *et al*., 2009a).

In 1986, Lamprecht isolated for the first time *Ca*. *pauciramosa* from *Medicago truncatula*. More lately, this pathogen has been identified as causal agent of disease on *Rododendron* spp., *Azalea* spp., *Eucalyptus* spp. and *Protea* spp. (Botha and Crous, 1992).

*Ca*. *pauciramosa* is a polycyclic fungal species, common in many countries in the world, such as Australia, New Zeeland, Brazil, Colombia, Mexico, and South Africa. In the following years, this pathogen has been reported for the first time in North America on *Erica capensis* (Koike *et al*., 1999).

In Europe, and in particular in Italy, the presence of *Ca*. *pauciramosa* has been reported and confirmed on *Polygala myrtifolia* (Polizzi and Crous, 1999).

Subsequently, this *Calonectria* species has been identified as responsible for disease on *Fejioa sellowiana*, *Arbutus unedo*, *Acacia retinodes* and *Dodonaea viscosa* (Polizzi and Catara, 2001).

Nowadays, it is a pathogen widely spread in the nurseries of the South of Italy, and especially in the eastern of Sicily. In 1996, Polizzi showed that *Ca*.

*pauciramosa* were able to cause several symptoms, such as leaf spots, defoliation and stem blight, on bottlebrushes, blue eucalyptus, red eucalyptus, melaleuca, myrtle and *Metrosideros* spp., which are the most susceptible species belonging to the family of *Myrtaceae* (Polizzi, 1996).

Furthermore, in South Africa and in Australia *Ca*. *pauciramosa* has been reported as causal agent of disease on forest plants in nurseries (Crous, 2002). In addition, this pathogen has been also reported as causal agent of disease on horticultural crops in Italy and in the USA (Schoch *et al*., 2001a; Crous, 2002; Polizzi *et al*., 2006a, 2007b, 2009a,c; Vitale *et al*., 2009a).

Female fertility in populations of *Ca*. *pauciramosa* from various geographical regions has been used to determine the ratio of mating types present (Schoch *et al*., 2001a). Thanks to these data it was possible to assert that *Ca*. *pauciramosa* was endemic to South America given that the ratio of both mating types approached 1:1 (Schoch *et al*., 2001a).

Thanks to DNA sequence comparisons and mating studies on *Ca*. *pauciramosa* isolates from South Africa and Colombia, it was possible to demonstrate some variation amongst isolates (Lombard *et al*., 2010a).

The use of DNA sequence comparisons and mating studies demonstrated that there was variation among *Ca*. *pauciramosa* isolates from South Africa and Colombia. Thanks to these data and those of Schoch *et al*. (2001), it was possible to assert that some cryptic species could be considered closely related to *Ca*. *pauciramosa*. As a matter of fact, three new cryptic species, such as *Ca*. *colombiana* sp. nov. from Colombia, *Ca*. *polizzii* sp. nov. from Italy and *Ca*. *zuluensis* sp. nov. from South Africa, have been identified, based on the DNA sequence data and mating compatibility (Lombard *et al*., 2010b), but they were previously treated as *Ca*. *pauciramosa* (Lombard *et al*., 2010a).

Although, until some years ago, the use of the keys of Crous and Wingfield (1994) and Schoch *et al*. (1999) were essential for the identification of *Calonectria* spp., nowadays, considering the difficulties in morphological identification, new species of this genus are characterized on basis of several molecular approaches, such as 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 and Simoneau, 2004), restriction fragment length polymorphisms (RFLP) (Crous *et al*., 1993b; Crous *et al*., 1995; Crous *et al*., 1997b; Jeng *et al*., 1997; Victor *et al*., 1997; Risède and Simoneau, 2001) and DNA hybridisation (Crous *et al*., 1993b, 1995, 1997b; Victor *et al*., 1997).

The morphological characters that must be taken into account for the distinction among different species are the measures of perithecia, conidia, terminal vesicle and conidiophores (Perally 1991a; Crous *et al*. 1992).

In 2002, Crous described the morphological features typical of this species. He showed that perithecia of *Ca*. *pauciramosa* are solitary or in groups, orange to red-brown: in section, apex and body are orange to red-brown, base dark red-brow, subglobose to ovoid, with a diameter of 170-300 µm and height of 250-400 µm. The perithecia wall consists of an outside layer of textura globulosa, 20-50 µm wide, an inner layer of textura angularis of 5-10 µm wide, becoming thin-walled and hyaline towards the center. Outer cells are 40-55 x 15-35µm, while inner cells are 5-10 x 3-5 µm. Asci are 8-spored, clavate, with dimensions of 70-140 x 8-25 µm, tapering to a long thin stalk. Ascospores are aggregated in the upper third of the ascus, hyaline, guttulate, fusoid with rounded ends, slightly curved, 1-septate, not or slightly constricted at the septum with mean dimensions of 35 x 6.5 µm. Macroconidiophores consist of a stipe, that is septate, hyaline, 120-230 µm long. Each stipe ends with a vesicle of variable shape from ellipsoidal to pyriform and with diameter of (5-) 7-9 (-11) µm. Each stipe is a sterile extension from which the bundle of branches fertile started. The primary branches are aseptate or with only one septum (12-45 x 5-6 µm), while secondary and tertiary branches are aseptate with dimensions of 15-20 x 5-6 µm and 12-15 x 5-6 µm, respectively. The terminal branches produce 2-6 phialides, with shape variable from doliiform to reniform, hyaline, without septum, with dimensions of 10-13 x 2.5-4 µm, with a small enlargement at the apex (Crous, 2002).

The characteristics of conidia are: cylindrical, hyaline, rounded at both ends, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by

colourless slime. The mean dimensions of conidia are 50 x 4.5 µm The microconidiophores are unknown. The fungus differentiates chlamydospores throughout the medium. They are dark brown in colour and aggregated to form microsclerotia. Chlamydospores represent the survival structures. The teleomorph produces perithecia subglobose to ovoid with height of 250-400 µm and with of 170-300 µm, with a variable colour from orange to red to brown. Asci are clavate, 70-140 µm, with filiform stipe, contain 8 ascospores, hyaline, fusoid, septate, guttulate and slightly curved with rounded tip (Crous, 2002).

At optimal growth temperature of 25 °C, colonies of *Ca*. *pauciramosa* are characterized by a rapid growth up to 35–40 mm diameter after 7 d on MEA (Crous, 2002). Colonies of *Ca*. *pauciramosa* are characterized by abundant white aerial mycelium (Rayner, 1970). The colony is fulvous color in the lower surface and red sepia in the upper (Crous, 2002).

### 2.7.3. *Calonectria polizzii*

*Calonectria polizzii* has been reported for the first time in Sicily (Italy) on *Arbutus unedo* and *Callistemon citrinus* in 1997, but its pathogenicity was not confirmed. More lately, it has been reported as causal agent of disease on ornamental plants in Tunisia and more sporadically in Italy (Lombard *et al*., 2011; Aiello *et al*., 2013). Lombard *et al*. (2011) confirmed for the first time its pathogenicity. It is able to cause leaf spots, crown and root rot on ornamental plants, such as *Callistemon* spp., *Dodonaea viscosa*, *Metrosideros* spp. and *Myrtus communis*. Moreover, *Ca*. *polizzii* was considered less virulent than *Ca*. *mexicana*, *Ca*. *pseudomexicana* and *Ca*. *tunisiana* (Lombard *et al*., 2011).

The large presence of this pathogen on several new plant hosts in Italy was confirmed by means of DNA analyses (Vitale *et al*., 2013b).

*Ca*. *polizzii* resides in *Ca*. *scoparia* complex, where it can be distinguished from the other members by its smaller macroconidial dimension (Lombard *et al*., 2010b). No information is available on its teleomorph.

Morphologically, it is characterized by having conidiophores with a stipe bearing a penicillate suite of fertile branches, stipe extensions and terminal vesicles. Stipe is septate, hyaline, smooth, with dimension of 58–108 × 5–7 µm.

Stipe extensions are 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. Conidiogenous apparatus is 27–57 µm long and 28–51 µm wide. The primary branches are aseptate or 1-septate, with dimensions of 15–35 × 4–6 µm, secondary branches aseptate, with dimensions of 12–26 × 3–5 µm and tertiary branches aseptate, with dimensions of 10–15 × 4–5 µm. Each terminal branch produces 2–6 phialides, that are 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 have been not seen (Lombard *et al*., 2010b).

Lombaerd *et al*. (2010b) found variation in $\beta$-tubulin sequence data for this species, as described by Schoch *et al*. (2001a) in the study on female fertility of *Ca*. *pauciramosa*.

Morphologically, *Ca*. *polizzii* is similar to *Ca*. *zuluensis* and *Ca*. *pauciramosa*. Furthermore, it can be distinguished from *Ca*. *pauciramosa* by its smaller 1-septate macroconidia (Lombard *et al*., 2010b). The macroconidia of *Ca*. *polizzii* (av. 37 × 4 µm) are smaller to those of *Ca*. *pauciramosa* (av. 50 × 4.5 µm). 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). Based on these studies and on morphological, biological and phylogenetic characteristics it is possible to distinguish this species from *Ca*. *pauciramosa* (Lombard *et al*., 2010b).

### 2.7.4. *Calonectria pseudomexicana*

*Calonectria pseudomexicana* L. Lombard, G. Polizzi and Crous, sp. nov. has been identified for the first time as causal agent of leaf spots on *Callistemon* sp. in Tunisia (Lombard *et al*., 2011). Afterwards, its pathogenicity has been confirmed and this new species is capable to cause symptoms on *Callistemon* spp. (Lombard *et al*., 2011).

*Ca*. *pseudomexicana* resides in the *Ca*. *scoparia* complex (Schoch *et al*., 1999). It is closely related to *Ca*. *mexicana*, based on phylogenetic inference, and morphologically resemble. In detail, *Ca*. *pseudomexicana* is characterized by having four or fewer conidiophores while *Ca*. *mexicana* has five (Lombard *et al*., 2011; Schoch *et al*., 1999).

Conidiophores of this pathogen consist of a stipe bearing penicillate suites of fertile branches, stipe extensions and terminal vesicles. The stipe are septate, hyaline, smooth with dimension of 38–69 × 5–9 µm; stipe extensions 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 diameter with papillate apex (Lombard *et al*., 2011).

Conidiogenous apparatus has a length of 38–68 µm and a wide of 32–64 µm; primary branches are aseptate or 1-septate with dimensions of 21–43 × 4–7 µm, secondary branches are aseptate with dimensions of 13–26 × 4–7 µm and tertiary and additional branches (–4) are aseptate, with dimensions of 10–18 × 2–6 µm. Each terminal branch produces 2–6 phialides, which are doliiform to reniform, hyaline, aseptate, 6–14 × 2–6 µm; apex with minute periclinal thickening and inconspicuous collarette (Lombard *et al*., 2011).

Conidia are cylindrical, rounded at both ends, straight, (40–)43–48(–49) × (4–5–6 µm (av. =45 × 5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Its megaconidia and microconidia are unknown (Lombard *et al*., 2011).

Colonies of *Ca*. *pseudomexicana* grow fast at 24 ºC on MEA (Malt Extract Agar). Its colonies are sienna to bay on surface, reverse sienna after 7 d, with a moderate white aerial mycelium and sparse to moderate sporulation. Chlamydospores extend throughout medium (Lombard *et al*., 2011).

### 2.7.5. *Calonectria tunisiana*

*Calonectria tunisiana* L. Lombard, G. Polizzi and Crous, sp.nov. has been isolated for the first time from *Callistemon* spp. and *Metrosideros excelsus*. Following pathogenicity trials showed the ability of this pathogen to cause the same symptoms (Lombard *et al*., 2011).

Morphologically, this pathogen is close to *Ca*. *mexicana* and *Ca*. *pseudomexicana*, from which it can be distinguished by its shorter extension. In addition, its conidiophores (-3) form fewer fertile branches than *Ca*. *mexicana* (-5) and *Ca*. *pseudomexicana* (-4) (Schoch *et al*., 1999).

The description of *Ca*. *tunisiana* adds a new species on *Calonectria scoparia* complex (Schoch *et al*., 1999).

Conidiophores are characterized by a stipe bearing penicillate suites of fertile branches, stipe extensions and terminal vesicles. Stipe are septate, hyaline, smooth, 42–95 × 7–11 µm. Stipe extensions are 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 diameter with papillate apex (Lombard *et al*., 2011).

Conidiogenous apparatus is 40–68 µm long and 30–66 µm wide, with primary branches aseptate or 1-septate, 17–41 × 5–7 µm, secondary branches are aseptate with dimensions of 10–22 × 4–7 µm , while tertiary branches are aseptate and 9–18 long and 4–5 µm wide. Each terminal branch produces 2–6 phialides, which are doliiform to reniform, hyaline, aseptate, with dimensions of 8–13 × 3–5µm (Lombard *et al*., 2011).

Its conidia are cylindrical, rounded at both ends, straight, with dimensions of (43–)47–51(–53) × 4–6 µm (av. = 49 × 5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. In this *Calonectria* species megaconidia and microconidia are unknown (Lombard *et al*., 2011).

*Ca*. *tunisiana* is characterized by a fast growth at 24 °C on MEA (Malt Extract Agar); its colour is variable from sienna to bay on surface, with sparse white aerial mycelium with sparse sporulation. Its chlamydospores extend throughout the medium (Lombard *et al*., 2011).

## 2.8. Calonectria disease control

Considering the epidemiological characteristics of *Calonectria* species, as mentioned so far, the management of diseases caused by this pathogen is very complicated. It is possible put different strategies in place to control these diseases.

Some authors reported that management of Calonectria disease in nursery, in greenhouse or in field is effective only with preventative applications of fungicides (Crous, 2002; Henricot *et al*., 2008; Aiello *et al*., 2013). Reduction of primary inoculums, use of resistant and disease-free plant species or cultivar propagation, removal of infected plants and utilization of uncontaminated potting medium are control strategies that, associated with good nursery practices, are able to control infections caused by these plant pathogens (Crous, 2002).

In addition to chemical control, alternative control strategies have been proposed.

Chase and Poole, in 1987, asserted that potting medium, compaction and pH are capable to influence petiole root caused by *Cylindrocladium spathiphyllum*. In detail, on the one hand high pH values reduce the build-up if disease, but on the other the onset of disease is favoured by warm, humidity, lower pH and higher soil compaction. At least, soil pH could play a positive role in disease control when temperatures were suboptimal. Temperature comprised between 25 and 30 °C can increase the disease, but when temperature reaches 32°C a decrease in the risk of infection (Chase and Poole, 1987).

One of the methods that could be used to control CBR and red collar (RCR) is the use of resistant varieties linked to cultural practices, such as reducing primary inoculums (Berner *et al*., 1988; Sidebottom and Beute, 1989). Pruning low branches, mulching under trees and planting grass between trees are cultural practices that have allowed the reduction of infections of *Cy*. *colhounii* on laves of custard apple (Hutton and Sanewski, 1989).

Considering that optimum temperature of *Calonectria* species is 25°C, high thermal levels next to 35 °C resulted in a decrease of infections of CBR on groundnut, to completely block the pathogen. As a matter of fact, it has been

proposed that the change of soil temperature could represent an effective cultural practice for controlling Calonectria infections (Sidebottom and Beute, 1989).

More recently, the use of soil solarization or biological control agents could be considered effective sustainable strategies for improve disease control (Vitale *et al*., 2012, 2013a).

### 2.8.1. Chemical control

The use of fungicides is very important for management of disease caused by several fungal plant pathogens, among which also *Calonectria*.

*Calonectria* species have been associated with a wide range of disease symptoms on numerous plants host worldwide (Crous, 2002). These species cause several damages on ornamental plants (Polizzi and Crous, 1999; Polizzi *et al*., 2006).

Currently, the chemical management is necessary to control these pathogens for healthy production of young plants. In the chemical management, fungicides were selected based on their ability to control *Calonectria* species.

A low level of disease control with fungicides can be due to several causes including low application rate, wrong choice of the fungicide on the target pathogen, improper timing or application method and excessive rainfall (Damicone and Smith, 2009). Protection from fungicides is temporary because they are subject to weathering and breakdown over time. Accordingly, they also must be reapplied to protect new growth when disease threatens. Moreover, it is important taking into consideration the phenomenon of fungicides resistance. The continued application of fungicides, belonging to the same group, can increase the risk of developing resistance and its accumulation in the field.

Although several active ingredients have been used in control *Calonectria* infections, some authors reported that to be effective, they must be applied before infection in preventative treatments and in a sufficient spray volume to achieve through coverage of the plant or treated area (Barnard, 1984; Crous, 2002; Aiello *et al*., 2013).

Analyzing the use of different fungicides in controlling infections caused by *Calonectria* spp., several experiments were carried out to test the effectiveness of benomyl.

Treatment with benomyl, applied as drench, showed an effective control of seedling and cutting wilt caused by *Cy. scoparium* and *Cy. floridanum* on azalea (Horst and Hoitink, 1968; Engelhard, 1971).

Bertus (1976) detected that foliar applications of fungicides, such as benomyl, carbendazim or thiabendazole, were successful to control *Cy. scoparium* on *Acacia flexuosa*. At the same time, soil drench with carbendazim and thiofanate-methyl eradicate the disease on *Banksia marginata* (Bertus, 1976).

Benomyl treatments, applied on mother plants of azalea 9-12 days before cuttings, were effective in controlling pot root caused by *Cylindrocladium* spp. In addition, it is important to emphasize that cuttings were dipped in this solution prior to planting, in order to control infections (Roos, 1980, 1981).

Foliar applications with benomyl and chlorothalonil allowed a good control of *Cy. scoparium* on *Eucaliptus grandis* and *E. robusta*, when these fungicides were used in combination with methyl bromide fumigation (Barnard, 1984). Soil drench with benomyl has yielded good results in controlling rot caused by *Cy. scoparium* on azalea, *Dianthus*, *Gerbera* and *Erica* (Niebisch and Kelling, 1986).

The germination of *Cy. pteridis* Walf was inhibited for up to 11 days on leaves of *Eucalyptus cloeaziana* with treatment with benomyl (Bedendo and Krugner, 1987).

Good results in reducing disease caused by *Cy. spathyphylli* on spathyphyllum was obtained with preventative applications with benomyl, triflumizole and prochloraz, in association with good nursery practices, such as reduction of primary inoculums, removing infected plants, using uncontaminated potting medium (Chase, 1987; Chase and Poole, 1987).

Treatments with benomyl linked to chlorothalonil and bordeaux mixture showed a good activity in control of *Ca. rigidiuscula* on cocoa (Delgado, 1991). Jayasinghe and Wijesundera (1995) proved that foliar applications with benomyl, mancozeb, metalaxyl+mancozeb, oxadixyl+mancozeb were able to

control leaf spots caused by *Cy*. *reteaudii* on *Eugenia*. *Cy*. *spathulatum*, responsible for leaf infection on *Ilex paraguanensis*, was effectively controlled with treatments with benomyl and captan (Gricoletti and Auer, 2003).

Although benomyl has proved a good efficacy in controlling Calonectria disease, its frequent use should be avoided since it can induce a high selection of resistant isolates (Alfenas *et al*., 1988). Crous (2002) suggested that is important to apply fungicides with different mode of actions in rotation.

The onset of resistant strains of *Calonectria* has also been reported for other fungicides, such as benzimidazoles and prochloraz (Polizzi and Vitale, 2001; Vitale *et al*., 2009b; Guarnaccia *et al*., 2012, 2014).

In addition to benomyl, several fungicides were assessed to evaluate their efficacy in controlling Calonectria disease.

Soil drench of Phytoactin L-318, as well as foliar applications of calcium copper chloride, ferric dimethyldithiocarbamate and 75 % Ntrichloromethyl-thiophthalimide showed a good control of *Cy*. *scoparium* on *Picea pungens* Englm. and *P*. *mariana* Mill. (Bugbee and Anderson, 1963a).

Infections caused by *Cy*. *scoparium* on various conifer species have been controlled with foliar applications of bordeaux mixture or ferric dimethyldithiocarbamate (Cox, 1953). In addition he suggested that a good control of this pathogen was obtained by applying bordeaux, manzate, zineb, ferban and thiram (Cox, 1953). In another experiment, applications of ferbam, zineb and fentinacetate on leaves reduced infections of *Cy*. *scoparium* on *Eucaliptus* spp. (Reis and Chaves, 1967).

Foliar applications with bordeaux mixture, carbendazim and chlorothalonil have yielded good results in controlling leaf spots caused by *Cy*. *colhouni* on *Eucaliptus* (Nair and Jayasree, 1986).

A good efficacy in reducing leaf spots and severe defoliation caused by *Cy*. *scoparium* on *Myrtus* spp. was observed when plants were treated with copper oxycloride. In the same experiment, it was noticed that benomyl, chlorothalonil, prochloraz and ditianon were not able to control the disease (Polizzi and Azzaro, 1996).

In an experiment it was evaluated the efficacy of 22 fungicides against *Cylindrocladium quinqueseptatum*, *Cy*. *ilicicola*, *Cy*. *floridanum*, *Cy*. *parvum* and *Cy*. *camelliae*, causal agents of several diseases of *Eucalyptus* in Kerala. The results showed that the effectiveness of fungicides varied according to the *Calonectria* species involved (Sharma and Mohanan, 1991).

The effectiveness of 9 chemical compounds was tested *in vitro* in order to show their ability in reducing conidial germination and mycelial growth of *Cy*. *buxicola*. Prochloraz, propiconazole, thiophanate-methyl and carbendazim + flusilazole were capable to inhibit totally the mycelia growth for 15 days, whereas tolylfluanide, mancozeb, chlorothalonil and fludioxonil + cyprodinil, applied at low rates, were able to inhibit conidial germination for 16 hours (Brand 2006).

In 2006, Ferreira *et al*. demonstrated that some fungicides, such as epoxiconazole, epoxiconazole + pyraclostrobin and tebuconazole were capable to inhibit conidial germination and mycelial growth of *Cy*. *candelabrum in vitro* experiment and foliar infection severity *in vivo*. Moreover, they showed a translaminar action and a good persistence in the leaves.

Preventive treatments with prochloraz and tebuconazole showed their ability in reducing root rot caused by different strains of *Cy*. *pauciramosum* on *Polygala myrtifolia*. In the same experiment, it was possible to note that treated plants had a fresh and dry weight of roots higher than those of untreated, and that tebuconazole provided symptoms of stunting with marked reduction of leaf edges on treated plants (Polizzi, 2000). In another experiment, prochloraz applied at interval of 10-12 days proved a high performance in controlling root rot of *Polygala myrtifolia* (Polizzi, 2000).

In an assay to control infections caused *Cy*. *pauciramosum* on cuttings of *Callistemon citrinus* var. 'Captain Cook', treatments with tebuconazole caused stunting symptoms, plants shorter and with a low number of shoot per plants than the other; in addition, plants treated with copper hydroxide showed symptoms of necrotic and dark red spot on young leaves and delay of blooming (Vitale *et al*., 2003).

Two cultivars, such as '*baetica*' and '*lusitanica*', of *Myrtus communis* were used as host plants to evaluate the efficacy of some fungicides in management of infections caused by *Cy*. *pauciramosum* on leaves. Foliar treatments with copper hydroxide showed a good efficacy on *Myrtus communis* cv. *'baetica'*, that showed at the same time a lower susceptibility than cultivar '*lusitanica*'. In both cultivars, prochloraz proved a poor efficacy, probably due to its variable activity (Vitale *et al*., 2003).

The frequent use of fungicides, such as benomyl, carbendazim, chlorothalonil, copper compounds, prochloraz, and thiophanate-methyl for controlling Calonectria disease have induce a high selection of resistant isolates, as reported in Italy and Brazil (Alfenas *et al*., 1988; Polizzi *et al*., 2001; Vitale *et al*., 2009b). In detail, *Ca*. *morganii* was found sensitive to benzimidazoles, while *Ca*. *pauciramosa* was reported as resistant to benzimidazoles with minimum inhibitory concentration between 10 and 100 $\mu$g ml-1 (Polizzi *et al*., 2001; Vitale *et al*., 2009b).

More recently, isolates belonging to *Ca*. *polizzii*, *Ca*. *tunisiana*, *Ca*. *mexicana* and *Ca*. *pseudomexicana* have been identified resistant to MBCs with MIC values >100 µg a.i./ml (Guarnaccia *et al*., 2012).

In a total of 105 isolates, those collected more recently (2005 to 2009) had a reduced prochloraz sensitivity, as indicated by greater values for the effective dose to reduce growth by 50%, than those collected earlier (1993 to 1996) (Guarnaccia *et al*., 2014).

Reduced sensitivity is caused by a genetic mutations, and more narrowly, it results from a single gene or multiple gene mutation. Single gene mutation confers resistance to site-specific fungicides, indeed mutations in multiple genes confer resistance to multi-site inhibiting fungicides. Mechanisms of resistance include alteration of the target site, reduced fungicide uptake, active export of the fungicide outside fungal cells, and detoxification or breakdown of the fungicide (Damicone and Smith, 2009).

*Cy*. *ilicicola*, causal agent of soybean black root rot, was controlled by carbendazim+diethofencarb and dazomet, which were capable to inhibit the mycelia growth and spore germination of this pathogen at very low rates. In

greenhouse, carbendazim+diethoferncarb, applied by drenching 2 days before or after inoculation, were capable to control soybean black root rot. Moreover, this disease could be controlled by tebuconazole, applied in curative treatment (Park *et al*., 2007).

More recently, new active ingredients have been tested for controlling Calonectria disease. In management of Cylindrocladium root rot on blueberry, fludioxonil significantly reduced lesion incidence and lesion length, when fungicide was applied at the cutting time (Haralson *et al*., 2007, 2013).

*In vitro* kresoxim-methyl, azoxystrobin and mancozeb were the most effective fungicides which are capable to inhibit conidia germination of *Cy*. *buxicola* more than 96%, while its mycelial growth was inhibited more than 96% by kresoxim-methyl, myclobutanil and penconazole (Šafránková *et al*., 2013).

The use of tebuconazole, fosetyl-Al, Cu hydroxide, thiophanate-methyl, prochloraz + cyproconazole, trifloxystrobin, azoxystrobin and prochloraz, applied in a preventative treatment, showed a good activity in control leaf spots caused by both *Ca*. *morganii* and *Ca*. *pauciramosa* in nurseries, while use of cyproconazole, propamocarb + fosetyl Al and K phosphite is discouraged (Aiello *et al*., 2013).

Also, the use of K phosphite, fosetyl-Al, prochloraz + cyproconazole, cyproconazole, Cu hydroxide, thiophanate-methyl, tryfloxystrobin and azoxystrobin provided a significant reduction of crown and root rot caused by *Ca*. *pauciramosa* on feijoa plants (Aiello *et al*., 2013).

Furthermore, fosetyl-Al, prochloraz + cyproconazole and tebuconazole showed the best activity also in controlling leaf spot on bottlebrush caused by *Ca*. *polizzii*, *Ca*. *pseudomexicana*, *Ca*. *tunisiana* and *Ca*. *mexicana* (Aiello *et al*., 2013).

In 2013, Henricot and Wedgwood showed that a mixture of epoxiconazole + kresoxim-methyl + pyraclostrobin (Opponent) was the best treatment in controlling infetions caused by *Cylindrocladium buxicola* on English booxwood, when applied preventatively 3 days before inoculation. In addition, curative treatments with chlorothalonil, boscalid + pyraclostrobin and prochloraz proved good results in controlling booxwood blight.

For controlling diseases caused by *Calonectria pseudonaviculata*, causal agent of leaf spot and stem lesion resulting in defoliation and dieback of boxwood, 20 different active ingredients were evaluated for their ability to inhibit conidial germination and mycelial growth. Moreover, it was determined the concentration that suppressed fungal growth to 15% of that on unamended media. This trial showed that mycelia growth was inhibited by thiophanate-methyl, fludioxonil, pyraclostrobin, trifloxystrobin, kresoxim-methyl, mancozeb and that chlorothalonil also had activity against mycelial growth. Conidial germination was inhibited by pyraclostrobin, trifloxystrobin, and kresoxim-methyl as well as fludioxonil, mancozeb, chlorothalonil, and boscalid. Furthermore, fludioxonil + cyprodinil had a lower $EC_{85}$ than the same rate of fludioxonil alone, suggesting that cyprodinil had activity against mycelial growth. Comprehensively, quinoxyfen, etridiazole, fenhexamid, hymexazol, famoxadone, and cymoxanil did not inhibit either *Ca*. *pseudonaviculata* conidial germination or mycelial growth (LaMondia, 2014a).

Shortly after, LaMondia (2014b) confirmed that myclobutanil, thiophanate-methyl, fludioxonil, pyraclostrobin, kresoxim-methyl and chlorothalonil (previously tested *in vitro*) as well as propiconazole had significant activity in controlling boxwood blight both in greenhouse and containers nursery experiments.

The fungicidal activity of herbicide containing glyphosate was evaluated both *in vitro* on *Calonectria crotolarie* and *in vivo* for controlling red crown rot on soybean. This experiment showed that glyphosate could be used both as a preplant herbicide for weed control and as fungicide for controlling disease caused by *Ca*. *crotolarie* (Berner *et al*., 1991).

*Cylindrocladium* Leaf Blight (CLB), caused by *Cy*. *quinqueseptatum*, is a serious disease that attacks *Eucalyptus* seedlings in nurseries and plantation (Sehgal *et al*., 1969). Systemic fungicides such as benomyl, thiram and carbendazim are capable to inhibit totally colonies of *Cy*. *quinqueseptatum in vitro* (Chaudhary, 2014). Some decades before, it was demonstrated a good efficacy of carbendazim, thiram and fenfuram in cotrolling infections of *Cy*. *scoparium* and *Cy*. *clavatum* on *E*. *tereticornis* (Rattan and Dhanda, 1985).

Since chemical control of *Cylindrocladium* Leaf Blight (CLB) is prohibitively expensive and impractical in plantation (Sharma and Mohanan, 1992; Chaudhary, 2014), a long-term solution could be the screening of resistant provenances or species. Furthermore, it was observed that the susceptibility rating of a provenance to different species of *Cylindrocladium* was related to the eucalypt species or subgenus (Sharma and Mohanan, 1991, 1992).

### 2.8.2. Soil fumigation and solarization

In ornamental plant nurseries, the spread of Calonectria diseases is due to the use of infected plants and soil. Accordingly, it is necessary to take precautions during the growing stage, such as seeding, rooting and post-transplanting in order to obtain healthy plants (Vitale *et al*., 2013b). For this purpose, one of the most important practices is the use of inoculum-free soil that could represent an alternative strategy to chemical control in reducing Calonectria infections in nursery (Silveira, 1996).

The species of the genus *Calonectria* are polycyclic fungi that are able to differentiate microsclerotia on senescing plant tissues. These microsclerotia can overwinter as resisting structures on cultural debris released in the soil and represent the primary inoculum of the pathogen (Crous, 2002). Microsclerotia can survive in the soil for 15 years even without susceptible crops (Thies and Patton, 1970; Sobers and Littrell, 1974; Phipps and Beute, 1979), but they can't survive in soil with lower water content (Sung *et al*., 1980).

Some experiments proved that fungicides used in the chemical control of Calonectria disease were not able to suppress survival of microsclerotia (Sharma and Mohanan, 1991).

Soil fumigation with methyl bromide, chloropicrin, metham-sodium and dazomet is one method that showed good results in reducing soil populations of some *Calonectria* spp. (Pickel, 1940; Jaunch, 1943; Crous, 2002).

Furthermore, few experiments were conducted to determine the efficacy of soil fumigation in suppressing microsclerotia of *Calonectria* from the soil.

In 1953, Cox reported that soil fumigation with formaldehyde and chloropicrin allowed to reduce infections of *Cy*. *scoparium* on conifer young plants.

The number of microsclerotia of *Cy*. *scoparium* has been reduced in the soil after treatment with mylone and methyl bromide (Thies and Patton, 1971).

It was reported that fumigation with 67% of methyl bromide and 33% of chloropicrin was capable to control infections of *Cy*. *floridanum* on *Liriodendron tulipifera* (Cordell *et al*., 1971).

Soil fumigation proved good results in controlling Cylindrocladium black rot (CBR) in peanut, as reported by Bayley and Matyac in 1989.

In 1990, Phipps suggested that it was possible to reduce the onset of CBR in groundnuts by planting resistant cultivars after soil fumigation. In particular, this result can be reached when soil is treated with metham-sodium and 1,3-dichloropropene supplemented with methyl isothiocyanate.

Further experiments demonstrated that treatments with metham-sodium resulted in partial control of pod and root rot, caused by *Cy*. *crotalariae* on susceptible cultivars of peanut. Moreover, the same study showed that the combination between metham-sodium and moderately resistant genotype of peanut resulted in reduction of pod and root rot and intermediate levels of inoculum density (Cline and Beute, 1986). Unfortunately, resistant plants are not available in all agronomical cultivars, so that the use of metham-sodium represents a valid alternative to control CBR of peanut (Cline and Beute, 1986).

Good results in controlling *Cy*. *scoparium* was obtained when compost and dazomet were applied in field (Lyons *et al*., 1997).

Until last years, no information was available about soil fumigation for controlling *Calonectria* spp. present in Europe and in the Mediterranean basin (Vitale *et al*., 2013b).

In the last decades, the modern agriculture enhanced the productions, but at the same time, it increased the onset of soil-borne disease and pests. Methyl bromide has been widely used in the past for the disinfection of soils. Moreover, the phase-out of this chemical fumigant, identified as an ozone depleting substance under the provisions of the Montreal Protocol Agreement, has led to find out new methods for controlling plant pathogens. In the last years, the use of metham-sodium and metham potassium (as liquid formulation) and dazomet (as granular formulation) resulted efficacy in control both soil-borne pathogens

and nematodes. These fumigants could replace methyl bromide for the disinfection of soil and substrates in nurseries (Vitale *et al*., 2013b). Nevertheless, the true efficacy of these fumigants is compromised by several factors, among which the most important are incorrect application methods, unfavourable environmental conditions and high level of emission to the atmosphere. Relating to the second point, these fumigants must be applied when soil temperatures and moisture allow a complete efficacy of the treatment.

The high level of emission of pollutant molecules to the atmosphere, which can range from 20% to 90% of the total applied fumigant, is the greatest issue related to the use of fumigants. In order to reduce this problem, it is possible to employ measures, according to the provisions of the European legislation in the Directive 2009/128. This document established a framework for Community action to achieve the sustainable use of pesticides.

The application of fumigants in IPM programs could represent a contradiction.

Drip application, simultaneous application of organic amendments or other chemical compounds (e.g. ammonium thiosulfate or potassium thiosulfate), the reduction of conventional application rates and the use of impermeable films are different measures which allow a sustainable use of fumigants. Furthermore, the integrated approach is based on the application in the soil of beneficial microorganisms that can protect the plant from harmful organisms that could survive to the fumigation treatments. Ślusarski *et al*. (2012) claimed that this approach is also feasible from a biological point of view, since the fumigation with reduced doses does not create a "biological vacuum" in the soil. Also, the addition of biological antagonists in an environment with reduced number of pathogens after the fumigation, increases their effect, also with respect to natural beneficial microorganisms (Ślusarski *et al*., 2012).

The use of plastic films is an important strategy for the sustainable use of fumigants, because they allow at the same time to apply low rates of fumigants, increasing their efficacy and reducing the gas emissions to the atmosphere. For this purpose are currently underway experimental trials, involving the use of two different impermeable film, such as virtually impermeable film (VIF) and totally impermeable film (TIF).

TIF is a relatively new barrier that has been shown to apply easily and retain fumigant better than VIF (Ajwa, 2008; Chow, 2008). This is a fivelayer film with two thin ethylene vinyl alcohol layers (EVAL) embedded in three layers of standard polyethylene film (Chow, 2008).

Several authors have developed tests to compare the different effectiveness of these two plastic tarps in soil fumigation.

It was demonstrated that soil fumigation with VIF has reduced methyl bromide emission to near-zero levels, if it was compared to a soil fumigation with a high-density polyethylene film (HDPE). In detail, the total global methyl bromide emission has been reduced from 32 Gg/yr to less than 1 Gg/yr (Yates *et al*., 1998).

Several experiments were carried out to evaluate the ability of these permeable tarps to reduce the fumigant emissions. In California and in Florida, it was demonstrated that covering raised beds with low permeable tarp, and in particular with TIF than VIF, can reduce significantly fumigant emissions (1,3-dichloropropene and chloropicrin) from the beds (Qin *et al*., 2013). Moreover, the highly retine fumigant amount under TIF may have a greater potential to transport to uncovered furrows contributing to high emission loss in coarse textured soils. Accordingly, different factors such as fumigant application depth, fumigant application method, raised bed configuration, soil moisture, and soil texture should be take into account in soil fumigation, especially for achieving low level of emission with the use of low permeable film (Qin *et al*., 2013).

A trial carried out in California demonstrated that the use of VIF didn't prove weed control and fruits yield in strawberry crop. Instead, it was possible to reduce up to 50% of rates normally used with a standard tarp and getting a good efficacy of treatment by using TIF as plastic tarp (Gao *et al*., 2014).

A trial conducted in Virginia by using dimethyl disulfide (DMDS), as fumigant, and VIF and TIF, as low permeable films, showed that it was possible to reduce fumigant used rates from 40% to 50% under TIF compared with VIF (McAvoy and Freeman, 2013).

In conclusion, it is possible to assert that the application of low permeable films such as virtually impermeable film (VIF) and totally impermeable film (TIF)

could increase fumigant retention. In detail, this phenomenon has a positive effect as a reduction in the amount of fumigant needed for effective pest control, decreasing buffer zone requirements, reducing fumigant emissions, and improving pest control. Particular attention should be paid to the application of TIF then VIF, for its structural characteristics. Thus, knowledge of these gas-tight tarps is important, taking into consideration the fact that their use has become mandatory in soil fumigation, as set out in Regulation (EC) N° 359/2012.

Even though all these measures can be put in place, nowadays the use of these metham-sodium, metham-potassium and dazomet is limited. This was established by the European Regulation N° 1107/2009 that provides the evaluation of crop protection products through more stringent hazard-based cut-off criteria (Colla *et al*., 2013).

Each member state shall adopt restrictions on the use of fumigants by 31 December 2014, as explained in the following Regulations.

As regards metham, Commission implementing regulation (EU) N° 359 of 25 April 2012 approves this active substance, in accordance with Regulation (EC) N° 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending the Annex to Commission Implementing Regulation (EU) N° 540/2011. According to part A of this regulation, metham is subjected to a serious of limitations. In detail, it can be used as nematocide, fungicide, herbicide and insecticide authorised for application as soil fumigant prior to planting, allowing one application every third year on the same field. This active substance can be applied in open field by soil injection or drip irrigation and in greenhouse by drip irrigation only, associating to it the use of gas-tight plastic film. Besides, the maximum application rate shall be 153 kg/ha (corresponding to 86,3 kg/ha of MITC) in case of open field applications. Overall, one of the most important aspects is that this fumigant must be applied only by authorized professional users.

European Commission established in the Commission Directive 2011/53/UE of 20 April 2011 that the active substance dazomet can be applied only once every

three years. In addition, metham and dazomet are active substances authorized until to 30 June 2022 and 31 May 2021, respectively.

Since the continued use of chemical fumigants could cause the environmental damage, another control measures to reduce primary inoculums of *Calonectria* spp. is represented by soil solarization. It is a method of non-chemical disease control that employs solar radiation to heat moistened soil using a plastic film and raises soil temperatures to levels harmful to resisting structures of soil-borne pathogens (Katan, 1981).

The effectiveness of solarization depends mainly on exposure time to solar radiation and for how long the temperature is maintained at optimum levels in the 0-30 cm rooting layer of soil (Katan and De Vay, 1991). It was demonstrated that 4-6 weeks of solarization during summer were usually able to control effectively many of soil-borne diseases (Stapleton and De Vay, 1982; Stapleton, 1990; Katan and De Vay, 1991).

Recently, it has been proved that the short-term soil solarization, using mulching plastic films such as ETFE, is effective for *Calonectria* microsclerotia suppression in nurseries (Vitale *et al*., 2012).

### 2.8.3. Biological control of *Calonectria* spp.

Calonectria species cause serious damage on various ornamental plants, so the use of chemical treatments is necessary for controlling diseases caused by these pathogens. Moreover, the continued use of fungicides, such as benzimidazoles or prochloraz, should be avoided for its high selection of benzimidazole or prochloraz resistant-strains (Alfenas *et al*., 1988; Vitale *et al*., 2009b; Guarnaccia *et al*., 2012, 2014). In addition, according to European legislation on the "Sustainable Use of Pesticides", the modern agriculture should focus its efforts to improve the use of biological control agents (BCAs) or non-chemical treatments in alternative to chemical ones in nurseries.

To respond to these needs, antagonistic microorganisms could represent a very important management strategy, to be applied in place of or in combination with chemical compounds. Furthermore, the use of these biological antagonists

requires the knowledge of different factors (environmental and biological), that can influence their effectiveness.

It is important to emphasize that hosts, pathogens and environment interact with each other, up to influence the disease severity. In detail, some environmental factors, such as soil moisture and temperature, have an important role in the "disease triangle", especially during the growing stage (seeding, rooting and transplanting) (Kunieda-de-Alonso, 1997; Stirling and Stirling, 1997).

Most of the antagonist microorganisms used in biological control belong to the genus *Trichoderma* (Kubicek and Harman, 1998; Harman and Kubicek, 1998).

Although several bio formulates containing different *Trichoderma* spp., such as *T. asperellum* V1, *T. harzianum* strains Rifai T22 and ICC012, *T. viride* ICC080, are effective against specific pathogens in soil treatments (Hjeljord and Tronsmo, 1998), they show a variable efficacy for controlling Calonectria infections (Harman, 2000; Polizzi and Vitale, 2002; Daughtrey and Benson, 2005; Vitale *et al*., 2012).

In the greenhouse, the management of disease caused by *Cylindrocladium* spp. on spathiphyllum is possible when *T. harzianum* T-22 is applied at interval of 7 days (Harman, 2000).

The efficacy of *T. harzianum* T-22, compared with the activity of prochloraz, was evaluated for controlling crown and root rot caused by *Ca. pauciramosa* on *Polygala myrtifolia*. This experiment showed that the biological control agent resulted effective to control infections and to improve the plant growth, compared to the untreated control and to the chemical treatments (Polizzi and Vitale, 2002).

Some decades ago, it was studied that damping-off of seedlings on *Eucalyptus*, caused by several species of *Calonectria*, can be controlled by the antagonistic activity of *Trichoderma* and fluorescent *Pseudomonas* (Blum and Lin, 1991).

In the last year, for the biological control of Cylindrocladium leaf blight (CLB) caused by *Cy. quinqueseptatum* on *Eucalyptus*, it was evaluated the ability of several biological control agents such as *T. virens*, *T. koningii*, *T. album* and *T. harzianum*. All antagonists were capable to inhibit colony growth of this

pathogen, but *T. koningii* turned out to be more efficacy than others (Chaudhary, 2014).

The ability of six isolates of *Trichoderma*, belonging to five different species, was assessed for controlling damping-off of *Eucalyptus grandis* seedlings caused by *Cy. scoparium* and *Rhizoctonia solani*. All six isolates showed a good activity in controlling both pathogens, when they were previously mixed with the soil for 15 o 30 days. In addition, the eucalyptus bark compost revealed its ability in controlling both pathogens when it was used alone (Garcia and Ferreia, 1997).

Several isolates belonging to the genus *Trichoderma*, such as *T. harzianum*, *T. viride*, *T. koningii*, *T. polysporum*, *T. hamatum* and *T. virense*, were capable to inhibit mycelial growth and microsclerotia of *Cy. floridanum in vitro*, thanks their ability to produce 6-n-pentyl-2H-pyran-2-one under laboratory conditions (Dumas *et al*., 1996).

Several experiments were carried out both *in vitro* and *in vivo* to evaluate the antagonistic activity of different strains of *T. harzianum* against several *Calonectria* species.

*In vitro* experiment mycelial growth of *Cy. pteridis*, causal agent of root necrosis on banana and plantain, was controlled by *T. harzianum* strain C184. (Ngueko and Tong, 2002).

*T. harzianum* T-22, applied to soil and plants, failed to control collar and root rot of *Myrtus communis* subspecies *tarentina* caused by natural infections of *Ca. pauciramosa* linked to *Phytophtora palmivora* (Vitale *et al*., 2003).

It was proved that the ability of *Cy. pauciramosum* to colonize carnation leaves and to produce microsclerotia was reduced significantly by antagonistic activity of *T. harzianum* T-22 (Vitale and Polizzi, 2005).

Damping-off of seedlings of *E. grandis* and *E. tereticornis* caused by *Rizhoctonia solani* and *Cy. quinqueseptatum* was controlled effectively with soil treatments with *T. harzianum* (Mohanan, 2007).

Other studies were carried out to test the CEN262 isolate belong to *T. harzianum*. The results of these trials showed the effectiveness of this isolate in controlling *Cy. scoparium* both *in vitro* and *in vivo* (de Carvalho Filho, 2009).

An experiment was carried out to evaluate the effectiveness of *T. harzianum* strain T-22 against several *Ca. pauciramosa* isolates. *In vitro* the biological control agent was capable to inhibit the microsclerotia production on carnation leaf agar (CLA) of most isolates, although antagonist activity showed variable effects among the tested isolates. Moreover, 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. *In vivo* on red clover and, in nurseries trial, on feijoa sellowiana *T. harzianum* T-22 showed variable effects related to the degree virulence of different isolates. Overall, this biological antagonist showed a good activity in reducing microsclerotia production on carnation leaf and the incidence and severity of collar and root rot on both selected hosts (Vitale *et al*., 2012).

More recently, in an experiment for studying red clover as model plants, it was assess the activity of several fungicides and biological control agents for reducing infections caused by *Ca. morganii* and *Ca. pauciramosa* on the host-model plant *Trifolium pratense*. It was found that propamocarb + fosetyl-Al, azoxystrobin, cyproconazole, K phosphite and fosetyl-Al were the most effective fungicides in reducing *Ca. morganii* infections. Besides *Clonostachys rosea* and *Penicillium oxalicum* were effective in controlling *Ca. morganii* infections, while *Bacillus subtilis* QST713 and *Streptomyces griseoviridis* K61 were not effective (Aiello *et al*., 2012).

Among all microorganisms antagonists applied in the control of Calonectria disease, there are also some bacterial species belonging to the genus *Bacillus* and *Pseudomonas*.

The mycelial growth of *Cy. colhounii*, causal agent of blueberry spot disease, was inhibited by *B. subtilis* strain B7, isolated from the forest field soil (Fan *et al*., 2008).

Several *Bacillus* strains have proved good results in controlling infections caused by *Ca. spathiphylli* on banana and *Ca. morganii* on *E. grandis* (Bettiol *et al*., 1988; Santos *et al*., 1993; de Wit *et al*., 2009).

Six strains of *Pseudomonas putida* showed their antagonistic activity against several *Cylindrocladium* isolates. In detail, *Pseudomonas putida* strain 93.1

totally inhibits mycelial growth of the fungus. This study proved that fluorescent pseudomonas represents a viable alternative for controlling banana root infections (Sutra *et al*., 2000).

Five bacterial strains belonging to the genus *Pseudomonas*, *Pantoea* and *Corynebacterium* showed their antagonistic activity against *Cy. scoparium* (Burch and Sarathchandra, 2006).

Furthermore, the use of biological control agents could be integrated with the application of mycorrhizal fungi, which showed a good decrease of the disease caused by *Calonectria* spp. In detail, *Suillus brevipes* were capable to inhibit the growth of four root pathogens, such as *Armillaria mellea*, *R. solani*, *Cy. parvum*, *Cy. scoparium*, *Fusarium oxysporum* and *F. solani* (Natarajan and Govindasamy, 1990).

Root infections incited by *Cy. spathiphylly* on banana (*Musa acuminata*) were attenuated when plants were preinoculated with arbuscular mychorrhizal fungi of the genus *Glomus* (Declerck *et al*., 2002). The activity of ectomycorrhizal fungi was evaluated for controlling root rot of conifers in forest nurseries caused by *Cy. floridanum*. In this trial, Morin *et al*. (1999) revealed that *Tricholoma* spp. and especially *Paxillus involutus* and *Hebeloma cylindrosporum* were able to inhibit growth of this pathogen *in vitro*.

*Cy. scoparium*, causal agent of damping-off of red pine (*Pinus resinosa*) was controlled by *Phaeoteca dimorphospora*. In addition, this fungal antagonist increased by 10% the germination of seeds of red pine coated with its microconidia *in vitro*. In greenhouse conditions, *P. dimorphospora* stimulated the population of *T. harzianum*, increasing by 100-500 times the number of propagules, whereas the population of *Cy. scoparium* decreased rapidly and was not detectable one month after sowing (Yang *et al*., 1995).

In addition, it was evaluated the efficacy of three different extracts from *Ginko biloba* for inhibiting *in vitro* the mycelia growth of *Cy. colhouni*. In this experiment, ethanolic extract showed the best inhibiting effect with its minimum efficacy of 37.4%, followed by petroleum ether extracts with a minimum efficacy of 23.7%, and the minimum efficacy of fresh extracts is 18.4%. (Feng *et al*., 2007).

## 3. Integrated Pest Management

In the last century, agriculture has undergone a drastic change, abandoning the concept of subsistence farming. The twentieth century industrial revolution changed the situation radically. The exponential increase in population has prompted an increasingly high level of food production. The introduction of fertilizers, machinery, better quality seeds, irrigation, plant protection products and good farming practices have significantly boosted productivity. At the same time, agriculture enhances its technological level, aiming to obtain high quality, nutritionally rich and safe agricultural products. This is the concept of "green revolution".

Plant protection plays an important role in development of modern agriculture. Since the early 1900s, fungicides have been widely used by farmers. Moreover, nowadays, the evolution of chemical, toxicological and environmental sciences acknowledged the incidence of undesired environmental effects, contamination of foodstuffs and risks for human health. Accordingly, it was proper and necessary to introduce a system of rules, which would regulate the use of these chemical compounds. From this assessment, the concept of Integrated Pest Management (IPM) started to be developed.

The first expression of Integrated Pest Management (IPM) appeared 30 years ago (Kogan, 1998). It is possible to find about 67 definitions of IPM in the worldwide literature. One of the most important concepts of IPM, that takes into account multiple definitions, is that proposed by Kogan in 1998. He stated that *"IPM is a decision support system for the selection and use of pest control tactics*, *singly or harmoniously coordinated into a management strategy*, *based on cost/benefit analyses that take into account the interests of and impacts on producers*, *society*, *and the environment"* (Kogan, 1998).

*"Integrated control represents the system of pest regulation which takes into account respective environment and population dynamics of harmful species and utilizes all suitable techniques and methods in the most effective combination to maintain pest population under the threshold of harmfulness"* is the definition given by FAO.

According to the International Organisation for Biological and Integrated Control of Noxious Animals and Plants (IOBC-IOLB), IPM has been defined as follows: *''Integrated control represents procedure (method) which utilizes all economically*, *ecologically and toxicologically acceptable methods for keeping the pests under the threshold of harmfulness with preferential and meaningful utilization of natural restricting factors"*.

One of the basic concepts of IPM is to anticipate or prevent the onset of disease. IPM establishes the applications of appropriate pest management techniques such as enhancing natural enemies, using semiochemicals, planting pest-resistant crops, adopting cultural management, paying close attention to the use of pesticides (Bajwa and Kogan, 2002).

Its aim is to limit the onset of disease, by applying at first measures with low environmental impact and ending with chemical pesticides with direct mode of action. In addition, it is important to resort to the use of pesticides only if the epidemiologically relevant infestation levels or economic thresholds are exceeded (Bajwa and Kogan, 2002). In addition, chemical treatments must be performed by applying minimum possible rates.

In particular, at first it is necessary applying preventative measures which take into consideration plant behaviours and environmental conditions favourable to pest development. Among these preventative measures there are choice of crop, crop rotation, correct fertilization, soil management, seed choice. If all these measures don't afford an adequate protection of plants, and the economic threshold level is reached, it is necessary to implement the repressive or curative phase. Nevertheless, care must be taken, because at first it should be applied biological products and, if it is indispensable, using chemical products (ND IPM Texas Pest Management Association; www.PestControlCanada.com).

Analyzing the Articles 6, 7, 8 of the Directive 2009/128/EC, one of the aims of the application of IPM strategies is the reduction of number and frequency of chemical treatments. This mode of action allows to employ measures that limit the onset of fungicide resistant strains. Thanks to these recent European regulations it is possible to reach these targets.

All these concepts have been taken into account by European Commission, which adapted the Directive 2009/128/EC on "Sustainable Use of Pesticides", became applicable since 14th December 2011. In the article 3 of this Directive it is explained literarily the definition of IPM, as follow: *'integrated pest management' means careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimise risks to human health and the environment. 'Integrated pest management' emphasises the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms"*.

In the Article 14 of the Directive 2009/128/EC it was established that IPM is mandatory for all EU members states from 1th January 2014.

It is important to emphasize that agrochemicals are subject to the general regulations of chemical substances, mainly concerning hazard classification, labelling and packaging, according to what is laid down in Regulation (EC) N° 1107/2009 of 21 October 2009.

Accordingly, as said before, the modern agriculture is oriented towards an integrated pest management, in order to detect alternative inputs to synthetic chemicals for controlling pests and diseases. Among these alternatives there are those referred to as biological controls.

### 3.1. Introduction: definition of biological control

The first proposal for biological control dates back about fifty years ago. Subsequently, in 1965 a symposium held in Berkley in 1965 was entitled: "*Ecology of soil-borne plant pathogens; prelude to biological control*" (Baker and Snyder, 1965).

Many different definitions of biological control were proposed. In particular, Cook and Baker (1983) affirmed that "*biological control is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man*".

European legislation on the “Sustainable Use of Pesticides” provides that the modern agriculture should implement the use of non-chemical management strategies, such as biological control, that could be considered a viable alternative method to chemical control.

Since 80 years, biological control of soil-borne pathogens by introduced microorganism has been studied, but only since last decades researches have focused their attentions on biological control and the companies have developed programs for the production of biological control agents (BCAs) as commercial products.

Moreover, nowadays, it is necessary to control plant diseases to maintain the quality of production. It is possible thanks to different approaches that may be used to prevent, mitigate or control plant disease. In the last decades, the modern agriculture has made an excessive use of chemical fertilizer and pesticides, in order to improve the production of plants and control plant disease. Accordingly, this excessive use of pesticides caused the environmental pollution, which has led to considerable changes in the use of pesticides in agriculture. In addition, at the same time there is a demand from society for healthy foods with less chemical residues. In response to this need, some researchers have studied carefully to develop alternative measures to synthetic chemicals for controlling plant disease. This theory is the basis of the modern control programs of plant disease, known as integrated pest management (Pal and McSpadden Gardener, 2006).

In plant pathology, the term biological control, or its abbreviated synonymous “biocontrol”, has been applied to the use of microbial antagonists to control diseases as well as the use of host-pathogen to control weed population (Pal and McSpadden Gardener, 2006). Moreover, this term can be applied to indicate the natural product extraction of fermented from various sources. These formulations could be considered as a simple mixture of natural ingredients with specific activities or complex mixtures with multiple effects on the host as well as the target pest or pathogen (Pal and McSpadden Gardener, 2006).

More broadly, biological control could be considered as the suppression of pathogenic or parasitic activities of one organism by more other organisms (Pal

and McSpadden Gardener, 2006). With regards to plant disease, biological control includes the cultural practices such as the rotation and planting of disease resistant cultivars. Moreover, the inducing host resistance could be considered a form of biological control. In detail, biological control indicates the utilization of microbial inoculants in order to suppress a single pathogen by a single antagonist, or by a complex of antagonists, and a single type or several class of plant disease (Pal and McSpadden Gardener, 2006).

In natural soil a wide range of microorganism lives and competes with one another for space and nutrient. Some of these cause plant diseases, whereby they are pathogens: while others are beneficial to plant by working against the pathogens or by supporting plant health. Considering the low availability of these microorganisms in the natural soil, it is necessary to introduce their use in integrated pest management, in order to control plant disease (Nederhoff, 2001).

In plant pathology, but not only, the organism, that allows suppressing the pathogen, is referred to as the biological control agent (BCA).

The term biological control includes not only the use of antagonist microorganisms but also cultural practices which contribute to decreasing disease incidence or disease severity (Alabouvette *et al*., 2006).

It is important to emphasize that hosts, pathogens and environment interact with each other, up to influence the disease severity. In detail, some factors of the environment, such as soil moisture and temperature, have an important role in the "disease triangle". Moreover, more organisms that live in the soil with the pathogen are often ignored (Stirling and Stirling, 1997).

### 3.2. BCAs: mechanisms of action

The biological control agents have different mechanisms of action, such us competition for nutrients and root exudates, competition for space or infection space of the roots, induction resistance in the plant and mycoparasitism (Pal and McSpadden Gardener, 2006).

In order to understand the mechanism of biological control, it is helpful to know the different mechanisms of interaction of plant and pathogen with a wide range

of organisms (Pal and McSpadden Gardener, 2006). However, organisms must have a direct or indirect contact, in order to interact with the host.

In 1953, Odum proposed different types of interaction, such as mutualism, commensalism, protocooperation, neutralism, competition, amensalism, parasitism and predation: some of these can be used in plant pathology and others in entomology.

In biological control, mutualism can be considered an association between two or more species, thanks to both species benefit. In soil mutualism interaction occurs between plant and mycorrhizal fungi, or, for example bacteria of the genus *Rhizobium*, that can reproduce either in the soil or through their mutualistic association with legume plants. Thanks to these types of mutualism the plants are most fortified, increasing the nutrition and stimulating hot defences (Pal and McSpadden Gardener, 2006).

One of the most important mechanisms of biological control is competition: in this case non-pathogen competes with the pathogen for nutrient in and around the host plant. Accordingly, there is a competition between species with a consequent reduction of growth, activity and fecundity of the interacting organism (Pal and McSpadden Gardener, 2006).

The most important expression of this phenomenon occurs at the leaf surface, where the antagonist reduces infection because it avoids the germination of fungal spore. In addition, competition for nutrients, especially for carbon, is common in soil which is an oligotrophic milieu; it is considered responsible for the well-known phenomenon of fungistasis (Lockwood, 1977) during which it occurs the inhibition of fungal spore germination in soil. Furthermore, it is possible that the BCA competes with the pathogen also for minor elements, such as iron, and not only for carbon in soil. It is the typical mode of action of *Pseudomonas fluorescens*, which are able to limit the growth of pathogenic fungi and reduce disease incidence or severity (Schippers *et al*., 1987; Bakker *et al*., 1991; Lemanceau and Alabouvette, 1993; Loper and Henkels, 1997).

Another mechanism of biological control is parasitism. This type of association is a symbiosis in which there is a prolonged relationship between two phylogenetically unrelated organisms (Pal and McSpadden Gardener, 2006).

Subsequently, one of the two organisms, the parasite, which is usually the physically smaller of the two, benefits and the host is harmed to some measurable extent. In detail, the parasite extends hyphal branches toward the target host, coils around and attaches to it with appressorium-like bodies, and punctures its mycelium (Chet and Baker, 1981; Goldman *et al*., 1994). During this phase occurs an interaction between the parasite and fungal host, including the detection of chemical gradients and mycelial surface features (Handelsman and Stabb, 1996). This last phenomenon has been explained by observing that *Trichoderma* coils around *Pythium ultimum* hyphae but not plastic threads of a similar diameter (Dennis and Webster, 1971b).

In hyperparasitism, a specific biological control agent attacks the pathogen and, subsequently, kills it or its propagules (Pal and McSpadden Gardener, 2006). This type of biological control is characterized by four major classes of hyperparasites, such as obligate bacterial pathogens (*Pasteuria penetrans* that is a nemotocide), hypoviruses, facultative parasites and predators. In this mechanism of biological control, hypoviruses are hyperparasites. The most important representation of this last mode of action (with good results in controlling the chestnut blight) is represented by the virus that infects *Cryphonectria parasitica*, a fungus causing chestnut blight, which causes hypovirulence, a reduction in disease-producing capacity of the pathogen (Milgroom and Cortesi, 2004).

In addition to all these antagonists, some parasites fungi of plant pathogens could be used in biological control, such as *Acremonium alternatum*, *Acrodontium crateriforme*, *Ampelomyces quisqualis*, *Cladosporium oxysporum*, and *Gliocladium virens* (Kiss, 2003).

In contrast to hyperparasitism, some fungi exhibit predatory behaviour under nutrient-limited conditions. This type of association is known as predation and it is a non-specific pathogen. In this case, the pathogen can produce enzyme that attack the cell walls of fungi, such as some species of *Trichoderma*. But, in few case, for example when fresh bark is used in composts, *Trichoderma* spp. do not directly attack the plant pathogen, *Rhizoctonia solani*, because there is a high concentration of readily available cellulose (Pal and McSpadden Gardener,

2006). Accordingly, when bark is decaying, the low level of cellulose allows the activation of the chitinase genes of *Trichoderma* spp. and the consequent production of chitinase, which parasitizes *R. solani* (Benhamou and Chet, 1997).
One of the most important ability of the BCAs is production of antibiotics, which are microbial toxins that, even at low concentrations, are able to poison or kills microorganisms.
Some biological control agents are able to produce different type of molecules, some of these have been show to be effective at suppressing plant pathogens (Fravel, 1988; Loper and Lindow, 1993; Weller and Thomashow, 1993). These molecules can be both antibiotics *sensu strictu* and bacteriocines or enzymes, such as cell wall degrading enzymes, and volatile compounds with an antifungal activity (Alabouvette *et al*., 2006). Furthermore, in order to be effective in controlling plant pathogen, antibiotics have to be produced in sufficient quantities near the pathogen. *In situ* production of antibiotics by several different biocontrol agents has been measured (Thomashow *et al*., 2002).
Several antagonists produce different types of secondary metabolites, which are effective against different species of fungal pathogens (Alabouvette *et al*., 2006). Moreover, this phenomenon is very common in many microorganisms, which are used as BCAs, such as fluorescent *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp. and *Trichoderma* spp. It has been demonstrated that some strains of *Trichoderma* spp. are able to produce different types of secondary metabolites (Sivasithamparam and Ghisalberti, 1998), including antibiotics (Howell *et al*., 1988) and cell wall degrading enzymes (Kubicek and Penttila, 1998; Lorito, 1998).
It is important to underline that the antifungal activity of antagonistic microorganism is expressed by the secondary metabolites and not only by a single metabolite produced by the strain (Alabouvette *et al*., 2006).
In biological control it can leverage the activities of different antagonists, such as *Trichoderma* species, which are capable to control some root disease, and some non-pathogenetic *Fusarium* species which may suppress Fusarium wilt. Furthermore, some strain of bacteria, such as *Bacillus subtilis*, *Pseudomonas* spp., could be used to control a large number of root disease.

**Plant Growth Promotion**

A good control of disease can be obtained by exploiting the ability of antagonistic microorganisms of increasing plant growth. In particular, this activity is important during the early stage of life cycle, when plant is most susceptible to the disease. Thanks to the interaction with bio agents, plants develop the root system, being able to absorb a greater amount of nutrients, made available by the activity of microorganisms (Chaube *et al*., 2003).

**Induction of host resistance**

One of the indirect effects caused by the biological control agents is the induction of resistance. Several environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability, and chemical ones, produced by soil and plant-associated microbes can induce a local or systemic host defence of plant (Pal and McSpadden Gardener, 2006). Nowadays, plant pathologists define, the first of these pathways, systemic acquired resistance (SAR), that is mediated by salicylic acid (SA), a compound which is frequently produced following pathogen infection and typically leads to the expression of pathogenesis-related (PR) proteins, among of which there are different enzymes, which can act directly to lyse invading cells, reinforce cell wall boundaries to resist infections, or induce localized cell death (Pal and McSpadden Gardener, 2006).

The plants can react to chemical molecules from natural or synthetic origin, inoculation of pathogenic or non-pathogenic organism and physical stress, by expressing defence reactions leading to systemic induced resistance (SIR) (Alabouvette *et al*., 2006).

The contact between plant pathogen and host plant determine the elicitation of mechanism defence by the host plant. This molecular recognition immediately initiates a cascade of molecular signals and the transcription of many genes. Subsequently, the plant produces a variety of defence molecules, such as phytoalexine, pathogenesis-related (PR), proteins and reinforcement of cell walls (Van Loon, 2000). A typical example of SIR is the hypersensitive reaction induced by a virulent pathogen on a resistant plant cultivar. Moreover, it is

important to stress that the hypersensitive reaction, which results in the death of the infected cells, inducing, as consequence, the stop of the development of the biotrophic pathogens.

Many BCAs, such as the fluorescent pseudomonads and *Trichoderma* species (Harman *et al*., 2004b), are capable to induce systemic resistance in plant. In detail, the fluorescent pseudomonads have been selected for their plant growth promoting capacity (Kloepper *et al*., 1993; Van Loon *et al*., 1998; McSpadden Gardener *et al*., 2005).

One of the most important aspect of the use of BCAs in biological control, is the ability of pathogens do not develop resistance against biological product. During the last decades the excessive use of fungicides has led to the emergence of fungal strains resistant to different chemical molecules. Comprehensively, a good management of diseases, caused by different *Calonectria* species, has been obtained thanks to the use of fungicides, such as benzimidazoles (Barnard, 1984, Chase, 1987; Nan *et al*., 1992; Kucharek and Atkins, 1993). Moreover, it is important to remember that the continued use of these fungicides has led to the selection of resistant strains belonging to the genus *Calonectria* (French and Menge, 1978; Alfenas *et al*., 1988). In detail, it has been demonstrated the selection of several resistant strains both of *Cy. scoparium* and *Cy. pauciramosum* to benomyl and carbendazim (Alfenas *et al*., 1988; De Prest and Poppe, 1988; Polizzi, 2000; Polizzi and Vitale, 2001; Vitale *et al*., 2009b). More lately, Guarnaccia *et al*. (2012, 2014) proved the onset of prochloraz resistant strains in *Calonectria* species. Accordingly, one of the aims of the use of biological control in agriculture is to limit the high risk of selection resistant strains to fungicides. Although BCAs produce several types of antibiotics, the populations of pathogens aren't able to develop resistance to these secondary metabolites. This phenomenon is due to two reasons. On the hand biological antagonists produce more than one antibiotics, and resistance to multiple antibiotics occur at very low frequency; on the other total exposure of population of pathogen to antibiotics is low because they are localized to the root (Handelsman and Stabb, 1996).

The efficacy of biological control could be improved if the different mode of actions is associated with each other, expressed simultaneously or successively. Most of the effective BCAs possess several modes of action and may be responsible for the control of different diseases on the same host or the same disease on several hosts (*Pseudomonas* spp. or *Trichoderma* spp.) (Alabouvette *et al*., 2006).

Although research has progressed in the knowledge of the modes of action of these biological control agents (BCAs), practical application often fails to control disease in the fields, because they are used as a chemical product. In detail, they have to be applied in accordance with their ecological requirements.

In order to being effectiveness, biological control strategies require knowledge-intensive management (Baker, 1987; Cook, 1993; Heydari *et al*, 2004; Shan-Smith and Burns, 1997). Subsequently, it is most important to understanding when and where biological control is profitable, in such a way that it can be put into the integrated pest management systems (Cook, 1993; Heydari *et al*., 2004; Shan-Smith and Burns, 1997).

Considering the concept, according to which the biological control is only not the application of BCAs, the use of appropriate cultural practices is the first phase of an integrated pest program, in order to promote the health crop (Cook, 1993; Heydari *et al*., 2004; Shan-Smith and Burns, 1997). Proper use of tillage and a careful management of soil fertility can limit plant stress, reducing the onset of plant disease (Cook, 1993). Moreover, in nurseries and greenhouse it is possible to control temperature, light, moisture and soil composition, even if this practice cannot wholly eliminate disease problems (Paulitz and Belanger, 2001).

The BCAs can be applied in different ways, in order to obtain a good control of several fungal plant pathogens. In detail, *Trichoderma harzianum* and *Pseudomonas fluorescens* (but also others antagonistic fungi and bacteria) can be applied directly to the infection court at high population level, but also in seed coating (Cook, 1993; Heydari and Misaghi, 2003; Heydari *et al*., 2004). Moreover, antagonists can be use to protect fruits in storage, e.g. *Pseudomonas fluorescens* (De Capdeville *et al*., 2002; El-Ghaouth *et al*., 2000; Janisiewicz

and Korsten, 2002; Janisiewicz and Peterson, 2004), and applied to soil at the site of seed placement (Heydari and Misaghi, 2003).

One of the most important aspects, but that requires further scientific investigation, is that biological control agents are not able to develop resistance to fungicides (Di Francesco and Mari, 2014). The possibility of applying in combination BCA and chemical formulations may offer the advantage of reducing the rates of fungicide and antagonist, thus contributing to management of fungicide resistance, helping to minimize the residue and the cost of biocontrol treatment (Lima *et al*., 2011).

## 4. Thesis aims

Nowadays, the ornamental industry has greatly increased its importance in agriculture of Southern Italy. The continued interest in this commercial sector induced the nurserymen to introduce new species of ornamental plants from other countries, increasing the risk of introduction of new plant pathogens.

Among these, the species of the genus *Calonectria* play an important role, representing a serious threat to the cultivation of ornamental plants. In detail, *Ca*. *morganii* and *Ca*. *pauciramosa* were the first species of this genus identified as causal agents of plant disease in southern Italy (Polizzi and Crous, 1999, Polizzi *et al*., 2006a, 2007b, 2009a,b,c). More recently, another species, such as *Ca*. *polizzii*, has been reported as pathogen of ornamental plants in Europe (Lombard *et al*., 2010b). All the three species are able to cause a large number of serious symptoms, such as crown rot, collar rot and root rot, leaf spots, damping-off and cutting rot, on tree, shrubs and cut flower belonging to *Anacardiaceae*, *Aquifoliaceae*, *Araceae*, *Araliaceae*, *Arecaceae*, *Asteraceae*, *Buxaceae*, *Ericaceae*, *Fabaceae*, *Myrtaceae*, *Polygaceae*, *Rhamnaceae* and *Rosaceae* (Massey, 1917; Anderson, 1919; Storey, 1964; Aragaki *et al*., 1972, 1988; Peerally, 1974; de Prest and Poppe, 1988; Carrai and Garibaldi, 1990; Uchida and Kadooka, 1997; Litterick and McQuilken, 1998; Polizzi and Crous, 1999; Henricot *et al*., 2000; Polizzi, 2000; Polizzi and Catara, 2001; Crous, 2002; Henricot and Culham, 2002; Henricot and Beales, 2003; Poltronieri *et al*., 2004; Lane *et al*., 2006; Polizzi *et al*., 2006a,b; Pérez-Sierra *et al*., 2006, 2007;

Polizzi *et al*., 2007a,b; Vitale and Polizzi, 2007; Aghajani *et al*., 2008; Henricot *et al*., 2008; Hirooka *et al*., 2008; Vitale *et al*., 2008; Polizzi *et al*., 2009c; Vitale *et al*., 2009a; Lechat *et al*., 2010).

Thus, analyzing the situation of ornamental nurseries in southern Italy, and in particular in Sicily, these *Calonectria* species cause several economic damages in some important crops, such as *Polygala myrtifolia*, *Metrosideros* spp., *Callistemon* spp. and other host plants. Accordingly, it is necessary resorting to the use of chemical treatments to control Calonectria diseases (Polizzi and Crous, 1999; Polizzi, 2000; Crous, 2002; Aiello *et al*., 2013).

Although different chemical compounds are reported as effective in controlling Calonectria disease, some authors reported that only preventive measures were able to control infections caused by *Calonectria* spp. on ornamental plants (Crous, 2002; Henricot *et al*., 2008; Aiello *et al*., 2013).

Although the use of fungicides belonging to benzimidazoles and sterol demethylation inhibitors (DMIs) demonstrated a good efficacy in controlling Calonectria disease, their use should be avoided since their continued use led to the selection of MBCs and DMIs-resistant strains (French and Menge, 1978; Alfenas *et al*., 1988; Polizzi and Vitale, 2001; Vitale *et al*., 2009b; Guarnaccia *et al*., 2012, 2014).

On the one hand, it is possible to limit the risk of onset resistant strains by using fungicides with low risk of resistance or different mode of action. On the other, also as set out in Directive 2009/128/EC on the "Sustainable use of pesticides", the modern agriculture should be directed toward a high use of biological control agents, reducing at the same time, the use of chemical compounds. Accordingly, increasing the application of antagonistic microorganisms, it is possible to limit the selection of fungicide resistant strains. Moreover, it is important to highlight that biocontrol has positive effects on plant growth, allowing to reduce the application of chemical fertilizers and increasing the degree of plant resistance to diseases.

Few authors report the efficacy of biological control to reduce infections caused by different species of *Calonectria*. In particular, species belonging to the genus *Trichoderma*, *Bacillus* and *Pseudomonas* have been tested for their antagonistic

activity against *Calonectria* species (Harman, 2000; Polizzi and Vitale, 2002; Bettiol *et al*., 1988; Blum and Lin, 1991; Santos *et al*., 1993; Dumas *et al*., 1996; Garcia and Ferreia, 1997; Sutra *et al*., 2000; Ngueko and Tong, 2002; Vitale *et al*., 2003; Burch and Sarathchandra, 2006; Fan *et al*., 2008; de Carvalho Filho 2009; de Wit *et al*., 2009; Aiello *et al*., 2012; Chaudhary, 2014). In detail, strain T22 of *T. harzianum* has given important results in controlling crown and root rot of *Polygala myrtifolia* caused by *Ca. pauciramosa* (Polizzi and Vitale, 2002). The same antagonist proved high performances against *Ca. pauciramosa* in reducing both microsclerotia production *in vitro* and collar and root rot *in vivo* on feijoa sellowiana and red clover (Vitale *et al*., 2012).

More recently, in an experiment for studying red clover as model plants, it was assessed the activity of several fungicides and biological control for reducing infections caused by *Ca. morganii* and *Ca. pauciramosa* on the host-model plant *Trifolium pratense*. It was found that propamocarb + fosetyl-Al, azoxystrobin, cyproconazole, K phosphite and fosetyl-Al were the most effective fungicides in reducing *Ca. morganii* infections. Besides *Clonostachys rosea* and *Penicillium oxalicum* were effective in controlling *Ca. morganii* infections, while *Bacillus subtilis* QST713 and *Streptomyces griseoviridis* K61 were not effective (Aiello *et al*., 2012).

Analyzing these few study about the biocontrol of *Calonectria* spp. and also as set out in Directive on the "Sustainable use of fungicides", as mentioned above, it is critically important to develop IPM programs that involve extensive use of biological products in order to make more and more current the concept of "green revolution". Whereas the importance of protecting plants from disease in order to maintain the high level of quality and production, the use of BCAs could represent a viable alternative to chemical control in management programs of pathogens.

Nevertheless, the use of BCAs is limited by several environmental and biological factors as well as the mode of application. In fact, they are often applied as fungicides, without taking into account the conditions of soil temperature and moisture, the ability of these antagonist microorganisms to colonize soil and leaf surface, which could influence the ability of these

microorganism to control pathogens (Pal and McSpadden Gardener, 2006). Thus, the development of IPM programs requires the knowledge of all the factors mentioned so far.

Considering an integrated control strategy to reduce plant diseases, greater attention must be paid to the phenomenon of the resistance of BCAs to fungicides (Di Francesco and Mari, 2014). The possibility of integrate antagonistic microorganisms and chemical formulates may offer the advantage of applying low doses, increasing the time of application between treatments, thus helping to minimize the residue, the cost of biocontrol treatment and reducing the risk of onset of fungicide resistance strains (Lima *et al*., 2011).

Based on these concepts, the detection of new integrated pest management programs is closely related to the relationship between BCAs, the host plant, the specific plant pathogens and the environmental conditions (Pal and McSpadden Gardener, 2006).

According to what said so far, the first aim of this thesis was to asses several fungicides and biological control agents for controlling infections caused by different species of *Calonectria* in ornamental plants. In particular, several experiments have been performed with the aim of drawing up IPM programs for reducing infections of *Ca*. *morganii* and *Ca*. *pauciramosa* in ornamental plants nurseries, observing the regulations imposed recently by the European Commission.

Considering mycoparasitism activity (Pal and McSpadden Gardener, 2006) and the production of non-volatile metabolites of some antagonistic microorganisms (Dennis and Webster, 1971a), a preliminary study was performed to evaluate *in vitro* the potential biocontrol activity of several antagonists against different *Calonectria* species in reducing the mycelial growth. This experiment was conducted by applying the dual culture and cellophane culture.

Subsequently, the first experiment *in vivo* was performed in order to determine which chemical and biological treatments have the best performances for management leaf spot caused by *Ca*. *morganii* on *Callistemon viminalis*.

Since in the last years other species of the genus *Calonectria* have been found as causal agents of disease in ornamental plants, one of the aim of this thesis was to found which fungicides and BCAs (applied alone and in combination) had the best effectiveness in controlling leaf spots caused by six different *Calonectria* species on two different host plants. *Ca*. *mexicana*, *Ca*. *morganii*, *Ca*. *pauciramosa*, *Ca*. *polizzii*, *Ca*. *pseudomexicana* and *Ca*. *tunisiana* were the species assessed in this experiment, while the host plants used were *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook". The effectiveness of each treatment was determined in relation to the different *Calonectria* species used and the different susceptibility of the host plant to the same pathogen.

In addition, in the third experiment *in vivo* it was evaluate the ability of several fungicides and BCAs, applied alone and in combination, for controlling stem rot caused by artificial infection of *Ca*. *morganii* on *Dodonaea viscosa*.

In the last decades, fungal species of the genus *Calonectria* have been reported as causal agents of disease on *Polygala myrtifolia*. In detail, *Ca. pauciramosa* was identified as responsible for crown and root rot on this host in Europe (Polizzi and Crous, 1999). As for other *Calonectria* species, chemical control of disease caused by *Ca. pauciramosa* is necessary for reducing infections in nurseries. For this purpose, it is really important to found IMP programs that could allow the management of this pathogen. Therefore, a further experiment of my thesis project was performed to test the best fungicides and BCAs, applied alone and in combination, for controlling crown and root rot caused by artificial infections of *Ca. pauciramosa* on *P. myrtifolia*.

One of the limiting factors in nurseries during the growing stage, rooting and transplanting is the use of infected soil (Vitale *et al*., 2013b). Thus, it is important avoid the presence of soil-borne pathogens in the soil. Among these, there are the species belonging to the genus *Calonectria*. They are polycyclic fungi. In addition, an important feature of this genus is that its species are able to differentiate chlamydospores and microsclerotia that are chains of chlamydospores, on senescing plant tissue. These microsclerotia can survive in

soil or in plant tissue for very long time, by representing the primary inoculum of pathogen.

Whereas species of the genus *Calonectria* are among the most troublesome and difficult pathogens to control, of all soil-borne pathogens that attack nurseries, field and greenhouse, the reduction of primary inoculum of this nectrioid is an important goal in management strategies of pathogens. Various methods have been proposed to achieve this target. One of these is soil solarization. In last year, Vitale *et al*. (2013a) asserted that soil solarization, performed in greenhouse during summer (with temperature above 35°C), allows to reduce microsclerotia of *Ca*. *pauciramosa* and *Ca*. *morganii*. Nevertheless, the application of this method is limited because it is highly dependent on the environmental conditions (temperature, soil moisture), the purchase and the cost of disposing of plastic films. Furthermore, in the modern agriculture, soil fumigation can represent a viable alternative method to achieve reduction of primary inoculum of soil-borne pathogens. Few information is viable about this method for suppressing microsclerotia of *Calonectria* spp. from substrate.

Although the use of fumigants in IPM strategies can be a contradiction, it is important to point out that their application at low doses does not create biological vacuum (Ślusarski *et al*., 2012). Accordingly, their simultaneous application with antagonistic microorganisms could be a valid strategy of integrated control (Ślusarski *et al*., 2012).

Thus, one of the goals of this thesis was to test the efficacy of two different chemical fumigants for reducing or suppressing microsclerotia of *Calonectria* spp. from the substrate. The purpose of this study was to determine the potential activity of metham-sodium and dazomet in suppressing primary inoculum, when applied at label and sub label rates. The ultimate goal would be to identify the lowest application rate capable to kill microsclerotia of *Calonectria*, thus complying with the latest European standards.

The recent measures by the European Union impose restrictions on the use of fumigants. Regulation 359/2012 establishes that the active substance metham is approved until to 30 June 2022, but its use is limited to one application every

three years on the same field and its maximum application rate is 153 kg/ha (corresponding to 86,3 kg/ha of MITC) in case of open field applications.

In the Commission Directive 2011/53/UE of 20 April 2011 it is established that the use of active substance dazomet is limited to one applicatio every three years and its use is permitted until to 31 May 2021. Comprehensively, it is recommended the use of gas-tight plastic films, immediately after the application of fumigants.

According what explained in all these regulations, from 1$^{th}$ January 2015 modern agriculture must be oriented toward the application of doses lower and lower. In order to limit the emission of pollutant molecules to the atmosphere and maintain the efficacy of treatment with fumigants, it is possible to apply plastic tarps for covering the fumigated soil. This concept was applied in one of the experiments of my thesis, in order to evaluate and compare the ability of two different plastic films, such as virtually impermeable film (VIF) and totally impermeable film (TIF) in improving the retention of both fumigants, increasing the effectiveness of their sub label rates in reducing microsclerotia of *Calonectria* spp.

## 5. *In vitro* antagonism of BCAs against *Calonectria* species.

### 5.1. Materials and methods

**Fungal cultures**

Five isolates of the genus *Trichoderma*, such as *Trichoderma harzianum* T22, *T. harzianum* T67, *T. reseei* T34, *T. atroviride* P1 and *T. koningii* 8009, one isolate of *Clonostachys rosea* and *Penicillium oxalicum* were assessed as antagonists *in vitro* against the following species belonging to the genus *Calonectria*: *Calonectria morganii* (GP0), *Ca. pauciramosa* (MY5), *Ca. polizzii* (TMC6), *Ca. mexicana* (TDV1), *Ca. pseudomexicana* (TCROU1), *Ca. tunisiana* (TCL1) and *Ca. ilicicola* (LN3).

Fresh cultures of each isolate were obtained by transferring agar plugs from stock cultures onto potato dextrose agar (PDA) plates. Cultures were incubated at 25 °C in the dark for 7 days before use.

**Dual culture technique**

*In vitro* mycoparasitism activity of each *Trichoderma* spp. and *C. rosea* against each *Calonectria* spp. was studied by following the method described by Morton and Stroube in 1955. A mycelial disc of 5 mm in diameter was cut with a cork-borer from the edge of colony of 7 day old cultures of each antagonist and inoculated at one side of the Petri dish with sterilized Potato Dextrose Agar (PDA). Simultaneously, a mycelial disc of 5 mm in diameter of the test *Calonectria* spp. was inoculated at the opposite side of the same Petri dish. A control Petri dish was inoculated only with a mycelial disc of pathogen tested. Each pairing was replicate three times in a completely randomized experimental design. The plates were incubated in a growth chamber at temperature of 25 °C. Data were collected 4 and 7 days after incubation period. The antagonistic activity of each *Trichoderma* spp. and *C. rosae* was evaluated by measuring radial growth of pathogen as percentage inhibition (I%). This value was calculated by applying the following formula (Vincent and Budge, 1990):

$$I\% = [(dC - dT)/dC] \times 100,$$

where dC is the diameter of colony of pathogen in control, dT is the diameter of colony of pathogen in dual culture.

The mycoparasitism activity of each *Trichoderma* spp. and *C. rosea* against each *Calonectria* species was also determined 4 and 7 days after the incubation by applying two different scales (Bell *et al*., 1982). In detail, after 4 days, it was used a 0-to-4 rating scale, as follow: 0- antagonist colonizes completely the pathogen and covers the whole surface of the medium, 1- antagonist colonizes two-thirds of the surface of medium, 2- antagonist and pathogen colonize each half of the surface of medium, 3- antagonist colonizes one-thirds of the surface of medium, 4-antagonist completely covered by the pathogen; while after 7 days data were collected by using a 0-to-7 rating scale: 0- pathogen overgrowth >1 cm, 1- pathogen overgrowth upon the antagonist mycelium 0.5 < > 1 cm, 2- overgrowth of pathogen <0.5 cm, 3- no overgrowth of the pathogen, 4- antagonist traces on the pathogen mycelium, 5- antagonist colonizes half of the surface of pathogen, 6- antagonist overgrows two-thirds of the surface of pathogen, 7- antagonist overgrows completely to pathogen and covers the whole surface of the medium.

**Cellophane culture**

Cellophane culture is a technique that allows to study the ability of antagonist to produce non-volatile metabolites (Dennis and Webster, 1971a). Sterilized cellophane disc was laid gently on sterilized PDA medium. A plug of 5 mm in diameter, cut with a cork-borer from the edge of colony of 7 day old cultures of the antagonist, was transferred to the center of the dish over cellophane. Then, Petri dish with cellophane was incubated at temperature of 25 °C for two days. Then, cellophane together with fungal disc was gently removed. For control, a plate with cellophane was inoculated with only PDA disc. After that, a plug of 5 mm in diameter of the pathogen was inoculated on the same medium and precisely to the center of the plate. The plates were incubated at temperature of 25 °C for 5 days. For each treatment three replicates were maintained. After 5 days, the ability of each antagonist to produce non-volatile metabolites was evaluated by measuring the diameter of radial growth of pathogen and at last

calculating the percentage inhibition (I%) by applying the formula used in dual culture experiment.

## 5.2. Results

**Dual culture**

Data recorded four days after incubation in dual culture technique showed that all *Trichoderma* species proved a variable effectiveness in inhibiting the mycelial growth of all *Calonectria* isolates. In detail, the mycelial growth of GP0, LN3 and TDV1 was inhibited by all *Trichoderma* species. *T. reseei* T34 was the only *Trichoderma* species able to reduce the mycelial growth of TCROU1 (11.11%). All *Trichoderma* species, except for *T. harzianum* T22 (0%), are able to reduce the growth of colony of TCL1, with percentage inhibition ranging from 8.82 to 11.76%. Only the two isolates of *T. harzianum* are able to inhibit the growth of MY5 (percentage inhibition of 7.69% for both), while the mycelial growth of TMC6 is reduced only by *T. harzianum* T22 (12.5%) and *T. reseei* T34 (2.07%) (Table 1).

The results collected after seven days proved that all *Trichoderma* species developed mycoparasitism activity against all *Calonectria* species. In addition, it is important to note that there was always an increase in percentage inhibition (ranged from 4.95 to 44.26%) in data recorded after seven days of incubation compared to those collected after four days (ranged from 2.56 to 13.21%).

*C. rosea* proved a good mycoparasitism activity against GP0, LN3 and MY5 after four days of incubation, with percentage inhibition of 13.43, 1.89 and 4.62%, respectively. Moreover, data collected after seven days showed that this antagonist was able to inhibit only the mycelial growth of LN3 (2.25%), TCL1 (8.2%) and TCROU1 (14.85%), but no effect was found for other *Calonectria* species (Table 1).

The mycoparasitims activity of each *Trichoderma* species and *C. rosea* against *Calonectria* species was also determined by using two different scales. Data collected after four days of incubation showed that all *Calonectria* isolates placed in class 1, proving that each antagonist colonized two-thirds of the surface of medium. Unlike all the others, TMC6 placed in the class 3 in

interaction with *T. atroviride* P1. This proved that antagonist colonized one-thirds of the surface of medium.

After seven days, no overgrowth of the pathogen was found and in some cases traces of the antagonist on the pathogen were observed. For this reason, all *Calonectria* isolates reside in class 3 and 4 (Table 2). Only for LN3 it was found a pathogen overgrowth upon the antagonist mycelium comprised between 0.5 and 1 cm (class1) or overgrowth of the pathogen less than 0.5 cm (class 2).

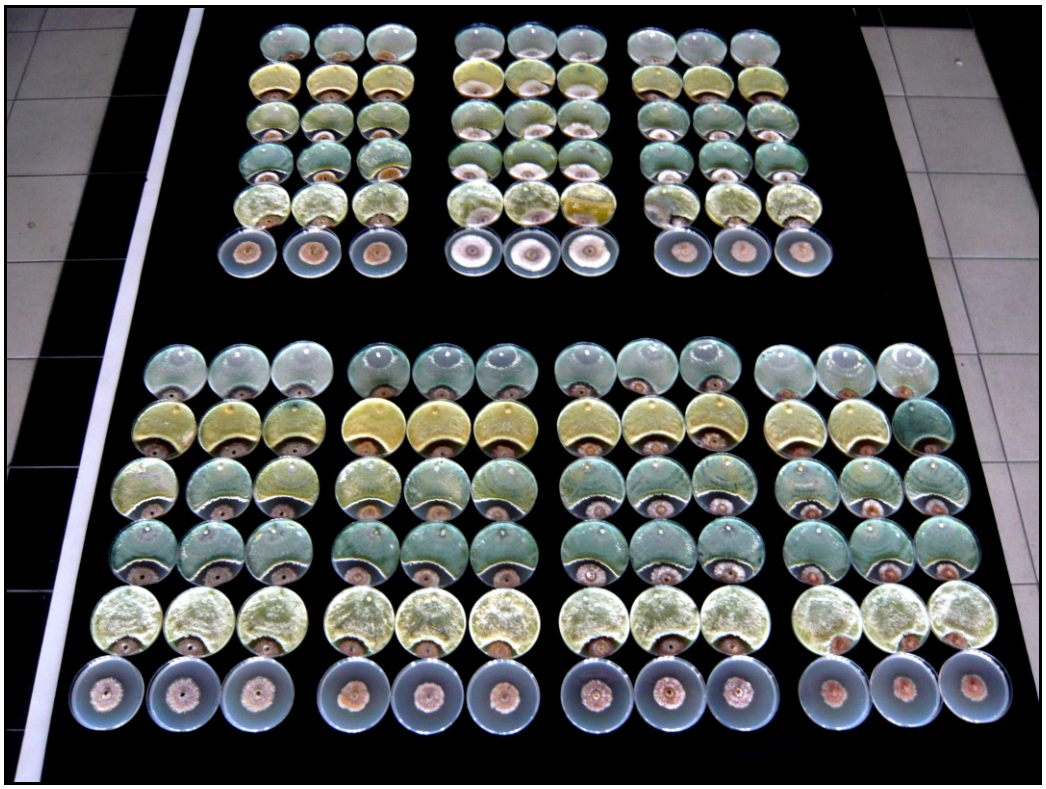

**Figure 4 - Mycoparasitism activity of *Trichoderma* spp. against several *Calonectria* spp.**

### Cellophane culture

The results proved that among *Trichoderma* species, *T. harzianum* T22, *T reseei* T34 and *T. atroviridae* P1 were not able to reduce the mycelial growth of GP0, TCL1 and TMC6. All isolates, except *T. harzianum* T22 and T67 and *T. atroviridae* P1, showed inhibitive activity on the mycelial growth of TDV1. In some cases, it is possible to observe that there is any effect of *T. koningii* and *T. harzianum* T67 against TCL1 and TMC6. No inhibitory effect was observed for both isolates of *T. harzianum* against MY5 as well as for *T. atroviridae* P1

against TDV1. The mycelial growth of LN3, MY5 and TCROU1 was averagely reduced by non-volatile metabolites produced by all *Trichoderma* species.

Among all *Calonectria* isolates, only LN3 and TCROU1 are significantly inhibited by the products of all *Trichoderma* species, with percentage inhibition ranging from 1.75 to 26.15%.

By analyzing the effectiveness of *P. oxalicum* in inhibiting the mycelial growth of *Calonectria* species, it is possible to assert that this antagonist is able to reduce the growth of colony of LN3 (7.02%) and TCROU1 (15.38%), but not for all other species.

At last, the non-volatile metabolites produced by *C. rosea* were able to inhibit the mycelial growth only of LN3 (6.14%) and TCROU1 (1.54%), while no effect was found in all other *Calonectria* species. All these data are reported in Table 3.

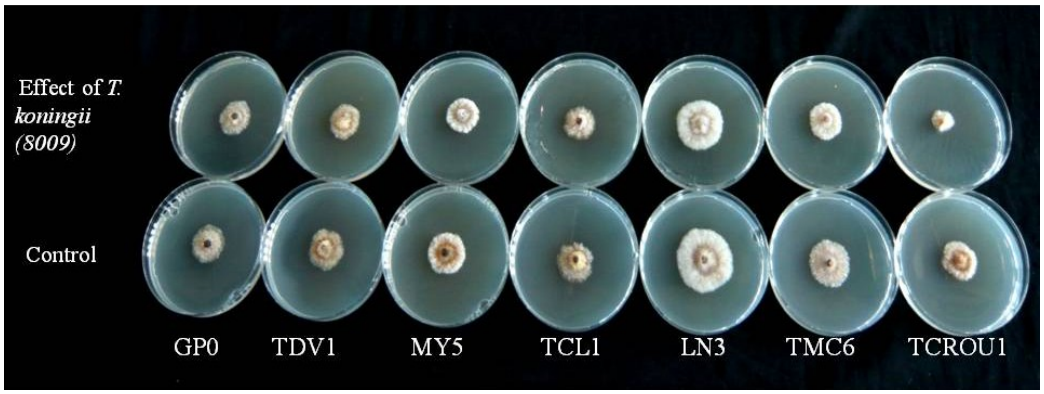


**Figure 5 - Effect of non-volatile metabolites produced by *Trichoderma koningii* (8009) on different *Calonectria* spp.**

**Table 1 - Mycoparasitism of several *Trichoderma* spp. against different *Calonectria* spp. (dual culture)**

| | Inhibition (%) after 4 days | | | | | | Inhibition (%) after 7 days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***T. harzianum* (T22)** | ***T. reseei* (T34)** | ***T. atroviride* (P1)** | ***T. koningii* (8009)** | ***T. harzianum* (T67)** | ***C. rosea*** | ***T. harzianum* (T22)** | ***T. reseei* (T34)** | ***T. atroviride* (P1)** | ***T. koningii* (8009)** | ***T. harzianum* (T67)** | ***C. rosea*** |
| **GP0** | 10.45 | 10.45 | 7.46 | 4.48 | 4.48 | 13.43 | 25.58 | 10.08 | 24.03 | 39.53 | 30.23 | -3.88 |
| **LN3** | 13.21 | 9.43 | 5.66 | 13.21 | 7.55 | 1.89 | 22.47 | 10.11 | 17.98 | 30.34 | 33.71 | 2.25 |
| **MY5** | 7.69 | 0 | -7.69 | -1.54 | 7.69 | 4.62 | 31.53 | 26.13 | 11.71 | 27.93 | 22.52 | -0.9 |
| **TCL1** | 11.76 | 8.82 | 8.82 | 8.82 | 0 | -1.47 | 44.26 | 26.23 | 9.84 | 40.98 | 19.67 | 8.20 |
| **TMC6** | 12.5 | 3.12 | 0 | 0 | -7.81 | -6.25 | 27.93 | 15.32 | 11.71 | 20.72 | 15.32 | -4.5 |
| **TCROU1** | -11.11 | 11.11 | -7.41 | -7.41 | -29.63 | -3.7 | 36.63 | 26.73 | 12.87 | 40.59 | 4.95 | 14.85 |
| **TDV1** | 8.97 | 2.56 | 2.56 | 5.13 | 7.69 | 0 | 37.88 | 24.24 | 21.21 | 36.36 | 27.27 | 0 |

**Table 2 - Mycoparasitism of several *Trichoderma* spp. against different *Calonectria* spp. (dual culture)**

| | Mycoparasitism rating (0–to-4) after 4 days | | | | | | Mycoparasitism rating (0–to-7) after 7 days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***T. harzianum* (T22)** | ***T. reseei* (T34)** | ***T. atroviride* (P1)** | ***T. koningii* (8009)** | ***T. harzianum* (T67)** | ***C. rosea*** | ***T. harzianum* (T22)** | ***T. reseei* (T34)** | ***T. atroviride* (P1)** | ***T. koningii* (8009)** | ***T. harzianum* (T67)** | ***C. rosea*** |
| **GP0** | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 4 | 3 | 4 | 3 | 3 |
| **LN3** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 3 |
| **MY5** | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 4 | 3 | 3 |
| **TCL1** | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 4 | 3 | 3 |
| **TMC6** | 1 | 1 | 3 | 1 | 1 | 1 | 3 | 3 | 3 | 4 | 3 | 3 |
| **TCROU1** | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 3 | 3 | 4 | 3 | 3 |
| **TDV1** | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 3 | 3 | 4 | 3 | 3 |

**Table 3 - Effects of non-volatile metabolites produced by several *Trichoderma* spp. on radial growth of different *Calonectria* spp. (cellophane culture)**

| Inhibition (%) | *T. harzianum* (T22) | *T. reseei* (T34) | *T. atroviride* (P1) | *T. koningii* (8009) | *T. harzianum* (T67) | *P. oxalicum* | *C. rosea* |
|---|---|---|---|---|---|---|---|
| **GP0** | -4,17% | -4,17% | -6,94% | 8,33% | 1,39% | -4,17% | 0,00% |
| **LN3** | 1,75% | 6,14% | 4,39% | 13,16% | 7,02% | 7,02% | 6,14% |
| **MY5** | 0,00% | 1,35% | 5,41% | 4,05% | 0,00% | -5,41% | -5,41% |
| **TCL1** | -17,91% | -11,94% | -2,99% | 0,00% | 0,00% | -16,42% | -16,42% |
| **TMC6** | -17,91% | -11,94% | -2,99% | 0,00% | 0,00% | -13,43% | -16,42% |
| **TCROU1** | 10,77% | 18,46% | 13,85% | 26,15% | 9,23% | 15,38% | 1,54% |
| **TDV1** | -5,26% | 3,95% | 0,00% | 6,58% | -1,32% | -3,95% | -9,21% |

### 5.3. Discussion

In conclusion, all these data collected from this preliminary *in vitro* antagonist study provide a framework for the biological control of *Calonectria* species. Although some *Trichoderma* species showed a variable mycoparasitism activity against *Calonectria* species after four days, all *Trichoderma* species are able to inhibit the mycelial growth of all *Calonectria* species after seven days, showing also an overgrowth on the pathogen colony. Thus, the time of incubation is an important factor that affects the ability of the antagonist to inhibit the mycelial growth of the pathogen. Therefore, *C. rosea* showed a variable mycoparasitism activity against different pathogen tested.

The non-volatile metabolites produced by different *Trichoderma* species, *P. oxalicum* and *C. rosea* proved a variable effectiveness in relation to the different *Calonectria* species involved. Only for LN3 and TCROU1 it is possible to note a good percentage inhibition of mycelial growth.

In conclusion, it is possible to assert that although all tested antagonistic species show a variable effectiveness both in mycoparasitism activity and in production of non-volatile metabolites, they can be taken into account for further studies of their efficacy *in planta*. Furthermore, these data provide important information for the selection of BCAs, and related commercial products, for evaluation under field conditions.

## 6. Biological and chemical control of Calonectria leaf spot on *Callistemon viminalis*

### 6.1. Materials and methods

A selected isolate of *Calonectria morganii* was used in these experiments as fungal agent able to induce leaf spot on *Callistemon viminalis*.

Fresh culture of this isolate was obtained by transferring agar plugs from stock cultures onto PDA plates. Then, a mycelial plug of five days old culture on PDA was placed on synthetic low-nutrient agar (SNA) dishes and incubated at 25 °C for 21 days. Conidial suspension was prepared by adding sterile distilled water (SDW) to the dishes, then gently rubbing the colony surface with a sterile loop and filtering through a triple layer of cheesecloth. Final spore suspension of 2.5 $\times 10^5$ conidia $ml^{-1}$ was determined using a haemocytometer.

Three experiments were performed to evaluate the ability of six commercial fungicides and seven bioformulates based on BCAs to control leaf spots caused by artificial infections of *Ca*. *morganii* on bottlebrush. Both chemical and biological products were employed at standard rates (Table 4). Fungicides treatments consisted of boscalid (Cantus®), boscalid+pyraclostrobin (Signum®), fluopyram (Luna Privilege®), fosetyl-Al (Aliette®), propamocarb+fosetyl-Al (Previcur Energy®) and trifloxystrobin (Flint®); instead, the BCAs, used in this study, were *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 (Amylo-X®), *B. subtilis* strain QST713 (Serenade Max®), *Streptomyces griseoviridis* strain K61 (Mycostop®), *Trichoderma atroviride* T-11+*T. asperellum* T-25 (Tusal®), *T. asperellum* TV1 (Xedavir®), *T. harzianum* T-22 (Trianum-P®) and *T. harzianum* strain ICC012+*T. viride* strain ICC080 (Radix®).

Each treatment was replicated three times in a randomized block design with a number per replicate up to 42 bottlebrush cuttings. The same number of untreated and inoculated and untreated and not-inoculated bottlebrush cuttings was used in each experiment.

In each experiment BCAs and fungicides were applied 24 and 3 hours before pathogen inoculation, respectively. They were prepared according to manufacturers recommendations and sprayed by a hand-pump up to run-off of

plants. Inoculum of *Ca*. *morganii* was sprayed on leaf surface of bottlebrush with a hand sprayer, by applying about 25-50 ml of conidial suspension for each replicate.

After inoculation bottlebrush cuttings were covered with plastic tunnels for 3 days and then maintained in greenhouse at temperature of about 25 °C.


**Figure 6 - *Callistemon viminalis* cuttings arranged in randomized blocks according to designed experimental scheme**

The ability of fungicides and BCAs in controlling leaf spots symptoms was evaluated 10 days after inoculation, by calculating disease incidence of infected plants ($DI_P$), disease incidence of infected leaves ($DI_L$) and symptoms severity (SS). The both DI values were calculated as a percentage of cuttings or leaves showing symptoms out of the total number of plants or leaves examined × 100. SS was calculated by using an empirical 1-to-5 rating scale that takes into account the percentage of infected foliar surface, as follow: 1 = healthy cuttings; 2 = 1 to 5%; 3 = 6 to 25%; 4 = 26 to 50%; 5 = more than 50% of infected leaf

area (Fig. 7). A single value was obtained by using the mean of 10 leaves per plant.

SS values were converted to mean disease rating (MDR) that was calculated as $\Sigma$ (the number of disease leaves in this class × the disease class) / number of leaves scored.


**Figure 7 - 1-to-5 rating scale used in these experiments**

**Statistical analysis**

Disease data obtained from the inoculation trials were subjected to analysis of variance according to parametric approaches (Statistica 10; StatSoft Inc., Analytical Software for Windows) with factorial treatment structure and interactions. All DI percentage data were transformed using arcsine ($\sin^{-1}$ square root x) prior to statistical analysis. The untransformed values of DI (%) are presented in tables and separated by Duncan test at $p$ = 0.01 and 0.05.

**Table 4 - Fungicides and BCAs employed in controlling leaf spot caused by *Calonectria morganii* on *Callistemon viminalis***

| *Active ingredient* | *Trade name* | *Manufacturer* | *Rates (g or ml/100 liters)* | *Formulation*[x] |
|---|---|---|---|---|
| Boscalid | Cantus | Basf Italia | 120 | 50 WG |
| Boscalid+pyraclostrobin | Signum | Basf Italia | 100 | 26.7+6.7 WG |
| Fluopyram | Luna Privilege | Bayer CropScience | 50 | 41.66 SC |
| Fosetyl-Al | Aliette | Bayer CropScience | 300 | 80 WG |
| Propamocarb + fosetyl-Al | Previcur Energy | Bayer CropScience | 250 | 47.2+27.6 SL |
| Trifloxystrobin | Flint | Bayer CropScience | 30 | 50 WG |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | Amilo-X | Biogard | 250 | 25 WG |
| *Bacillus subtilis* strain QST713 | Serenade Max | Basf Italia | 400 | WP |
| *Streptomyces griseoviridis* strain K61 | Mycostop | Bioplanet | 25 | 33.33 WP |
| *Trichoderma asperellum* TV1 | Xedavir | XedaItalia | 400 | 2.8 WP |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | Tusal | Certis | 250 | WG |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride strain* ICC080 | Radix | Certis | 250 | 2 + 2 WP |
| *Trichoderma harzianum* T-22 | Trianum-P | Koppert Biological Systems | 100 | 1.15 WP |

[x] Percentage of active ingredient; SC = suspension concentrate; SL=soluble concentrate; WG = water dispersible granule; WP = wettable powder.

## 6.2. Results

In all experiments $DI_P$ ranged from 34.1 to 83.3%, while $DI_L$ and SS ranged from 13.7 to 23.9% and from 1.21 to 1.3, respectively.

Through all experiments, boscalid+pyraclostrobin, fluopyram and *B. subtilis* strain QST713 revealed the most effective treatments in controlling Calonectria leaf spot. In detail, these treatments always reduced significantly $DI_P$ with values comprised between 0 and 10.5%, if compared with the untreated and inoculated control. $DI_L$ and/or SS were always reduced but significant results were detected for the first and third experiments.

In detail, the best performances in experiment I were observed for boscalid+pyraclostrobin and fluopyram, followed by *B. subtilis* strain QST713 (Table 5). Boscalid, propamocarb+fosetyl-Al, fosetyl-Al and *B. amyloliquefaciens* subsp. *plantarum* strain D747 showed an intermediate efficacy in controlling Calonectria leaf spot. Trifloxystrobin reduced significantly $DI_P$ and SS but it was not able to reduce $DI_L$. Otherwise, the worst performances were detected for *T. atroviride* T-11+*T. asperellum* T-25, *T. harzianum* strain ICC012+*T. viride* strain ICC080, *T. asperellum* TV1, *T. harzianum* T-22 and *S. griseoviridis* strain K61 (Table 5).

**Table 5 - Effects of fungicides and BCAs on disease incidence and symptoms severity of leaf spot caused by *Calonectria morganii* on bottlebrush (experiment I)**

| Treatments | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity [z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 e | 0.0 d | 1.00 e |
| Boscalid+pyraclostrobin | 2.3 e | 0.2 d | 1.00 e |
| Fluopyram | 4.6 e | 1.1 d | 1.01 e |
| *Bacillus subtilis* strain QST713 | 10.5 de | 1.8 d | 1.07 bcde |
| Boscalid | 14.6 cde | 2.7 cd | 1.03 de |
| Propamocarb+fosetyl-Al | 16.0 cde | 5.0 bcd | 1.06 cde |
| Fosetyl-Al | 17.2 cde | 4.1 cd | 1.05 cde |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 19.7 cde | 2.7 cd | 1.03 de |
| Trifloxystrobin | 23.2 bcde | 11.8 abc | 1.07 bcde |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 46.6 abcd | 15.0 ab | 1.19 abc |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080 | 51.4 abc | 15.8 ab | 1.19 abc |
| *Trichoderma asperellum* TV1 | 60.8 ab | 20.4 a | 1.26 a |
| *Trichoderma harzianum* T-22 | 64.4 ab | 15.5 ab | 1.18 abcd |
| *Streptomyces griseoviridis* strain K61 | 69.1 a | 16.9 ab | 1.22 ab |
| Untreated and inoculated control | 83.3 a | 23.9 a | 1.30 a |

[y] Averaged from 3 replicates each formed up to 42 *Callistemon viminalis* cuttings.
[z] Averaged from 3 replicates each formed up to 420 *Callistemon viminalis* leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

In experiment II, the best performances were observed for fluopyram, followed by boscalid+pyraclostrobin and *B. subtilis* strain QST713 (Table 6). Although significant differences were detected for these treatments if compared with controls only for the $DI_P$ at $p = 0.01$, the data for $DI_L$ and SS were always lower than remaining treatments and significant differences from relative controls were detected only at $p = 0.05$ (*data not shown*).

**Table 6 - Effects of fungicides and BCAs on disease incidence and symptoms severity of leaf spot caused by *Calonectria morganii* on bottlebrush (experiment II)**

| Treatments | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 b | 0.0 a | 1.00 a |
| Fluopyram | 0.0 b | 0.0 a | 1.00 a |
| Boscalid+pyraclostrobin | 1.6 b | 0.4 a | 1.00 a |
| *Bacillus subtilis* strain QST713 | 1.6 b | 0.2 a | 1.00 a |
| Boscalid | 10.3 ab | 3.3 a | 1.06 a |
| Propamocarb+fosetyl-Al | 18.2 ab | 7.0 a | 1.11 a |
| Fosetyl-Al | 22.2 ab | 11.3 a | 1.21 a |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 4.8 ab | 1.4 a | 1.02 a |
| Trifloxystrobin | 7.9 ab | 2.2 a | 1.03 a |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 14.3 ab | 7.6 a | 1.11 a |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080 | 18.2 ab | 6.9 a | 1.13 a |
| *Trichoderma asperellum* TV1 | 5.6 ab | 1.8 a | 1.03 a |
| *Trichoderma harzianum* T-22 | 26.2 ab | 13.4 a | 1.22 a |
| *Streptomyces griseoviridis* strain K61 | 11.9 ab | 5.3 a | 1.05 a |
| Untreated and inoculated control | 34.1 a | 13.7 a | 1.25 a |

[y] Averaged from 3 replicates each formed up to 42 *Callistemon viminalis* cuttings.
[z] Averaged from 3 replicates each formed up to 420 *Callistemon viminalis* leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

In the last experiment, fluopyram, boscalid+pyraclostrobin and *B. subtilis* strain QST713 showed the best performances in controlling Calonectria leaf spot, thus confirming previous results. Although significant differences were detected for all remaining treatments if compared with relative controls, the least efficacy was detected for propamocarb+fosetyl-Al, *S. griseoviridis* strain K61 and *T. harzianum* strain ICC012+*T. viride* strain ICC080. Intermediate performances were recorded for the remaining treatments (Table 7).

**Table 7 - Effects of fungicides and BCAs on disease incidence and symptoms severity of leaf spot caused by *Calonectria morganii* on bottlebrush (experiment III)**

| Treatments | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 e | 0.0 d | 1.00 c |
| Fluopyram | 1.3 de | 0.1 d | 1.00 c |
| Boscalid+pyraclostrobin | 0.0 e | 0.0 d | 1.00 c |
| *Bacillus subtilis* strain QST713 | 1.3 de | 0.1 d | 1.00 c |
| Boscalid | 4.0 cde | 1.2 bcd | 1.01 bc |
| Propamocarb+fosetyl-Al | 25.3 b | 4.5 b | 1.05 bc |
| Fosetyl-Al | 5.3 cde | 1.3 bcd | 1.02 bc |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 10.7 bcd | 1.5 bcd | 1.01 bc |
| Trifloxystrobin | 4.0 cde | 0.4 cd | 1.00 bc |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | 13.3 bcd | 2.7 bc | 1.04 bc |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080 | 20.0 bc | 3.9 b | 1.04 bc |
| *Trichoderma asperellum* TV1 | 12.0 bcd | 2.3 bc | 1.02 bc |
| *Trichoderma harzianum* T-22 | 20.0 bc | 4.5 b | 1.07 b |
| *Streptomyces griseoviridis* strain K61 | 13.3 bcd | 2.7 bc | 1.03 bc |
| Untreated and inoculated control | 78.7 a | 16.9 a | 1.21 a |

[y] Averaged from 3 replicates each formed up to 42 *Callistemon viminalis* cuttings.
[z] Averaged from 3 replicates each formed up to 420 *Callistemon viminalis* leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

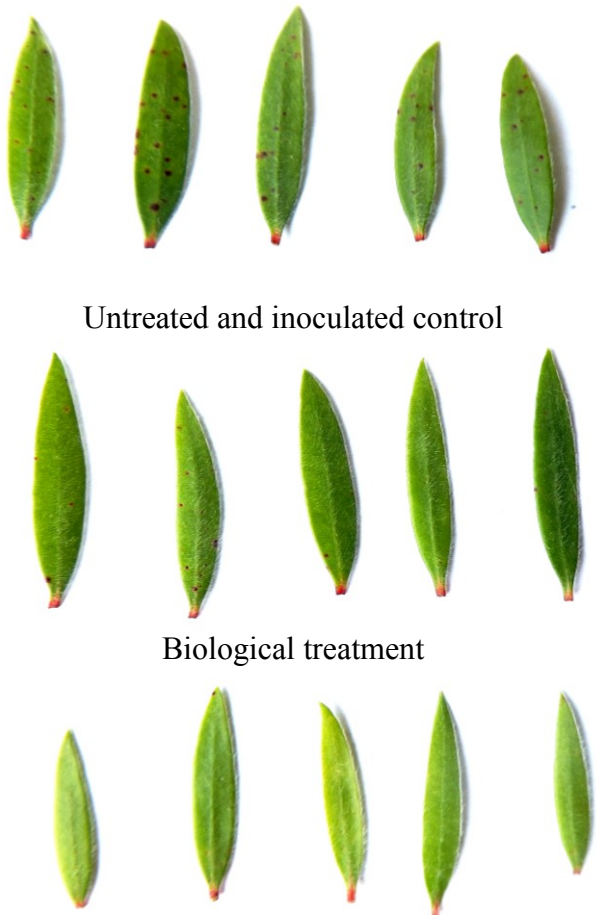


**Figure 8 - Different effectiveness between chemical and biological treatments**

### 6.3. Discussion

Boscalid+pyraclostrobin and fluopyram were the best fungicides proving high performances in controlling leaf spot caused by *Ca*. *morganii* on *C. viminalis*. Moreover, *B. subtilis* strain QST713 was the best BCA tested in these trials, being able to reduce significantly Calonectria infections. The performances of this BCA are very interesting, since they are statistically similar to those of boscalid+pyraclostrobin and fluopyram. All these experiments have shown a variability of effectiveness of treatments with boscalid, propamocarb+fosetyl-

Al, fosetyl-Al, trifloxystrobin and *B. amyloliquefaciens* subsp. *plantarum* strain D747, being able in some cases to reduce significantly Calonectria leaf spot.

Comprehensively other BCAs, such as *T. atroviride* T-11+*T. asperellum* T-25, *T. harzianum* strain ICC012+*T. viride* strain ICC080, *T. asperellum* TV1, *T. harzianum* T-22, *S. griseoviridis* strain K61provide encouraging results and thus they could be taken into account for further studies.

Based on these results it would be possible to develop IPM programs for controlling Calonectria leaf spot, that require the application of *B. subtilis* strain QST713 alone or in combination with effective fungicides.

## 7. Biological and integrated control of Calonectria leaf spot on *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook"

### 7.1. Materials and methods

In two experiments, five pathogenic isolates of the genus *Calonectria* were assessed for their ability to cause leaf spot symptoms on *Metrosideros excelsa* "aurea" (experiment I) and *Callistemon* "Captain Cook" (experiment II): *Ca*. *pauciramosa* (from *Callistemon citrinus*, Catania, Italy), *Ca*. *polizzii* (from *M*. *communis*, Tunis, Tunisia), *Ca*. *pseudomexicana* (from *Callistemon* sp., Tunis, Tunisia), *Ca*. *tunisiana* (from *C*. *laevis*, Tunis, Tunisia) and *Ca*. *mexicana* (from *D*. *viscosa*, Tunis, Tunisia). A representative isolate of *Ca*. *morganii* (CBS 120930 from *Callistemon* hybrid 'Rose Opal', Catania, Italy) was added in experiment conducted on *Callistemon* "Captain Cook".

Fresh cultures of each isolate were obtained by transferring agar plugs from stock cultures onto PDA plates. After 5 days, a mycelial disc of each isolate was transferred on SNA dishes. After incubation at 25 °C for 21 days, the plates were filled with SDW and then rubbed gently on the colony surface with a sterile loop. Final spore suspension of each isolate (about $3 \times 10^5$ conidia $ml^{-1}$) was filtered through a triple layer of cheesecloth and determined by using a haemocytometer.

Three fungicides, such as boscalid+pyraclostrobin (Signum®), cyprodinil+fludioxonil (Switch®), fosetyl-Al (Aliette®) and three BCAs, such as *B*. *amyloliquefaciens* subsp. *plantarum* strain D747 (Amylo-X®), *B*. *subtilis* strain QST713 (Serenade Max®), *T*. *atroviride* T-11+*T*. *asperellum* T-25 (Tusal®) were used to evaluate their efficacy in reducing Calonectria leaf spot both on *M. excelsa* "aurea" and on *Callistemon* "Captain Cook". Fungicides and BCAs were employed as commercial products and at standard used rates (Table 8).

Each treatment was replicated three times in a randomized block design with 28 metrosidero and 3 bottlebrush cuttings for replicate. The same number of untreated and inoculated and untreated and not-inoculated cuttings was used in each experiment.

In these experiments fungicides and BCAs were applied alone. In addition, cyprodinil+fludioxonil mixture was assessed in combination with each BCA, in order to evaluate the ability of BCA to reduce the application fungicide rates and the number of chemical treatments.

BCAs and fungicides were applied 24 and 3 hours before pathogen inoculation, respectively. They were applied by spraying to run-off using a hand sprayer. Inoculum of each *Calonectria* species was sprayed on leaf surface with a hand sprayer, by applying approximately 25 ml and 10 ml of conidial suspension for each replicate, respectively for *M. excelsa* "aurea" and bottlebrush.

After inoculation plants were maintained under plastic tunnels at 25 °C at about 70% of relative humidity for 3 days.

**Figure 9 - *Metrosideros excelsa* "aurea" cuttings arranged in randomized blocks according to designed experimental scheme**

The ability of each treatment in controlling leaf spots symptoms was evaluated 10 days after inoculation, by calculating disease incidence of infected plants ($DI_P$), disease incidence of infected leaves ($DI_L$) and symptoms severity (SS). Both DI data were obtained as a percentage value of cuttings or leaves showing

symptoms out of the total number of plants or leaves examined × 100. SS was calculated by using an empirical 1-to-5 rating scale that takes into account the percentage of infected foliar surface, as follow: 1 = healthy leaves; 2 = 1 to 5%; 3 = 6 to 25%; 4 = 26 to 50%; 5 = more than 50% of infected leaf area. A single value was obtained by using the mean of 5 and 15 leaves per plant, respectively for metrosidero and bottlebrush. SS values were converted to mean disease rating (MDR) that was calculated as $\Sigma$ (the number of disease leaves in this class × the disease class)/number of leaves scored.

**Statistical analysis**

All data obtained from the inoculation trials were subjected to analysis of variance according to parametric approaches (Statistica 10; StatSoft Inc., Analytical Software for Windows) with factorial treatment structure and interactions. All DI percentage data were transformed using arcsine ($\sin^{-1}$ square root x) prior to statistical analysis. The untransformed values of DI (%) are presented in tables and separated by Duncan test at $p = 0.01$.

**Table 8 - Fungicides and BCAs employed in controlling leaf spot caused by several *Calonectria* spp. on *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook"**

| *Active ingredient* | *Trade name* | *Manufacturer* | *Rates (g or ml/100 liters)* | *Formulation*[x] |
|---|---|---|---|---|
| Boscalid+pyraclostrobin | Signum | Basf Italia | 100 | 26.7+6.7 WG |
| Cyprodinil+fludioxonil | Switch | Syngenta Crop Protection | 80 | 37.5+25 WG |
| Fosetyl-Al | Aliette | Bayer CropScience | 300 | 80 WG |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | Amilo-X | Biogard | 250 | 25 WG |
| *Bacillus subtilis* strain QST713 | Serenade Max | Basf Italia | 400 | WP |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | Tusal | Certis | 250 | WG |

[x] Percentage of active ingredient; WG = water dispersible granule; WP = wettable powder.

## 7.2. Results

**Effects of chemical, biological and integrated treatments in controlling *Calonectria mexicana* infections on *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook"**

In experiment performed to evaluate the efficacy of chemical, biological and integrated means against *Ca*. *mexicana*, disease pressure of *Ca*. *mexicana* on *M*. *excelsa* "aurea" was moderate, being $DI_P$, $DI_L$ and SS equal to 39.3%, 11.7 % and 1.14, respectively.

In these conditions, treatments with cyprodinil+fludioxonil applied in combination both with *B. subtilis* strain QST713 and with *B. amyloliquefaciens* subsp. *plantarum* strain D747, proved a significant reduction of infections caused by *Ca*. *mexicana* on *M*. *excelsa* "aurea" compared with the control. Good results were also obtained for treatments with boscalid+pyraclostrobin and *B. amyloliquefaciens* subsp. *plantarum* strain D747 only for $DI_P$ (ranging from 22.6 to 23.8%), while no effect was observed for $DI_L$ and SS. *B. subtilis* strain QST713, fosetyl-Al, cyprodinil+fludioxonil combined with *T. atroviride* T-11+*T. asperellum* T-25, cyprodinil+fludioxonil and *T. atroviride T-11*+*T. asperellum* T-25 were ineffective in reducing infections compared with the control (Table 9).

**Table 9 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria mexicana* on *Metrosideros excelsa* "aurea"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 d | 0.0 d | 1.00 c |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 19.0 c | 4.5 c | 1.05 bc |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 20.2 c | 4.8 bc | 1.05 bc |
| Boscalid+pyraclostrobin | 22.6 bc | 8.8 abc | 1.10 ab |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 23.8 bc | 7.4 abc | 1.08 ab |
| *Bacillus subtilis* strain QST713 | 28.6 abc | 8.1 abc | 1.09 ab |
| (Cyprodinil+fludioxonil)+ (*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25) | 33.3 abc | 10.7 abc | 1.11 ab |
| Cyprodinil+fludioxonil | 34.5 abc | 9.8 abc | 1.11 ab |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | 35.7 abc | 10.7 ab | 1.11 ab |
| Fosetyl-Al | 44.0 a | 14.5 a | 1.15 a |
| Untreated and inoculated control | 39.3 ab | 11.7 a | 1.14 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 28 *M. excelsa* "aurea" cuttings.
[z] Averaged from 3 replicates each formed by 140 *M. excelsa* "aurea" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

High disease pressure was recorded on *Callistemon* "Captain Cook" inoculated with *Ca*. *mexicana* since $DI_P$, $DI_L$ and SS values of 100%, 97.8% and 4 were respectively detected.

In these conditions, treatments with cyprodinil+fludioxonil, applied in combination both with *B. subtilis* strain QST713 ($DI_P$, $DI_L$ and SS were 22.2%, 4.4% and 1.04, respectively) and *B. amyloliquefaciens* subsp. *plantarum* strain D747 ($DI_P$, $DI_L$ and SS were 33.3%, 5.9% and 1.06, respectively), confirmed once again the results of previous experiment (Table 10). For the remaining treatments no significant differences from relative control were detected for $DI_P$ parameter. Otherwise, they reduced significantly $DI_L$ and SS parameters when compared with relative controls. In detail, among these treatments the least effective one was *T. atroviride* T-11+*T. asperellum* T-25 (Table 10).

Comprehensively, *B. amyloliquefaciens* subsp. *plantarum* strain D747, boscalid+pyraclostrobin, *B. subtilis* strain QST713, fosetyl-Al, cyprodinil+fludioxonil combined with *T. atroviride* T-11+*T. asperellum* T-25,

cyprodinil+fludioxonil and *T. atroviride* T-11 +*T. asperellum* T-25 were ineffective for $DI_P$ for those at least all parameters were statistically different from untreated and inoculated controls (Table 10).

**Table 10 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria mexicana* on *Callistemon* "Captain Cook"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.00 d | 0.0 d | 1.00 c |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 22.2 cd | 4.4 cd | 1.04 c |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 33.3 bcd | 5.9 cd | 1.06 c |
| *Bacillus amyloliquefaciens subsp. plantarum* strain D747 | 66.7 abc | 12.6 c | 1.13 c |
| Boscalid+pyraclostrobin | 66.7 abc | 8.1 cd | 1.08 c |
| *Bacillus subtilis* strain QST713 | 77.8 abc | 17.8 c | 1.21 c |
| Fosetyl-Al | 77.8 abc | 22.2 c | 1.25 c |
| (Cyprodinil+fludioxonil)+*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | 77.8 ab | 23.0 c | 1.24 c |
| Cyprodinil+fludioxonil | 88.9 a | 14.8 c | 1.16 c |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | 100.0 a | 51.9 b | 1.87 b |
| Untreated and inoculated control | 100.0 a | 97.8 a | 4.00 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 3 *Callistemon* "Captain Cook" cuttings.
[z] Averaged from 3 replicates each formed by 45 *Callistemon* "Captain Cook" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

## Effects of chemical, biological and integrated treatments in controlling *Calonectria pauciramosa* infections on *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook"

Moderate disease pressure was detected in this experiment with artificial inoculation of *Ca. pauciramosa*. In these conditions, infections caused by *Ca. pauciramosa* on *M. excelsa* "aurea" were reduced significantly for all three disease parameters examined, according to Duncan test at $p = 0.01$, except for *T. atroviride* T-11+*T. asperellum* T-25.

Averagely, the most effective treatment was cyprodinil+fludioxonil (Table 11).

**Table 11 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria pauciramosa* on *Metrosideros excelsa* "aurea"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 c | 0.0 d | 1.00 b |
| Cyprodinil+fludioxonil | 8.3 b | 1.9 c | 1.02 b |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25*)* | 14.3 b | 4.0 bc | 1.04 b |
| Boscalid+pyraclostrobin | 14.3 b | 5.0 bc | 1.05 b |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 15.5 b | 5.0 bc | 1.06 b |
| *Bacillus subtilis* strain QST713 | 16.7 b | 4.0 bc | 1.04 b |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 17.9 b | 5.7 bc | 1.06 b |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 19.0 b | 5.7 bc | 1.06 b |
| Fosetyl-Al | 22.6 b | 6.9 bc | 1.08 b |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 25.0 ab | 7.9 b | 1.08 b |
| Untreated and inoculated control | 48.8 a | 19.3 a | 1.22 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 28 *M. excelsa* "aurea" cuttings.
[z] Averaged from 3 replicates each formed by 140 *M. excelsa* "aurea" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

The disease pressure induced by *Ca*. *pauciramosa* on *Callistemon* "Captain Cook" was detected with values of 100%, 77.8% and 2.26 for $DI_P$, $DI_L$ and SS, respectively. In this experiment, all treatments were effective in reducing significantly $DI_L$ (comprised between 3 and 27.4%) and SS (comprised between 1.03 and 1.34) but not $DI_P$, compared with the control. In detail, boscalid+pyraclostrobin and cyprodinil+fludioxonil combined with *B. amyloliquefaciens* subsp. *plantarum* strain D747 showed the best performances in controlling Calonectria disease on bottlebrush (Table 12). Although with low values of significance, cyprodinil+fludioxonil combined with *T. atroviride* T-11+*T. asperellum* T-25, cyprodinil+fludioxonil, cyprodinil+fludioxonil combined with *B. subtilis* strain QST713, *B. amyloliquefaciens* subsp. *plantarum* strain D747, *B. subtilis* strain QST713, fosetyl-Al and *T. atroviride* T-11+*T. asperellum* T-25 were able to reduce infections of *Ca*. *pauciramosa* on bottlebrush (Table 12).

**Table 12 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria pauciramosa* on *Callistemon* "Captain Cook"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 b | 0.0 d | 1.00 c |
| Boscalid+pyraclostrobin | 22.2 ab | 3.7 cd | 1.04 c |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 33.3 ab | 3.0 cd | 1.03 c |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25*)* | 44.4 ab | 11.1 bcd | 1.13 bc |
| Cyprodinil+fludioxonil | 66.7 ab | 13.3 bc | 1.13 bc |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 66.7 ab | 11.1 bcd | 1.12 c |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 77.8 ab | 27.4 b | 1.34 b |
| *Bacillus subtilis* strain QST713 | 77.8 ab | 19.3 bc | 1.21 bc |
| Fosetyl-Al | 77.8 ab | 18.5 bc | 1.21 bc |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | 88.9 a | 13.3 bc | 1.16 bc |
| Untreated and inoculated control | 100.0 a | 77. 8 a | 2.26 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 3 *Callistemon* "Captain Cook" cuttings.
[z] Averaged from 3 replicates each formed by 45 *Callistemon* "Captain Cook" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

## Effects of chemical, biological and integrated treatments in controlling *Calonectria polizzii* infections on *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook"

A significant reduction of infections caused by *Ca. polizzii* on *M. excelsa* "aurea" was always observed for all fungicides and BCAs tested, in conditions of moderate disease pressure ($DI_P$, $DI_L$ and SS were 58.3%, 21.7% and 1.25, respectively). Indeed, $DI_P$, $DI_L$ and SS of treated plants ranged from 8.3 to 16.7%, from 2.4 to 5.2% and from 1.03 to 1.05, respectively (Table 13).

**Table 13 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria polizzii* on *Metrosideros excelsa* "aurea"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 c | 0.0 c | 1.00 b |
| Boscalid+pyraclostrobin | 8.3 b | 2.4 bc | 1.03 b |
| *Bacillus subtilis* strain QST713 | 8.3 bc | 2.9 bc | 1.03 b |
| Cyprodinil+fludioxonil | 11.9 b | 3.8 b | 1.04 b |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 11.9 b | 4.8 b | 1.05 b |
| Fosetyl-Al | 13.1 b | 3.1 b | 1.03 b |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 14.3 b | 4.5 b | 1.05 b |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25) | 15.5 b | 3.8 b | 1.04 b |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 16.7 b | 5.2 b | 1.05 b |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 16.7 b | 5.0 b | 1.05 b |
| Untreated and inoculated control | 58.3 a | 21.7 a | 1.25 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 28 *M. excelsa* "aurea" cuttings.
[z] Averaged from 3 replicates each formed by 140 *M. excelsa* "aurea" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

When all treatments effects were tested against *Ca. polizzii* on *Callistemon* "Captain Cook" the $DI_P$, $DI_L$ and SS values of control plants were 100%, 85.9% and 3.3, respectively (high disease pressure) (Table 14).

In these conditions, boscalid+pyraclostrobin and cyprodinil+fludioxonil combined with *B. amyloliquefaciens* subsp. *plantarum* strain D747 were effective in reducing significantly both DI and SS compared with the control. A good efficacy in controlling *Ca. polizzii* infections on bottlebrush was also observed for cyprodinil+fludioxonil applied in combination with *T. atroviride* T-11+*T. asperellum* T-25, followed by cyprodinil+fludioxonil combined with *B. subtilis* strain QST713, cyprodinil+fludioxonil and fosetyl-Al (Table 14).

*T. atroviride* T-11+*T. asperellum* T-25, *B. amyloliquefaciens* subsp. *plantarum* strain D747, *B. subtilis* strain QST713 were able to reduce significantly $DI_L$ (ranged from 20.7 to 30.4%) and SS (ranged from 1.22 to 1.32) but not $DI_P$ (Table 14).

**Table 14 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria polizzii* on *Callistemon* "Captain Cook"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 e | 0.0 e | 1.00 b |
| Boscalid+pyraclostrobin | 11.1 de | 1.0 e | 1.01 b |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 22.2 de | 3.7 de | 1.04 b |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25) | 33.3 cde | 6.7 cde | 1.07 b |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 44.4 bcd | 6.7 cde | 1.07 b |
| Cyprodinil+fludioxonil | 44.4 bcd | 7.4 cde | 1.07 b |
| Fosetyl-Al | 55.6 bcd | 9.6 bcde | 1.10 b |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 77.8 abc | 20.7 bcd | 1.22 b |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 88.9 ab | 24.4 bc | 1.29 b |
| *Bacillus subtilis* strain QST713 | 100.0 a | 30.4 b | 1.32 b |
| Untreated and inoculated control | 100.0 a | 85.9 a | 3.30 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 3 *Callistemon* "Captain Cook" cuttings.
[z] Averaged from 3 replicates each formed by 45 *Callistemon* "Captain Cook" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

## Effects of chemical, biological and integrated treatments in controlling *Calonectria pseudomexicana* infections on *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook"

A moderate infection level caused by *Ca. pseudomexicana* was observed on *M. excelsa* "aurea", with values of 34.5%, 14.5% and 1.14, respectively for $DI_P$, $DI_L$ and SS.

A good control of Calonectria leaf spot was observed on plants of *M. excelsa* "aurea" treated with boscalid+pyraclostrobin ($DI_P$, $DI_L$ and SS were 9.5%, 2.6% and 1.03), followed by cyprodinil+fludioxonil, *B. subtilis* strain QST713, cyprodinil+fludioxonil combined with *T. atroviride* T-11+*T. asperellum* T-25 and cyprodinil+fludioxonil combined with *B. subtilis* strain QST713 ($DI_P$, $DI_L$ and SS ranged from 11.9 to 15.5%, from 3.6 to 5% and from 1.04 to 1.05, respectively) (Table 15).

Treatments with cyprodinil+fludioxonil in combination with *B. amyloliquefaciens* subsp. *plantarum* strain D747, *T. atroviride* T-11+*T. asperellum* T-25 and fosetyl-Al proved efficacy in reducing significantly $DI_L$

and SS, whereas no significant effect was observed for $DI_P$. Among all treatments, the application of *B. amyloliquefaciens* subsp. *plantarum* strain D747 was totally ineffective in controlling infections (Table 15).

**Table 15 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria pseudomexicana* on *Metrosideros excelsa* "aurea"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 c | 0.0 d | 1.00 c |
| Boscalid+pyraclostrobin | 9.5 b | 2.6 c | 1.03 c |
| Cyprodinil+fludioxonil | 11.9 b | 3.6 bc | 1.04 bc |
| *Bacillus subtilis* strain QST713 | 14.3 b | 5.0 bc | 1.05 bc |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25) | 14.3 b | 4.3 bc | 1.04 bc |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 15.5 b | 4.0 bc | 1.04 bc |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 16.7 ab | 5.9 bc | 1.06 bc |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | 20.2 ab | 5.0 bc | 1.05 bc |
| Fosetyl-Al | 21.4 ab | 6.2 bc | 1.06 bc |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 21.4 ab | 10.0 ab | 1.10 ab |
| Untreated and inoculated control | 34.5 a | 14.5 a | 1.14 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 28 *M. excelsa* "aurea" cuttings.
[z] Averaged from 3 replicates each formed by 140 *M. excelsa* "aurea" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

*Ca. pseudomexicana* proved a high virulence on *Callistemon* "Captain Cook", showing value of 100% for both DI and 4.46 for SS (Table 16).

In these conditions, all treatments were effective in reducing all three disease parameters examined except for *B. amyloliquefaciens* subsp. *plantarum* strain D747, fosetyl-Al, *B. subtilis* strain QST713 and *T. atroviride* T-11+*T. asperellum* T-25 exclusively for $DI_P$ (Table 16). This last treatment was the least effective in reducing Calonectria leaf spot. Otherwise, best results were recorded for cyprodinil+fludioxonil combined with *B. subtilis* strain QST713, cyprodinil+fludioxonil combined with *T. atroviride* T-11+*T. asperellum* T-25, cyprodinil+fludioxonil combined with *B. amyloliquefaciens* subsp. *plantarum* strain D747 and boscalid+pyraclostrobin (Table 16).

**Table 16 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria pseudomexicana* on *Callistemon* "Captain Cook"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 d | 0.0 e | 1.00 d |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 11.1 cd | 1.0 e | 1.01 d |
| (Cyprodinil+fludioxonil)+(*T. atroviride* T-11+ *Trichoderma asperellum* T-25) | 11.1 cd | 1.5 e | 1.01 d |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 22.2 cd | 3.7 e | 1.04 d |
| Boscalid+pyraclostrobin | 22.2 cd | 1.5 e | 1.01 d |
| Cyprodinil+fludioxonil | 44.4 bc | 3.0 e | 1.03 d |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 88.9 ab | 18.5 d | 1.24 d |
| Fosetyl-Al | 88.9 ab | 34.1 cd | 1.39 d |
| *Bacillus subtilis* strain QST713 | 100.0 a | 50.4 c | 2.11 c |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 100.0 a | 72.6 b | 3.04 b |
| Untreated and inoculated control | 100.0 a | 100.0 a | 4.46 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 3 *Callistemon* "Captain Cook" cuttings.
[z] Averaged from 3 replicates each formed by 45 *Callistemon* "Captain Cook" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

## Effects of chemical, biological and integrated treatments in controlling *Calonectria tunisiana* infections on *Metrosideros excelsa* "aurea" and *Callistemon* "Captain Cook"

A medium disease pressure was detected in the experiment in which *Ca. tunisiana* was inoculated on *M. excelsa* "aurea". In these conditions, the best performances in controlling Calonectria leaf spot were detected for boscalid+pyraclostrobin.

Good results in reducing all parameters examined were also obtained for all remaining treatments, except for $DI_P$ of *T. atroviride* T-11+*T. asperellum* T-25 (Table 17).

**Table 17 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria tunisiana* on *Metrosideros excelsa* "aurea"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 d | 0.0 d | 1.00 c |
| Boscalid+pyraclostrobin | 7.1 cd | 2.1 cd | 1.02 c |
| Cyprodinil+fludioxonil | 11.9 bc | 4.0 bc | 1.04 bc |
| *Bacillus subtilis* strain QST713 | 11.9 bc | 4.5 bc | 1.04 bc |
| Fosetyl-Al | 16.7 bc | 4.8 bc | 1.05 bc |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 17.9 bc | 5.2 bc | 1.05 bc |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 17.9 bc | 5.9 bc | 1.06 bc |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 19.0 bc | 9.8 b | 1.05 bc |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25*)* | 19.0 bc | 5.2 bc | 1.05 bc |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 30.9 ab | 9.5 b | 1.09 b |
| Untreated and inoculated control | 50.0 a | 22.1 a | 1.22 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 28 *M. excelsa* "aurea" cuttings.
[z] Averaged from 3 replicates each formed by 140 *M. excelsa* "aurea" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

In conditions of high disease pressure induced by *Ca. tunisiana* on *Callistemon* "Captain Cook" ($DI_P$, $DI_L$ and SS were 100%, 100% and 4.7, respectively), all treatments were able to reduce significantly both $DI_L$ (ranged from 1.5 to 80%) and SS (ranged from 1.01 to 2.7) of *Ca. tunisiana* infections compared with the control (Table 18). Some differences were detected among treatments in reducing $DI_P$. In detail, cyprodinil+fludioxonil in combination with *B. subtilis* strain QST713 showed the best performances in controlling Calonectria disease ($DI_P$, $DI_L$ and SS values were of 11.1%, 1.5% and 1.01, respectively), followed by cyprodinil+fludioxonil applied in combination both with *T. atroviride* T-11+*T. asperellum* T-25 and with *B. amyloliquefaciens* subsp. *plantarum* strain D747. Overall, applications of cyprodinil+fludioxonil, boscalid+pyraclostrobin, fosetyl-Al, *B. amyloliquefaciens* subsp. *plantarum* strain D747, *B. subtilis* strain QST713, *T. atroviride* T-11+*T. asperellum* T-25 reduced significantly $DI_L$ and SS, whereas no effect was observed in reducing $DI_P$ (Table 18).

**Table 18 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria tunisiana* on *Callistemon* "Captain Cook"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 d | 0.0 e | 1.00 d |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 11.1 cd | 1.5 e | 1.01 d |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25) | 22.2 bcd | 2.2 e | 1.02 d |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 22.2 bcd | 1.5 e | 1.01 d |
| Cyprodinil+fludioxonil | 44.4 abcd | 12.6 de | 1.29 cd |
| Boscalid+pyraclostrobin | 66.7 abc | 6.7 de | 1.07 cd |
| Fosetyl-Al | 77.8 ab | 22.2 cd | 1.24 cd |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 100.0 a | 28.9 cd | 1.37 cd |
| *Bacillus subtilis* strain QST713 | 100.0 a | 52.6 bc | 1.57 c |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 100.0 a | 80.0 b | 2.70 b |
| Untreated and inoculated control | 100.0 a | 100.0 a | 4.70 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 3 *Callistemon* "Captain Cook" cuttings.
[z] Averaged from 3 replicates each formed by 45 *Callistemon* "Captain Cook" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

## Effects of chemical, biological and integrated treatments in controlling *Calonectria morganii* infections on *Callistemon* "Captain Cook"

In the trial where *Ca. morganii* was inoculated only on *Callistemon* "Captain Cook", the combination between cyprodinil+fludioxonil and *T. atroviride* T-11+*T. asperellum* T-25 showed the best results in controlling Calonectria leaf spot where the high disease pressure was detected.

All remaining treatments were able to reduce significantly $DI_L$ and SS, whereas the same treatments were ineffective in reducing $DI_P$ (Table 19).

**Table 19 - Effects of chemical, biological and integrated treatments in controlling leaf spot caused by *Calonectria morganii* on *Callistemon* "Captain Cook"**

| Treatment | DI Infected plants[y] (%) | DI Infected leaves[z] (%) | Symptoms severity[z] (MDR) |
|---|---|---|---|
| Untreated and not-inoculated control | 0.0 c | 0.0 c | 1.00 b |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25*)* | 22.2 bc | 1.5 bc | 1.01 b |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 33.3 abc | 6.7 bc | 1.07 b |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 33.3 abc | 2.2 bc | 1.02 b |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 44.4 abc | 3.0 bc | 1.03 b |
| Cyprodinil+fludioxonil | 44.4 abc | 10.4 bc | 1.12 b |
| Boscalid+pyraclostrobin | 44.4 abc | 5.2 bc | 1.05 b |
| Fosetyl-Al | 66.7 abc | 19.3 b | 1.27 b |
| *Bacillus subtilis* strain QST713 | 66.7 abc | 13.3 b | 1.15 b |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 66.7 ab | 11.9 bc | 1.22 b |
| Untreated and inoculated control | 100.0 a | 80.7 a | 2.90 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 3 *Callistemon* "Captain Cook" cuttings.
[z] Averaged from 3 replicates each formed by 45 *Callistemon* "Captain Cook" leaves.
Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

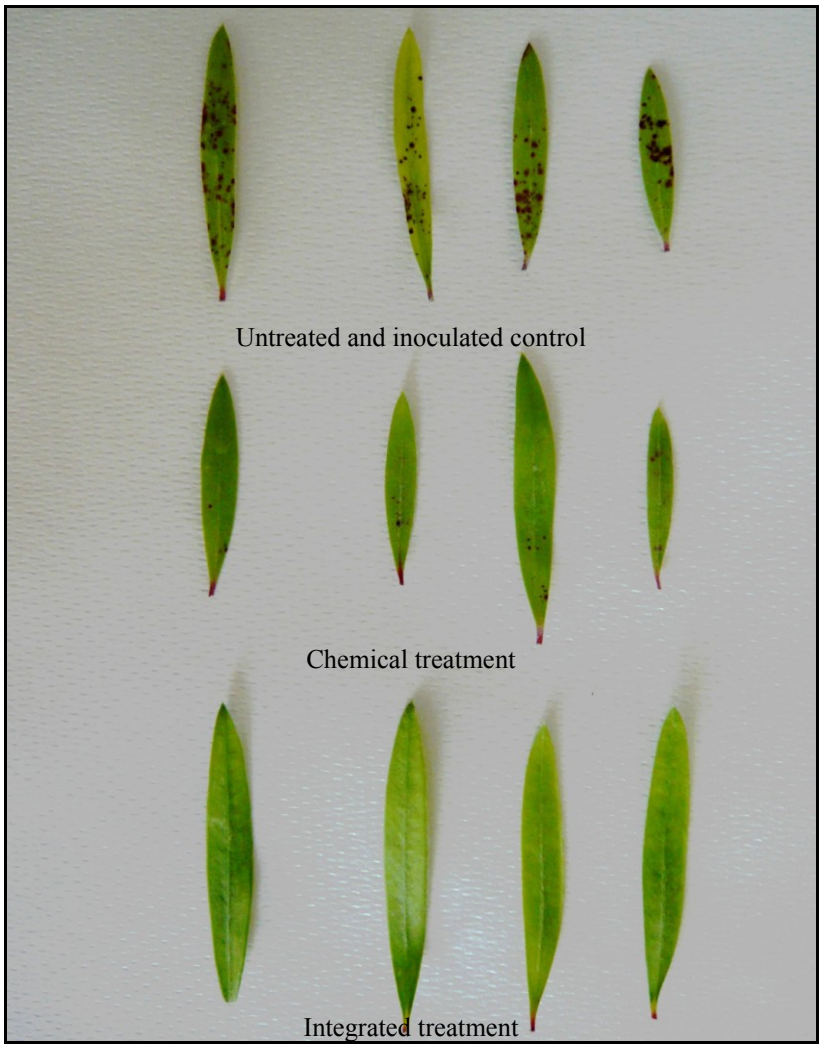


**Figure 10 - Efficacy of different treatments on *Callistemon* "Captain Cook"**

### 7.3. Discussion

These experiments provide variable results for all treatments in relation to different *Calonectria* species and host plants involved. By analyzing comprehensive results, boscalid+pyraclostrobin and cyprodinil+fludioxonil proved a good efficacy in reducing significantly infections caused by different *Calonectria* species both on *M. excelsa* "aurea" and *Callistemon*" Captain Cook". It is interesting to highlight that cyprodinil+fludioxonil results most effective when applied in combination with some BCAs than applied alone. Among BCAs applied alone, *B. subtilis* strain QST713 and *B. amyloliquefaciens* subsp. *plantarum* strain D747 were slightly effective in reducing Calonectria

leaf spot on both host plants, showing variable results. Moreover, all others BCAs employed in these trials were able to reduce $DI_L$ and SS compared to the controls, although they showed values of DI and SS lower than those mentioned above.

To the light of these findings, boscalid+pyraclostrobin and cyprodinil+fludioxonil could be suggested to control leaf spot caused by *Calonectria* species both on *M. excelsa* "aurea" and on *Callistemon* "Captain Cook". Furthermore, taking into consideration the efficacy of some BCAs and the need to comply with the restrictions on the sustainable use of fungicides, the application of *B. amyloliquefaciens* subsp. *plantarum* strain D747, *T. atroviride* T-11+*T. asperellum* T-25 and *B. subtilis* strain QST713 could be suggested in combination with cyprodinil+fludioxonil for developing integrated control programs for management of Calonectria leaf spot on ornamental plants, such as *M. excelsa* "aurea" and *Callistemon* "Captain Cook".

## 8. Biological and integrated control of Calonectria stem rot on *Dodonaea viscosa*

### 8.1. Materials and methods

**Assessment of *Calonectria morganii* virulence on *Dodonaea viscosa* seedlings**

A preliminary assay was performed to determine pathogenicity of *Calonectria morganii* on *Dodonaea viscosa* seedlings. To this aim, 56 seeds (28 for inoculation and 28 for control) were selected and sown in sterile peat. Twenty-one day after sowing, seedlings at 4-5$^{th}$ true life stage were inoculated as soil drench with 0.5 ml/pot of conidial suspension (about $2 \times 10^5$ conidia $ml^{-1}$). Control seedlings were inoculated with SDW. Consequently, they were maintained under plastic bags at 25°C for 3 days. Disease evaluation was performed 21 days after inoculation by calculating disease incidence (DI - as percentage of infected seedlings) and symptoms severity (SS). At the same time, in order to confirm the etiology of fungal infections, isolation attempts were performed in medium from stem infected tissue. Moreover, DI was calculated as the percentage of plants showing symptoms out of the total number of plant ×100 and SS by evaluating the length of stem infected tissue.

**Efficacy of chemical, biological and integrated treatments in controlling *Calonectria morganii* on *Dodonaea viscosa***

Fresh culture of *Ca*. *morganii* was obtained by transferring agar plug from stock cultures onto PDA plates. After 5 days, a mycelia disc of this isolate was transferred on SNA dishes and then incubated at 25 °C for 21 days. Conidial suspension was obtained by adding SDW to SNA dishes, dislodging the conidia gently rubbing the colony surface with a sterile loop and filtering the suspension through a triple layer of cheesecloth. Final spore suspension of about $2 \times 10^5$ conidia $ml^{-1}$ was determined using a haemocytometer.

In four nursery experiments the efficacy of 11, 21, 21 and 24 treatments were examined. Each treatment was determined to evaluate the efficacy of some active ingredients and comparing them with those already reported effective (standards treatments) in controlling Calonectria disease.

Nine commercial fungicides and eight bioformulates based on several BCAs were assessed for their ability in reducing stem rot on *D. viscosa* caused by artificial infection of *Ca. morganii*. Both chemical and biological products were employed alone and in combination, at standard used rates (Table 20).

The active ingredients tested in these trials were: boscalid+pyraclostrobin (Signum®), cyprodinil+fludioxonil (Switch®), etridiazole (Terrazole®), fluopyram (Luna Privilege®), fludioxonil (Geoxe®), fosetyl-Al (Aliette®), propamocarb+fosetil-Al (Previcur Energy®), thiophanate-methyl (Ranger Gold®) and trifloxystrobin (Flint®); instead, BCAs were *B. amyloliquefaciens* subsp. *plantarum* strain D747 (Amylo-X®), *B. subtilis* strain QST713 (Serenade Max®), *Pseudomonas chlororaphis* MA342 (Cedomon®), *S. griseoviridis* strain K61 (Mycostop®), *T. asperellum* TV1 (Xedavir®), *T. atroviride* T-11+*T. asperellum* T-25 (Tusal®), *T. harzianum* T-22 (Trianum-P®) and *T. harzianum* strain ICC012+*T. viride* strain ICC080 (Radix®).


**Figure 11 - *Dodonaea viscosa* seedlings arranged in randomized blocks according to designed experimental scheme**

In each treatment, 28 seedlings per replicate were used for the inoculation arranged in a randomized complete block design with three replicates. The same

number of untreated and inoculated and untreated and not-inoculated seedlings served as positive and negative controls, respectively.

In each experiment, treatments with BCAs were performed 21 days and repeated 3 days before pathogen inoculation. Fungicides were applied 1 day before pathogen inoculation. Biological and chemical treatments were repeated 6 and 8 days after inoculation, respectively. Each treatment was performed by spraying to run-off using a hand sprayer, by applying approximately 30 ml of suspension for each replicate.

About 0.5 ml of conidial suspension of *Ca. morganii* was inoculated as soil drench onto crown area of each *D. viscosa* seedling. Then, inoculated young seedlings were covered for 3 days under plastic tunnels and then maintained at 25 °C. Twenty-one days after pathogen inoculation DI and SS parameters were recorded. DI was calculated as a percent values resulting from number of symptomatic plants out of the total number of plants examined × 100. SS was evaluated by measuring the length (cm) of necrotic stem tissue.

**Figure 12 - Increasing scale of symptoms severity on stem of *Dodonaea viscosa* seedlings**

**Statistical analysis**

All data obtained from the inoculation trials were subjected to analysis of variance according to parametric approaches (Statistica 10; StatSoft Inc., Analytical Software for Windows) with factorial treatment structure and interactions.

Data from all experiments were analysed separately and the mean values of DI and SS for each treatment were calculated, averaging corresponding values determined for each replicate. All DI percentage data were previously transformed using arcsine ($\sin^{-1}$ square root x) prior to statistical analysis. The untransformed values of DI (%) are presented in the tables.

Mean separation of the percentage recovery data in each experiment was conducted using Fisher's least significance difference test at $p = 0.01$.

**Table 20 - Fungicides and BCAs employed in controlling stem rot caused by *Calonectria morganii* on *Dodonaea viscosa***

| *Active ingredient* | *Trade name* | *Manufacturer* | *Rates (g or ml/100 liters)* | *Formulation*[x] | *Fungicide use* |
|---|---|---|---|---|---|
| Boscalid+pyraclostrobin | Signum | Basf Italia | 100 | 26.7+6.7 WG | Experiment I, II, III, IV |
| Cyprodinil+fludioxonil | Switch | Syngenta Crop Protection | 80 | 37.5+25 WG | Experiment III, IV |
| Etridiazole | Terrazole | Certis | 400 | 25 EC | Experiment II, III |
| Fludioxonil | Geoxe | Syngenta Crop Protection | 50 | 50 WG | Experiment I, II, III |
| Fluopyram | Luna Privilege | Bayer CropScience | 50 | 41.66 SC | Experiment IV |
| Fosetyl-Al | Aliette | Bayer CropScience | 300 | 80 WG | Experiment I, II, III, IV |
| Propamocarb + fosetyl-Al | Previcur Energy | Bayer CropScience | 250 | 47.2+27.6 SL | Experiment II |
| Thiophanate-methyl | Ranger Gold | Diachem | 100 | 38.3 SC | Experiment II, III, IV |
| Trifloxystrobin | Flint | Bayer CropScience | 30 | 50 WG | Experiment IV |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | Amilo-X | Biogard | 250 | 25 WG | Experiment II, III, IV |
| *Bacillus subtilis* strain QST713 | Serenade Max | Basf Italia | 400 | WP | Experiment II, III, IV |
| *Pseudomonas chlororaphis* MA342 | Cedomon | Nufarm Italia | 500 | 8.7 EC | Experiment II, III, IV |
| *Streptomyces griseoviridis* strain K61 | Mycostop | Bioplanet | 25 | 33.33 WP | Experiment II, III, IV |
| *Trichoderma asperellum* TV1 | Xedavir | XedaItalia | 400 | 2.8 WP | Experiment I, II, III, IV |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | Tusal | Certis | 250 | WG | Experiment I, II, III, IV |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080 | Radix | Certis | 250 | 2 + 2 WP | Experiment II, III, IV |
| *Trichoderma harzianum* T-22 | Trianum-P | Koppert Biological Systems | 100 | 1.15 WP | Experiment I, II, III, IV |

[x]Percentage of active ingredient; EC = emulsifiable concentrate; SC = suspension concentrate; SL=soluble concentrate; WG = water dispersible granule; WP = wettable powder.

## 8.2. Results

**Assessment of *Calonectria morganii* virulence on *Dodonaea viscosa* seedlings**

The *in vitro* preliminary assay proved the pathogenicity of *Ca*. *morganii* on *Dodonaea viscosa* seedlings. In detail, this isolate was able to induce symptoms on 100% of seedlings with evident stem rot symptoms. Furthermore, the re-isolation of *Ca*. *morganii* from stem infected tissue fulfilled proof pathogenicity. As expected, not-inoculated seedlings did not develop disease symptoms. Accordingly, considering these data, this plant was chosen as model plant for studying the effects of chemical, biological and integrated treatments to control Calonectria stem rot.

**Figure 13 - Stem rot on *Dodonaea viscosa* seedlings caused by artificial infection of *Calonectria morganii***

**Efficacy of chemical, biological and integrated treatments in controlling *Calonectria morganii* on *Dodonaea viscosa***

In all nursery experiments DI of infected plants ranged from 47.6 and 83.3 %, while SS values ranged from 0.75 and 1.5 cm. All fungicides and BCAs, both applied separately and in combination, were effective in controlling stem rot of *D. viscosa* caused by *Ca. morganii*, according to Fisher test at $p = 0.01$.

In experiment I, all fungicides and BCAs were applied alone. In addition all fungicides were applied in combination with *T. harzianum* T22. This BCA was tested in combination for its already note ability in reducing infections caused by *Calonectria* spp. (Polizzi and Vitale, 2002; Vitale *et al*., 2003; Vitale *et al*., 2012). In this experiment, all treatments reduced significantly both DI and SS. In detail, boscalid+pyraclostrobin showed the best performances in reducing infections caused by the pathogen for both disease parameters examined followed by fludioxonil, fosetyl-Al combined with *T. harzianum* T-22 and *T. atroviride* T-11+*T. asperellum* T-25 (Table 21).

DI and SS values, ranging from 45.2 to 54.8% and from 0.6 to 0.9 cm, respectively, were recorded for fosetyl-Al, fludioxonil combined with *T. harzianum* T-22, *T. harzianum* T-22, *T. asperellum* TV1, boscalid+pyraclostrobin combined with *T. harzianum* T-22. Comprehensively, these DI and SS values were significantly higher than boscalid+pyraclostrobin, thus showing a lesser efficacy in controlling Dodonaea stem rot (Table 21).

**Table 21 - Effects of chemical, biological and integrated treatments on disease incidence and symptoms severity of stem rot caused by *Calonectria morganii* on *Dodonaea viscosa* (Experiment I)**

| Treatment | Disease incidence[y,z] (%) | Symptoms severity[y,z] (cm) |
|---|---|---|
| Untreated and not-inoculated control | 0.0 e | 0.0 e |
| Boscalid+pyraclostrobin | 27.4 d | 0.4 de |
| Fludioxonil | 32.1 cd | 0.4 de |
| Fosetyl-Al+*Trichoderma harzianum* T-22 | 34.5 cd | 0.5 cd |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | 36.9 bcd | 0.4 de |
| Fosetyl-Al | 45.2 bc | 0.9 bc |
| Fludioxonil+*Trichoderma harzianum* T-22 | 46.4 bc | 0.7 bcd |
| *Trichoderma harzianum* T-22 | 52.4 b | 0.9 b |
| *Trichoderma asperellum* TV1 | 52.4 b | 0.6 bcd |
| (Boscalid+pyraclostrobin)+*Trichoderma harzianum* T-22 | 54.8 b | 0.8 bc |
| Untreated and inoculated control | 82.1 a | 1.5 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Data are means of three replicates, each containing 28 *Dodonaea viscosa* seedlings.
[z] Values followed by the same letters within a column are not significantly different according to Fisher test at $p = 0.01$ for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis. Untransformed data are presented.

In experiment II, although all treatments showed a very good activity in controlling *Ca*. *morganii* infections, boscalid+pyraclostrobin and fludioxonil were the most effective among those tested, with values of 13.1% and 0.11 cm, respectively for DI and SS, followed by *S. griseoviridis* strain K61 treatment (Table 22).

A certain efficacy in reducing both disease parameters examined was averagely observed also for propamocarb+fosetyl-Al combined with *S. griseoviridis* strain K61, *B. amyloliquefaciens* subsp. *plantarum* strain D747, thiophanate-methyl, *B. subtilis* strain QST713, *T. atroviride* T-11+*T. asperellum* T-25, propamocarb+fosetyl-Al combined with *T. harzianum* T-22, *T. harzianum* T-22, *P. chlororaphis* MA342, propamocarb+fosetyl-Al combined with *B. subtilis* strain QST713, propamocarb+fosetyl-Al, propamocarb+fosetyl-Al combined with *T. atroviride* T-11+*T. asperellum* T-25, etridiazole, *T. harzianum* strain ICC012+*T. viride* strain ICC080 with a decreasing efficacy, respectively (Table 22).

Propamocarb+fosetyl-Al combined with *B. amyloliquefaciens* subsp. *plantarum* strain D747, fosetyl-Al and *T. asperellum* TV1 reduced always significantly the Dodonaea stem infections although with DI and SS values averagely higher than remaining treatments (Table 22).

It is important to report that the plants treated with propamocarb+fosetyl-Al showed symptoms of phytotoxicity (Fig. 14). Therefore, this chemical compound has not been used in the following experiment.

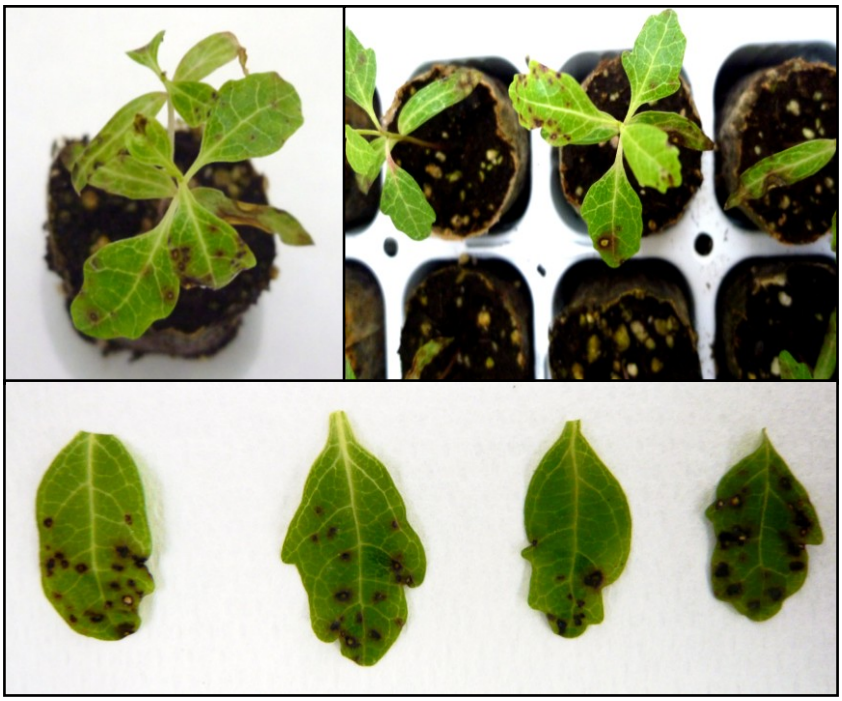

**Figure 14 - Symptoms of phytotoxicity on young leaves of *Dodonaea viscosa* seedlings caused by propamocarb+fosetyl-Al**

**Table 22 - Effects of chemical, biological and integrated treatments on disease incidence and symptoms severity of stem rot caused by *Calonectria morganii* on *Dodonaea viscosa* (Experiment II)**

| Treatment | Disease incidence[y,z] (%) | Symptoms severity[y,z] (cm) |
|---|---|---|
| Untreated and not-inoculated control | 0.0 f | 0.0 f |
| Boscalid+pyraclostrobin | 13.1 e | 0.11 ef |
| Fludioxonil | 13.1 e | 0.11 ef |
| *Streptomyces griseoviridis* strain K61 | 13.1 e | 0.13 def |
| (Propamocarb+fosetyl-Al)+*Streptomyces griseoviridis* strain K61 | 15.5 de | 0.19 cdef |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 16.7 cde | 0.20 cdef |
| Thiophanate-methyl | 16.7 cde | 0.13 def |
| *Bacillus subtilis* strain QST713 | 17.9 cde | 0.17 cdef |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 19.0 cde | 0.17 cdef |
| (Propamocarb+fosetyl-Al)+*Trichoderma harzianum* T-22 | 19.3 cde | 0.20 cdef |
| *Trichoderma harzianum* T-22 | 21.4 bcde | 0.23 bcde |
| *Pseudomonas chlororaphis* MA342 | 21.4 bcde | 0.24 bcde |
| (Propamocarb+fosetyl-Al)+*Bacillus subtilis* strain QST713 | 21.7 bcde | 0.26 bcde |
| Propamocarb + fosetyl-Al | 25.0 bcde | 0.36 bc |
| (Propamocarb+fosetyl-Al)+ (*Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25) | 27.4 bcd | 0.35 bc |
| Etridiazole | 27.4 bcd | 0.30 bcde |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080 | 28.6 bcd | 0.32 bcd |
| (Propamocarb+fosetyl-Al)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 29.8 bc | 0.43 b |
| Fosetyl-Al | 30.9 bc | 0.34 bc |
| *Trichoderma asperellum* TV1 | 34.5 b | 0.40 bc |
| Untreated and inoculated control | 83.3 a | 0.84 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Data are means of three replicates, each containing 28 *Dodonaea viscosa* seedlings.
[z] Values followed by the same letters within a column are not significantly different according to Fisher test at $p = 0.01$ for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis. Untransformed data are presented.

Therefore, propamocarb+fosetyl-Al was replaced with cyprodinil+fludioxonil in the third experiment. This fungicide mixture was assessed alone and in combination with BCAs, that previously showed the best results. All treatments that include cyprodinil+fludioxonil, applied alone or in combination with BCAs, proved the best performances in controlling both DI and SS of stem rot, with values ranging from 3.6 to 5.9% and from 0.02 to 0.06 cm, respectively (Table 23). A good reduction of both disease parameters was also observed for fludioxonil and boscalid+pyraclostrobin, followed by thiophanate-methyl.

Moreover, *B. subtilis* strain QST713, *B. amyloliquefaciens* subsp. *plantarum* strain D747, fosetyl-Al, *S. griseoviridis* strain K61, *P. chlororaphis* MA342, *T. atroviride* T-11+*T. asperellum* T-25, *T. asperellum* TV1, *T. harzianum* T-22 showed an intermediate efficacy. Etridiazole followed by *T. harzianum* strain ICC012+*T. viride* strain ICC080 revealed the least effective treatments in reducing infections caused by *Ca*. *morganii*, with values comprised between 33.3 and 38.1% for DI and between 0.52 and 0.64 cm for SS (Table 23).


**Figure 15 - Differences of symptoms severity between treated and untreated and inoculated plants**

**Table 23 - Effects of chemical, biological and integrated treatments on disease incidence and symptoms severity of stem rot caused by *Calonectria morganii* on *Dodonaea viscosa* (Experiment III)**

| Treatment | Disease incidence[y,z] (%) | Symptoms severity[y,z] (cm) |
|---|---|---|
| Untreated and not-inoculated control | 0.0 f | 0.00 g |
| (Cyprodinil+fludioxonil)+*Trichoderma harzianum* T-22 | 3.6 ef | 0.02 g |
| (Cyprodinil+fludioxonil)+*Streptomyces griseoviridis* strain K61 | 3.6 e | 0.02 g |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 5.9 e | 0.06 fg |
| Cyprodinil+fludioxonil | 5.9 e | 0.05 g |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 5.9 e | 0.04 g |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25) | 5.9 e | 0.04 g |
| Fludioxonil | 7.1 e | 0.06 fg |
| Boscalid+pyraclostrobin | 7.1 e | 0.07 fg |
| Thiophanate-methyl | 9.5 de | 0.08 efg |
| *Bacillus subtilis* strain QST713 | 21.4 cd | 0.22 defg |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 22.6 bc | 0.29 cdef |
| Fosetyl-Al | 22.9 bc | 0.34 cd |
| *Streptomyces griseoviridis* strain K61 | 23.8 bc | 0.31 cde |
| *Pseudomonas chlororaphis* MA342 | 26.2 bc | 0.37 cd |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 27.4 bc | 0.37 cd |
| *Trichoderma asperellum* TV1 | 28.6 bc | 0.36 cd |
| *Trichoderma harzianum* T-22 | 33.3 bc | 0.33 cd |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080 | 33.3 bc | 0.52 bc |
| Etridiazole | 38.1 b | 0.64 b |
| Untreated and inoculated control | 79.8 a | 1.23 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Data are means of three replicates, each containing 28 *Dodonaea viscosa* seedlings.
[z] Values followed by the same letters within a column are not significantly different according to Fisher test at $p = 0.01$ for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis. Untransformed data are presented.


**Figure 16 - Difference between treated and untreated and inoculated plants**

In the last (4th) experiment, cyprodinil+fludioxonil was tested in combination with all BCAs used, since encouraging data were obtained in previous experiment (Table 24). This fungicide mixture confirmed once again its ability in reducing Calonectria infections both when applied alone and/or in combination. The best performances in controlling Dodonaea stem rot was obtained for the combination of cyprodinil+fludioxonil with *T. atroviride* T-11+*T. asperellum* T-25 (absence of symptoms). In addition, good results were recorded for cyprodinil+fludioxonil combined with *T. asperellum* TV1, cyprodinil+fludioxonil combined with *S. griseoviridis* strain K61, thiophanate-methyl, cyprodinil+fludioxonil combined with *P. chlororaphis* MA342, cyprodinil+fludioxonil, cyprodinil+fludioxonil combined with *T. harzianum* T-

22, cyprodinil+fludioxonil combined with *T. harzianum* strain ICC012+*T. viride* strain ICC080, cyprodinil+fludioxonil combined with *B. amyloliquefaciens* subsp. *plantarum* strain D747, cyprodinil+fludioxonil combined with *B. subtilis* strain QST713 and *S. griseoviridis* strain K6 (Table 24).

Treatments with *B. subtilis* strain QST713, fluopyram, *T. asperellum* TV1, boscalid+pyraclostrobin, *T. atroviride* T-11+*T. asperellum* T-25, *P. chlororaphis* MA342, *T. harzianum* strain ICC012+*T. viride* strain ICC080 and *T. harzianum* T-22 showed an intermediate efficacy in controlling Calonectria infections.

*B. amyloliquefaciens* subsp. *plantarum* strain D747, trifloxystrobin and fosetyl-Al provided the lowest reductions of both disease parameters although data are significant if compared with the untreated and inoculated control. All these data were reported in Table 24.

**Table 24 - Effects of chemical, biological and integrated treatments on disease incidence and symptoms severity of stem rot caused by *Calonectria morganii* on *Dodonaea viscosa* (Experiment IV)**

| Treatment | Disease incidence[y,z] (%) | Symptoms severity[y,z] (cm) |
|---|---|---|
| Untreated and not-inocuated control | 0.0 h | 0.0 g |
| (Cyprodinil+fludioxonil)+(*Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25) | 0.0 h | 0.0 g |
| (Cyprodinil+fludioxonil)+*Trichoderma asperellum* TV1 | 1.2 gh | 0.01 fg |
| (Cyprodinil+fludioxonil)+*Streptomyces griseoviridis* strain K61 | 1.2 gh | 0.01 efg |
| Thiophanate-methyl | 2.4 fgh | 0.02 defg |
| (Cyprodinil+fludioxonil)+*Pseudomonas chlororaphis M*A342 | 3.6 efg | 0.03 defg |
| Cyprodinil+fludioxonil | 3.6 efg | 0.03 defg |
| (Cyprodinil+fludioxonil)+*Trichoderma harzianum* T-22 | 4.8 defg | 0.05 bcdefg |
| (Cyprodinil+fludioxonil)+(*Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080) | 4.8 efg | 0.05 bcdefg |
| (Cyprodinil+fludioxonil)+*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 5.9 def | 0.03 cdefg |
| (Cyprodinil+fludioxonil)+*Bacillus subtilis* strain QST713 | 7.1 cdef | 0.05 bcdefg |
| *Streptomyces griseoviridis* strain K61 | 7.1 cdef | 0.07 bcdefg |
| *Bacillus subtilis* strain QST713 | 9.5 bcde | 0.08 bcdefg |
| Fluopyram | 10.7 bcde | 0.11 bcdefg |
| *Trichoderma asperellum* TV1 | 10.7 bcde | 0.11 bcdefg |
| Boscalid+pyraclostrobin | 10.7 bcde | 0.09 bcdefg |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 11.9 bcde | 0.10 bcdefg |
| *Pseudomonas chlororaphis* MA342 | 14.3 bcd | 0.12 bcde |
| *Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080 | 14.3 bcd | 0.14 bcd |
| *Trichoderma harzianum* T-22 | 16.7 bc | 0.12 bcde |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 16.7 bc | 0.15 bc |
| Trifloxystrobin | 17.9 bc | 0.15 b |
| Fosetyl-Al | 19.0 b | 0.16 b |
| Untreated and inoculated control | 47.6 a | 0.75 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Data are means of three replicates, each containing 28 *Dodonaea viscosa* seedlings.
[z] Values followed by the same letters within a column are not significantly different according to Fisher test at $p = 0.01$ for disease incidence and symptoms severity. Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis. Untransformed data are presented.

## 8.3. Discussion

*Ca*. *morganii* is one of the most common *Calonectria* species reported on ornamental plants in Sicily, being able to cause serious damages (Vitale *et al*., 2013b). Therefore, the management of this pathogen is very important. These experiments provide useful information on the development of IPM programs for controlling *Ca*. *morganii* on young plants. A preliminary experiment proved

the susceptibility of *D. viscosa* to *Ca. morganii* infections. *D. viscosa* was previously reported as host plant since leaf spot caused by *Ca. mexicana*, *Ca. pauciramosa*, *Ca. polizzii*, *Ca. pseudomexicana* and *Ca. tunisiana* was reported (Polizzi and Catara, 2001; Lombard *et al*., 2011). Therefore, considering its fast growing characteristic, its high germination rate and its susceptibility to *Ca. morganii*, *D. viscosa* was chosen as model plant to evaluate the efficacy of chemical, biological and integrated treatments. These results proved that all fungicides and BCAs tested were able to reduce significantly Calonectria infections, both in individual and in integrated applications. Moreover, different levels of significance among treatments were found. Among all treatments, cyprodinil+fludioxonil applied alone and in combination with BCAs showed the best performance in controlling Calonectria disease. These results proved that cyprodinil+fludioxonil could be inserted in IPM programs, by applying it in combination with different commercial formulates containing *Trichoderma* spp., *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp.

Good results have been obtained also for BCAs applied alone, as reported for *S. griseoviridis* strain K61, *T. atroviride* T-11+*T. asperellum* T-25, *T. harzianum* T-22 and *B. subtilis* strain QST713.

Although propamocarb+fosetyl-Al showed good results in controlling disease, its use should be avoided since it causes phytotoxicity on young leaves of *D. viscosa*. Among all chemical compounds tested, boscalid+pyraclostrobin proved good results in controlling infections.

Although good results in controlling Dodonaea stem rot were observed for thiophanate-methyl, its use should be avoided since its selective pressure could induce selection of resistant strains within *Calonectria* genus (Polizzi and Vitale, 2001; Vitale *et al*., 2009b). In conclusion, based on these encouraging results it is possible to develop management programs for an integrated control of stem rot caused by *Ca. morganii* on *D. viscosa* seedlings, complying with the latest guidelines on sustainable use of pesticides.

## 9. Biological and integrated control of *Calonectria pauciramosa* on *Polygala myrtifolia*

### 9.1. Materials and methods

Two experiments were carried out on *Polygala myrtifolia* cuttings to assess the efficacy of chemical, biological and integrated treatments in controlling artificial infections caused by *Ca. pauciramosa*, causal agent of crown and root rot.

Fresh culture of *Ca. pauciramosa* was obtained by transferring agar plug from stock cultures onto PDA plates.

*Ca. pauciramosa* was grown on SNA dishes at 25 °C for 21 days. Conidial suspension was obtained by flooding the dishes with SDW, gently rubbing the colony surface with a sterile loop and filtering the suspension through a triple layer of cheesecloth into a flask to collect conidia. Final spore suspension of $3 \times 10^5$ conidia $ml^{-1}$ was determined using a haemocytometer.

The efficacy of 17 and 12 treatments were studied in experiment I and II, respectively.

A total of four commercial fungicides, such as boscalid+pyraclostrobin mixture (Signum®), fosetyl-Al (Aliette®), prochloraz (Octave®), propamocarb+fosetyl-Al mixture (Previcur Energy®) and six bioformulates based on several BCAs, such as *B. amyloliquefaciens* subsp. *plantarum* strain D747 (Amylo-X®), *B. subtilis* strain QST713 (Serenade Max®), *S. griseoviridis* strain K61 (Mycostop®), *T. atroviride* T-11+*T. asperellum* T-25 (Tusal®), *T. harzianum* strain ICC012+*T. viride* strain ICC080 (Radix®) and *T. harzianum* T-22 (Trianum-P®) were used to evaluate their efficacy in reducing crown and root rot caused by *Ca. pauciramosa* on *P. myrtifolia*.

Fungicides and BCAs were prepared according to manufacturers recommendations (Table 25) and sprayed by a hand-pump up to run-off on plants, by applying approximately 240 ml of suspension for each replicate.

According to a management schedule previously established, in the experiment I fungicides and BCAs were applied 24 and 48 hours before pathogen inoculation, respectively. After that, chemical and biological treatments, applied alone, were repeated every 2 weeks. When fungicides were applied in combination with

BCAs, chemical and biological treatments were performed at 14- and 15-day intervals, respectively.

In the experiment II, fungicides and BCAs were applied 24 and 48 hours prior to pathogen inoculation, respectively. Subsequently, chemical and biological treatments were repeated every 15 and 7 days, respectively.

About 1 ml of conidial suspension of *Ca. pauciramosa* was inoculated as soil drench onto crown and root area of *P. myrtifolia* plants in both experiments.

Subsequently, inoculated plants were maintained for 7 days under plastic tunnels at 25 °C.

In both experiments, each treatment was replicated three times with 72 plants for replicate, according to a randomized completely block design. The same number of untreated and inoculated plants and untreated and not-inoculated plants served as positive and negative controls, respectively.

The efficacy of treatments was evaluated by referring to disease incidence (DI) of Calonectria infections. This parameter was obtained as a percentage values resulting from number of infected plants out of the total number of plants × 100.

In addition, only in the experiment II the treatment effects were evaluated on the length, number and weight of young polygala shoots. Recovery frequency from symptomatic tissues was always detected to confirm DI data.

**Figure 17 - *Polygala myrtifolia* cuttings arranged in randomized blocks according to designed experimental scheme**

## Statistical analysis

All data obtained from these experiments were subjected to analysis of variance according to parametric approaches (Statistica 10; StatSoft Inc., Analytical Software for Windows) with factorial treatment structure and interactions.

Data from all experiments were analysed separately and the mean values of DI for each treatment were calculated, averaging corresponding values determined for each replicate. All DI percentage data were previously transformed using arcsine ($\sin^{-1}$ square root x) prior to statistical analysis. The untransformed values of DI (%) are presented. Mean separation of the percentage recovery data in each experiment was conducted using Fisher test at $p = 0.01$ and 0.05.

**Table 25 - Fungicides and BCAs employed in controlling crown and root rot caused by *Calonectria pauciramosa* on *Polygala myrtifolia***

| *Active ingredient* | *Trade name* | *Manufacturer* | *Rates (g or ml/100 liters)* | *Formulation*[x] | *Fungicide use* |
|---|---|---|---|---|---|
| Boscalid+pyraclostrobin | Signum | Basf Italia | 100 | 26.7+6.7 WG | Experiment I, II |
| Fosetyl-Al | Aliette | Bayer CropScience | 300 | 80 WG | Experiment I |
| Prochloraz | Octave | Basf Italia | 120 | 46.1 WP | Experiment II |
| Propamocarb+fosetyl-Al | Previcur Energy | Bayer CropScience | 250 | 47.2+27.6 SL | Experiment I |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | Amilo-X | Biogard | 250 | 25 WG | Experiment I |
| *Bacillus subtilis* strain QST713 | Serenade Max | Basf Italia | 400 | WP | Experiment I, II |
| *Streptomyces griseoviridis* strain K61 | Mycostop | Bioplanet | 25 | 33.33 WP | Experiment I, II |
| *Trichoderma atroviride* T-11+ *Trichoderma asperellum* T-25 | Tusal | Certis | 250 | WG | Experiment II |
| *Trichoderma harzianum strain* ICC012+*Trichoderma viride* strain ICC080 | Radix | Certis | 250 | 2 + 2 WP | Experiment I |
| *Trichoderma harzianum* T-22 | Trianum-P | Koppert Biological Systems | 100 | 1.15 WP | Experiment II |

[x] Percentage of active ingredient; SL=soluble concentrate; WG = water dispersible granule; WP = wettable powder.

### 9.2. Results

The infection level induced by *Ca. pauciramosa* inoculation on *P. myrtifolia* was high. In these conditions, boscalid+pyraclostrobin was the most effective treatment in reducing DI, according to Fisher test at $p = 0.01$, followed by fosetyl-Al (Table 26). A certain efficacy in reducing DI was averagely observed for *B. subtilis* strain QST713, *T. harzianum* strain ICC012+*T. viride* strain ICC080, fosetyl-Al combined with *B. subtilis* strain QST713, propamocarb+fosetyl-Al combined with *T. harzianum* strain ICC012+*T. viride* strain ICC080, fosetyl-Al combined with *S. griseoviridis* strain K61, fosetyl-Al combined with *T. harzianum* strain ICC012+*T. viride* strain ICC080 and *B. amyloliquefaciens* subsp. *plantarum* strain D747 (DI comprised between 65 and 71.11%) with a decreasing efficacy, respectively. Otherwise, no significant effect was observed for remaining treatments (Table 26).

**Figure 18 - Symptoms of crown and root rot caused by *Calonectria pauciramosa* on *Polygala myrtifolia* cuttings**

**Table 26 - Effects of chemical, biological and integrated treatments in controlling crown and root rot caused by *Calonectria pauciramosa* on *Polygala myrtifolia***

| Treatment | Disease incidence[y,z] (%) |
|---|---|
| Untreated and not-inoculated control | 0.00 h |
| Boscalid+pyraclostrobin | 30.97 g |
| Fosetyl-Al | 56.56 f |
| *Bacillus subtilis* strain QST713 | 65.00 ef |
| *Trichoderma harzianum strain* ICC012+*Trichoderma viride* strain ICC080 | 65.77 def |
| Fosetyl-Al +*Bacillus subtilis* strain QST713 | 67.39 cdef |
| (Propamocarb+fosetyl-Al) +(*Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080) | 68.19 cdef |
| Fosetyl-Al +*Streptomyces griseoviridis* strain K61 | 68.40 cdef |
| Fosetyl-Al + (*Trichoderma harzianum* strain ICC012+*Trichoderma viride* strain ICC080) | 68.58 cde |
| *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 71.11 bcde |
| (Propamocarb+fosetyl-Al) +*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 | 75.11 a-e |
| (Propamocarb+fosetyl-Al) +*Streptomyces griseoviridis* strain K61 | 76.44 abcd |
| Propamocarb+fosetyl-Al | 77.20 abc |
| *Streptomyces griseoviridis* strain K61 | 80.05 ab |
| Fosetyl-Al +*Bacillusamyloliquefaciens* subsp. *plantarum* strain D747 | 81.36 ab |
| (Propamocarb+fosetyl-Al) +*Bacillus subtilis* strain QST713 | 84.56 a |
| Untreated and inoculated control | 84.90 a |

Fungicide+BCA indicates that fungicide was applied in combination with BCA.
[y] Averaged from 3 replicates each formed by 72 *Polygala myrtifolia* cuttings.
[z] Values followed by the same letters within a column are not significantly different according to Fisher test ($p = 0.01$) for disease incidence. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown.

In the experiment II infection levels induced by *Ca. pauciramosa* on *P. myrtifolia* was very high. In these conditions, boscalid+pyraclostrobin combined with *B. subtilis* strain QST713, boscalid+pyraclostrobin, boscalid+pyraclostrobin combined with *T. harzianum* T-22, boscalid+pyraclostrobin combined with *T. atroviride* T-11+*T. asperellum* T-25 were the most effective treatments in reducing DI (ranging from 39.7 to 48%), according to Fisher test at $p = 0.01$ (Table 27).

Prochloraz, boscalid+pyraclostrobin combined with *S. griseoviridis* strain K61 and *S. griseoviridis* strain K61 were slightly lesser effective than previous ones in controlling Calonectria infections.

By analyzing agronomic data not significant differences were recorded among treatments for number of shoots. Otherwise, boscalid+pyraclostrobin combined with *B. subtilis* strain QST713 followed by prochloraz and boscalid+pyraclostrobin combined with *S. griseoviridis* strain K61 showed the best performances in increasing the length and shoot weight according to Fisher test only at $p = 0.05$, although with variable results (Table 27).

**Table 27 - Effects of chemical, biological and integrated treatments in controlling crown and root rot caused by *Calonectria pauciramosa* on *Polygala myrtifolia***

| Treatment | Disease incidence[w,y,z] (%) | Agronomic data on shoot[x,y,z] | | |
|---|---|---|---|---|
| | | Number | Length | Weight |
| Untreated and not-inoculated control | 0.0 eD | 5.82[ns] | 10.44 aA | 98.60 aA |
| (Boscalid+pyraclostrobin)+(*Bacillus subtilis* strain QST713) | 39.7 dC | 5.98[ns] | 10.06 abA | 84.47 abcAB |
| Boscalid+pyraclostrobin | 45.1 cdC | 5.73[ns] | 9.08 abcA | 80.83 abcAB |
| (Boscalid+pyraclostrobin)+ (*Trichoderma harzianum* T-22) | 47.6 cdeC | 5.32[ns] | 7.97 cA | 61.83 cB |
| (Boscalid+pyraclostrobin)+(*Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25) | 48.0 cdeC | 6.15[ns] | 9.58 abcA | 80.37 abcAB |
| Prochloraz | 52.8 cdeBC | 4.78[ns] | 10.07 abA | 79.27 abcAB |
| (Boscalid+pyraclostrobin)+(*Streptomyces griseoviridis* strain K61) | 54.0 cdeBC | 6.27[ns] | 10.36 abA | 92.23 abAB |
| *Streptomyces griseoviridis* strain K61 | 66.2 bcdBC | 5.37[ns] | 8.40 bcA | 60.60 cB |
| *Trichoderma atroviride* T-11+*Trichoderma asperellum* T-25 | 67.1 bcABC | 4.85[ns] | 8.92 abcA | 65.77 cB |
| *Bacillus subtilis* strain QST713 | 69.1 bcABC | 5.28[ns] | 9.23 abcA | 73.10 bcAB |
| *Trichoderma harzianum* T-22 | 77.2 abAB | 5.15[ns] | 9.00 abcA | 64.37 cB |
| Untreated and inoculated control | 90.9 aA | 4.88[ns] | 7.82 cA | 62.73 cB |

Fungicide +BCA indicates that fungicide was applied in combination with BCA.
[w] Averaged from 3 replicates each formed by 72 *Polygala myrtifolia* cuttings.
[x] Averaged from 3 replicates each formed by 20 *Polygala myrtifolia* cuttings.
[y] Same letters within each column denote not significant differences among treatments according to Fisher least significant difference test. Small letters indicate a significativity at $p = 0.05$, whereas capital letters denote significativity for $p = 0.01$.
[z] Values followed by the same letters within a column are not significantly different according to Duncan test ($p = 0.01$) for disease incidence. Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis. Untransformed data are shown. ns = not significant data.

The high recovery frequency of *Ca. pauciramosa* from symptomatic tissues confirmed the etiology of infections.

### 9.3. Discussion

These findings provide encouraging information for controlling crown and root rot caused by *Ca. pauciramosa* on *P. myrtifolia*. As reported in previous experiments, boscalid+pyraclostrobin confirmed its efficacy in controlling Calonectria infections. In detail, this fungicide mixture applied in combination with BCAs, such as *B. subtilis* strain QST713 and *S. griseoviridis* strain K61, showed very good results both in controlling the disease and in favouring the growth of young plants. Good results were also obtained for fosetyl-Al and prochloraz. However this last fungicide is not recommended for its already reported activity to induce selection of resistant strains within the genus *Calonectria* (Guarnaccia *et al*., 2012, 2014).

Averagely, treatments with *B. subtilis* strain QST713 could offer slight efficacy in reducing crown and root rot disease, although with variable results.

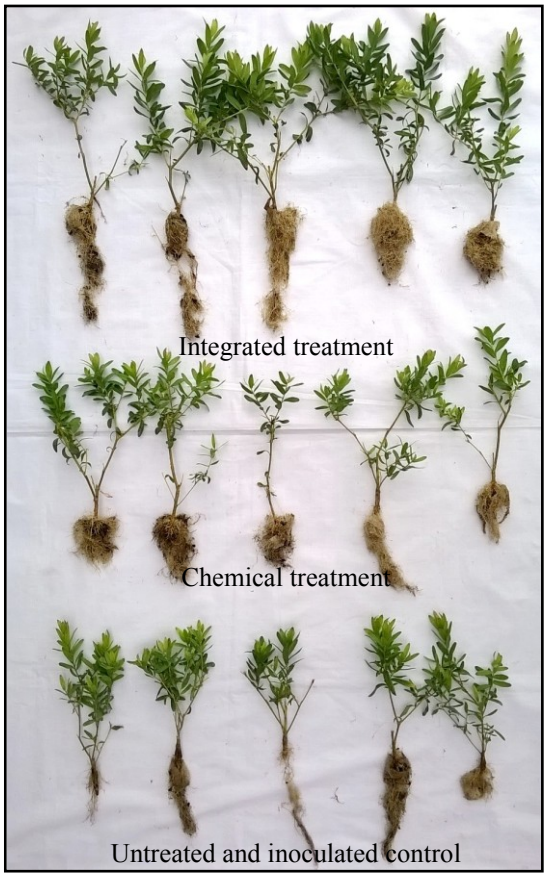


**Figure 19 - Different effects of treatments on young plants**

## 10. Effects of label and sub-label rates of dazomet and metham-sodium on survival of *Calonectria* spp.

### 10.1. Materials and methods

**Pathogen isolates**

Seven representative isolates including *Ca*. *ilicicola* (CBS 129186), *Ca*. *mexicana* (CBS 130353), *Ca*. *morganii* (CBS 119669), *Ca*. *pauciramosa* (DISTEF-G173), *Ca*. *polizzii* (CBS 130351), *Ca*. *pseudomexicana* (CBS 130354) and *Ca*. *tunisiana* (CBS 130357) were used in microcosm assays and two representative isolates including *Ca*. *pauciramosa* (DISTEF-G173) and *Ca*. *polizzii* (CBS 130351) were used in two field tests. All isolates were incubated for 4 weeks at 25 ± 1°C on carnation leaf agar (CLA) for microsclerotia production.

**Fumigant effects on microsclerotia survival and viability**

Three experiments were carried out to assess the efficacy of two chemical fumigants in reducing or suppressing primary inoculum of *Calonectria* species.
The commercial fumigants evaluated were dazomet (Basamid Granulat® 99% a.i., registration holder is Kanesho Soil Treatment SPRL/BVB, Brusseles, Belgium) and metham-sodium (Divapan® 42.8%=510 g/L a.i., registration holder is Taminco Italia S.r.l., Milano, Italy). Two carnation leaf segments (about 6 cm long) colonized by microsclerotia were placed in nylon mesh bags and buried (about 15 cm depth) in plastic containers and plots containing soil. In all experiments, the soil temperature was measured at 15 cm depth in each tray and plot.
In experiment I, performed in microcosm, dazomet was applied at 200 kg/ha, 400 kg/ha and 500 kg/ha and mixed uniformly into the soil before the bags were buried, while metham-sodium was applied at 400 l/ha, 700 l/ha and 1000 l/ha as soil drench. After the treatments the containers were covered with Virtually Impermeable Film (VIF, Ecobrom®, AgriPlast S.r.l., Vittoria, Ragusa, Italy) and placed in single-span steel greenhouse located in Giarre, Catania province. Untreated infested soil served as a control. Each treatment was replicated three

times in a completely randomized design. This assay was performed three times through 2011 (on June and November) and in 2012 (on February).

Unlike the first, the experiments II and III were performed in open field located in Riposto, Catania province. Each experimental plot consisted of a layer of soil of 2.5 m × 5.0 m (40 cm height) disposed on a cement bed. Untreated infested plot served as a control. Each treatment was replicated three times, according to a randomized completely block design.

In the experiment II, dazomet and metham-sodium were applied at the same rates used in microcosm. After the treatments the experimental plots were covered with Virtually Impermeable Film (VIF, Ecobrom®, AgriPlast S.r.l., Vittoria, Ragusa, Italy). This experiment was repeated twice in 2012 (on June and October).

The experiment III was conducted to further reduce the application rates of both fumigants. In detail, dazomet was applied at 100 kg/ha, 160 kg/ha and 200 kg/ha and mixed uniformly into the soil before the bags were buried, while metham-sodium was applied at 250 l/ha, 350 l/ha and 400 l/ha as soil drench. After the treatments the experimental plots were covered with two different plastic films. Virtually Impermeable Film (VIF, Ecobrom®, AgriPlast S.r.l., Vittoria, Ragusa, Italy) and Totally Impermeable Film (TIF™ Eval™, Kuraray America INC., Pasadena, Texas, USA) were the two plastic tarps tested in this experiment.


**Figure 20 - Experiment in open field before (a) and after treatment (b)**

The purpose of this last experiment was to determine which of these two plastic films had the higher retention capacity of the fumigant compared to each other, by evaluating the ability of fumigant in reducing microsclerotia survival. This assay was conducted three times during 2013 (on July and November) and in 2014 (on May).

Data were collected 21 days after treatment. All nylon bags were retrieved from the soil. Nine leaf pieces (about 6.5 mm long), obtained by cutting each leaf segment, were washed with sterile water and placed onto PDA, supplemented with streptomycin sulphate (at 100 mg $l^{-1}$), and maintained for 1 week at 25 °C. The percentage of leaf pieces from which pathogen colonies developed was used to determine the survival of *Calonectria* species after recovery from soil. Moreover, the infection ability of microsclerotia after soil treatment was tested on red clover (*Trifolium pratense* L.) seedlings (Vitale *et al*., 2012). To this aim, in each plot arranged in the field experiment, additional two leaf segments colonized by each isolate used, were buried as described above. The retrieved carnation leaf portions were cut into smaller pieces and mixed into sterile soil in aluminium trays. These aluminium trays, each containing about 50 red clover seeds, were placed in a growth chamber and brought to water field capacity. Crown and root rot symptoms showed on red clover seedlings were recorded 10 days after seeding.

**Statistical analysis**

All data obtained from the inoculation trials were subjected to analysis of variance according to parametric approaches (Statistica 10; StatSoft Inc., Analytical Software for Windows) with factorial treatment structure and interactions. Recovery percentage data were transformed using arcsine ($sin^{-1}$ square root x) prior to statistical analysis. The untransformed values of recovery percentage are presented in tables and separated by Fisher's least significant difference test at $p = 0.01$ and 0.05.

## 10.2. Results

### Microcosm experiments

In this experiment all fumigants at used rates were highly effective in reducing or suppressing microsclerotia of *Calonectria* species according to Fisher test at $p = 0.05$. On June with soil temperatures comprised between 23.9 and 28.8 °C, the viability of all *Calonectria* microsclerotia was totally inhibited (*data not shown*). Otherwise, on November with soil temperatures comprised between 19.7 and 24.2 °C, viable microsclerotia were recovered only from soil fumigated at the lowest rate of metham-sodium (Table 28). In experiment on February, with soil temperatures comprised between 9.1 and 13.2 °C, metham-sodium was totally effective in suppressing microsclerotia only at the higher rate while dazomet shows a partial efficacy at the lowest rate (Table 29). However, significant differences in the viable recovery of microsclerotia among tested isolates were detected. On November *Ca*. *morganii* was suppressed at all rates of both fumigants tested. On February, only *Ca*. *morganii* was not recovered as viable inoculum at the intermediate rate of metham-sodium, while the lowest rate of dazomet was not effective in total suppression of *Ca*. *pauciramosa*, *Ca*. *polizzii* and *Ca*. *mexicana* microsclerotia (Table 29).

**Table 28 - Recovery (%) of *Calonectria* spp. isolates from infested soil after treatments in November 2011 in microcosm (experiment I)**

| *Calonectria* spp. [x,y] | *Ca*. *ilicicola* | *Ca*. *mexicana* | *Ca*. *morganii*[z] | *Ca*. *pauciramosa* | *Ca*. *polizzii* | *Ca*. *pseudomexicana* | *Ca*. *tunisiana* |
|---|---|---|---|---|---|---|---|
| MS (1000 l/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| MS (700 l/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| MS (400 l/ha) | 7.4 bC | 9.9 bCD | 0A | 0.6 aA | 1.9 bB | 13.6 bD | 6.8 bC |
| DZ (500 kg/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| DZ (400 kg/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| DZ (200 kg/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| Untreated soil | 100 c | 100 c | 100 | 100 b | 100 c | 100 c | 100 c |

MS: Metham-sodium; DZ: Dazomet

[x] Data averaged from three replications, each from 54 values.

[y] Means followed by different small letters within each column (efficacy of treatment) and by different capital letters within each row (pathogen response to treatment) are significantly different according to Fisher's least significance difference test ($p = 0.05$).

[z] Since error mean square is zero, homogeneous groups cannot be computed and, consequently letters within this column for post-hoc analysis of significant differences are omitted.

**Table 29 - Recovery (%) of *Calonectria* spp. isolates from infested soil after treatments in February 2012 in microcosm (experiment I)**

| *Calonectria* spp.[x,y] | *Ca. ilicicola* | *Ca. mexicana* | *Ca. morganii*[z] | *Ca. pauciramosa* | *Ca. polizzii* | *Ca. pseudomexicana* | *Ca. tunisiana* |
|---|---|---|---|---|---|---|---|
| MS (1000 l/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| MS (700 l/ha) | 40.1 cD | 46.9 cE | 0 A | 22.8 cC | 21.6 cBC | 19.1 bB | 42.6 bD |
| MS (400 l/ha) | 98.1 dB | 100 dC | 100 C | 100 dC | 88.9 dA | 100 cC | 100 cC |
| DZ (500 kg/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| DZ (400 kg/ha) | 0 a | 0 a | 0 | 0 a | 0 a | 0 a | 0 a |
| DZ (200 kg/ha) | 16.6 bE | 10.5 bC | 0 A | 12.3 bD | 3.7 bB | 0 aA | 0 aA |
| Untreated soil | 100 e | 100 d | 100 | 100 d | 100 e | 100 c | 100 c |

MS: Metham-sodium; DZ: Dazomet
[x] Data averaged from three replications, each from 54 values.
[y] Means followed by different small letters within each column (efficacy of treatment) and by different capital letters within each row (pathogen response to treatment) are significantly different according to Fisher's least significance difference test ($p = 0.05$).
[z] Since error mean square is zero, homogeneous groups cannot be computed and, consequently letters within this column for post-hoc analysis of significant differences are omitted.

## Open field experiments

In experiment II conducted in open field, by applying the same fumigant rates used in microcosm, all treatments proved high performances in suppressing microsclerotia of both *Calonectria* species, with soil temperature comprised between 25 and 31°C. Indeed, no viable inoculum was recovered from all fumigated plots (Tables 30).

**Table 30 - Recovery (%) of *Ca. pauciramosa* and *Ca. polizzii* from infested soil after fumigation in October (1st) and November (2nd) 2012 in open field (experiment II)**

| Treatment | *Ca. pauciramosa* | *Ca. polizzii* |
|---|---|---|
| **Trial** | **First and second trials combined [y,z]** | **First and second trials combined [y,z]** |
| MS (1000 l/ha) | 0.0 | 0.0 |
| MS (700 l/ha) | 0.0 | 0.0 |
| MS (400 l/ha) | 0.0 | 0.0 |
| DZ (500 kg/ha) | 0.0 | 0.0 |
| DZ (400 kg/ha) | 0.0 | 0.0 |
| DZ (200 kg/ha) | 0.0 | 0.0 |
| Untreated soil | 100 | 100 |

MS: Metham-sodium; DZ: Dazomet
[y] Since error mean square is zero, homogeneous groups cannot be computed and, consequently letters within each column for post-hoc analysis of significant differences are omitted.
[z] Data are means of three replicates, each containing 54 infected carnation leaf pieces.
Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis; untransformed data are presented. $p$ = significant probability values associated with Fisher test.

## Open field experiments at reduced rates

In experiment conducted on July (2013), with soil temperatures comprised between 24.6 and 41.9°C, no viable inoculum was recovered from all fumigated plots (Table 31).

**Table 31 - Recovery (%) of *Calonectria pauciramosa* and *Calonectria polizzii* from infested soil after chemical fumigation performed under TIF and VIF barriers in open field (July 2013) (Experiment III)**

| | TIF | | VIF | |
|---|---|---|---|---|
| **Treatment** | ***Ca. polizzii*[y,z]** | ***Ca. pauciramosa*[y,z]** | ***Ca. polizzii*[y,z]** | ***Ca. pauciramosa*[y,z]** |
| MS (400 l/ha) | 0.0 | 0.0 | 0.0 | 0.0 |
| MS (350 l/ha) | 0.0 | 0.0 | 0.0 | 0.0 |
| MS (250 l/ha) | 0.0 | 0.0 | 0.0 | 0.0 |
| DZ (200 kg/ha) | 0.0 | 0.0 | 0.0 | 0.0 |
| DZ (160 kg/ha) | 0.0 | 0.0 | 0.0 | 0.0 |
| DZ (100 kg/ha) | 0.0 | 0.0 | 0.0 | 0.0 |
| Untreated soil | 100.0 | 100.0 | 100.0 | 100.0 |

MS: Metham-sodium; DZ: Dazomet

[y] Since error mean square is zero, homogeneous groups cannot be computed and, consequently letters within each column for post-hoc analysis of significant differences are omitted.

[z] Data are means of three replicates each containing 54 infected carnation leaf pieces. Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis; untransformed data are presented. $p$ = significant probability values associated with Fisher test.

On November 2013 (with soil temperature comprised between 15.7 and 31.8°C), the higher and intermediate rates of metham-sodium and dazomet suppressed totally microsclerotia viability of both *Calonectria* species under both TIF and VIF barriers (Table 32). The lowest rate of dazomet reduced significantly recovery percentages of both pathogens under TIF, and these values were also not significant different from other treatments. The lowest rates of dazomet and metham-sodium applied under VIF barrier also reduced significantly recovery percentages of both viable *Calonectria* inocula at $p$ = 0.01. For *Ca. pauciramosa* these treatments showed values significant lowest than remaining ones. The lowest rate of dazomet produced recovery of viable *Ca. polizzii* inocula significant different from all fumigation treatments at $p$ = 0.05, except for the lowest rate of metham-sodium, that was not significant different from all fumigated plots (Table 32). In addition, the recovery percentages of *Calonectria* microsclerotia were always lower from fumigated plots covered with TIF than from those covered with VIF.

**Table 32 - Recovery (%) of *Ca*. *pauciramosa* and *Ca*. *polizzii* from infested soil after chemical fumigation performed under TIF and VIF barriers in open field (November 2013) (experiment III)**

| | TIF | | VIF | |
|---|---|---|---|---|
| **Treatment** | ***Ca*. *polizzii*[y,z]** | ***Ca*. *pauciramosa*[y,z]** | ***Ca*. *polizzii*[y,z]** | ***Ca*. *pauciramosa*[y,z]** |
| MS (400 l/ha) | 0.0 bB | 0.0 bB | 0.0 cB | 0.0 cC |
| MS (350 l/ha) | 0.0 bB | 0.0 bB | 0.0 cB | 0.0 cC |
| MS (250 l/ha) | 0.0 bB | 0.0 bB | 9.9 bcB | 24.7 bB |
| DZ (200 kg/ha) | 0.0 bB | 0.0 bB | 0.0 cB | 0.0 cC |
| DZ (160 kg/ha) | 0.0 bB | 0.0 bB | 0.0 cB | 0.0 cC |
| DZ (100 kg/ha) | 13.0 bB | 9.3 bB | 17.9 bB | 36.4 bB |
| Untreated soil | 100.0 aA | 100.0 aA | 100.0 aA | 100.0 aA |

MS: Metham-sodium; DZ: Dazomet

[y] Same letters within each column denote not significant differences among treatments according to Fisher least significant difference test. Small letters indicate a significativity at $p = 0.05$, whereas capital letters denote significativity for $p = 0.01$.

[z] Data are means of three replicates each containing 54 infected carnation leaf pieces. Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis; untransformed data are presented.

The last trial was performed on May (2014), with soil temperatures ranging from 20.2 to 33.2°C. In this trial, all rates of metham-sodium and the highest rate of dazomet totally suppressed viability of *Calonectria* microsclerotia (Table 33).

Although the intermediate rates of dazomet did not totally suppress viability of *Ca*. *polizzii* microsclerotia, this value (20.4%) was not significant different from all remaining fumigated plots.

The lowest rate of dazomet always reduced significantly percentage of viable inoculum (ranging from 1.2 to 19.1%) retrieved for both *Calonectria* species under both gas-tight tarps, but produced significant differences from other fumigation treatments only for *Ca*. *pauciramosa* under both TIF and VIF barriers (Table 33).

**Table 33 - Recovery (%) of *Ca. pauciramosa* and *Ca. polizzii* from infested soil after chemical fumigation performed under TIF and VIF barriers in open field (May 2014) (experiment III)**

| | TIF | | VIF | |
|---|---|---|---|---|
| **Treatment** | ***Ca. polizzii*[y,z]** | ***Ca. pauciramosa*[y,z]** | ***Ca. polizzii*[y,z]** | ***Ca. pauciramosa*[y,z]** |
| MS (400 l/ha) | 0.0 bB | 0.0 cC | 0.0 cB | 0.0 cC |
| MS (350 l/ha) | 0.0 bB | 0.0 cC | 0.0 cB | 0.0 cC |
| MS (250 l/ha) | 0.0 bB | 0.0 cC | 0.0 bB | 0.0 cC |
| DZ (200 kg/ha) | 0.0 bB | 0.0 cC | 0.0 bB | 0.0 cC |
| DZ (160 kg/ha) | 0.0 bB | 0.0 cC | 20.4 bB | 0.0 cC |
| DZ (100 kg/ha) | 1.2 bB | 16.7 bB | 19.1 bB | 18.5 bB |
| **Untreated soil** | 100.0 aA | 100.0 aA | 100.0 aA | 100.0 aA |

MS: Metham-sodium; DZ: Dazomet

[y] Same letters within each column denote not significant differences among treatments according to Fisher least significant different test. Small letters indicate a significativity at $p = 0.05$, whereas capital letters denote significativity for $p = 0.01$.

[z] Data are means of three replicates each containing 54 infected carnation leaf pieces. Arcsine ($\sin^{-1}$ square root $x$) transformation was used on percentage data prior to analysis; untransformed data are presented.

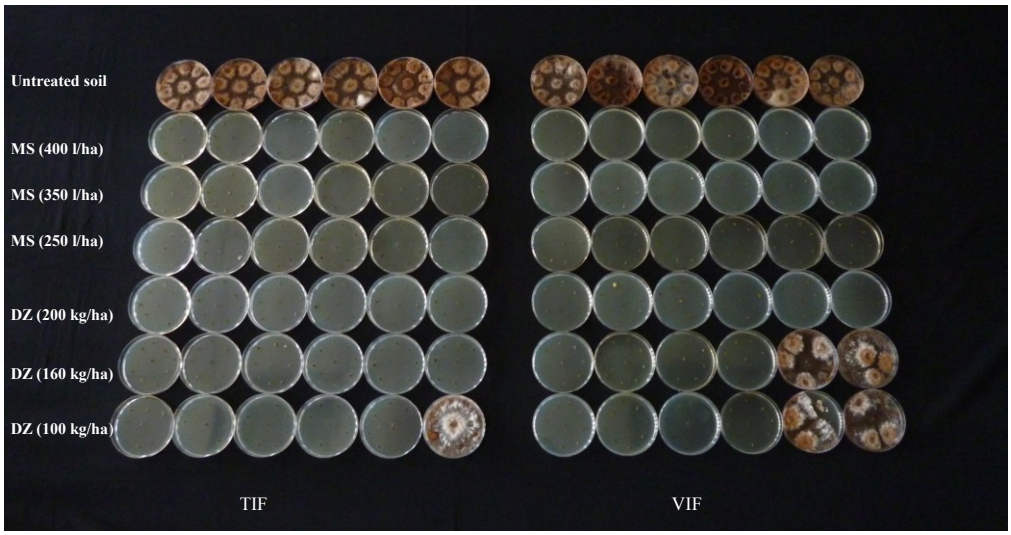


**Figure 21 – Recovery percentage of *Calonectria polizzii* after fumigation treatment on May 2014**

Overall, for the full set of trials performed at reduced dosages a very highly significant effect of treatment was always observed both for *Ca. pauciramosa* and *Ca. polizzii* recovery. Otherwise, interactions among different factors, treatment×trial×film, treatment×film, trial×film and treatment×trial were always not significant only for *Ca. polizzii* but significant for *Ca. pauciramosa* (Table 34). Thus, in figure 22 average recovery percentages of viable inoculum from different treated plots was reported only for *Ca. polizzii*.

**Table 34 – Effect of interactions among treatment, trial and film used on recovery percentage of *Calonectria* microsclerotia**

| | *Recovery percentage*[z] | | | | | |
|---|---|---|---|---|---|---|
| | *Calonectria polizzii* | | | *Calonectria pauciramosa* | | |
| | **df** | ***F*** | ***p* value** | **df** | ***F*** | ***p* value** |
| Treatment | 6 | 337.9326 | 0.00 **** | 6 | 631.0638 | 0.00 **** |
| Trial | 2 | 2.4460 | 0.092786 ns | 2 | 9.5123 | 0.0002 *** |
| Film | 1 | 2.7746 | 0.099495 ns | 1 | 6.7583 | 0.011 * |
| Treatment × trial | 12 | 1.8387 | 0.054634 ns | 12 | 5.8485 | 0.00 **** |
| Trial × film | 2 | 1.0407 | 0.357700 ns | 2 | 6.2625 | 0.0029 ** |
| Treatment × film | 6 | 0.6870 | 0.660582 ns | 6 | 2.8171 | 0.015 * |
| Treatment × trial × film | 12 | 0.8053 | 0.644108 ns | 12 | 2.6128 | 0.0053 ** |

[z] *F* test of fixed effects, df = degree of freedom, *p* value associated to *F*; ns = not significant data.
****=very highly significant interaction; ***=highly significant interaction; **=significant interaction; *=low significant interaction.

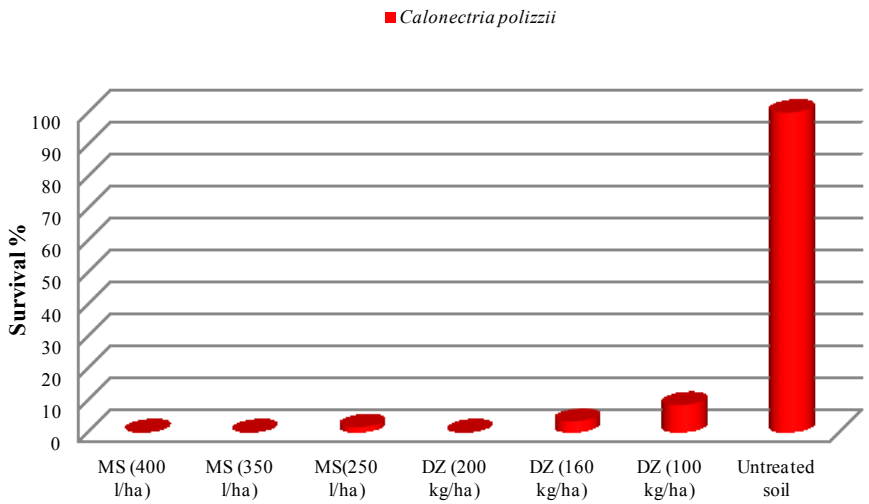


**Figure 22 - Recovery (%) of *Calonectria polizzii* from infested soil after chemical fumigation performed combined for all trials performed and film used**

Otherwise, interactions among examined factors are always significant for recovery of viable inocula of *Ca. pauciramosa*, thus suggesting for this pathogen a different effect correlated to film used (higher effect of TIF than VIF) and to trial (different environmental conditions).

All these results were further confirmed by data referred to the microsclerotia infection ability of each *Calonectria* species on red clover seedlings. Calonectria

infections were only reproduced from the viable inocula retrieved from all untreated plots, but also from plots where fumigation was less effective.

### 10.3. Discussion

Calonectria diseases represent a heavy threat for ornamental plant production in south Italy. Potential use of soil solarization or BCAs for their control has been addressed in some scientific papers (Vitale *et al*., 2012; Vitale *et al*., 2013b). Currently, chemical control is the most used approach to manage Calonectria diseases. However, the use of benzimidazoles (MBCs) and sterol demethylation inhibitors (DMIs) should be avoided since these fungicides can induce a high selection of resistant isolates (French and Menge 1978; Alfenas *et al*., 1988; Vitale *et al*., 2009b; Guarnaccia *et al*., 2012, 2014). Little or no information has been published on efficacy of both fumigants tested in these experiments against Calonectria diseases, except for metham-sodium (Phipps, 1990; Cline and Beute, 1986) and a preliminary study on dazomet efficacy (Enebak *et al*., 1989). Otherwise, the effects of fumigants are unknown on the *Calonectria* spp. present in Europe and the Mediterranean basin (Vitale *et al*., 2013b). Thus, these experiments provide new information on the efficacy of both fumigants applied at label and sub-label rates for suppressing *Calonectria* microsclerotia in nursery.

These fumigants could be employed for the disinfection of substrate in microcosm. In fact, based on these results they are able to reduce significantly microsclerotia, although temperature plays a key role in the efficacy of treatments.

The soil fumigation with these chemical compounds is also possible in open field. Indeed, the application of metham-sodium and dazomet, at the same rates used in microcosm, showed a totally suppression of *Calonectria* microsclerotia from the soil.

According to the European legislation on the “Sustainable Use of Pesticide”, the modern agriculture is oriented towards the application of chemical compounds at lower and lower rates. The latest European regulations prescribe the application of gas-tight tarps in association with soil fumigation. Thus, the use

of TIF may offer substantial benefits compared to VIF. In fact, the third experiment was directed to demonstrate which of the two plastic films used had a higher retention capacity. The results proved that TIF had a greater effectiveness than VIF, being able to increase the efficacy of treatment at lowest rates for *Ca*. *pauciramosa*. Indeed, the application of lowest rate of metham-sodium under TIF barrier allowed the total suppression of *Calonectria* microsclerotia compared to the same treatment under VIF barrier. In addition, when fumigant was not totally effective in suppressing microsclerotia, the recovery percentage of *Calonectria* microsclerotia from soil covered with TIF was always lower compared to that recorded from soil covered with VIF.

By analyzing the results, combining all treatments data through trials and film used, it was proved that there is no effect of trial and film on the effectiveness of treatments in suppressing *Ca*. *polizzii* microsclerotia. This could be due to higher sensitivity to fumigation treatments of *Ca*. *polizzii* than *Ca*. *pauciramosa*. Therefore, the application of metham-sodium and dazomet at lowest rates in association with TIF would achieve good results in substrate disinfestation. Mulches with these gas-tight films could offer several benefits, such as a reduction of the application rates more and more (up to 250 l/ha and 100 Kg/ha for metham-sodium and dazomet, respectively), maintaining the effectiveness of fumigation treatment. Also, this would further reduce the emission of pollutant molecules in the atmosphere. The achievement of these targets would comply with the latest European regulations.

An important result, emerged from one of these trials, is that there is a good effect of soil temperature in increasing the efficacy of fumigation treatments.

At last, it is possible to assert that these fumigants show a wide activity range and could represent a valid alternative for the disinfection of soil and substrates used in pot-grown plants production. In addition, these studies provide important information on the proper application of fumigants, such as the association with TIF, in compliance with all European standards.

## 11. Conclusions

The ornamental nursery occupies a strategic role in the agriculture sector, in which the management of plant pathogens deserves special attention. By the way, *Calonectria* spp. are among the most pathogens to control, of all soil-borne pathogens that attack nurseries. Therefore, it is necessary to find alternative measures to chemical control that could allow an effective control of Calonectria diseases.

Nowadays, according to latest European standards, it is mandatory IPM programs for the proper management of plant disease. Biological control agents (BCAs) play a key role in IPM programs offering the possibility to manage the phenomenon of resistance to fungicides that is an important limiting factor in chemical control. In management of plant disease, these BCAs can be applied alone or in combination with fungicides. In this last case, the application of BCAs can increase the efficacy of fungicide, reducing its application rate and the frequency between treatments. This is the fundamental concept of IPM.

Since 1$^{th}$ January 2014, the IPM is become mandatory for controlling plant diseases. Therefore, it is important to identify effective BCAs that could be inserted in IPM programs for controlling Calonectria disease.

These studies provide important information about the insertion of integrated treatments in management schedules.

On the one hand these data confirmed the efficacy of chemical treatments in controlling Calonectria disease, on the other interesting results were observed for biological and integrated treatments. Indeed the application of fungicides, such as boscalid+pyraclostrobin and cyprodinil+fludioxonil, showed the best performances in controlling Calonectria diseases.

The crucial aspect was that integrated application of these fungicides with BCAs showed better results than single application. Comprehensively, this integrated control would reduce the application rates of fungicide, the frequency and the total number of chemical treatments. Considering these findings, the use of cyprodinil+fludioxonil and boscalid+pyraclostrobin, that are broad-spectrum fungicides, offer the possibility to control at the same time different pathogens.

In addition, data of these experiments proved that *B. subtilis* strain QST713, *B. amyloliquefaciens* subsp. *plantarum* strain D747, *S. griseoviridis* strain K61 and *T. atroviride* T-11+*T. asperellum* T-25 showed variable results in reducing Calonectria infections on different ornamental plants.

Since one of the limiting factor in the cultivation of ornamental crops is the use of infested soil during the growing stage in nursery (Vitale *et al*., 2013b), the fumigation for disinfection of soil represents a valid alternative for suppressing *Calonectria* microsclerotia from soil. The findings of these studies show encouraging results about the effectiveness of metham-sodium and dazomet in suppressing *Calonectria* microsclerotia from soil. The application of these fumigants at label rates showed good efficacy in reducing *Calonectria* microsclerotia. Overall, the results detected in this thesis showed the efficacy of these fumigants applied at sub-label rates up to 5-fold reduced. Indeed, the viability of *Calonectria* inocula was often reduced by fumigation treatments. In addition, the application of fumigants in association with TIF or VIF barriers improves the efficacy of soil fumigation. These studies showed that TIF has a retain capacity of fumigants higher than VIF, allowing the reduction of fumigant application rates and at the same time a low emission of pollutant gas.

In conclusion, the findings detected during this research have enabled us to provide an overview about the integrated management of Calonectria disease on ornamental plant during the growing stage in nursery.

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