UNIVERSIDADE DE LISBOA
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THE ROLE OF MYCORRHIZAE IN MEDITERRANEAN ECOSYSTEM REVEGETATION

Patrícia Maria Ferreira Correia

DOUTORAMENTO EM BIOLOGIA
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Tese orientada por:
Professora Maria Amélia Botelho Paulo Martins Campos Loução
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DECLARAÇÃO

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26 de Abril de 2006

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SUMMARY

The reestablishment of a functional soil microbial community, in particular arbuscular mycorrhizal fungi (AMF), is crucial for successful plant establishment in ecosystem restoration trials. AMF soil inoculation is suggested for these extreme situations. However, little is known about its beneficial effects on woody Mediterranean plants. The overall aim of this research was to provide more information concerning the addition of commercial AMF inoculum in nursery-grown plants, as an approach to overcome constraints to Mediterranean quarry restoration, and to evaluate its effects on the composition of the indigenous AMF community remaining in the soil after disturbance. Effectiveness of indigenous AMF communities from disturbed soil prior to inoculation, and from undisturbed nearby *maquis* soil were also assessed. Three specific questions were addressed:

1. Does disturbance act as a selective force, shifting AMF community composition by selecting less infective and less effective AMF species?

2. Do changes in species diversity and abundance of AMF communities, driven by disturbance and commercial inoculum addition, induce changes on native plant growth benefits? Are those changes plant species dependent?

3. Do changes in AMF communities, driven by disturbance and commercial inoculum addition, induce changes on plant-root endophyte interactions?

Overall, the results led to the following conclusions:

1. Disturbance had a negative effect on AMF propagule density and infectivity as measured through sorghum root colonization, although this effect was not related to AMF effectivity in woody plants.

2. Adding commercial AMF inoculum or using disturbed soil containing only indigenous AMF species, were both efficient in promoting growth for all studied woody plant species. Plants were able to increase diversity and infectivity of AMF communities, although plant species-specific variations were observed.

3. Native plant-root endophytical fungal (mycorrhizal and non mycorrhizal) feedback did not affect plant growth. Plant biomass variation was associated with changes in AMF species abundance.

KEYWORDS: arbuscular mycorrhizae, commercial mycorrhizal fungi inoculum, Mediterranean woody plants, restoration, soil disturbance

RESUMO

Actualmente, a recuperação de ecossistemas degradados directa ou indirectamente pela acção humana, como por exemplo de pedreiras, é cada vez mais importante. Durante a exploração das pedreiras o solo é perturbado, sendo retirado e armazenado até ser reutilizado nas áreas a revegetar. Esta perturbação reduz a viabilidade dos microganismos do solo, e a erosão dos solos durante o armazenamento leva à perda de nutrientes. Assim, a qualidade microbiológica do solo e as espécies de plantas selecionadas para revegetar serão factores condicionantes do sucesso da revegetação. É actualmente aceite que as micorrizas arbusculares (AM) são microrganismos fundamentais na recuperação de ecossistemas perturbados, dado que estas simbioses mutualistas podem aumentar a capacidade das plantas para tolerar e sobreviver a situações adversas. Estas associações possibilitam à planta um aumento de nutrientes enquanto o fungo beneficia dos hidratos de carbono provenientes da actividade fotossintética da planta.

Vários estudos sugerem a manipulação dos fungos micorrízicos em planos de revegetação. Para tal são recomendados dois tipos de avaliação sequencial: 1) estudo de infectividade dos fungos micorrízicos que possam ainda existir no solo perturbado, isto é, capacidade dos fungos para colonizarem as raízes das plantas; 2) estudo da efectividade destes fungos, isto é, da promoção do crescimento das plantas. Se ambas as avaliações forem negativas, deverá efectuar-se a adição de inóculo micorrízico externo, como estratégia para ultrapassar as limitações que a redução de fungos micorrízicos possa trazer ao desenvolvimento das plantas durante a revegetação. Esta aplicação de inóculo micorrízico poderá ser feita directamente no campo ou durante o processo de produção em viveiro das plantas a usar na revegetação. Se, pelo contrário, a comunidade de fungos micorrízicos for ainda infectiva, é então sugerida a sua manipulação indirecta através do uso de herbáceas ou de plantas de crescimento rápido, para aumentar o número de propágulos micorrízicos no solo. Ambas as estratégias se baseiam em estudos que demonstram a existência de uma relação linear entre a densidade de propágulos no solo, a colonização das raízes e o aumento do benefício da associação micorrízica para as plantas. No entanto, a investigação actual sobre as associações micorrízicas, tem demonstrado que tal linearidade não é universal. A associação micorrízica nem sempre é verdadeiramente mutualista, podendo inclusivé ser prejudicial ao crescimento das plantas, dependendo das espécies de fungos envolvidas. Não é possível, portanto, generalizar a função benéfica destes fungos para todas as espécies de plantas. O resultado da simbiose pode ainda variar com as espécies das plantas envolvidas, e com as condições abióticas e bióticas do sistema em estudo. Dada a complexidade de factores que fazem variar esta relação, torna-se difícil

prever o sucesso da manipulação das fungos micorrízicos para favorecem o crescimento das plantas numa revegetação.

A investigação desta tese procurou avaliar a funcionalidade das associações micorrízicas estabelecidas entre diferentes comunidades fúngicas e plantas lenhosas mediterrânicas. De acordo com conhecimentos científicos recentes, a avaliação do sucesso das associações micorrízicas é conseguida através do efeito que os fungos exercem no crescimento das plantas e, reciprocamente, do efeito que as plantas exercem na comunidade nativa de fungos micorrízicos. Neste trabalho seleccionaram-se três comunidades de fungos micorrízicos: (i) comunidade existente num solo perturbado pelas actividades extractivas e de armazenamento numa pedreira; (ii) comunidade resultante da adição de inóculo micorrízico comercial à comunidade existente no solo perturbado; (iii) comunidade encontrada no solo de uma área de *maquis* não perturbada, próxima da pedreira, e onde naturalmente crescem as espécies de plantas estudadas. As comunidades fúngicas seleccionadas para este estudo têm, assim, relevância para possíveis manipulações de inóculo micorrízico em planos de revegetação.

As plantas seleccionadas foram de espécies lenhosas mediterrânicas, pertencentes a estádios serais da sucessão diferentes e consequentemente com diferentes estratégias adaptativas: *Ceratonia siliqua* (alfarrobeira), *Myrtus communis* (murta) e *Lavandula stoechas* (alfazema). Pretendeu-se especificamente responder às seguintes questões:

1. Será o efeito da perturbação no solo uma força selectiva, capaz de induzir diferenças na composição da comunidade fúngica, alterando assim a infectividade e efectividade dos fungos micorrízicos? (*Capítulos 2* e *3*)

2. Será que diferenças nas comunidades de fungos micorrízicos, devidas à perturbação do solo e à adição de inóculo comercial, induzem diferentes benefícios no crescimento de plantas mediterrânicas? Dependerá esse efeito da espécie da planta? (*Capítulo 3*)

3. As diferentes comunidades de fungos micorrízicos irão alterar as interacções que existem entre as plantas e os seus fungos endófitos das raízes? (*Capítulo 4*)

Os resultados mais relevantes obtidos em resposta a estas questões resumem-se em seguida.

No *Capítulo 2*, o solo perturbado foi comparado com o solo não perturbado, para responder à pergunta 1. Foram utilizadas as três espécies de plantas hospedeiras acima mencionadas e, como planta-referência de crescimento rápido, foi usado o sorgo, muito comum em estudos de avaliação de infectividade de fungos micorrízicos. Os resultados mostraram que a perturbação teve um efeito negativo na densidade de propágulos e, simultaneamente, na infectividade determinada com o sorgo, mas não com as plantas mediterrânicas. Pode-se então concluir que a perturbação altera a densidade de propágulos micorrízicos mas não a sua capacidade de colonizar plantas mediterrânicas.

No *Capítulo 3*, e ainda para responder à pergunta 1, confirmaram-se as diferenças entre a diversidade das comunidades de fungos micorrízicos seleccionadas. De acordo com o inicialmente previsto, a comunidade de fungos micorrízicos resultante da perturbação do solo apresentou a menor densidade e diversidade de esporos, enquanto a comunidade do solo não perturbado apresentou maior diversidade. A adição de inóculo comercial induziu alterações na comunidade ao nível da densidade de esporos. Para responder à pergunta 2, o desenho experimental deste capitulo foi delineado para comparar as três comunidades fúngicas e a sua efectividade nas plantas mediterrânicas. Os resultados deste estudo não mostraram aumento do crescimento das plantas mediterrânicas devido à adição de inóculo micorrízico comercial ao solo. Pelo contrário, a comunidade de fungos do solo perturbado promoveu mais o crescimento das plantas esclerófilas (alfarrobeira e murta), comparativamente à espécie semi-decídua (alfazema), do que a comunidade de fungos do solo não perturbado. Neste estudo, as alterações da densidade de propágulos micorrízicos no solo não induziram alterações da efectividade desses fungos na promoção do crescimento das plantas. Por outro lado, as plantas mediterrânicas foram capazes de manter a infectividade do inóculo comercial ao longo de um ano, tendo a murta e a alfazema aumentado a capacidade infectiva da comunidade de fungos micorrízicos do solo não perturbado. Foi evidenciado que o "benefício" das diferentes plantas mediterrânicas na communidade fúngica dependeu da espécie de planta usada.

No *Capítulo 4* procurou-se responder à terceira pergunta. Tem sido sugerido por vários autores que o inóculo micorrízico adicionado ao solo tem a capacidade de actuar como agente bioprotector. Assim, seria esperado que o inóculo comercial adicionado ao solo mediasse as interacções entre as plantas e os fungos endófitos da raiz (micorrízicos e não micorrízicos). No entanto, a adição de *Glomus intraradices* não alterou a diversidade e/ou abundância dos fungos micorrízicos da comunidade do solo perturbado, nem dos fungos não micorrízicos endófitos da raiz. Pode então ser sugerido, pelo menos neste estudo, que *G. intraradices* não actuou directamente como agente bioprotector. Observou-se, pelo contrário, um efeito negativo da comunidade conspecífica de microrganismos do solo no crescimento de murta. Aparentemente, esta redução do crescimento das plantas de murta está inversamente associada à variação de abundância de espécies nativas de fungos micorrízicos (*G. geosporum* e *G. constrictum*) e não à presença de fungos patogénicos nas raízes.

Como conclusão geral pode afirmar-se que a variabilidade observada no crescimento das plantas em função das diferentes comunidades de fungos micorrízicos, e no recíproco efeito das diferentes espécies de plantas nas comunidades fúngicas, se relaciona mais com as espécies de plantas e de fungos envolvidas do que com o efeito da perturbação ou da adição de inóculo comercial micorrízico.

PALAVRAS-CHAVE: inóculo de fungos micorrízicos; micorrizas arbusculares; perturbação do solo; plantas mediterrânicas; revegetação.

Chapter 1 - GENERAL INTRODUCTION

1. MYCORRHIZAE – AN OVERVIEW

1.1. DEFINITION

It was A.B. Frank in 1885 who used the term mycorrhiza for the first time. Since then, research progressed towards the description and characterization of the morphology and function of these "organisms" (see Koide and Mosse, 2004). Gerdemann (1970) defined mycorrhiza as a mutualistic symbiotic association between soil-borne fungi and plants. It is generally accepted that these symbiotic organisms have co-evolved since the first terrestrial plants appeared (Simon *et al.*, 1993; Taylor *et al.*, 1995; Gonçalves and Martins-Loução, 1998; Brundrett, 2002). Mycorrhizal symbioses are the most widespread among natural communities of terrestrial plants and among plant groups: in almost all liverworts and hornworts, in more than 95% of pteridophytes, in all gymnosperms and about 82% of the angiosperns (Brundrett, 2002).

This association is usually considered as mutualistic (see Johnson *et al.*, 1997), due to the highly interdependent relationship established between both partners, in which the host plant receives mineral nutrients via the fungal mycelium, and the biotrophic fungus obtains carbon compounds from the host's photosynthesis (Harley and Smith, 1983; Smith and Read, 1997).

Read (2002) categorized mycorrhiza in six major types according to their morphology, structural and functional attributes related to the fungi, and plant taxa forming the symbiosis (Fig.1.1). The present study focuses on the arbuscular mycorrhiza type (AM) (re-named by Walker, 1995), the most widespread association, which will be simply referred here as "mycorrhiza". In this type of mycorrhiza, the fungus develops unseptated extra- and intra-radical hyphae, intercellular arbuscules and, in some genera, vesicles. Arbuscule formation encompasses the profuse branching of hyphae after penetration of the plant cell wall. This fungal structure is closely surrounded by the plasmalemma and the large surface area thereby created between both symbionts allows the bidirectional transfer of metabolites and nutrients to and from the fungus (Gianninazzi-Pearson *et al.*, 1996; Smith and Read, 1997). Vesicles are apical or intercalary swellings of hyphae containing lipids, which function as the energy storage "organs" of these fungi. The AM colonization begins with hyphae that arise from different propagules: spores, soil mycelium network and mycorrhizal root fragments (Bonfante and Peroto, 1995; Smith and Read, 1997). As the internal colonization spreads to the youngest part of the roots, the extra-radical hyphae grow outwards into the surrounding soil. The extensive mycelium network developed will create a bridge across the nutrient depletion zone surrounding the roots and gain access to slowly mobile nutrients such as P from the bulk soil (Jakobsen, 1995).

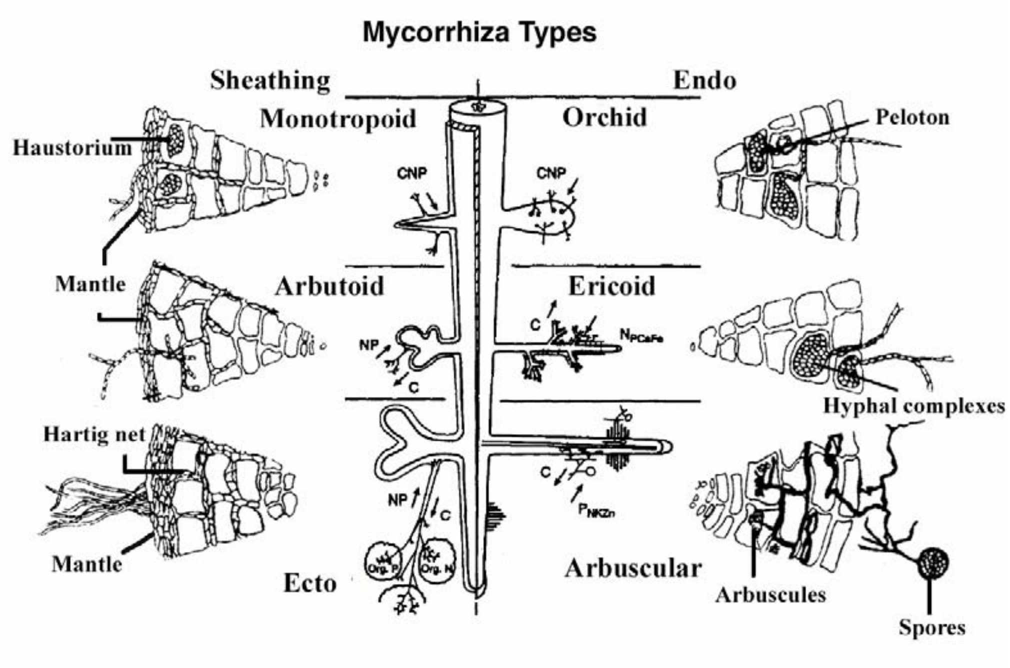

Figure 1.1. The diagnostic structural features of the six recognised types of mycorrhizae. Two basic categories are designated, one in which the root surface is sheathed in a fungal mantle (SHEATHING), and one lacking a mantle but in which hyphae proliferate internally (ENDO). The defining structures of each type are fungal pegs (MONOTROPOID), Hartig net and intracellular penetration (ARBUTOID – also seen in the subtype "ectendo"), Hartig net, mantle, external mycelial network (ECTO), peloton (ORCHID), hyphal complexes in hair roots (ERICOID) and arbuscles or hyphal coils (ARBUSCULAR). The most important nutrient acquisition pathways are shown. (*in* Martins-Loução, 2002).

1.2. ARBUSCULAR MYCORRHIZAL FUNGI

1.2.1. Taxonomy

Arbuscular mycorrhizae are a monophyletic group of fungi that evolved some 400 to 460 millions of years ago, and radiated into the groups we know today as early as 350 to 400 millions of years ago (Simon *et al.*, 1993). At the present time, and based on molecular analysis, AMF are classified as Glomeromycota, a distinct group of the Zygomycota, which were shown to be polyphyletic (Schüβler *et al.*, 2001). Initially, the taxonomy of the group was based on the morphological features of spore walls. The choice of spores was largely due to the obligate biotrophy of these symbionts, which cannot be cultured in the absence of their host plant (Morton and Bentivenga, 1994). Therefore, to fully evaluate AMF diversity it is necessary that the fungi achieve their reproductive phase and sporulate. This could explain the small degree of morphological diversity in comparison to the apparent high genetic diversity (Sanders, 1999). The 156 AMF species currently described are taxonomically classified into the following categories:

Phylum : Glomeromycota (Schüβler *et al.*, 2001);
  Order: Glomales (Morton and Benny, 1990);
    Suborders: Glomineae and Gigasporineae (Morton and Benny, 1990);
      Families: Glomaceae, Acaulosporaceae, Gigasporineae (Morton and Benny, 1990), Archaesporaceae, Paraglomaceae (Morton and Redecker, 2001)
        Genera: *Glomus, Acaulospora, Entrophospora, Gigaspora, Scutellospora* (Walker, 1987, 1992), *Archaepospora* and *Paraglomus* (Morton and Redecker, 2001)

According to recent classification studies, *Geosiphon* sp. was added to Glomerycota. This species is non-mycorrhizal, but forms a symbiosis with cyanobacteria (*Nostoc*), and it is thought to be a basal lineage of the Glomales (Schüβler, 1999). There is indication that *Geosiphon* sp. may be less distant from characteristic Glomales than some deeply branched AMF taxa (Redecker *et al.*, 2000), which have been placed in the new families Archaesporaceae and Paraglomaceae.

Improved biochemical and molecular methods have shown that diversity may be greater in natural soils than thought until now, but the relationship between morphological and molecular diversity is still unclear (Clapp *et al.*, 2002).

The taxonomic and evolutionary relatedness between organisms is illustrated in phlylogenetic trees. The three classical families of the Glomales (Glomaceae, Acaulosporaceae and Gigasporineae) defined with morphological criteria (Morton and Benny 1990), are supported by molecular data (Simon *et al.*, 1993). Nevertheless, there is little support for the two sub-orders Glomineae and Gigasporineae (Schüβler, 1999), and within families the relationship among species is unclear (Clapp *et al.*, 2002). Molecular analysis (ribosomal DNA based) has proven to be advantageous comparatively to morphology-based taxonomy to consistently identify taxa present in the roots of plants (Clapp *et al.*, 2002). In studies by Helgason *et al.* (1998, 2002), the dominant AMF taxa in the roots were those that were neither grown in culture nor found in the soil as spores, and the AMF diversity was unexpectedly large. These studies suggest that many more AMF species exist than those that have already been described.

Molecular studies that describe AMF communities in plant roots provide a degree of information about fungal identity, but it is unclear whether such information can be used to explain functional differences in AMF life-strategies or plant growth (van der Heijdeen, *et al.*, 2004). This subject has been under debate. Hart and Reader (2002a,b) experimentally demonstrated that there is a link between functional traits in AMF and taxonomy at the family level. Additionally, Munkvold *et al.* (2004) showed that there is high functional diversity within a single AMF species with respect to P-uptake. All these drawbacks need to be clarified in order to understand the meaning of AMF diversity and functioning. Apparently, functional diversity is independent of taxonomic diversity.

Spore-based characters are still the basis for Glomalean taxonomy at the species level. However, they provide little or no information on AMF life-history traits associated with abundance and architecture of fungal components, their rate of formation and longevity, and their cost to the plant host when in the symbiosis. All these features are important to the understanding at various levels, from the molecular to the ecological ones, and they are generally independent of morphological determinants (Morton and Bentivenga, 1994). On the other hand, molecular analysis has only just begun, and a streamline methodology to allowhigher throughput of samples is needed.

1.2.2. Mycorrhizal symbiosis functioning

Reciprocal exchange of nutrients between symbionts is the central mechanism in mycorrhizal functioning (Jakobsen *et al.*, 2002). Most studies of mycorrhizal functioning are made from the point of view of plant benefit from the symbiosis. AM symbiosis influences several aspects of plant physiology, such as mineral nutrition, tolerance to biotic and abiotic stresses and plant protection (Brundrett, 2002), triggering a better plant growth and development. If the plant reflects such benefits from the association, the AMF is defined as *effective*.

The primary importance of mycorrhizae for plants results from the enlargement of the nutrient absorption surface by fungal hyphae, which enhances the roots' capacity of nutrient absorption (Schüepp *et al.*, 1987; Koide, 1991; Smith and Read, 1997). Potentially, this can maximize the acquisition of nutrients which in turn may increase primary production. Mycorrhizae can also considerably enhance nutrient mobilization, which is particularly important for low mobile ions like phosphate and orthophosphate (Pearson and Jakobsen, 1993; Jakobsen, 1995; Smith *et al.*, 2003). Mycorrhizae have also been shown to be important in the mobilization of: zinc, copper, iron and manganese (Ross and Harper, 1970; Gilmore, 1971; Marshner and Dell, 1994; Azaizeh *et al.*, 1995; Liu *et al.*, 2000); soluble inorganic nitrogen, namely nitrate and ammonium (Barea, 1991; Johansen *et al.*, 1994; Martins-Loução *et al.*, 2000; Yoshida and Allen, 2001, 2004; Cruz *et al.*, 2004) and, as recently shown, organic nutrients (Koide and Kabir, 2000; Hodge *et al.*, 2001).

Other plant benefits which are not only directly associated with a better plant nutrition but also with physiological mechanisms (hormonal signaling, changes in root:shoot ratio root,) include, for example, enhanced recovery from water stress (Allen and Allen, 1986; Duan *et al.*, 1996; Augé, 2001; Querejeta *et al.*, 2003) and tolerance to salinity (Ruiz-Lozano and Azcón, 2000; Cantrell and Linderman, 2001; Carvalho *et al.*, 2003) and metals (Leyval *et al.*, 1997; Weissenhorn *et al.*, 1994; Davies *et al.*, 2001).

The mycorrhizal symbiosis can provide protection against biotic stress, mainly to root fungal pathogens (Newsham *et al.*, 1995; Klironomos, 2000; Borowicz, 2001). Nevertheless, some reports have shown that the symbiosis can also reduce the incidence of leaf pathogen (Lingua *et al.*, 2002) or bacteria (Garcia-Garrido and Ocampo, 1988, 1989), and that the detrimental effects of nematodes (Van der Putten *et al.*, 1993).

The antagonistic interactions between parasitic and mycorrhizal fungi are not exerted with the same effectiveness by all AMF species. It depends on the pathogens, the plant species and the environmental conditions involved (see review by Whipps, 2004). The studies on the AMF mode of action for promoting plant protection revealed that several mechanisms can be operative for each AM fungus – plant – pathogen combination, and that any such interaction should be considered to be dynamic as a continuum of possible modes of action. Whipps (2004) identified several modes of action (Table 1.1). Associated microorganisms may complement the activity of AMF (Budi *et al.*, 1999; Barea *et al.*, 2002). The interactions of AMF with these associated soil microorganisms are not restricted to plant pathogens; the extra-radical mycelium has been shown to establish both beneficial and deleterious relations with other fungi, bacteria, nematodes and arthropods (Fitter and Garbaye, 1994; Klironomos and Ursic, 1998). Interactions between soil invertebrates and mycorrhiza seem to be an important mechanism by which these organisms structure plant communities (Klironomos and Ursic, 1998; Gange, 2001).

The AMF symbiosis changes the pattern of root exudation (Marschner *et al.*, 1997; Pinior *et al.*, 1999). In addition, the development of the fungal soil mycelium serves as a carbon source to soil microbial communities and introduces physical modifications into the environment surrounding the roots. These changes affect the microbial populations in the rhizosphere of the mycorrhizal plant both quantitatively and qualitatively (Barea *et al.*, 2002; Antunes *et al.*, 2006).

Table 1.1. Potential modes of action of AMF involved in disease biocontrol (*in* Whipps, 2004).

	Reference
Direct competition or inhibition	
Competition for photosynthate or carbon in or on root	Graham 2001; Larsen and Bødker 2001; Morandi *et al.* 2002
Competition for exudates external to the root	Schwab et al. 1984; Bansal and Mukerji 1994; Bago et al. 1996; St-Arnaud et al. 1997; Filion et al. 1999, 2003; Norman and Hooker 2000
Competition for infection sites or space on roots	Liu 1995; Cordier et al. 1998; Fusconi et al. 1999; Vigo et al. 2000; Matsubara et al. 2001; Morandi et al. 2002
Quality and quantity of exudates from roots or mycorrhizal fungi inhibit pathogens (potentially including low levels of antibiotics or defence compounds)	Schwab et al. 1984; Bansal and Mukerji 1994; St-Arnaud et al. 1995; Filion et al. 1999, 2003; Norman and Hooker 2000
Competitive interactions with pathogens in soil	Garcia-Garrido and Ocampo 1989; St-Arnaud et al. 1994, 1995, 1997; Filion et al. 1999; Norman and Hooker 2000
Enhanced or altered plant growth, nutrition, and morphology	
Increased nutrient uptake (particularly phosphorus), increased trace elements, drought tolerance, decreased toxicity to salt an heavy metals (alleviation of abiotic stress)	Hooker et al. 1994; Linderman 1994; Karagiannidis et al. 2002
Altered root branching and root morphology	Norman et al. 1996; Fusconi et al. 1999
Hormonal changes (e.g., abscisic acid, auxins, cytokinins, ethylene)	Allen et al. 1980, 1982; Danneberg et al. 1993; Dugassa et al. 1996; Hirsch et al. 1997; Torelli et al. 2000
Damage compensation	Cordier et al. 1996; Pozo et al. 2002*a*
Biochemical changes associated with plant defence mechanisms and induced resistance	
Phenolics and phytoalexins	Morandi 1996
Amino acid levels (e.g., arginine, proline)	Baltruschat and Schönbeck 1972; Giovannetti et al. 1991
Internal structural barriers (e.g., lignins, callose, hydroxyproline-rich glycoproteins)	Benhamou et al. 1994; Matsubara et al. 1995; Cordier et al. 1998; Pozo et al. 2002*b*
Defence-related proteins (e.g., pathogenesis-related proteins, β-1,3-glucanases, chitosanases, chitinases, peroxidases, phenylalanine ammonia lyase, chalcone synthase, superoxide dismutase)	Dugassa et al. 1996; Morandi 1996; Pozo et al. 1996, 1998, 1999, 2002*a*, 2002*b*; Mohr et al. 1998; Slezack et al. 2000; Guillon et al. 2002
Increased DNA methylation and respiration	Dugassa et al. 1996
Systemic induced resistance	Cordier et al. 1996, 1998; Pozo et al. 2002*b*
Development of an antagonistic microbiota	
Bacteria and fungi	Meyer and Linderman 1986*a*; Secilia and Bagyaraj 1987; Thomas et al. 1994; Citernesi et al. 1996; St-Arnaud et al. 1995,1997; Andrade et al. 1997, 1998; Hodge 2000; Vázquez et al. 2000; Filion et al. 2003

1.2.3. Effect on soil structure

Soil structure depends on the type and size of aggregates, and is an important component of the nutrient cycling system. The AMF (roots and extra-radical hyphae) act as driving factors for macroaggregates (>250 μm) stability, while microbial and plant residues, polysaccharides produced by bacteria and inorganic materials stabilize microaggregates (<250 μm) (Tisdall, 1994). The formation of macroaggregates can prevent wind and water erosion, and both the quality and the size distribution of soil aggregates can affect soil porosity. Together, these properties can influence soil physical, chemical and biological processes, through effects on the accessibility of carbon, shelter, water, oxygen and nutrients to soil biota (Miller and Jastrow, 1992).

AMF are considered to be primary soil aggregators through direct or indirect effects. The direct effects involve the AMF acting as binding agents. A positive relationship between AMF soil hyphae (some can grow as long as 90 mm, *in* Camel *et al.*, 1991) and aggregate stabilization has been identified (Andrade *et al.*, 1998; Jastrow *et al.*, 1998). Indirect effects on water soluble aggregate (WSA) stabilization were described by Rillig *et al.* (1999), via the production of glomalin-related soil protein (GRSP). However, the underlying mechanisms associated with this system are not fully understood. The percentage of WSA depends on root and AMF hyphal lengths and also on interspecfic interactions (Piotrowski *et al.*, 2004).

1.3. COST-BENEFIT OF THE SYMBIOSIS

The cost-benefit analysis can be used to evaluate the benefits provided by mycorrhizal associations (enhanced mineral nutrients uptake) against the costs (carbon supplied by the host). Arbuscular mycorrhizal plants have been estimated to allocate 4-20% more photosynthates to roots compared to non-mycorrhizal plants (Jakobsen and Rosendahl, 1990; Smith and Read, 1997). The growth of the obligate biotrophic fungi in roots relies on carbon transferred across interfaces between the plant and the fungus, which means that the symbiosis is always obligatory for the AMF, whereas for the individual plant it is usually facultative. Some plants, however, can fully depend on the association, at least at some soil fertility levels (Brundrett, 1991).

1.3.1. Plant mycorrhizal dependence *vs.* plant responsiveness

Mycorrhizal dependence should not be confused with host plant responsiveness to mycorrhiza as was explained by Janos (1988). Mycorrhizal dependence measurements are intended to accurately assess the need of plant to form mycorrhizal associations. Host plant responsiveness to mycorrhiza, which is used as a measure of fungal effectiveness, is influenced by the host plant, AM fungus and soil fertility level. The plant response to inoculation changes if different AM fungal species are used as inoculants, or if edaphic conditions are altered (Sieverding, 1991).

1.3.2. Mycorrhiza association - from mutualism to parasitism

For the majority of plants, the benefits of exchanging photosynthates for mineral nutrients outweigh the costs. However, this "trade" of nutrients is not always mutualistic (i.e., beneficial for both symbionts), and under some circumstances the net cost of the symbiosis exceeds the net benefit (Francis and Read, 1995). The full spectrum of plant responses to the formation of mycorrhizae can range from mutualism to parasitism (Johnson *et al.*, 1997; Klironomos, 2003; Jones and Smith, 2004). Several factors can determine that variation, just as plant taxa vary in mycorrhizal dependency, and fungal taxa vary in mycorrhizal effectiveness. However, it cannot be concluded that a certain AMF genotype is always parasitic, because the cost-benefit balance in a pair of host plant-AMF species can change over time according to edaphic or seasonal factors (Fig. 1.2). Lapointe and Molard (1997) demonstrated that plant benefit from the presence of mycorrhizae varies during the life cycle of *Erythronium americanum*: in fall, mycorrhizae were more costly for the plant in terms of carbohydrate reserves.

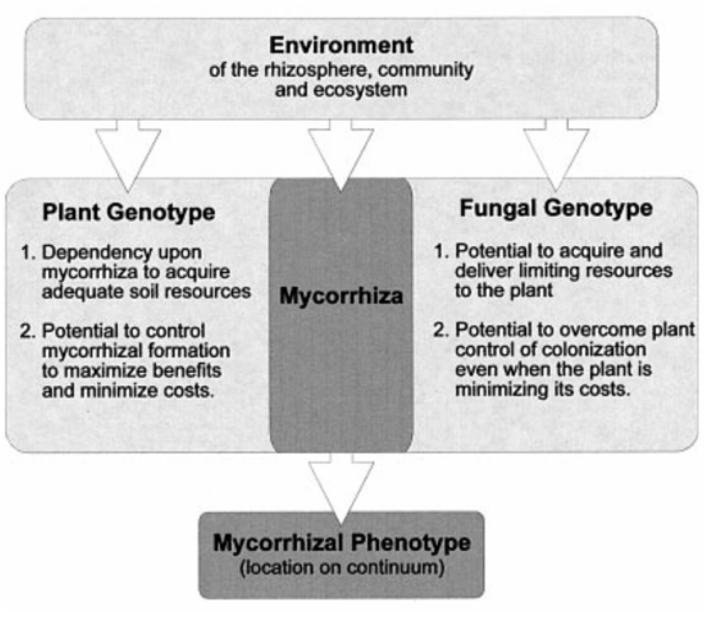

Figure 1.2. Mycorrhizal phenotypes are manifestations of the interaction between plant and fungal genotypes and environmental conditions. These factors determine the functioning of mycorrhizae along the mutualism-parasitism continuum (*in* Johnson *et al.*, 1997).

In the extreme situation of the mycorrhizal association, where the plant or fungus become parasitic, the partner is then defined, in ecological terms, as a "cheater" (Janos 1987; Smith and Smith, 1996; Martins-Loução, 2002). A cheater in a symbiosis is the individual that receives the benefits of mutualism but does not reciprocate (Soberon and Martinez del Rio, 1985). The explanation for this occurrence remains unclear and is probably related to the outcome of the symbiosis. The host–fungus interface of mycorrhizal associations may have evolved, in part, as a mechanism to limit cheating by tightly coupling the costs and benefits of the exchange for both partners. This process seems to be primarily controlled by the plants through different mechanisms, which can escape from their obligations in the associations (facultative) (Smith and Smith, 1996; Pinior *et al.*, 1999; Redman *et al.*, 2001; Brundrett, 2002). Plant mechanisms for preventing unwanted colonization may not be specific enough to distinguish cheaters from beneficial fungi. The most effective mechanism for plants to stop fungi from absorbing photosynthates without providing benefits is by suppressing root colonization, particularly by arbuscules. Klironomos *et al.* (1993) found that hyphal coils were prevalent but arbuscules were rare in maple forests. Pawlowska *et al.* (1996) reported the mycorrhizal status of plant species, where AMF fungi were present in the roots forming vesicles, but no arbuscules were found.

1.3.3. Preferential associations between host plant-AMF species

In 1983, Hayman demonstrated that certain fungi can be more effective than others to stimulate plant growth, or have no influence at all. Other studies also revealed such a wide range of outcomes (e.g., Gianinazzi and Gianinazzi-Perason, 1986; Bethlenfalvay *et al.*, 1989; McGonigle and Fitter, 1990; Sylvia *et al.*, 1993; Wilson and Hartnett, 1998; Graham and Abbot, 2000; Klironomos, 2003). Recently, Klironomos *et al.* (2000) demonstrated that two AMF species (*G. etunicatum* and *G. intraradices*) induced plant biomass differently, and suggested that those responses were due to differences in the carbon cost for plants maintaining the symbiosis with different fungi. Those differences can be related to variation in intra- and inter-radical growth rates of each AMF species (Hart and Reader, 2002a,b) and hence, to differing needs for plant carbon, or to different abilities to obtain it. In addition, different AMF vary greatly in the amount of phosphorous transported to the plant, and plant identity is an important determinant of the amount of phosphate transported (Jakobsen *et al.*, 2002). Conversely, the effects of a change in photosynthate availability in the plant due to herbivory affect the mycorrhizal relationship differentially depending on the species of fungus involved or the AMF community composition (Klironomos *et al.*, 2004)

The differential gains that plants receive from a mycorrhizal association in relation to AMF taxa, can be found either at family level (Hart and Reader, 2002a) or at species level (Sanders and Fitter, 1992; Bever *et al.*, 1996; Klironomos, 2003). These differences in the outcome of the mycorrhizal symbiosis were recently interpreted based on functional compatibility and diversity concepts (Ravnskov and Jakobsen, 1995; Klironomos, 2000), and even on the presence of some level of "specifity" (Sanders, 2002, 2003).

Even though the mycorrhizal association has been described as non-specific, given the AMF species ubiquitous distribution and the capability to infect a wide range of plants, there is evidence of host-specificity. Results from recent studies have revealed a differential host plant-AMF species combination, which may imply some "preference" or even "specificity" among arbuscular mycorrhizal associations (McGonigle and Fitter, 1990; Bever, 2002). Nevertheless, it has not been shown whether plant or AMF species are capable of selecting a suitable partner. By definition, specificity implies compatibility between two organisms, with molecular recognition between them. For arbuscular mycorrhizae this specificity concept has to be differently interpreted, considering the genotype (of both symbionts) and the environmental features involved (Sanders, 2002).

1.4. ROLE OF MYCORRHIZAE IN MAINTAINING PLANT COMMUNITY DIVERSITY, LINKING ABOVE AND BELOW GROUND

Since a single plant can form mycorrhizae with many fungi (Tommerup, 1988; Helgason *et al.*, 1998), and a single fungus can connect many plants (Allen, 1996), the number of possible combinations of symbionts is significant, and so is the variety of possible outcomes from the symbiosis. This fact, associated with the patchy distribution of AMF species within communities (Klironomos *et al.*, 1993, 1999; Carvalho *et al.*, 2003), may play a role in determining plant community composition.

1.4.1. Effect of AMF communities on plant communities

The presence *vs*. absence of AMF (e.g.: Grime *et al.*, 1987; Gange *et al.*, 1993; Hartnett and Wilson, 1999; O'Connor *et al.*, 2002) and AMF community composition (*e.g.*, Streitwolf-Engel *et al.*, 1997; van der Heijden *et al.*, 1998a, b; Kiers *et al.*, 2000) can greatly influence the structure and productivity of plant communities. Grime *et al.* (1987)

and Gange *et al*. (1993) showed a positive relation between mycorrhizal and plant diversity, i.e. the presence of AMF would increase plant diversity. However, Hartnett and Wilson (1999) and O'Connor *et al*. (2002) showed an inverse relation between mycorrhiza and plant diversity. The positive relation between AM and plant diversity may be due to an increase in plant eveness through the transfer of assimilates from dominat species to the other species via the external mycelium. Hartnett and Wilson (1999) proposed that the differential host species response to fungal colonization limits plant species evenness, when mycorrhizal plant species become dominant, and no changes in the total biomass of plant community occur. O'Connor *et al*. (2002) observed in a semiarid herbland, that the behavior of individual dominant mycorrhized plant species, was a strong determinant of community structure. The suppression of mycorrhizal activity with equivalent mycorrhizal responsiveness but different capacities to exploit the symbiosis for nutrients uptake or alleviation of water stress, resulted in changes in plant competition and redistribution of plant biomass within the community. In addition, studies at the AMF community level showed that, not only the presence or absence of AMF inoculum, but also the diversity and identity of AMF induced differential effects in plant growth, being determinant of plant diversity and productivity (van der Heijden *et al*., 1998). These authors also found that plant biomass increased with increasing AMF diversity, although productivity saturated after a certain level of AMF species diversity was reached. So these results support the redundancy hypothesis (see Hart *et al*., 2001). It has been suggested that the underlying mechanisms by which AMF promote growth of such mycorrhizal dependent plant species can be used to explain how AMF promote plant diversity (van der Heijden, 2002). However, the mechanisms are not yet explicit.

1.4.2. Effect of host plants on AMF communities

AMF community composition can be influenced by host plant species (e.g., Johnson et al., 1991; Bever et al., 1996; Bever, 2002), by plant community composition (Eom et al., 2000; Burrows and Pfleger, 2002; Lovelock et al., 2003), and by soil factors such as soil moisture gradient (e.g., Anderson et al., 1984), fertility (Johnson, 1993), namely N concentrations (Egerton-Wharburton and Allen, 2000), textural differences (Allen et al., 1998), and pH (Moutoglis and Widden, 1996). Host plants may be one of the most important factors regulating AMF species composition, mainly through carbon allocation to roots, since every phase in AMF life history (colonization, hyphal spread in the roots and sporulation) is directly influenced by plant roots. Herbaceous plant species were shown to induce changes in AMF community composition through significant differences in species richness and evenness (Eom et al., 2000). While tropical tree species were shown to induce only changes in relative abundance of a few common AMF species (Lovelock et al., 2003). These findings suggest that roots from different plant species offer different environments for reproduction, growth or survival of different AMF species or genera.

1.4.3. Host plant-AMF interactions: feedback dynamics relation

Bever *et al.* (*e.g.*, 1997, 1999) suggested a feedback model as the underlying mechanism behind the plant-AMF mutual interactions. Theoretically, a feedback response occurs between interacting organisms when one organism (A) affects the growth of another (B), which in turn has a positive or negative effect on the performance of the first organism (A); these relations can be self-promoting (positive feedback) or self-limiting by affecting the growth of other organism (negative feedback) (Bever *et al.*, 1997; Hart *et al.*, 2003) (Fig 1.3).

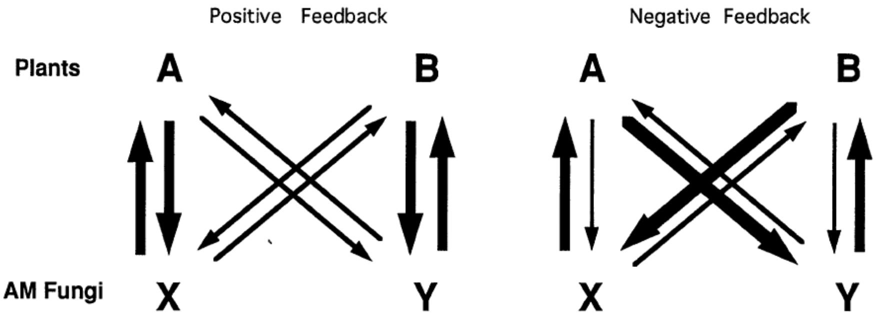

Figure 1.3. Feedback due to changes in composition of the community of soil mutualists. The direction of benefit delivered between two plants species, A and B, and their fungal mutualists, X and Y, are indicated by arrows, with the thickness of the arrow indicating the magnitude of benefit. *Positive feedback*, the presence of plant A will result in an increase in the frequency of fungus X relative to fungus Y, which will then increase the rate of growth of plant A relative to plant B. *Negative feedback*, the presence of plant A will result in a decrease in the frequency of fungus X relative to fungus Y, which will then decrease the rate of growth of plant A relative to plant B. (*in* Bever *et al.*, 1997).

Exemplifying for the mycorrhizal association, positive feedback occurs when certain host plant species gradually "select" AMF assemblages that can optimize their growth, while negative feedback occurs when "selected" AMF have such heavy carbon demands that actually impair host growth. These findings have wider implications at community level. Through the positive feedback approach, the AMF community would increase the growth rates of a particular host plant species that could become the dominant species, leading to a decline in plant species diversity. Through the negative feedback approach, the AMF community would decrease the relative growth rates of the most abundant plant species through a weaker mutualism relation, leading to the coexistence of competing plant species (Kiers *et al.*, 2000; Klironomos, 2002; Bever, 2003). Additionally, the benefit that certain plant species receive from its AMF community can degrade over time, and this process leads to the dissolution of the mutualism and to an evolutionary feedback dynamics over time (Bever *et al.*, 2002).

The previous findings describing that differential host plant-AMF species interactions may regulate each organism growth and fitness, and consequently induce shifts at plant-AMF community level, were recently extended to more complex interactions within plant-AMF-soil microorganisms. These interactions can influence plant community diversity and dynamics, by facilitation of invasive plant species or by mediating plant competition (van der Putten *et al.*, 1993; Mills and Bever, 1998; Packer and Clay, 2000; Klironomos 2002; Callaway *et al.*, 2004), enhancing the importance of mycorrhizal associations at the ecosystem level. Reynolds *et al.* (2003) suggested that positive feedback between plants and soil microbes plays a central role in early successional communities, while negative feedback contributes both to species replacements and to diversification in later successional communities.

1.5. EFFECT OF DISTURBANCES ON MYCORRHIZAE, PARTICULARLY IN MEDITERRANEAN ECOSYSTEMS

Arbuscular mycorrhizae are vulnerable to disturbance and it has been frequently shown that disturbance causes a reduction in AMF community composition and functioning. A more detailed review of the available studies shows that there is large variation in the results of the effect of disturbance on AMF populations: some report a great loss of AMF viable propagules, spores (Miller *et al.*, 1985; McGee, *et al.*, 1997) and hyphal network (Jasper *et al.*, 1989a,b; Evans and Miller, 1990; McGonigle and Miller, 1993), and their subsequent infectivity (Evans and Miller 1988; Jasper *et al.*, 1989c; Abbott and Robson, 1991; Lovera and Cuenca, 1996; McGonigle and Miller, 1996); other studies only show a reduction in propagule number, and not in the AMF infectivity (Jasper *et al.*, 1991; Miller *et al.*, 1995, Gavito and Miller, 1998). This variability in AMF responses to disturbance is probably dependent on (i) the severity of the disturbance, as well as on (ii) the abiotic and biotic conditions prior to disturbance, (*e.g*,. soil nutrient concentrations, the AMF community composition and the successional stage of the plant community composition) (Miller and Jastrow, 1992; McGonigle and Miller, 1993).

i) Disturbance severity is increased by frequency and intensity of the action (Abbott and Gazey, 1994). Examples of severely disturbed ecosystems may include those with recently experienced volcanic activity, as well as those used for the extraction of aggregates, such as quarries and gravel pits. After disturbance, soil resources may not be limiting, but AMF inoculum potential may be reduced or eliminated, limiting the establishment of mycotrophic plants (Hart and Klironomos, 2002). Research on Mt. St. Helen, after the 1980 eruption, showed that the landscape was devoid of AMF, and only after AMF inoculum was reintroduced into the system by the activities of animals, a wide variety of mycorrhizal-dependent plants began to establish (Allen, 1991). Surface mining is another example of the most severe disturbances; three degradation factors are inherent to this activity: removal of growing plants, soil disturbance and topsoil storage. Despite the large impact of disturbance caused by heavy machinery (destroying plant roots and soil structure), stockpiling the soil is the most detrimental process. Stockpiling causes a negative effect on soil aggregation, litter decomposition and microorganisms diversity and survival, including AMF (Waaland and Allen, 1987; Stahl *et al.*, 1988). Although mycelium, their living biomass is destroyed, AMF are able to resist to the most extreme condition, since spores can remain dormant for a long time. In stockpiled soil, the inoculum potential decreases because top soil is continuously diluted with deeper profile soil, which is poorer in AMF infective propagules (Nehl *et al.*, 1999). However, the major negative effect in AMF infectivity is due to the reduction of extraradical hyphal network, which is very sensitive to physical disturbance. Disturbance in general, and soil stockpiling in particular, has different effects on different AMF species inducing shifts in AMF community composition (Abbott and Robson, 1991). This is probably due to different rates of sporulation within AMF species (Klironomos and Hart, 2002). For example, Visser *et al.* (1984) show that disturbance induced a change in the dominant fungi species, from *G. fasciculatum* to *G. mossae*.

(ii) *Abiotic and biotic components prior to disturbance*. Edaphic parameters can also influence the effect of disturbance. For example, the stockpiled storage in semiarid conditions can reduce the AMF inoculum potential more than in moister environments. According to the hypothesis proposed by Jasper *et al.* (1991), AM infectivity is less affected by soil disturbance if there was high inoculum density prior to disturbance. However, McGonigle and Miller (2000) reported that high inoculum density did not prevent the negative effect of disturbance on AMF colonization. They suggested that if those colonization differences appeared following soil disturbance they were probably dependent on the interactions between the environment and plants species.

Mediterranean plant communities are continually subject to natural stresses: lower temperature when there is more moisture in the soil, and drier wheather during the growing season, together with low nutrient and water availabilities, and heterogeneous spatial distribution of AMF inoculum and vegetation (Carvalho *et al.*, 2003). In addition, Stahl and Smith (1984) suggested that AMF species diversity and spore density decreased with increased aridity in semi-arid grasslands, which agrees with other studies reporting low AMF propagule density in semi-arid areas (*e.g.* McGee, 1989; Requena *et al.*, 1996; Ferrol *et al.*, 2003). Stutz and Morton (1996) contradicted these findings demonstrating that AMF diversity in a semi-arid region was similar to the one found in other plant communities after three cycles of trap culturing. Nevertheless, the spore density found in this study cannot be related to species abundance in the field. In addition, mycelium is the main infective propagule in Mediterranean ecosystems (Requena *et al.,* 1996; Bashan *et al.*, 2000) and remains infective after the Mediterranean natural cycles of soil dryness (Jasper *et al.*, 1987, 1993; Braunberger *et al.,* 1996). Together, these findings support the idea that AMF communities are vulnerable to soil disturbance in these ecosystems comparatively to others.

During ecosystem recovery from disturbance, changes take place in plant populations (secondary succession). There is evidence that in some situations mycorrhizal inoculum may be a factor influencing the rate of succession. During this process, non-mycorrhizal plants are gradually replaced by obligate mycorrhizal plants later in succession (Miller, 1987; Allen, 1991; Francis and Read, 1994; Barni and Siniscalco, 2000), and the AMF spore abundance and species richness increase along the successional gradient (Allen, 1991; Johnson *et al.*, 1991; Gemma and Koske, 1997). In semiarid ecosystems, where natural secondary succession is very slow (Bradshaw, 1983), the influence of mycorrhiza in controlling plant diversity may be vitally important. Plant communities can be subject to dramatic seasonal and interannual fluctuations, and may rely on AMF high biodiversity to maintain stability (Grime, 1997).

1.6. MYCORRHIZAL ROLE IN THE RESTORATION OF DEGRADED MEDITERRANEAN ECOSYSTEMS

Several studies performing large scale field restoration assays demonstrated that manipulation of AMF community guarantee the success of plant establishment, overcoming AMF propagule limiting factor (Cuenca *et al*., 1998; Dodd *et al.,* 2002; Caravaca *et al*., 2004). Through appropriate AMF inoculum management in the restoration process, it is possible to recover not only the vegetation but also the biological and physico-chemical soil properties (Miller and Jastrow, 2000; Jeffries and Barea, 2001, Jeffries *et al*., 2003; Caravaca *et al*., 2005).

Reeves *et al.* (1979) and Janos (1980) had already suggested that increasing AMF population would possibly overcome the negative effect of disturbance on AMF, and therefore accelerate natural secondary succession. However, Miller and Jastrow (1992) alert to the fact that AMF manipulation would not facilitate the skipping of successional stages, but would rather prevent the stagnation of community development.

In the early 1990's some protocols appeared to ameliorate the disturbance effect, suggesting AMF inoculum management for restoration (Dodd and Thomson, 1994; Jasper, 1994) and agricultural purposes (Sieverding, 1991). All of them began with an evaluation of indigenous AMF infectivity in disturbed soil, as well as a test of AMF effectiveness. If both evaluation yielded poor results, different AMF inoculum manipulations were suggested, but the most recommended one was the addition of AMF inoculum.

Sieverding (1991) and Jasper (1994) suggested two manipulation strategies: (i) manipulation of indigenous AMF communities or (ii) soil inoculation with selected AMF species (Fig. 1.5).

i) through indirect selective practices, like using appropriate cropping plant species, it is possible to build up the indigenous inoculum. This approach is recommended when indigenous AMF infectivity is low but with adequate effectivity. The advantages of this strategy are related to the presumed adaptation of these AMF to the native plants and to environmental conditions. Nevertheless, if only AMF propagule density is increased, no changes will occur in the diversity of the disturbed AMF community, and the existing AMF community may or may not be effective in promoting plant establishment and growth.

ii) through direct soil inoculation with allochthonous AMF species. Inoculation would imply a significant change in indigenous AMF community, increasing the diversity and density of AMF species in disturbed soil.

Dodd and Thomson (1994) proposed a protocol to identify the responsive sites for mycorrhizal inoculation, accompanied by another protocol for isolating and screening the most effective mycorrhizal species/isolate, in order to artificially boost the indigenous inoculum by using a range of baiting and plant culture techniques. Other authors agree on the importance of considering the sources of mycorrhizal fungi in any screening and selection trail. Also, it may be ideal to specifically isolate candidate organisms from target sites such as those of future use (Abbott and Gazey, 1994; Requena *et al.*, 1997, 2001; Lovato *et al.*, 1999). However, none of the AMF management protocols suggests to test the commercial inoculum effectivity on native plants prior to restoration programs, nor the evaluation of the effects on the remaining AMF community composition after inoculation.

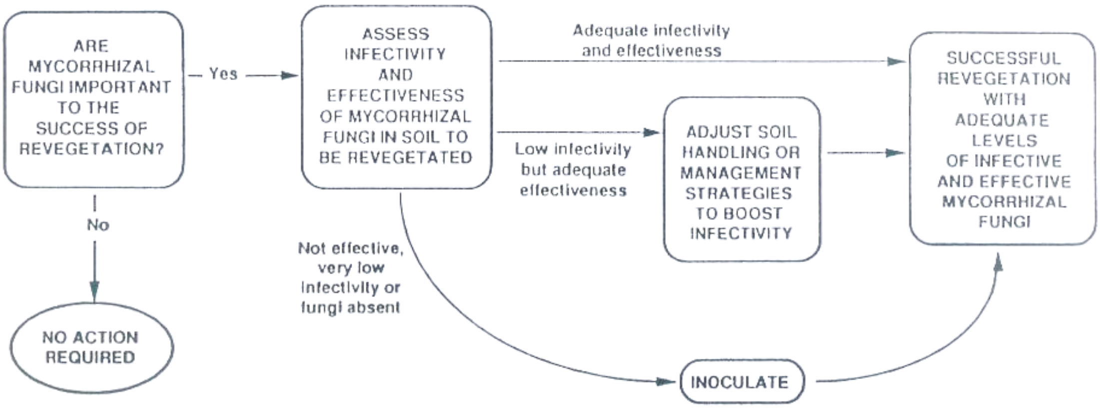

Figure 1.4. A schematic representation of a protocol for managing mycorrhizal fungi in soils to be revegetated. (*in* Jasper, 1994).

If AMF inoculum is missing in the disturbed soil, the *addition of commercially available inoculum* is suggested. The basic criteria for selection and characterization of AMF inoculants is the ability to achieve improved plant growth (Abbott and Gazey, 1994; Estaún *et al.*, 2002; Vosátka and Dodd, 2002; Gianinazzi and Vosátka, 2004) and/or that they act as agents for disease control (Smith and Read, 1997; Varma and Hock, 1999; Barea *et al.*, 2002; Gianinazzi and Vosátka, 2004). AMF can be considered as a biocontrol agent because it was shown that AMF reduced the detrimental effects of pathogens in plants beyond the additive effects resulting from an improved plant nutrition (Borowicz, 2001). Sylvia *et al.* (1993), in a complex experiment involving wide range of soils (with different levels of P content), AMF isolates and two host plant species, showed that two *Glomus* isolates were effective for the majority of soils. These findings were relevant for those involved in producing AMF inoculum at commercial scale, demonstrating that AMF could adapt to different edaphic conditions, and that it is possible to select effective isolates. However, the strategy of adding commercial inoculum has been over-generalized and suggested for application, independently of the ecosystem type and disturbance severity, without being pre-tested for effectiveness in the target plants. In general, it is assumed that regardless of their diversity, AMF species act as homogeneous functional groups in promoting plant benefit for any host-plant species. Recent studies on mycorrhizal ecology have shown that this assumption is not verified in all cases. Different AMF-plant host pair combinations have different outcomes in symbiosis effectivity. Gianinazzi and Vosátka (2004) suggested strong collaboration between inoculum producers, plant growers and researchers to improve the understanding of AMF ecophysiology, in order to achieve an efficient AMF manipulation, since it is difficult to predict the symbiosis outcome in terms of plant growth and health.

Little is known about the stability of plant growth response to commercially available AMF inoculum over a range of host plants species and soil environments. Some studies have been recently published concerning the improvement of plant growth in Mediterranean ecosystems through direct field inoculation with allochthonous AMF species (Herrera *et al.*, 1993; Bhatia *et al.*, 1998; Requena *et al.*, 2001; Barni and Siniscalco, 2000; Caravaca *et al.*, 2003a, 2005). Herrera *et al.* (1993) showed that AMF helped to establish native woody plants in a long-term Mediterranean revegetation study. The author emphasized the importance of mycorrhizal biotechnology for nursery of native plant species for revegetation programs, because the symbiosis enhances the ability of these plants to become established and cope with natural stresses in such ecosystems. Despite these allochthonous AMF species are usually selected for their extreme aggressiveness (see Graham and Abbot, 2000), their effectiveness in promoting native Mediterranean plants growth is sometimes unsuccessful, inoculation with indigenous AMF species proving to be more effective when plants are outplanted (Requena *et al.*, 2001; Caravaca *et al.*, 2003a; 2005; Alguacil *et al.*, 2005;). In addition, the variation of inoculation effectiveness, regardless of the AMF inoculum source, is plant species dependent (Caravaca *et al.*, 2003a) and the reciprocal is also verified, which means different native species promote diferential mycorrhizal propagule production (Palenzuela 2002; Azcon-Aguillar *et al.*, 2003; Caravaca *et al.*, 2003b). Other studies in Mediterranean ecosystems that involved soil inoculation did not find a positive relation between the increment of soil AMF propagules through inoculation and better plant establishment or plant productivity, either using AMF inoculum from greenhouse cultures (Richter and Stutz, 2002; Bell *et al.*, 2003) or commercial inoculum (Werner *et al.*, 2001; Clemente *et al.*, 2004).

Nevertheless, the scarce references in literature referring the effects of AMF commercial inoculum on woody Mediterranean plant growth, and on indigenous AMF community, are even more limited in the case of commercial inoculum abilities to compete for Mediterranean woody plant roots with non mycorrhizal fungi.

2. OBJECTIVES AND RATIONALE OF THE RESEARCH

AMF are important elements of terrestrial ecosystems, and thus considered as key components for a successful restoration, linking above and below ground communities. However, inconsistent results in plant growth benefit have been documented concerning the application of AMF soil inoculum, ranging from positive (see Lekberg and Koide, 2005) to null (eg: Bell *et al.*, 2003) and to detrimental outcomes from the symbiosis (eg: Modjo and Hendrix, 1986; Hendrix *et al.*, 1992). Nevertheless, the fundamental concept of AMF inoculum management approaches relies on the assumption that there is a positive linear relationship between the presence and/or abundance of AMF soil propagules and plant productivity, and that AMF are functionally redundant in their effects on a host, not considering the AMF species diversity effect and the complexity of different interactions of AMF with plant species, microbial communities and soil abiotic characteristics.

Little is known about the possible underlying mechanisms behind the inconsistent results of inoculation. Considering recent scientific concepts like the relation between AMF diversity and plant productivity, the mycorrhizal functioning in the mutualism-parasitism continuum, the evidence of functional compatibility between pairs of host plant-AMF species, and the plant-AMF feedback interaction the *major aims of this thesis* were:

i) to provide more detailed information about the effects of introduced AMF species, as commercial inoculum, on plant growth of different Mediterranean woody plant species;

ii) to improve the understanding of the role of introduced AMF species as mediators between the Mediterranean woody plants growth and indigenous AMF community diversity and functioning.

Specifically, the following questions were addressed:

1. Does disturbance act as a selective force, shifting the AMF community composition by selecting less infective and effective AMF species? (*Chapter 2*)

2. Do changes in species diversity and abundance of AMF communities, driven by disturbance and commercial inoculum addition, induce changes on native plant growth benefits? Are those changes plant-species dependent? (*Chapter 3*)

3. Do changes in AMF communities, driven by disturbance and commercial inoculum addition, induce changes on plant-root endophyte interactions? (*Chapter 4*)

Since the essential component of this work is the evaluation of the effects of AMF inoculum application and the interpretation of the underlying mechanism of its action, a situation where the use of commercial inoculum is undoubtedly recommended was selected (Jasper, 1994; Requena *et al.*, 1996, 2001; Caravaca *et al.*, 2004): the restoration of a very disturbed site in Mediterranean ecosystems.

An innovating experimental system was set up to achieve the former goals and to obtain more conclusive results from the comparison of different AMF community functions. The use of removal experiments (with or without AMF) and the manipulation of AMF species, building "artificial" communities, were avoided. The control of other variables which may also induce plant growth differences, such as soil nutrient levels and soil microbial community, was also sought. More detailed information on how these conditions were achieved is given in Materials and Methods of *Chapters 3* and *4*.

In this study, different AMF communities were used: i) the AMF community from a disturbed soil, and ii) the AMF community from a disturbed soil *plus* that from an undisturbed soil. Comparing these two AMF communities it would be possible to infer the outcome of the addition of commercial inoculum by itself. The effects of those communities on plant growth were further compared with those of the AMF community in an undisturbed soil, retrieved from a surrounding well-preserved scrub.

To compare the different AMF community functions, plant biomass, root colonization and AMF soil inoculum were consistently used as key estimators of symbiosis effectiveness for plant and AMF performance. Root colonization represents the contact interface between the two symbionts. For a more accurate assessment of symbiosis functionality, internal mycelial structures - arbuscules, vesicles, and hyphae - were also evaluated, following previous recommendations (Hart and Reader, 2002b).

Underlying hypotheses and expected results

In *Chapter 2*, different methodological approaches were used to test whether disturbance has a selective effect on AMF inoculum and on plant growth. It was expected that disturbance acted as a selective force on the AMF community, reducing propagules density and diversity, comparatively to the AMF community from the undisturbed soil.

Consequently, root colonization by AMF would be reduced (McGonigle and Miller, 1996) (Fig. 1.5). In addition, the growth benefit for individual plants would decrease (Fig. 1.5), although it was expected that the rate of benefit reduction would depend on the host plant species. Given that AMF differ in their symbiotic function and that plant species differ in their responses to AMF, then communities with higher AMF species richness could mean more functions fulfilled and more opportunities for beneficial relationships to develop. Since disturbance may induce a shift in AMF community, reducing diversity, it is hypothesized that plants would grow better in the presence of the AMF community from the undisturbed soil. Moreover, the tested plants were from a middle - late successional stage, and according to Allen (1991) and Johnson *et al.* (1991) it would be expected that these plants would grow better with late successional AMF communities.

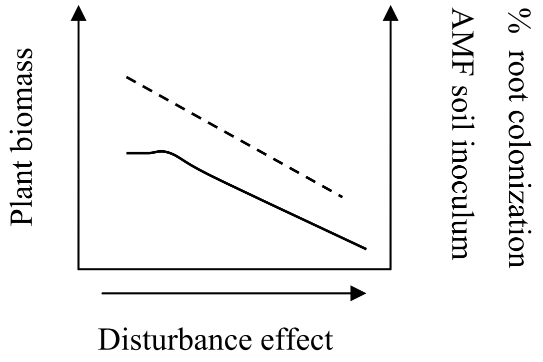

Figure 1.5. Schematic representation of hypothetical disturbance effect for the dependent variables: plant biomass (---), AMF soil inoculum and percentage root colonization (—). The hypothesized variability of AMF variables starts with a plateau, corresponding to the well established AMF soil community in the undisturbed soil and to the root colonization of native plants living in it.

Chapter 3 presents the experiment designed to test whether inoculum of AMF communities from a disturbed soil with or without AMF commercial inoculation, in comparison with AMF communities from undisturbed area, led to different host plant growth. The effects of plant species on AMF soil inoculum was also tested. For the hypothesis formulation, two approaches are possible:

i) The concept that the addition of AMF commercial inoculum to promote plant growth depends only on AMF soil propagules density, assumes that AMF act as a homogeneous functional group, regardless of their species diversity. This means that biodiversity is assumed to be functionally redundant even with a low number of species. Therefore, in this thesis it is hypothesized that target plant species colonized by the AMF community from the undisturbed native area would present similar growth as compared to plants colonized by the AMF community from the disturbed soil previously inoculated with commercial inoculum (Fig 1.6).

(ii) If there is functional compatibility between pairs of plant-AMF species and if AMF community diversity influences plant productivity, then a differential plant growth among the three tested woody species is expected, relatively to the AMF communities treatments.

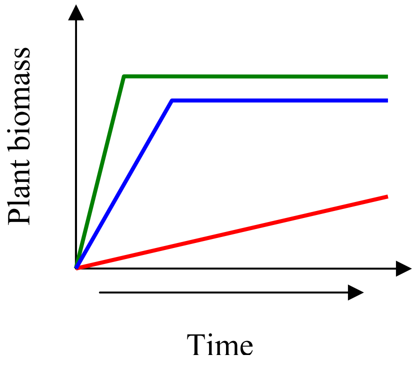

Figure 1.6. Schematic representation of hypothetical plant growth responsiveness to three different AMF communities: AMF community from the **undisturbed soil**, AMF community from the disturbed soil **inoculated with commercial inoculum**, AMF community from the **disturbed soil**. It is assumed that AMF biodiversity is functionally redundant.

It is hypothesized that each plant species responsiveness to AMF activity (*sensu* Wilson and Hartnett, 1998) would be largely variable since each community can be composed of several species. Therefore, it is possible that some mutualistic preferential association occurs between native plant species and certain adapted indigenous AMF species; this would be revealed as a reciprocal benefit for both symbionts measured as an increased plant growth and inoculum potential, although the rate of inoculum build-up could be plant species-specific. The beneficial plant response of the addition of commercial inoculum would be plant species-specific.

In *Chapter 4*, it was tested whether individual plant species induce shifts in soil fungal communities (conspecific *vs*. heterospecific communities), and whether plant growth is increased or decreased by these communities. As part of this objective, the possible capacity of AMF species from commercial inoculum to alter the feedback mechanism of plant-root fungal endophyte interactions was also investigated.

It was expected that the addition of commercial inoculum would reduce the negative feedback among the interactions of conspecific plant-root endophyte community. Since AMF species from commercial inoculum are usually previously selected for their aggressive infectivity abilities, it is hypothesized that they would act as a biocontrol agent, either by increasing competitiveness for root space against other root endophyte fungi, or by inducing plant physiological changes and morphological root differences. Since not all AMF species exert the protection function similarly, it was expected that plant growth increased as a consequence of pathogens infection amelioration, and changes would occur differentially for each plant species according to AMF communities and non-mycorrhizal root fungi species diversity.

3. EXPERIMENTAL FRAMEWORK

Mediterranean climate is characterized by dry and hot summers contrasting with wet and relatively cold winters. Mediterranean-type ecosystems correspond to a transitional region between temperate and tropical ecosystems and have a restricted distribution, (Mediterranean Basin, California, Chile, South Africa and some southern areas in Australia) (Di Castri *et al.*, 1981). The complex biological diversity of these ecosystems is a result of a "recent" evolution and adaptation to climatic changes, together with several selective driving forces: biogeography, geology, ecology and history (Blondel and Aronson, 1999). The historical patterns of resources management in the Mediterranean Basin often resulted in their overexploitation, with profound impact on structure and composition of the vegetation. The exceptional richness of plant species is partially a result of long-standing human activities (fire-setting, clear-cutting, grazing by domestic livestock, etc.) (Naveh, 1975; Blondel and Aronson, 1999; Vallejo *et al.*, 2006). Ecosystem heterogeneity ("mosaic effect") caused by the combination of those factors acts not only as a consequence but also as a main evolutionary factor for Mediterranean species. Abiotic (*e.g.* water, nutrients) and biotic (*e.g.* plant and microbial distributions), variables that typically have a patchy distribution in these ecosystems (*e.g.* Joffre and Rambal, 1993; Cruz *et al.*, 2002; Carvalho *et al.*, 2003) .

Generally, vegetation is a dense scrub (named maquis in the Mediterranean Basin) dominated by woody evergreen shrubs with sclerophyllous leaves, and summer semi-deciduous shrubs. There may be an overstory of small trees, as well as an understory of annuals and herbaceous perennials. Sclerophyllous shrubs are typical of middle-late successional stages. They present deep root systems and leaves with low surface-tovolume ratio and thick cuticles, features that favor the control of transpiration and are considered adaptations to summer drought and/ or to low soil nutrient availability, as is the case in Mediterranean-type ecosystems (Correia and Catarino, 1994; Blondel and Aronson, 1999; Werner, 2000). Semi-deciduous shrubs dominate in the earlier successional stages. They present shallow root systems and loose part of their leaves before summer drought, thus reducing their transpirational surface during that season (Correia *et al.*, 1987, 1992; Kyparissis & Manetas, 1993; Werner, 2000).

The extraordinary plant diversity (25 000 plant species) found in the Mediterranean Basin (Quézel *et al.*, 1999) is accompanied by a high diversity of mycorrhizal types. The maquis comprises plant species able to form almost all types of mycorrhizae (Brundrett, 1991; Puppi and Tartaglini, 1991). Examples among woody species are *Quercus coccifera* (ectomycorrizal mycorrhizae), *Arbustus unedo* (arbutoid mycorrhizae), *Erica arborea* (ericoid mycorrhizae); the most common are the species establishing arbuscular mycorrizae such as *Pistacia lentiscus* and *Olea europaea* var. *sylvestris*. The above ground spatial complexity of this community is increased by the presence of different vegetation layers (trees, shrubs and herbaceous), creating a plant root mixed system from different plants species hosting different mycorrhizal fungi.

Selected sites and plants

Part of the soil samples (*disturbed soil*) used throughout the study were retrieved from a limestone quarry at Outão (SECIL company) where revegetation projects have been taking place since 1983. It is one of the largest limestone quarries in the region and is located within the Arrábida Natural Park, in Portugal (38º 29’ 46’’ N, 8º 57’ 00’’ W). The reference area – from where *undisturbed soil* was collected - was adjacent to the quarry and is representative of a late seral native Mediterranean plant community (Fig. 1.7), suffering only from minor disturbance from peripheral mining activities (*e.g.* dust).

Serra da Arrábida (38º 27-30’ N, 8º 55’-9º 02’ W) is a small chain of limestone outcrops with a maximum elevation of 500m, which receives official protection since the 18th century, and is a Natural Park since 1976. The soils are classified as Mediterranean red soils, established on a Jurassic limestone. A true soil profile is almost absent. The natural vegetation in this area consists of a well-preserved Mediterranean *maquis*, dominated by evergreen sclerophyllous shrubs and summer semi-deciduous shrubs, respectively in later and earlier successional stages (Catarino *et al.*, 1982; Correia, 1988; Werner, 2000; Clemente, 2002). These plants are adapted to drought stress and low-nutrient availability (Correia, 1988; Werner, 2000).

The climate is Mediterranean, with a mean annual rainfall of 650 mm and mean annual temperature of 16ºC at the closest meteorological station (Setúbal) (data from Correia and Catarino, 1994). Soil is commonly denominated as "terra rossa" (red soils) and contains a large proportion of hard limestone and other calcareous rocks, mostly shallow. Generally, soils are poor in nutrients and have a neutral to basic pH (Correia, 1988).

Figure 1.7. Aspect of the reference undisturbed site, in Serra da Arrábida (A), of the greenhouse facilities where the experiments were carried out, in Praias do Sado (B), and plant species used – carob (C), myrtle (D) and lavender (E).

The experimental studies were carried house in the nursery facilities of the company EDP, at Praias do Sado, about 10 km from Serra da Arrábida (Fig. 1.7). Plants and seeds of Serra da Arrábida provenance, as well as maintenace of the growing plants, were provided by this nursery. For the purposes of this thesis, the selected native Mediterranean plant species were *Ceratonia siliqua* L., *Myrtus communis* L.[1], both regularly used, among others, for revegetation purposes at SECIL quarry. A third species belonging to the local flora was chosen, *Lavandula stoechas* L. The three target plant species (Fig. 1.7) form arbuscular mycorrhizal associations (Correia and Martins-Loução, 1996; Azcon and Barea, 1997; Matosevic *et al.*, 1997), but present different plant life traits and different drought resistance strategies (Correia, 1988; Oliveira and Peñuelas, 2000; Clemente *et al.*, 2005) (Table 1.2). Root morphology and physiological characteristics condition plant ability to acquire nutrients and cope with natural stress, and may reduce plants dependency on mycorrhiza (Brundrett, 1991, 2002). Plants with low root branching frequency and few (or short) root hairs appear to be more dependent on mycorrhiza for mineral nutrients uptake. Several other factors, like seed size with the correspondent nutrient resources can also indirectly affect mycorrhiza formation and benefit the plant on early developmental stages.

Table 1.2. Characteristics of the plant species used in the present study.

PLANT SPECIES	GROWTH FORM	FUNCTIONAL GROUP	ROOT SYSTEM	SEED SIZE	SUCESSIONAL STAGE	MYCORRHIZAL DEPENDENCE
Ceratonia siliqua	tree	sclerophylls	deep, profuse secondary roots	large	late seral	facultative
Myrtus communis	shrub	sclerophylls	deep, highly branched	medium large	middle-late	facultative
Lavandula stoechas	shrub	summer semi-deciduous	shallow	small	early-middle	obligate

Ceratonia siliqua (carob tree) belongs to the Caesalpinoideae family. Although it is a legume, it is unable to establish any rhizobium strain association, and therefore to fix atmospheric nitrogen (Martins-Loução, 1985). It is a long-lived, slow-growing evergreen tree species, up to 10 m, well adapted to nutrient-poor soils and to the Mediterranean climate. Flowers are produced in summer-autumn, and fruits become mature one year later. It develops an extensive root system penetrating deeply in the soil where water is probably available. Compared to other Mediterranean plant species, carob presents a water-spending strategy, with high photosynthetic activity and growth during spring, when water supply is not limiting (Nunes *et al.*, 1992; Ramalho *et al.*, 2000). However it also displays some drought resistance mechanisms, including leaf and branch shedding under severe drought stress (Salleo and LoGullo, 1989; Correia and Martins-Loução, 1993). Growth of carob plants is positively correlated with nitrogen source and amount (Cruz *et*

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[1] Species nomenclature according to Franco (1971- 1984).

al., 1993; Correia and Martins-Loução, 1993, 1995, 2004). Carob plants are always mycorrhized, regardless of age, site of growth or soil type (Correia and Martins-Loução, 1996).

Myrtus communis (mirtle) belongs to the Myrtaceae family. It is a densely branched evergreen woody shrub, up to 1-2 m, with a profusely branched roots and a deep tap root. Vegetative growth occurs in spring, flowering in summer and fruiting in autumn-winter. Strategies to face drought stress include control of leaf transpiration through stomatal closure (Losch *et al.*, 1982). These shrubs are able to resprout after cutting or fire. The nursery production of myrtle is difficult because of the transplant stress of young seedlings, but the use of mycorrhiza (*G. intraradices*) increases plant growth and reduces the transplant stress, allowing renewed growth after 60 days (Matosevic *et al.*, 1997).

Lavandula stoechas (lavender) belongs to the Labiatae family. It is a drought semi-deciduous shrub up to 1m tall, with a short main woody stem that is profusely and densely branched. It produces stalked inflorescences in spring. Lavender regenerates exclusively by seed germination after disturbances. Plants have main taproot. Leaves of lavender are more drought-sensitive than those of sclerophyllous shrubs (Nogues and Baker, 2000). It has been demonstrated that this species achieves maximal growth when mycorrhized (Azcón and Barea, 1997).

Chapter 2 - ASSESSING ARBUSCULAR MYCORRHIZAL FUNGI INFECTIVITY AND EFFECTIVITY PRIOR TO QUARRY RESTORATION

1. INTRODUCTION

Quarries represent some of the most difficult ecosystems to restore. The main reason for this difficulty is that the original soil is completely removed from the site and is often stockpiled while the site is being quarried, then reintroduced prior to restoration planting. However, stockpiling may harm the resident soil microbiota, because erosion and the absence of plant cover lead to reductions in microbial biomass. This is a major concern because native plants depend on soil microbiota, such as arbuscular mycorrhizal fungi.

Arbuscular mycorrhizal fungi (AMF) occur in most soils and influence directly the establishment of plant communities (van der Heijdeen *et al.*, 1998a). These associations contribute to plant nutrition, particularly plant uptake of poorly mobile soil nutrients such as phosphorous (Smith and Read, 1997) and to improved water relations where water is growth limiting (Sanchez-Diaz and Honrubia, 1994). A reduction in soil AMF inoculum may endanger the native plants establishment, especially under harsh environmental conditions, like in Mediterranean ecosystems. To determine the need of AMF inoculation for successful restoration, some studies suggest that it may be cost-effective to test the stockpiled soil for AMF infectivity potential, before landscape inoculation (Jasper, 1994; Dodd and Thomson, 1994).

AMF spores are able to survive soil stripping and stockpiling (Jasper *et al.*, 1987; 1988; Miller and Jastrow, 1992), but the extraradical hyphal network is very sensitive to any physical disturbance (Jasper *et al.*, 1989b; Evans and Miller, 1990; McGonigle and Miller, 1993; Requena *et al.* 1996). However, it as been shown that AMF families differ in terms of mycelial size (Hart and Reader, 2002a) and on colonization life-history strategies (Klironomos and Hart, 2002). These authors, for example, found that members of the Gigasporacea family were more successful to initiate colonization when spores were used. These findings indicate differences in vulnerability to disturbance within AMF groups. It is highly expected that shifts in AMF community composition, abundance and subsequent infectivity will occur due stockpiling disturbance.

Soil disturbance effects on AMF community can be characterized by assessing either propagule density or AMF inoculum infectivity. The former approach consists in a direct measurement of spores or hyphae. The latter approach is an indirect evaluation using a bait plant bioassay. Infectivity measurement through this approach is defined by Plenchette *et al.* (1989), as the degree to which new seedlings are infected by AMF after being exposed to the soil. Both methods can be valuable, although this last determination is cited as the most accurate since it accounts for all possible interactions between AMF and biotic and abiotic components of the host-plant rhizosphere (Brundrett *et al.*, 1995). Obligate mycorrhizal-dependent plant species are usually the bait plants chosen for the bioassay infectivity evaluation, because it has been shown that variations on their root colonisation represent proportional changes of the soil AMF inoculum regardless of the AMF species. Therefore, this method provides the researchers with an easy and fast way to evaluate variation of the AMF amount in the tested soil. Possible constrains for the general use of those bait plants species instead of native plants adapted to target ecosystem, is the recently recognized that host-plant AMF preferential compatibility influences AMF trap culture results (Liu and Wang, 2003).

The present study focused on a disturbed quarry ecosystem within a Portuguese Mediterranean ecosystem. The aim of this study was to evaluate the influence of disturbance caused by soil stockpiling on AMF infectivity. More specifically, the study was set up to address whether the number of AMF propagules and their infectivity were affected by disturbance, and also whether both mentioned approaches for AMF soil infectivity evaluation were related with AMF soil infectivity for native plant species.

We hypothesized a negative effect of disturbance on AMF community and, consequently on their infectivity on woody native plants, although a direct relation with AMF propagules density is not expected. This hypothesis is based on other studies showing that a change in percent root colonization is not linearly related with the number of AMF propagules in the soil, rather it is influenced by other variables, namely soil characteristics, and AMF and host plant species involved (McGonigle, 2001; Allen, 2001). Finally, for restoration purposes, it is expected that the evaluation of AMF infectivity is more accurate and meaningful when using native species as bait plants.

2. MATERIALS AND METHODS

Study site and field sampling

This study was conducted in a quarry within the boundaries of Serra da Arrábida's Natural Park (see *Chapter 1* for further details). The study involved a comparison of disturbed (stockpiled) and undisturbed soil. Both soils are clay-loam. The disturbed soil was collected from stockpiled soil in the quarry, and the undisturbed soil was collected from a well-preserved site within the Mediterranean maquis surrounding the quarry. The disturbed soil had been stripped six years ago and then stockpiled. Until the beginning of this study, there had been no plant cover on this soil. Chemical properties of the soils are summarized in Table 2.1. Both types of soil have similar nutrition levels (with a very low phosphorus concentration) but they differ significantly in physical structure and organic matter content.

Table 2.1. Soil chemical analyses. Values for each soil result from the mix of 5 core samples.

SOIL SITE	P_2O_5 PPM	K_2O PPM	MG PPM	ORGANIC MATTER (%)	PH
Undisturbed	8	100	95	2.75	8.2
Disturbed	8	79	125	0.29	8.5

In March 2001, soil cores were collected from eighteen different and randomly chosen (but at least 2 m apart) points in the stockpile and in the undisturbed site. Each of the eighteen sampling units consisted of four intact soil cores (∅ 9 cm; 15 cm long) taken adjacent to each other. Each of these units represented a replicate *per* disturbance regime soil, and each soil core within a sampling unit represented a plant species replicate. A total of 144 soil cores were collected (18 sampling units x 4 host plants cores x 2 disturbance treatments). Only nine sampling units were actually used for the bioassay measurements, since the remaining nine units were used to screen the beginning of root colonization. Close to each sampling unit we also collected bulk soil for the extraction of fungal propagules.

Direct counting of AMF propagules

In this study only spores and hyphae were assessed as AMF propagules.

Spores were extracted from 30 g of each soil sample by wet sieving followed by 60% sucrose gradient centrifugation (Daniels and Skipper, 1982). The total number of spores was counted under a dissecting microscope (45x). To assess viability, 30 spores per disturbance replicate were randomly collected, placed in an iodonitrotetrazolium (INT) solution (1 mg ml^{-1}) and left at room temperature for 48 h (Walley and Germida, 1995).

For the extraction and quantification of AMF extraradical hyphae, two soil samples (5 g fresh weight each) were taken from each of the nine cores (one per sampling "unit") of both soil types. Extraradical hyphae were extracted from the soil by a modified (Miller *et* *al.*, 1995; Carvalho *et al.*, 2003) procedure. Individual soil samples were suspended in 495 ml of water and homogenized in a blender for 1 min. The suspension was decanted and then stirred with an electronic stir bar. One 20-ml aliquot was removed from halfway between the beaker edge and the vortex and diluted in 80 ml of water. This mixture was stirred again and one 10-ml aliquot was transferred to a filter holder with a nitrocellulose membrane filter (47-mm diameter and 1.2-µm pore size). Trypan blue stain (0.05%) was added and after 5 min the stained suspension was drawn through the filter using vacuum suction. The filter was cut in half and placed on microscope slides. After dried, the filters were covered with low viscosity immersion oil. Extraradical mycelium was estimated by the gridline intersect method, recording 140 fields of view using a 10 x 10 squared grid eyepiece reticule and viewed at x 200 magnification. Only aseptate hyphae with a characteristic "knobby" appearance and dichotomous branching were considered as AMF hyphae. Total hyphal length was calculated based on Tennant (1975) and converted to m g^{-1} dry soil on the basis of soil moisture content measurements.

To assess hyphal viability, the previous procedure was repeated, but instead of using Trypan blue, NBT vital stain was added, and the sample incubated overnight at 27 ºC. Viable hyphae length was determined using the same gridline method as described above.

Sorghum bioassay

Sorghum was chosen as the test plant for the bioassay analysis since it is one of the most recommended bait plants (Franson and Bentlenfalvay, 1989; Morton, personal communication, 2005). Sorghum seeds were surface-sterilized and pre-germinated. More than one seed was sown in each soil core. All except one were removed after the appearance of the first pair of leaves. In the other three additional soil cores from each soil disturbance replicate, three plants were grown. Those plants were used to estimate the harvest time based on their colonization rate. Plants were checked for colonization on a weekly basis. After 7 weeks, all sorghum plants were harvested, root and shoot were detached and fresh weight and total root length were determined. In this study, total fresh weight will be referred as fresh biomass. Root/shoot ratio was also determined. Total root length was assessed through digital image analysis with WinRhizo software (Regent Instruments Image Analysis Systems, Canada). The three samples of the entire root systems for each soil core were cleared and stained using a modified procedure by Phillips and Hayman (1970). The roots were cleared for 30 min in a 10% KOH solution at 90 ºC. After rinsing, the roots were placed in 10% HCl solution for 10 min and stained with a glycerol-trypan blue solution (0.05%) at 70 ºC for 20 min. Percent colonization was assessed by Giovannetti and Mosse (1980) intersection method at x 45-100 magnification and expressed as percentage of root length colonized and length of colonized roots. Infection units originated from entry points were counted at x 200 magnification (Franson and Bethlenfalvay, 1989) and the results were expressed as infection units per root length. In the bioassay approach, percent root colonization of the bait plant is assumed to reflect the number of total infective propagules (spores, mycelium and colonized roots from other plants).

Woody target plants bioassay

To determine indigenous AMF infectivity and effectivity for woody native Mediterranean plant species another bioassay was conducted, as the one described above for sorghum plants species. For this purpose we chose three plants species that are able to form AM, do naturally occur in the quarry undisturbed surrounding areas and which are potential target species for revegetation trials: carob (Ceratonia siliqua L.), myrtle (Myrtus communis L.) and lavender (Lavandula stoechas L.) (see Chapter 1 for further details).

Soil cores from the same sampling units used in the sorghum bioassay were used for these native plant species (nine replicates for each plant species).

Seeds were pre-germinated and surface-sterilized. Prior to germination, myrtle seeds were placed under running water for 1-2 days and carob in 100% sulphuric acid for 10 min (Martins-Loução *et al.*, 1996). One pre-germinated seed from each host was transplanted into each soil core, and survival was assessed. All seedlings survived except for one carob seedling in an undisturbed soil core. During Spring, the cores with seedlings were randomly placed on greenhouse benches. The harvesting time was different for each plant species. Lavender was colonized at the 8th week, myrtle at the 10th week and carob at the 17th week. Differences in the harvesting date were due to plant species different times required to initiate root colonization. Every couple of weeks a replicate of each plant species of the remaining nine sampling units was harvested and roots checked for colonization. The measurements conducted after harvesting were the same as the ones described for sorghum plants above.

Clearing and staining protocols had to be adapted, particularly incubation time, which was increased due to the woody characteristics of these plants roots. Lavender and myrtle roots were incubated for 50 min in KOH (10%) at 90ºC, while carob roots were incubated for 70 min. To remove lignins from myrtle and carob roots, they were immersed in H_2O_2 (10 vol.) for 30 min.

Statistical analyses

AMF propagules data (number of spores, hyphal lengths) and sorghum root colonization were analyzed by T-test to evaluate differences in AMF soil potential due to disturbance. Spearman rank-correlations coefficients were calculated to relate (a) viable propagules with AMF soil infectivity measured through percent sorghum root colonization in the bioassay, (b) AMF infectivity (% root colonization, % root length colonization and number of root entry points) with AMF viable propagules, to determine the most adequate AMF soil inoculum quantification method, (c) native host plant root colonization with AMF amount in the soil (% native plant species root colonization *vs*. propagules *vs*. AMF infectivity), and (d) growth of native plants with their AMF root colonization.

The results from the native plant bioassay were analyzed by analysis of variance (ANOVA) to test for the effects of soil disturbance and native plant species. Significant results ($P<0.05$) were analyzed by Kruskall-Wallis non-parametric test. Prior to analyses, % root colonization data were arcsin square root-transformed, root/shoot data were square root plus one transformed and fresh biomass data were log-transformed (Zar, 1984). SPSS software was used for all statistical analysis (Field, 2003).

3. RESULTS

All AMF propagules density was significantly reduced in the disturbed soil. Total hyphal length was 91.3% reduced by disturbance ($T_{16} = 5.49$, $P<0.0001$), while total soil-born spores decreased in 61.9 % ($T_{16}= 4.23$, $P<0.001$) (Fig. 2.1). Propagules viability was largely affected. In the undisturbed soil, viable spores represented 40% of the total spores, while in the disturbed soil viable spores represented only 14.5%. Similar proportionalities were found in the percentage of viable hyphal length relatively to the total hyphal length: 32.7% in the undisturbed soil and only 14.6% in the disturbed soil (Fig. 2.1). There was a significant positive relation ($r= 0.632$; $P=0.005$) between hyphal length and number of spores, although a strong negative relationship was found when viable propagules were correlated ($r= - 0.986$; $P<0.0001$).

At harvesting time, the frequency of soil cores with colonized roots was 100% for lavender and myrtle, while carob frequency was 50% in disturbed soil cores and 44.4% in undisturbed soil cores.

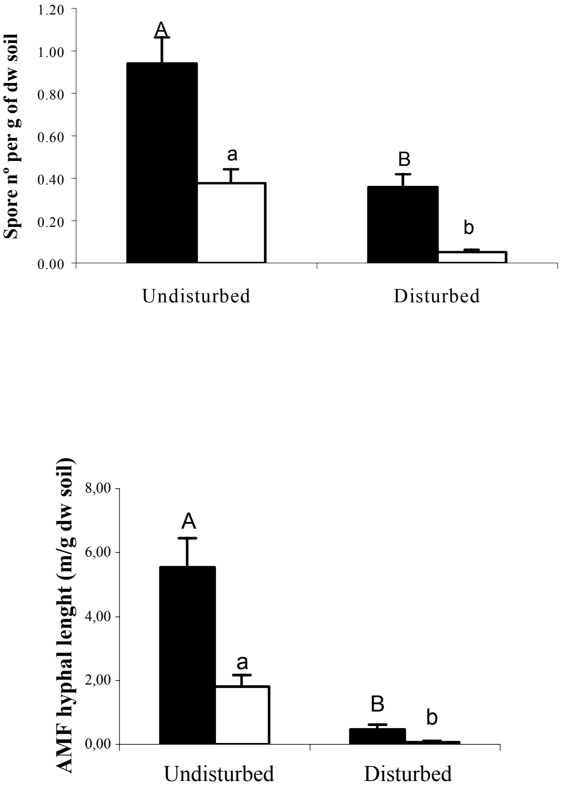

Figure 2.1. Direct count of AMF propagules (black bars) and their viability (white bars) in undisturbed and disturbed soil. Values (mean±SE of 9 replicates) followed by the same lower cases represent no significant differences (*P*> 0.05) in the viable number of AMF propagules: number of spores and hyphal length.

AMF infectivity, estimated by the bioassay method, also decreased significantly with soil disturbance (Fig 2.2), but in a different ratio from the propagules. Both measured bioassay dependent variables, such as percentage of AMF colonization and number of entry points were positively correlated (r= 0.697; *P*<0.0001). Thus, the percentage of sorghum root colonization will be hereafter assumed as representing AMF infectivity.

The results achieved by the two methods used to quantify AMF soil inoculum potential led to similar conclusions on the negative effects of disturbance. Even so, comparing the disturbance impact on soil AMF inoculum potential through a direct counting of propagules, points out to a more severe effect then using the bioassay approach.

Sorghum root colonization in the bioassay was correlated with total spores (r=0.534; *P*<0.05) and hyphal length (r=0.655; *P*<0.005), demonstrating its meaning for AMF soil infectivity evaluation. Viable extraradical hyphal length was positively correlated with AMF infectivity (r=0.642; $P<0.005$), while viable spores were negatively correlated (r = -0.829; $P<0.05$).

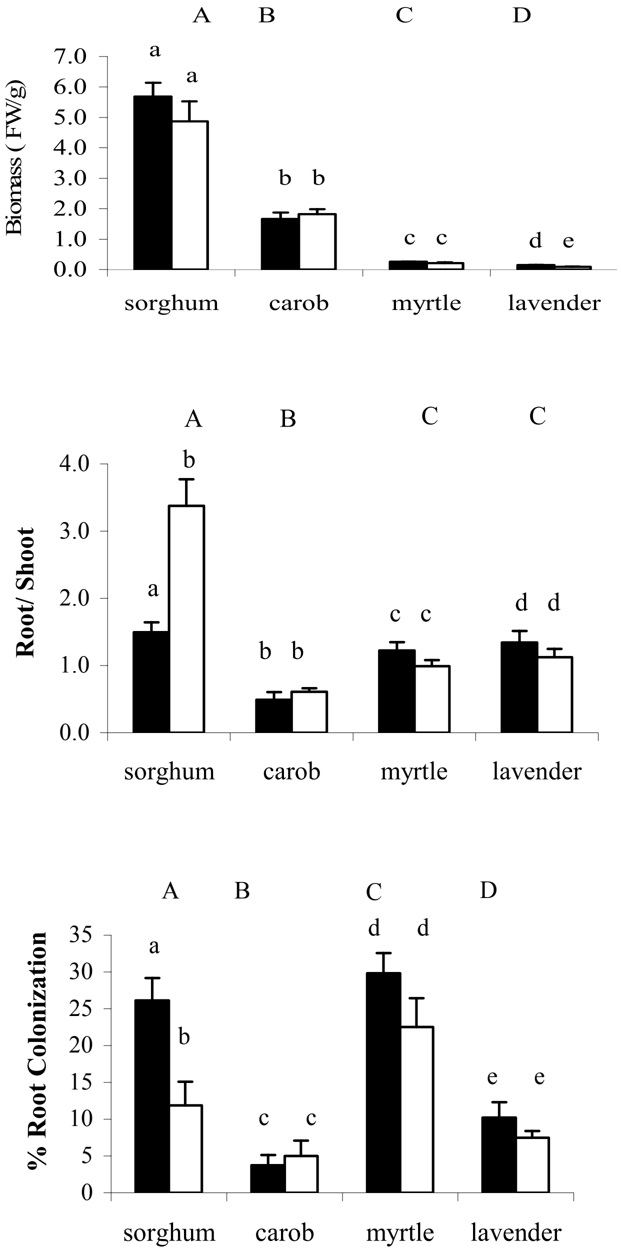

Figure 2.2. Biomass expressed as fresh weigh (g), and root/shoot ratio for the different host plants and their respective root colonization percentage under disturbed (white bars) and undisturbed treatments (black bars). Lower cases represent the disturbance effect on the host plants; values (means±SE of 9 replicates) followed by the same small letter are not significantly different at $P<0.05$ (t-Test). Upper cases represent the plant species effect; values (means±SE of 18 replicates) followed by the same cap letter are not significantly different at $P<0.05$ (Tukey *post-hoc* test).

Percentage root colonization was measured to assess the AMF infectiveness in the native plant. Despite the fact that all native plants were AM colonized (Fig 2.2), no significant differences in native plant roots colonization were found, caused by disturbance. Changes in AMF propagules were not correlated with native plants AMF root colonization. Also no relation was found between AMF infectivity, assessed through sorghum root colonization, and percent root colonization of any native host plant bioassay. These results indicate that native plants are not good indicators of changes in AMF inoculum. Only sorghum plants are in agreement with the hypothesis that a significant propagules reduction leads to a reduction of colonization (T_{16}= 3.309, $P<0.005$) (Fig 2.2).

Analysing Fig 2.2, it is notorious that plant host species is the determinant factor in plant responses rather than the disturbance regime, sorghum being the exception, as mentioned above. It was surprising to find no significant differences ($P>0.05$) between myrtle and sorghum in the percent root colonization since myrtle is a woody plant. There were no significant differences in biomass ($P>0.05$) neither for native plants species between disturbed and undisturbed soil, nor for sorghum (Fig 2.2). Moreover, no correlations were found between percentage root colonization and biomass for all the plants species, suggesting that plant biomass in early development stages is not directly related with AMF infectivity. However, sorghum presents a positive significant correlation between root colonization and root/shoot ratio (r=0.08; $P<0.05$). This result corroborates the significant differences found for sorghum root/shoot ratio parameter due to disturbance.

4. DISCUSSION

Our data shows that AMF soil inoculum potential assessed either by direct propagules count or indirectly through sorghum bioassay, was negatively affected by disturbance similarly to what was found in other disturbed Mediterranean ecosystems (Jasper *et al.*, 1991; Cuenca and Lovera, 1992; Requena *et al.*, 1996).

The negative correlation found between the viable hypha length and spores number could be interpreted as trade-offs between hyphae and spore production, so AMF tend to invest more in the mycelium network than in new spores, in agreement with Requena (1996) who concludes that with Mediterranean soils the main infective AMF propagules in the ecosystems is the extra-radical hyphal. However, these propagules were also the most reduced ones during mining activities and stockpiling the soil in agreement with other studies (Jasper *et al.* 1987; 1993). Consequently, the reductions in the mycelium network and spores density were directly responsible for the reduction in AMF soil infectivity when measured by the root colonization of bait plant. Others have largely described this close relation for some obligately mycorrhiza dependent herbaceous trap plants (Plenchete *et al.*, 1989; Franson and Benthelefalvay, 1989; Sieverding, 1991). Therefore, our results give support to previous works suggesting bait plant bioassay as a reliable and feasible method to compare viable propagules density differences in the soils due to disturbance (Brundrett *et al.*, 1995; 1996a,b; Requena *et al.*, 1996). However, for restoration management it is not so important to assess the exact AMF inoculum changes due to disturbance, but rather the ability of the remaining AMF present in the disturbed soil to colonize and to establish effective mycorrhizal associations with the native target plants.

In this study, it was shown that AMF infective propagules were largely reduced due to disturbance caused by stockpiling the soil. Still, the remaining inoculum is enough for a successful colonization of woody Mediterranean plant species, although no differences were found when both soil disturbed *vs*. undisturbed were compared. Nevertheless, there were differences in root colonization extension within host plant species and on the time required to begin colonization. Differences in the colonization extension within plant species could be related to a possible differential "control" on nutrient exchanges, especially the carbon plant resources to sustain the symbiosis, while changes in the colonization rate could be more related to the energy sources of propagules and the type of propagules that are able to colonize roots (Klironomos and Hart, 2002; Hart and Reader, 2002b). In addition, Brundrett and Kendrick (1990) suggested an explanation for woody plants slower colonization rate comparatively to crops, based on morphological root limitations, caused by exodermis suberization or extreme lignifications, which creates a physical barrier for the fungi. Moreover, an inconsistent disturbance effect on roots colonization, depending on AMF density prior to disturbance has also been shown (McGonigle and Miller, 2000).

Although this study was not particularly addressed to associate AMF infectivity with AMF effectiveness due to disturbance, some considerations can be made concerning the early plant growth benefit. Differences in AMF infectivity were not related to differences in AMF effectivity for the woody native plant species, since there were differences in sorghum root colonization but not in sorghum biomass whereas differences in lavender were found for biomass but not for root colonization. These results, together with the significant reduction of the root/shoot ratio observed in sorghum, show that this species invests on root biomass when there is a reduction of AMF soil propagules density, and that sorghum is mycorrhizal-dependent. Despite lavender did not show any difference in root/

shoot ratio, it is an obligate mycorrhizal-dependent plant (Azcon and Barea, 1997). It is suggested that besides abiotic experimental conditions, individual plant species features like mycorrhizal dependence or other physiological characteristics, could determine plant mycorrhizal responsiveness (Brudrett, 1991; van der Heijdeen, 2002), and that would be more important in the AMF effectivity evaluation than differences in AMF inoculum like initially proposed for this study. Moreover, these differences in AMF infectivity and effectivity could result from shifts in AMF community composition or from the interaction with other soil micrорganisms. However, this hypothesis can not be ascertained in the present investigation, because whole soil inoculum was used and only AMF propagules densities were evaluated.

Given that, the hypothesized reduction in AMF propagules and AMF soil infectivity measured by the sorghum bioassay were confirmed but no reductions in woody native plants root colonization extension were found, when disturbed soil was compared with the undisturbed soil. Thus, the evaluation of the disturbance effect on AMF soil infectivity, measured through native woody plant bioassay is different from that obtained from a sorghum bioassay. These findings reveal that AMF infectivity results will depend on the methodological approach used and on the species used as trap plant.

Overall, the present results suggest that both AMF infectivity and AMF effectivity need to be considered when evaluating the effects of disturbance on the functioning of AMF, since infectivity and effectivity variables may not always be correlated. A bioassay using sorghum or other highly mycorrhiza-dependent plant as bait would be accurate when the aim of the study is to compare AMF communities, for example, the presence and abundance of AMF viable propagules. However, to predict the needs of commercial inoculum for application in a quarry restoration trial, it is more appropriate to use native plants for evaluation of the mycorrhizal status.

Chapter 3 - USING INDIGENOUS AND COMMERCIAL ARBUSCULAR MYCORRHIZAL FUNGI TO GROW NATIVE PLANTS FOR MEDITERRANEAN ECOSYSTEM RESTORATION

1. INTRODUCTION

Natural colonization and redevelopment of severely disturbed ecosystems are typically slow, although an assisted-reconstruction has the potential to speed up the process (Bradshaw, 1983). A limitation for successful ecosystem restoration is the establishment of native plants, particularly those found in mature communities (Kleijn, 2003). In restoration settings, plant community establishment relies on the availability of suitable and functional soil microbial communities, in particular of arbuscular mycorrhizal fungi (AMF) (Allen, 1991; Sylvia and Williams, 1992; Herrera *et al.*, 1993; Jasper, 1994; Moynahan *et al.*, 2002). These fungi can enhance plant community rehabilitation, primarily by increasing the ability of native plants to establish under and cope with stressful conditions, particularly in Mediterranean ecosystems (*e.g.*, low availability of nutrients and drought) (Brundrett, 1991; Barea *et al.,* 1997; Caravaca *et al*., 2003a; Augé, 2001). However, along with vegetation, AMF are also negatively affected by severe disturbance, with density and AMF species richness being typically reduced (Janos, 1980; Jasper *et al.*, 1991; McGonigle and Miller, 2000). Sites targeted for restoration will most likely require an increase in the biomass of effective AMF taxa (Sieverding, 1991). Two strategies for enhancing AMF have been proposed: (a) planting native host plant species that promote the growth of indigenous AMF (Dodd *et al.*, 1991; Sieverding, 1991; Karasawa *et al.*, 2001; Azcón-Aguillar *et al.*, 2003; Caravaca *et al.*, 2003b); and (b) introducing AMF inoculum (either from indigenous or commercially-available cultures) directly into the target soil (Herrera *et al.*, 1993; Dodd and Thomson, 1994; Jasper, 1994; Haselwandter and Bowen, 1996; Caravaca *et al.*, 2002, 2003a). Mycorrhizal biotechnology is also being integrated in nurseries specialized in growing native plants for revegetation purposes, because the symbiosis enhances the ability of these plants to establish when out-planted, and to cope with natural stresses (Dodd and Thomson, 1994; Jasper, 1994; Haselwandter and Bowen, 1996).

It is not always clear which strategy is the most appropriate, but the decision can be assisted by understanding some basic aspects of the biology of indigenous AMF in the system subject to restoration (Sieverding, 1991; Dodd and Thomson, 1994; Jasper, 1994).

Generally, the first step will be to evaluate the indigenous AMF *infectivity* in plant roots and the *effectivity* in promoting growth of target plants. If both criteria score low, then AMF inoculation is typically recommended to quickly increase AMF biomass in the soil. On the other hand, if the indigenous AMF populations that remained in the soil following disturbance (even if densities are low) are effective, all that may be necessary is to grow suitable plant species as promoters to build up the existing AMF community.

Promoting the growth of indigenous AMF inoculum is usually regarded to be a superior strategy, since indigenous AMF may hold some adaptations to native plants (Enkhtuya *et al*., 2000; Lovelock and Ewel, 2005) and to local environmental conditions (Carvalho *et al*., 2004; Johnson *et al*., 1992). In addition, evidences have been accumulating in recent literature showing a differential compatibility, or even “specificity”, between particular pairs of plant-AMF species that may affect the symbiosis outcome (McGonigle and Fitter, 1990; Sanders and Fitter, 1992; Bever *et al*., 1996; Klironomos, 2003). Furthermore, it has been demonstrated that individual plant species or plant communities are able to change AMF community composition and propagules densities (Johnson *et al*., 1992; Eom *et al*., 2000; Bever, 2002; Burrows and Pfleger, 2002) However, studies that have tried to build up native inoculum in the field have reported inconclusive results (Azcón-Aguilar *et al*., 2003; Karawasa *et al.,* 2001). A major limitation to this approach is that the build-up of inoculum is a slow process and susceptible to stagnation, depending on the initial potentiality of the AMF inoculum to recovery plant community (Miller and Jastrow, 1992).

Since in restoration programs the goal is not to recover the original AMF community, but rather its “beneficial functionality” to ameliorate the new plant community establishment, the most common approach in plant nurseries and larger-scale restoration programs is the introduction of allochthonous AMF (commercially available) (Jeffries and Barea, 2001; Azcón and Barea, 1997; Gemma and Koske, 1997; Dodd *et al*., 2002). Re-introducing AMF inoculum is sometimes considered important in surface mining where the exploration process drastically decreases the viable populations of these fungi (Jasper *et al*., 1987, 1989b,c; Miller and Jastrow, 1992). In the revegetation of highly disturbed soils and in agricultural studies, the addition of allochthonous AMF will consecutively increase AMF density and diversity, followed by an increase in the establishment of native plant species (Sieverding, 1991; Herrera *et al*., 1993; Caravaca *et al*., 2003a, 2004). Contrarily, other studies did not find a positive relation between higher AMF soil inoculum and a better plant establishment or productivity (Richter *et al*., 2002).

AMF community diversity has been shown to influence plant community diversity and productivity (van der Heijden *et al.,* 1998a, b; Hartnett and Wilson, 1999; Kiers *et al.*, 2000; Bever *et al.,* 2003). However, the beneficial effect of particular AMF species used in field inoculations may be difficult to generalize from such results. AMF used in commercial inoculum are usually selected for being highly infective, but their effectiveness on plant growth depends largely on the fungal × plant interaction (Klironomos, 2003) and environmental conditions, the mycorrhizal symbiosis ranging from parasitic to mutualistic (Johnson *et al*., 1997). Nevertheless, it is equally important that the target plants are also effective in promoting and/or maintaining the density of commercial inoculum AMF species. Plants used in field out-planting will serve as mycorrhizal inoculum sources for other plant species (Haselwandter and Bowen, 1996; Carrillo-Garcia *et al.*, 1999; Azcón-Aguilar *et al.,* 2003).

In degraded semiarid ecosystems the re-establishment of adapted native plant species is a key step in restoration strategies (Vallejo *et al*., 2006). Within the framework of revegetation programmes currently being developed in a Portuguese limestone quarry, a number of drought-tolerant shrub species are being assayed in nurseries before being out-planted. The objective of this study was to compare the effectiveness of indigenous AMF community from disturbed soil with commercial inoculum in promoting plant growth during the nursery stage (Fig. 3.1). Furthermore, the ability of different plant species to enhance the density of indigenous AMF viable propagules and to maintain high AMF density following the addition of commercial inoculum in the disturbed soil was also tested (Fig. 3.1).

The underlying hypothesis was that commercial inoculum addition would be more beneficial to plant growth than using non-inoculated disturbed soil, because disturbance due to mining or quarrying drastically reduces the density of indigenous AMF, even though beneficial plant responses are expected to be plant species-specific. On the other hand, it was also hypothesized that plants would grow better in the presence of the AMF community from the undisturbed soil than with an AMF community from the disturbed soil, since disturbance may induce a shift in AMF community composition. Since AMF differ in respect to function and plant species differ in their responses to AMF then, communities with higher AMF species richness are expected to fulfil more functions and provide more opportunities for beneficial relationship to develop. A third hypothesis was that the ability to increase the AMF infective propagules in the soil would depend on the plant species rather than on the tested AMF community composition.

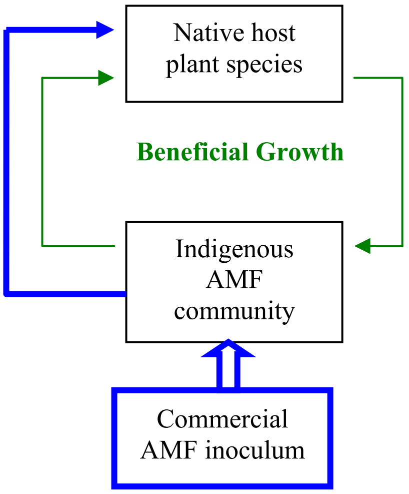

Figure 3.1. Schematic representation of the experimental working hypotheses.

2. MATERIALS AND METHODS

Soil analyses

The undisturbed soil samples were collected on January 2001 from the well-preserved *maquis* surrounding the quarry (Outão, SECIL) in Serra da Arrábida (see *Chapter 1* for further details). Random sampling points were selected to take account of soil heterogeneity and guarantee a maximum of AMF diversity (Carvalho *et al*., 2003). The disturbed soil samples were taken from a stockpile within the quarry, which resulted from mine stripping and had been stockpiled for six years, exposed to physical erosion and with reduced AMF propagules density most likely due to the absence of plant cover. Approximately one ton of soil was collected from each site. Both soils were clay-loamy, though exposed to different conditions. Despite both study soils had slight different nutrition levels (with a very low amount of phosphorous), they presented differences in physical structure and organic matter content (see Table 2.1). The differences between the disturbed soil and the soil from the undisturbed site were due to perturbation driven by soil removal and erosion during the pilling period.

Plant species

The plant species used in this study were commonly found in the undisturbed area adjacent to the quarry - *Ceratonia siliqua* L. (carob, tree), *Myrtus communis* L. (myrtle, shrub) and *Lavandula stoechas* L. (lavender, shrub). Carob seeds were scarified with sulfuric acid (H_2SO_4) according to Martins-Loução (1985), and myrtle and lavender seeds were scarified using tepid water for 24 h. The seeds from all plant species were surface sterilized and pre-germinated in trays with autoclaved vermiculite.

AMF treatments

Three AMF treatments were used for this nursery assay: indigenous AMF community from the disturbed soil (non-inoculated treatment); commercial AMF inoculum (*Glomus intraradices* Shenck & Smith) added to the disturbed soil (inoculated treatment); and indigenous AMF community from the undisturbed native area (undisturbed treatment).

In this study, the undisturbed treatment was intended to be an ecological reference, representing plant growth induced by the AMF community that naturally occurs in the *maquis*.

The commercial inoculum was purchased from Premier Tech (Riviere-du-Loup, Quebec, Canada), and consisted of a liquid suspension with 1 000 000 spores. The suspension was kept under agitation and aliquots taken until 4 000 spore sub-samples were obtained. Each of these sub-samples was used for individual pots from the inoculated treatment (66.7 spores *per* 100 g). Inoculum was added to the disturbed soil by placing it into a hole in the center of the pot where the seedlings would be planted. This approach was used to maximize direct contact of AMF with the roots.

Indigenous AMF taxa diversity

To characterize the indigenous AMF communities, and therefore confirm differences between them, the AMF species and respective abundance were recorded. AMF were identified using spores extracted directly from the field soil, or after an initial trap culturing procedure. Three soil samples per site were randomly collected from each of the big-bags containing the soil for the nursery assay, and AMF spores were extracted from the soil by wet sieving and sucrose centrifugation (Carvalho *et al*., 2003). To develop trap cultures, a mixture was prepared containing soil, sterilized sand, vermiculite and perlite (1:1:1:1). *Sorghum* sp. was planted on the soil mix and allowed to grow for two cycles of four months. In the middle of each cycle, shoots were cut and left to dry for a month. Cultures were kept in a cold chamber until they were used again. All spores were kept in a solution (10% Glycerol, 10% ethyl alcohol, 80% water) (C. Walker, personal information). All identifications were performed by C. Walker. The percentage of similarity between the two indigenous AMF communities was calculated using the *Kulezynski Index* for species abundance (Wolda, 1981).

Kulezynski Index = Σ (minimum of spores density from disturbed or control soils)/2 × (1/total spores in undisturbed soil + 1/total spores in disturbed soil)

Experimental design and set-up

The experiment was set up using a complete randomized factorial design with three independent variables: host plant species (3), AMF treatments (3) and harvest dates (2).

To guarantee that plant growth differences between the AMF treatments were only due to discrepancies in AMF communities, differences between other independent variables, which could also influence plant growth, were equalized (soil nutritional level and soil microbiological status). Details of soil chemical properties after soil homogenization, that constituted each soil treatment, are summarized in Table 3.1. To equalize the nutritional level and soil microorganisms, keeping the AMF communities from each soil intact, the procedure represented on Fig. 3.2 was followed.

Table 3.1. Chemical characteristics of the soil mixtures before use in this assay. Disturbed soil AMF treatment resulted from the mixture of disturbed soil with sterile soil from the undisturbed area. Another AMF treatment resulted from the mixture of soil from the undisturbed area with sterile disturbed soil. In inoculated disturbed soil both soils were sterilized. Soil analyses to evaluate the effect of sterilization for both soils did not present any alteration (data not shown).

SOIL PARAMETERS	DISTURBED + COMMERCIAL AMF	DISTURBED	UNDISTURBED
P_2O_5 (ppm)	8	8	8
K_2O (ppm)	96	94	100
Mg (ppm)	125	125	125
Org. Matter (%)	1.3	1.2	1.1
pH	8.4	8.3	8.3

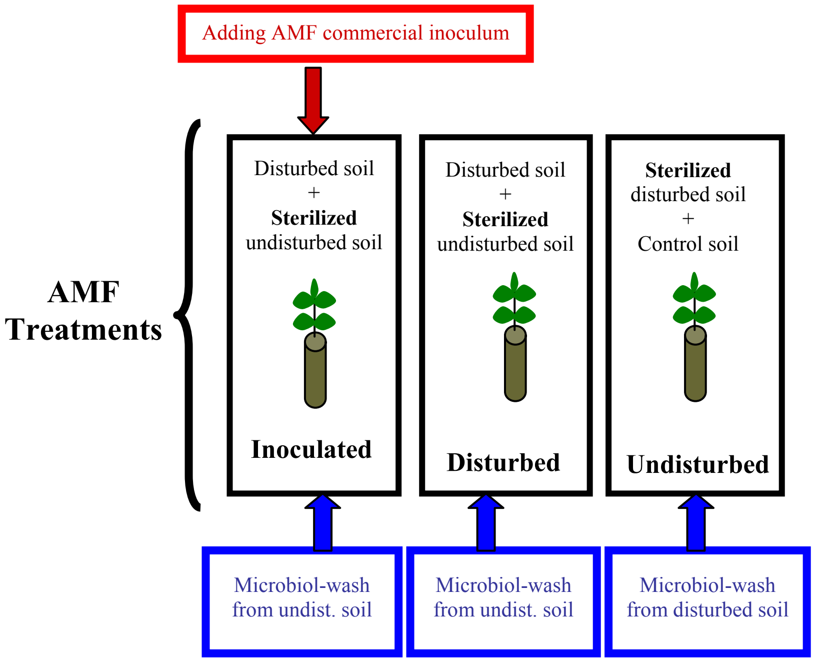

Figure 3.2. Schematic representation of experimental AMF treatments set-up.

In every pot, both undisturbed and disturbed soils were mixed in 1:1 proportion to level the nutritional differences between the soils. In all the pots one of the soils was sterilized, contributing only to the mineral component of the mixture. For example, in the non-inoculated AMF treatment sterilized soil from the undisturbed area was added to the disturbed soil, so that the only organisms alive belong to the disturbed community (Fig. 3.2). A suspension of *G. intraradices* spores was also added to the commercially-inoculated treatment. The undisturbed treatment resulted from the addition of sterilized disturbed soil to non-sterilized soil from the undisturbed area. Soils were sterilized by autoclave (121ºC, 1 h) for two consecutive days, and were then kept in a cold chamber (4ºC) until used. Soil mixture was homogenized using a concrete mixer machine previously bleached, and stones were subsequently removed from all soils by hand.

To level the microbial differences between the two soils, all pots were also inoculated with 250 mL of soil filtrate to control for non-AMF microorganisms. The filtrate was prepared by suspending 200 g of each soil in 1000 mL sterile water for 60 min. After shaking and decanting, the suspension was filtered twice (<20 mm, Whatman) (Klironomos *et al.*, 2004). The filtrate distribution followed the schematic representation shown in Fig. 3.2.

All plants were grown in 6 L pots in a nursery at Praias do Sado (see *Chapter 1* for further details). Pots were distributed randomly through the greenhouse and their relative position was changed in a random way every three months. Plants were watered when necessary. A drainage system was set up to avoid contact among running waters from different AMF treatments. Plants were not fertilized during the experiment.

Harvesting and data collection

To a subset of each plant species seedlings (n=12 *per* AMF treatment) plant height was measured when seedlings were transplanted to pots, and the stem marks were kept to measure the growth rates until the end of experiment. This plant growth time information was useful to determine the sampling periods for the destructive measurements: the first sampling period was at day 180, and the second at day 405. All plants species reduced growth rate or reached a *plateau* after one year in pots, probably due to root space constraint; therefore the measurements were stopped because they were no longer representative of plant growth response to AMF treatments.

At six and 12 months after the beginning of the experiment, six replicates *per* plant and AMF treatment were destructively sampled. The only exception was during the final harvest for lavender, due to high mortality (42% in the inoculated AMF treatment, 50% in the disturbed treatment and 58% in the undisturbed treatment). Soil was separated from the roots by hand, and roots were washed using water running through a sieve. For all plants, total root fresh weight was measured and a stratified sample of the youngest part of the root system was removed to determine mycorrhizal colonization. Total plant biomass was determined after drying for 48 h at 60ºC. Roots were cleared and stained with Trypan Blue (Correia and Martins-Loução, 1996). Root fragments were mounted in parallel on glass slides. For each plant × AMF treatment, three slides were prepared (containing 4-5 root fragments, approximately 3 cm long). The occurrence of AMF structures was assessed using a modified line intersection method (McGonigle and Fitter, 1990), where a minimum of 70 line intersections per slide were recorded.

AMF soil infectivity assay

AMF infectivity in the soil was assessed using a bioassay (Brundrett and Abbott, 1995). This allowed us to measure the ability of the different target plant species to enrich the soil rhizosphere with infective AMF propagules, building up the native AMF inoculum. *Sorghum* sp. was used as trap plant in the bioassays, because we previously determined a direct correlation between the number of viable propagules in the soil and the percentage root colonized (Correia *et al.*, 2004). Pre-germinated *Sorghum* seeds were sown into the soil immediately after plant roots were harvested, and were left to grow for four weeks. Mycorrhizal colonization was estimated by a grid-line intersection method (Giovannetti and Mosse, 1980).

Statistical analyses

To test the *AMF effectivity on plant growth* the following dependent variables were measured: plant height over time; total biomass; and root colonization. Plant height data were processed by analyses of variance for repeated measurements (ANOVAR). For each plant species, a mixed model with two independent variables was used (AMF treatments and plant species), where the dependent variable (plant height) had repeated measures along the experimental time (11 measurement dates). It was followed by Tukey *post-hoc* test when appropriate to detect differences between treatments per measuring date. Woody plant roots colonization and plant biomass data were processed using three-way analyses of variance (ANOVA) followed by Tukey *post-hoc* test. One-way ANOVAs for each host plant species at each harvest time were performed to find the source of variance found in the three-way ANOVA for AMF × Plant interaction effects.

To test for the *plant species effect on AMF community growth*, *Sorghum* root colonization from the bioassay were measured and data analyzed through three-way ANOVA followed by *Tukey post-hoc* test.

3. RESULTS

3.1. AMF DIVERSITY SURVEY

The stockpiling of soil significantly reduced AMF diversity and density (Table 3.2). *Glomus intraradices* was identified in the AMF community belonging to the late-seral undisturbed area, but was not found in the disturbed soil. Subsequently, the addition of commercial inoculum to the disturbed soil, containing only *G. intraradices*, did not even the diversity differences between the two AMF communities from the disturbed and undisturbed soil. In the disturbed soil, only *Glomus* genus was identified, while in the soil from the late-seral undisturbed area *Scutellospera, Sclerocystis, Achaeospora* and *Paraglomus* genera were also found. AMF species abundance similarities in the sampled

AMF communities were 44% (percentage of similarity calculated according to Wolda, 1981), indicating an alteration in AMF community composition due to disturbance and soil storage. Trap cultures were established to bait the whole AMF diversity, although only two more AMF *Glomus* morphotypes were found in the late-seral undisturbed area, as compared to the spores directly collected from the field. In both soils, the most abundant species was *G. etunicatum*. Other AMF species abundance differed 57%, according to the Kulenzynki indices. The major difference between the two sampled soils was in spores density: 154 ± 69 spores *per* 100g of undisturbed soil from an undisturbed area (mean ± SE; n= 3 mixed soil samples) and 21 ± 4 spores *per* 100g of non-inoculated disturbed soil.

Table 3.2. Indigenous AMF community composition from disturbed soil (early-seral AMF community) and undisturbed soil (late-seral AMF community). Spore density is expressed as the number of spores per 100g soil dry weight (n=3).

UNDISTURBED SOIL	**Spore density**	**DISTURBED SOIL**	**Spore density**
AMF SPECIES		**AMF SPECIES**	
Glomus constrictum Trappe	24.4	*Glomus constrictum* Trappe	2.2
Glomus etunicatum Becker & Gerdemann	14.4	*Glomus etunicatum* Becker & Gerdemann	5.6
Glomus geosporum (Nicol. & Broome) Walker	2.2	*Glomus geosporum* (Nicol. & Broome) Walker	2.2
Glomus mosseae” group” (Nicol. & Gerd.) Gerd. & Trappe	*	*Glomus mosseae*” group” (Nicol. & Gerd.) Gerd. & Trappe	4.4
Glomus etunicatum Becker & Gerdemann # 2	54.4	*Glomus etunicatum* Becker & Gerdemann # 2	6.7
Glomus intraradices Schenck & Smith	4.4		
Glomus coronatum Giovannetti	4.4		
Glomus cf. *diaphanum* Morton & Walker	5.6		
Glomus fasciculatum (Thaxter) Gerd. & Trappe emend. Walker & Koske	1.1		
Glomus sp. 1	1.1		
Glomus sp. 2	*		
Scutellospora calospora (Nicol. & Gerd.) Walker & Sanders	5.6		
Archaeospora trappei (Ames & Linderman) Morton & Redecker	7.8		
Paraglomus occultum (Walker) Morton & Redecker	4.4		
Sclerocystis rubiformis Gerd. & Trappe	20.0		
AMF spores (not identifiable)	4.4		

* Only identified from trap cultures.

3.2. AMF EFFECTIVITY ON PLANT GROWTH

Plant height over time

Mycorrhizal treatments had significant effects on myrtle growth (Table 3.3). The significant AMF treatment by time interaction (AMF × Time) indicated that the myrtle growth rate over time depended on the AMF communities present in the soil. For carob and lavender plants species no significant AMF treatment effect was found, demonstrating that differences induced on plant growth by AMF communities were also plant species dependent.

Data analysed in Table 3.3 are represented in Fig. 3.3 In the first 135 days, the inoculated disturbed soil seemed to be the most effective AMF treatment, inducing a faster plant growth rate for all target plant species. Time had a significant within-subjects effect for all tested plant species, independently of the AMF treatment applied (Table 3). Carob and myrtle followed similar growth rate patterns under the different AMF treatments over time. Lavender plants grew continuously in disturbed soil AMF community, becoming the tallest plants at the end of the experiment. Plants with commercial inoculum reached the height plateau at day 180.

Table 3.3. *F*-values with significance levels are given for repeated measure analyses of variance (ANOVAR) of the height of three target plant species grown in soils with three different AMF communities: from late-seral undisturbed area, non-inoculated disturbed soil and disturbed soil inoculated with commercial inoculum containing only *G. intraradices*.

SOURCE OF VARIATION	**Carob**	**Myrtle**	**Lavender**
WITHIN-SUBJECTS **(GREENHOUSE-GEISSER)**			
TIME	166.20**	350.26**	47.45**
TIME × AMF TREATMENT	1.95	4.09*	2.43
BETWEEN-SUBJECTS			
AMF TREATMENTS	2.61	12.91*	2.95

* $P < 0.05$; ** $P < 0.001$.

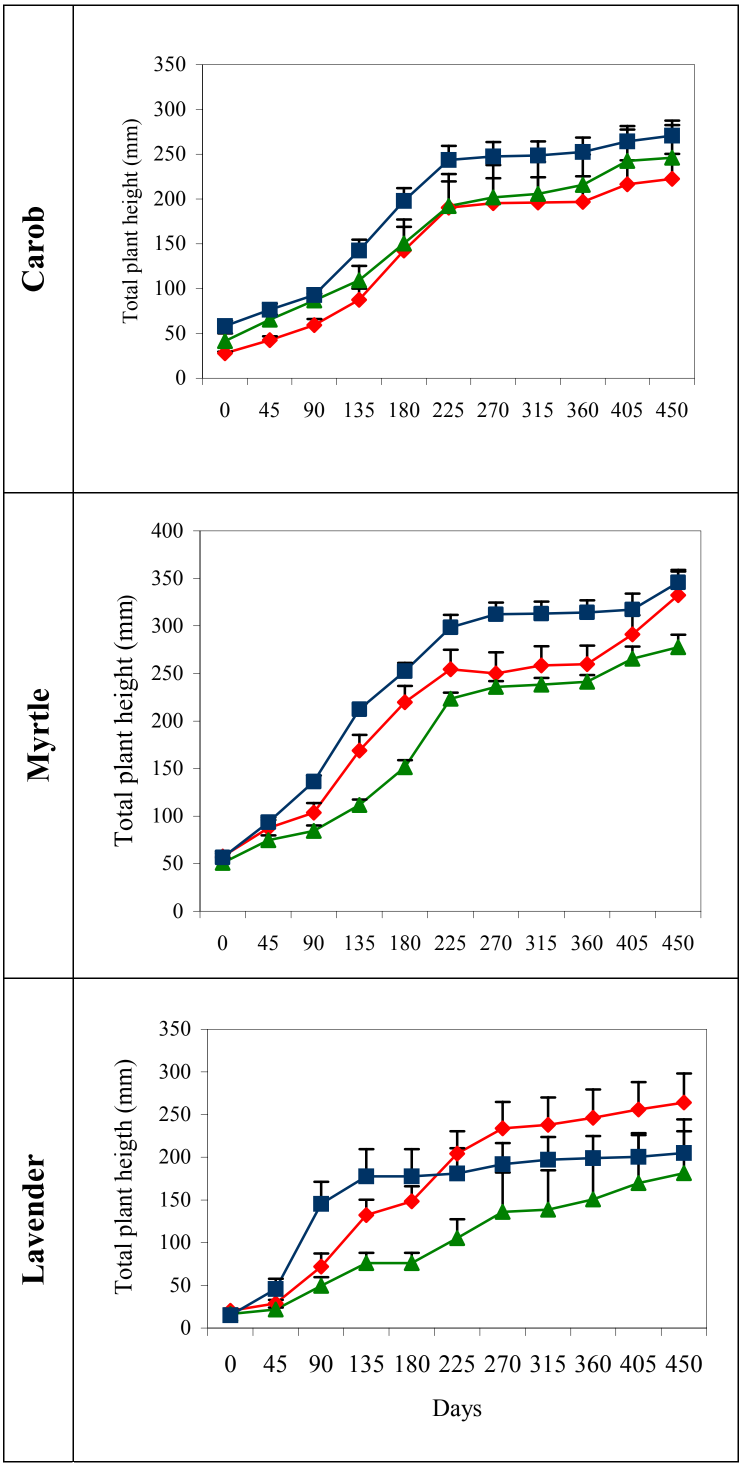

Figure 3.3. Time-course effect of the AMF treatments on the heights of three host-plant species. Seedlings were grown in soil containing different AMF communities: from late-seral undisturbed area (▲); non-inoculated disturbed soil (♦); and disturbed soil inoculated with commercial inoculum containing only *G. intraradices* (■).Vertical bars show the standard error of the mean at each date (n=12 plants).

3.3. ROOT COLONIZATION

Root colonization was significantly influenced by the AMF treatments, and differed with plant species and time (Table 3.4). Time had a positive significant effect in all AMF treatments, which was particularly evident for carob (Fig. 3.4). After six months of growth, carob plants presented similar low levels of root colonization despite of the large differences in AMF spore density and AMF species diversity between AMF treatments (Table 3.2). After 12 months, root colonization from the inoculated and non-inoculated AMF disturbed soil treatments increased significantly, while the AMF from undisturbed soil colonized the roots with a similar percentage of colonization at the two harvest times. Therefore, by the end of the experiment this treatment presented a significantly lower root colonization was than the others. Myrtle plants grown in the inoculated disturbed soil had higher percentage of root colonization than in the two non-inoculated soils, although it was not significantly different from the non-inoculated disturbed soil at 6-month harvest. For lavender, the inoculated disturbed soil presented higher percentage of root colonization than the two non-inoculated soils, although it was not significantly different between the two harvest dates.

Table 3.4. *F*-values with significance levels are given for three-way ANOVA of AMF soil community, plant species and harvest time effects on root colonization, plant biomass and soil AMF infectivity of three target plant species grown in soils with three different AMF communities: from late-seral undisturbed area, non-inoculated disturbed soil and disturbed soil inoculated with commercial inoculum containing only *G. intraradices.*

SOURCE OF VARIATION	ROOT COLONIZATION	PLANT BIOMASS	SOIL AMF INFECTIVITY
AMF df= 2	12.3***	11.2***	29.1***
Plant species df= 2	16.2***	30.6***	10.1***
Harvest Time df= 1	19.6***	67.0***	19.1***
AMF × Plant df= 4	4.1**	3.0*	2.7*
AMF × Time df= 2	0.6	2.1	5.8**
Plant × Time df= 2	3.2*	4.7*	1.2
AMF × Plant × Time df= 4	3.0*	1.9	0.3

$P<0.05$; **$P<0.005$; *** $P<0.001$

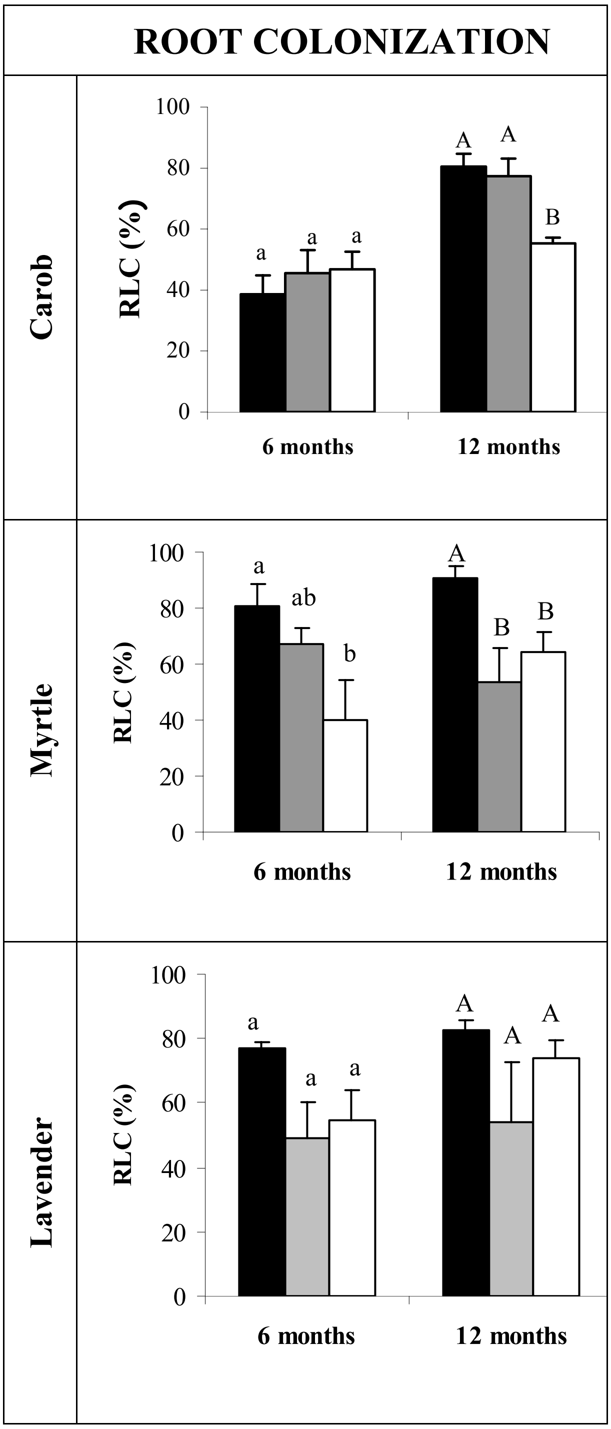

Figure 3.4. Root length colonization at plant harvest after 6 and 12 months of three target plant species grown in soils with three different AMF communities: (■) from late-seral undisturbed area; (■) non-inoculated disturbed soil; and (□) disturbed soil inoculated with commercial inoculum containing only *G. intraradices*. An one-way ANOVA for each host plant species at each harvest time was performed. Values (means ± SE of six replicates) followed by the same letter are not significantly different ($P<0.05$, Tukey test).

From microscopical observation, and based on *G. intraradices* typical morphological features (spores inside the roots) it was possible to confirm that the roots from the inoculated disturbed AMF treatment were mainly colonized by this species comparatively to other AMF species present in the disturbed soil. After six months, the woody Mediterranean plant roots were already heavily colonized and so remained throughout the experimental time. This result suggests that *G. intraradices* spores were able to persist in the disturbed soil and compete for the target plant roots with the indigenous AMF species from the disturbed soil.

3.4. PLANT BIOMASS

For plant biomass, there was a significant main effect of AMF treatments, plant species and harvest time ($P<0.001$), as well as a significant interaction between AMF treatments × plant species and plant species × time, showing that the AMF effectiveness in promoting plant growth was dependent on plant species, and varied along the time (Table 3.4). Carob biomass differences between the AMF treatments were only significant after 12 months of growth, while myrtle biomass responded sooner to the different AMF treatments (after 6 months) (Fig. 3.5). In general, myrtle and carob plants growing in disturbed soil with or without inoculation had significantly higher biomass than those growing in undisturbed soil (Fig. 3.5). For lavender, there were no significant biomass differences between AMF treatments (Fig. 3.5), mainly due to the high plant variability within each AMF treatment. The main effect interactions found by the three-way ANOVA were primarily induced by lavender biomass data and by the undisturbed AMF treatment data. The subsequent two-way ANOVA for each host plant species revealed no differences in plant biomass due to disturbed soil AMF inoculation.

3.5. PLANT EFFECT ON AMF COMMUNITY GROWTH

The capability of the target plant species to induce a soil enrichment with infective AMF propagules varied significantly ($P<0.001$) with plant species, AMF treatment and harvest time (Table 3.4). The significant AMF treatment × plant species interaction indicated that the enhancement of infective AMF propagules depended on the combination between each target plant species and each AMF community present in the soil. These results demonstrated the importance of choosing the appropriate plant species for the AMF

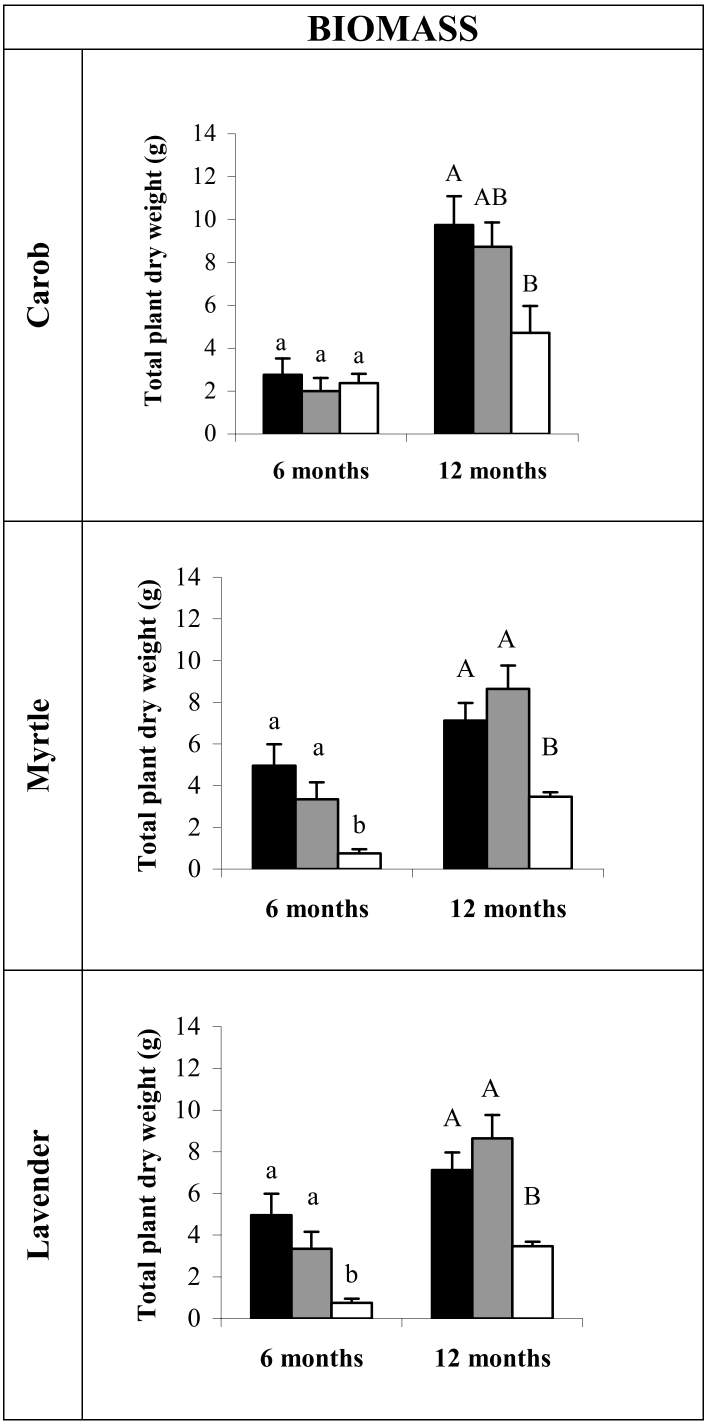

Figure 3.5. Plant biomass at plant harvest after 6 and 12 months of three target plant species grown in soils with three different AMF communities: (■) undisturbed soil; (■) non-inoculated disturbed soil and (□) disturbed soil inoculated with commercial inoculum containing only *G. intraradices*. A one-way ANOVA for each host plant species at each harvest time was performed. Values (means ± SE of six replicates) followed by the same letter are not significantly different (*P*<0.05, Tukey test).

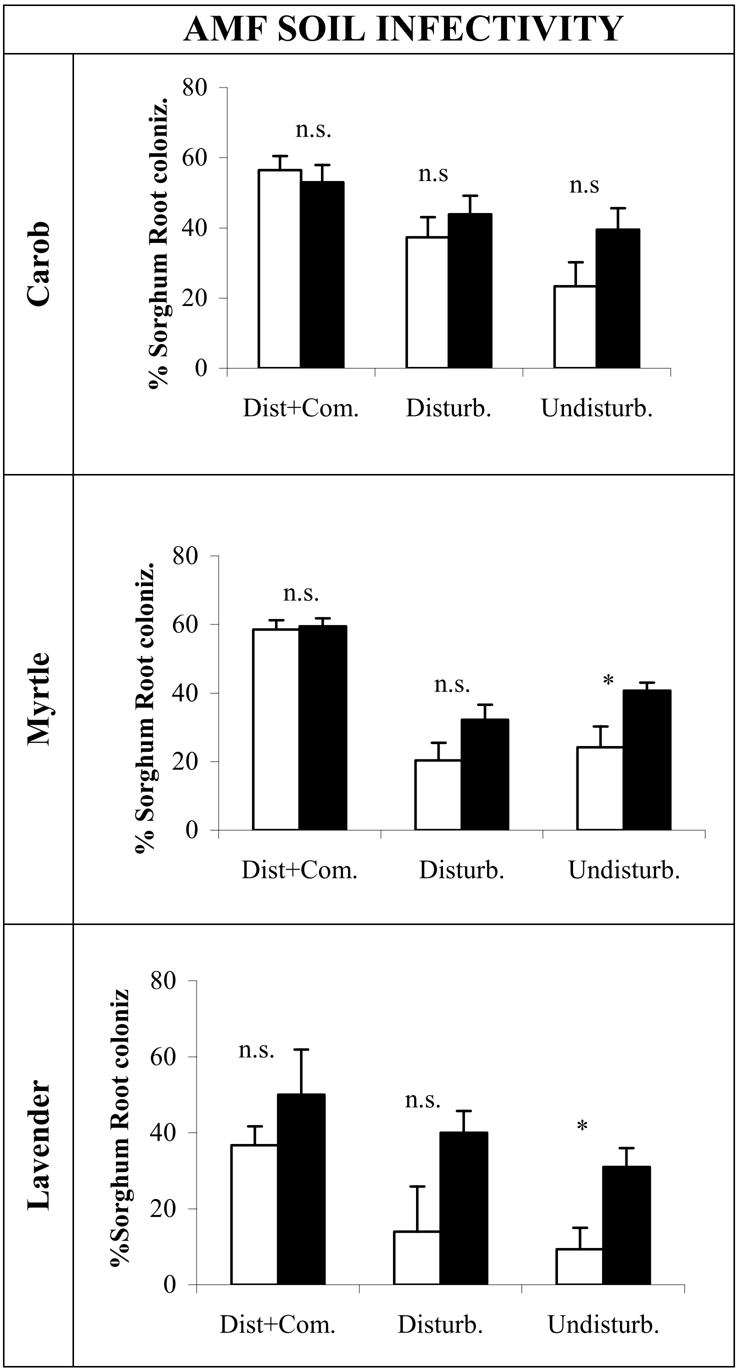

Figure 3.6. Variation of AMF soil infectivity promoted by three different host-plant species, measured by *Sorghum* bioassay. Open bars indicate harvest time after 6 months of plant growth and filled bars indicate harvest at 12 months of plant growth. A t-test for each host plant species grown in each AMF community tested was performed to compare the two harvest times. Values (means ± SE of six replicates) followed by an asterisk are significantly different ($P<0.05$).

community present in the soil to be restored. An AMF treatment × time significant interaction indicated that soil inoculum built up over time occurred naturally in the presence of every plant species, but this increment was dependent on the AMF community (Table 3.4, Fig.3.6). Myrtle and lavender plants were able to significantly increase the AMF community infectivity in the late-seral undisturbed soil (undisturbed treatment) over time. Myrtle and carob built up the different AMF community infectivity similarly, although carob did not significantly increase any AMF community. *Sorghum* root colonization on lavender soil rhizosphere after six months of growth presented the lowest absolute values, as compared to the other host plants. This was probably due to the smallest root plant biomass displayed by lavander (data not shown). By the end of the experimental time these plants achieved values similar to those of the other two host plant species, suggesting that root structure and biomass influence the natural AMF communities build up. Due to external AMF propagules addition, the inoculated disturbed treatment presented initially the highest soil infectivity, and that infectivity remained through the assay; no other AMF treatment increased infectivity values as high as those of the inoculated disturbed soil. The indigenous AMF treatments had the highest soil infectivity increment regardless of the host plant species.

4. DISCUSSION

AMF communities effect on plants growth

Disturbance had a negative effect on AMF community composition. The disturbed AMF community had lower AMF species diversity and abundance, although AMF infectivity for all woody native plants species was not reduced.

Regardless of the AMF treatment, height was significantly affected by time, indicating different growth patterns among plant species, probably related to their phenological characteristics. The height growth pattern was significantly affected by AMF treatment in myrtle, with an apparent positive effect of disturbed soil treatments in spring-summer (myrtle and lavender). During this period, the addition of the commercial inoculum seemed to be somewhat advantageous to plant height growth, but this benefit decreased along the experiment, probably because the cost of the symbiosis exceeded the benefit. This is probably due to an increase of carbon drainage from the plant to the fungus, since a significantly large extent of root colonization in inoculated myrtle was observed. In addition, there are reported data showing that *G. intraradices* has higher costs (carbon) for plants than gains (nutrients) (Klironomos *et al*., 2000).

In the evaluation of the AMF inoculation effectivity, the results from biomass evaluation support the null hypothesis: adding commercial AMF inoculum or using non-inoculated disturbed soil, containing only indigenous AMF species, were equally efficient in promoting plant growth (biomass).

Even though in this study increasing the diversity and density through AMF addition did not improve plant biomass, the symbiotic infectiveness and effectiveness were significantly dependent on the specific interaction between host plant species and AMF communities. Lavender plants responded similarly to the AMF treatments. Myrtle and carob presented lower root colonization and biomass when growing with the undisturbed AMF community, in contrast with the hypothesis that plants would perform better in this soil community. The results here presented confirm the hypothesis that plant growth (biomass) response was plant species-specific. Time did not change plant biomass response to the addition of commercial inoculum like in other studies (Fagbola *et al*., 2001; Kaliq and Sanders, 2000; Bell *et al*., 2003), but carob had a differential plant growth response between disturbed AMF community (non-inoculated and inoculated) and undisturbed AMF community along time.

In a study where myrtle seedlings were inoculated with indigenous endophytes from a Mediterranean area or with *G. intraradices*, Matosevic *et al*. (1997) found that plants grew more with the indigenous fungi, despite the fact that both treatments displayed similar levels of root colonization. Other studies in Mediterranean ecosystems, comparing plant response to indigenous and introduced AMF, did not find an additional growth benefit due to inoculation (Clemente *et al*., 2004; Gazey *et al.,* 2004; Pattinson *et al.,* 2004) and the results may have varied with tested host plant species and the indigenous AMF species already present in the disturbed soil (Palenzuela *et al*., 2003; Caravaca *et al*., 2003a, 2004, 2005).

Since it is impossible to separate cause and effects of these mutual plant-AMF interaction, to explain the results of the different plant species responses to the tested AMF communities, the aspects concerning both mycorrhizal symbionts have to be considered: (1) the woody native plants' life-strategy, and/or, (2) the hypothetical functional compatibility with different AMF communities.

The first aspect is based on differences in the phenological and morphological adaptations of the tested plants to the Mediterranean environmental conditions (poor soil nutrition, water stress and high temperatures). Carob trees and myrtle shrubs are evergreen species, whereas lavender shrubs are semi-deciduous species (see Table 1.2). Therefore, their biomass gain and main stem growth vary among the different seasons of the year, independently of the mycorrhizal colonization, and that was evident in the results concerning the variation of plant height growth over time. Evergreen sclerophyllous shrubs and trees grow intensively during a short period in spring, while semi-deciduous plants such as lavender grow all over the year, except for a short period during summer. Productivity and survival of Mediterranean plants are a result of complex growth adaptations that may act as confounding factors when plant growth response is evaluated to measure AMF beneficial inoculation. Moreover, plant dependency on mycorrhizae is also different: facultative for carob and myrtle, dependent in the case of lavender.

The second aspect, concerning the indigenous AMF community was based on recent studies that have shown some degree of specific adaptation of indigenous AMF species to their soil environment (Schultz *et al*., 2001), as well as some functional specificity between host plant ecotypes and their symbiotic fungal communities (Sanders, 2002; Ronsheim *et al*., 2001; Sylvia *et al*., 2003). In addition, Klironomos (2003) demonstrated that plants respond differently to local and foreigner AMF taxa, and that the growth response variability was increased when plants were inoculated with local AMF. These findings may explain why mutualistic symbiotic functionality of these indigenous AMF communities remained, inducing a plant biomass similar to the one found in the commercially inoculated disturbed soil, independently of the low AMF species diversity and abundance. Moreover, plants in close systems like pots or monocultures are referred as being able to increase the frequent occurrence of positive feedbacks by selecting AMF species that can optimize their own growth (Johnson *et al*., 1992; Burrows, 2001), while some aggressive AMF species like *G. intraradices* can impair host plant growth, stimulating negative feedbacks interaction. In conclusion, if functional compatibility between indigenous AMF and native plants existed, more effective associations were established within AMF community from the disturbed soil than with *G. intraradices*. Then it would be advantageous in nurseries promoting indigenous AMF inoculum with native plants, instead of inoculating with available commercial AMF inoculum.

The presented results do not support the expected optimal plant growth in the AMF undisturbed community for none of the tested plant species. Although the undisturbed soil had higher AMF species diversity and number of spores than the disturbed community, this did not result in the hypothesized enhancement of mutualistic preferential plant-AMF pair associations. Although the results of indigenous AMF community from disturbed soil point to some preferential association with native plants species, this is not likely extensible to the indigenous AMF species from undisturbed communities. One possible reason to explain these infectivity and effectivity differences between the tested non-inoculated indigenous AMF communities is based on theoretical AMF successional models (see Hart *et al.*, 2001), where disturbed soil AMF community represents an early successional community resulting from stockpiling soil disturbance, and the undisturbed soil represents undisturbed mid- late successional stage. The present results are consistent with previous studies that compared early- and late seral inoculum tree response (Kiers *et al.*, 2000; Allen *et al.*, 2003; Lovelock *et al.*, 2003). In nursery and field studies, Allen *et al.* (2003) showed that early-seral AMF inoculum consistently promoted growth benefit for six woody late-seral plants, while late-seral inoculum showed no benefit for restoration seedling establishment. They suggested early-seral inoculum is the most appropriated to be used in nurseries, because plants responded significantly to the inoculum with increased height in the first year. We suggested that host plant growth response to the AMF community from undisturbed soil cannot be considered as a reduced growth response comparatively to the other AMF treatments, but rather as a natural average growth response from the woody native plant interaction with their AMF community and other soil microorganisms. Although the diversity of non-mycorrhizal soil microorganisms was equalized in all treatments by using soil washing dilution, it is improbable that species diversity and abundance did not differ among the AMF community treatments. Thus, the possible different interactions among plant-AMF-soil organisms could explain the different results obtained among the tested AMF communities.

Plant effect on AMF community growth

As hypothesized initially, AMF inoculum increased over time in the target plant rhizosphere, though the increment rate depended on plant species-AMF community interaction. None of the tested plant species was able to increase the target AMF communities. A significant enhancement of infective soil propagules only occurred with the shrub species (myrtle and lavender) and the late-seral indigenous AMF community from undisturbed soil. These results were in agreement with previous studies showing that AMF communities were differently influenced by the host plant species (Johnson *et al.*, 1992; Eom *et al.*, 2000; Burrows and Pfleger, *2002*). According to Hart and Reader (2002a,b), differences found in AMF soil infectivity over time can be explained by differences in AMF community species composition. The early-seral indigenous AMF community of the disturbed soil contains only *Glomus* species due to disturbance pressure. The same authors demonstrated that *Glomus* species were the fastest and most extensive root colonizers, while other AMF taxa were slow root colonizers although they produced more extra radical mycelium. These findings may explain the significant AMF soil infectivity increment recorded in the undisturbed AMF treatment, which contains other indigenous AMF species besides *Glomus*. Previous studies in Mediterranean restoration that evaluated indigenous AMF inoculum also concluded that woody shrub species were able to enhance AMF propagules in their natural rhizosphere and that the increment rate differs among the tested plant species (Azcón-Aguilar *et al*., 2003; Caravaca *et al*., 2003b). This reinforces the suggestion for restoration projects that an appropriate host plant species selection can effectively build up the remaining indigenous AMF community (Azcón-Aguillar *et al*., 2003), which is important because mycelium network is likely to increase the structure stability of the disturbed soil (Miller and Jastrow, 2000), and can facilitate the establishment of mycorrhizal plant species dispersed into the site as seeds.

Conclusions and suggestions for AMF manipulation

In the present study plants did not benefit from AMF commercial inoculation, although no simple generalization concerning quarry restoration in Mediterranean ecosystems can be made. From a practical stand-point, for decision-makers in quarry restoration this study demonstrated the importance of conducting experimental assays similar to this, in order to determine whether the chosen AMF commercial inoculum will bring any advantages to the target plants, or whether the indigenous AMF propagules density does not limit the effective establishment of mycorrhizal associations. It is also important to test if the restoration target plants are suitable “nurse plants” to maintain and increase the AMF community in disturbed soil. Despite recent AMF ecology research attempts to show AMF functional specificity (Sanders, 2002, 2003), it is still not possible to predict the AMF growth effect on particular plant species under specific environmental conditions, not even for AMF species largely tested like *G. intraradices*. The use of commercial inoculum has unpredictable results, since it also depends on soil fertility, and on the ability of the introduced commercial AMF inoculum species to survive in the ecosystem and to be effective to the target restoration plant species.

In this plant nursery assay, it was also shown that *G. intraradices* was able to compete and persist among the indigenous AMF species from disturbed soil, although the outcome of the introduction in the field of this novel AMF species in the existing AMF community cannot be predicted. Further studies should be conducted to evaluate life strategies of specific AMF species commonly used as AMF commercial inoculum, assessing their persistence and competitive abilities (Hart *et al.*, 2001), in order to improve our capability to predict future implications for the restored plant and AMF community succession.

Chapter 4 - DOES AMF SOIL COMMUNITY MEDIATE PLANT-ROOT ENDOPHYTIC FUNGI INTERACTIONS?

1. INTRODUCTION

The structure and functional diversity of microbial communities in the soil are tightly related to plant productivity and community, thus establishing an important link between above and below ground processes in terrestrial ecosystems (Packer and Clay, 2000; Wardle, 2002; Bonkowski and Roy, 2005). Most of the biotic activity in the soil occurs in the rhizosphere (Fitter and Garbaye, 1994). Rhizospheric fungi are important regulators of plant productivity and of soil nutrient cycles (Allen, 1991; Klironomos *et al*., 1997). They are a diverse and ubiquitous set of heterotrophic microorganisms that can function as decomposers, pathogens, parasites and mutualistic symbionts. These plant-fungal interactions contribute to dynamic feedback interactions (eg: Bever, 2002; Klironomos, 2002; Reynolds *et al*., 2003; Wardle *et al*., 2004). Positive feedbacks occur when plant species change the rhizospheric microbial community in soils in a way that enhances the beneficial effects on the plants that cultured them. Negative feedbacks occur when plant species accumulate pathogenic microbes in their rhizospheres, creating conditions for a decreased performance of that plant species in comparison with others. Yet, negative feedback may promote species diversity in plant and soil communities (van der Putten *et al.,* 1993; van der Heijden *et al*., 1998a; Kiers *et al*. 2000; Klironomos, 2003; Callaway *et al.,* 2004), while positive feedback may either enhance (Molofsky *et al*., 2001) or reduce plant diversity (Bever*,* 1999). The mutualistic interaction in mycorrhizal symbiosis is generally considered to be a positive feedback, but it is possible that the interactions between plants and arbuscular mycorrhizal fungi (AMF) species generate a negative feedback (Bever, 1994). Differential effects of AMF on the growth of different plant species have already been demonstrated (Streitwolf-Engel *et al.* 1997; van der Heijden *et al*., 1998; Klironomos, 2003) and, conversely, that plants can affect density and composition of soil AMF communities (Johnson *et al*., 1992; Eom *et al.,* 2000; Burrows and Pfleger, 2002; Lovelock and Ewel, 2005).

Mycorrhizal fungi also interact with a wide range of other microorganisms in the rhizosphere and inside the roots, modifying the interactions of plants with pathogens, such as root-inhabiting nematodes and fungi, and with mutualists, such as nitrogen-fixing bacteria and other symbiotic fungi (Barea *et al.,* 2002; van der Putten, 2003; Reynolds *et* *al.,* 2003). For instance, Marschner *et al.* (1997) found that plants colonized by *Glomus intraradices* may reduce root exudates and thereby decrease the population density of certain bacterial groups, namely *Pseudomonas fluorescens*. The complexity of the interactions among plants, mycorrhizal fungi, and non mycorrhizal endophytic fungi (saprobes, parasites and decomposers) causes great difficulty to determine the contribution of each fungal functional group to plant growth. In addition, it has been shown that the diversity of root endophytic fungi is high. For example, 49 species of fungi were identified in the roots of a single grass plant, *Arrhenatherum elatius* (Vandenkoornhuyse *et al.*, 2002), and 82 genetically distinct endophytes were described in western white pine (Ganley *et al.*, 2004). The majority of non mycorrhizal endophytes appear to be neither parasites nor simple decomposers (Ganley *et al.*, 2004), existing symbiotically in host plants, also contributing to plant growth (Mucciarelli *et al.*, 2003). The results found by Lucero *et al.* (2006) in arid and semi-arid ecosystems, demonstrated that plant-fungal interactions can induce plant morphology and biomass differences when shoot and root endophytes from a plant species were transferred to other plant species and *vice-versa*. Possibly, different endophytes are responsible for the variability in performance among individual plants. Therefore, small trade-offs in plant fitness are due to general functional shifts at microbial level, implying at a larger scale significant differences in the outcome of plant communities competition (Bonkowski and Roy, 2005).

In this study, it was tested whether plant-root endophytic fungal (including both mycorrhizal and non mycorrhizal fungi) feedback occurs between woody Mediterranean plant and indigenous soil fungal community, and if changes in soil AMF community alter the plant-root endophytic fungal interaction (Fig. 4.1). This study will have broad implications for the success of a quarry revegetation, evidencing soil microbiology importance on soil selection and the possible advantages of introducing commercial AMF inoculum as a possible mediator on the plant-root endophytic fungal feedback. Since for quarry restoration almost all abiotic and biotic variables can be adjusted artificially, it becomes a good system to evaluate plant-microbial interactions. In this study, we selected two biotic factors that are relevant for plant production in the nurseries for restoration: (i) AMF communities from different soil origins used in restoration trials, and (ii) rhizospheric soil communities, obtained by pre-cultivating the different soils with different host plant species. This procedure relies on what has been largely suggested in the literature, *i.e.*, an increase of species-specific plant fungal pathogens can be responsible for detrimental plant growth, thus inducing plant-soil microbial negative feedback (Packer and Clay, 2000; Klironomos, 2002; Callaway *et al.*, 2004). Besides the mycorrhizal fungi, only the non mycorrhizal fungi were examined because the majority of root plant parasites and pathogens are fungi. To test the possible existence of plant-root endophytic fungal negative feedback, the biomass of a second generation of plants growing in soil communities pre-cultivated by conspecifics and heterospecifics were compared. To test if AMF mediate the feedback between plants and root endophytic fungi, the plant growth response to the previous plant-fungal interactions was compared between the different AMF communities.

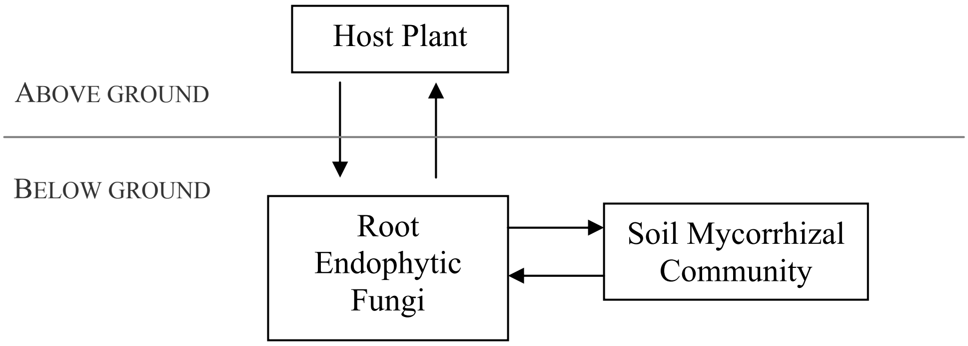

Figure 4.1. Schematic diagram of hypothetical plant-root endophytic fungal feedback mediated by the soil mycorrhizal community. Soil mycorrhizal community may mediate root non mycorrhizal endophytes by competing for the roots, as well as change plant chemical defenses. Moreover, root colonization by endophytic fungi will depend on each AMF species life traits from the soil community and on host plant-AMF species "preference". This diagram scheme will be followed to test for possible advantages in adding commercial AMF inoculum to the soil as a mediator of plant species-root endophytic fungal feedback. The plant benefits from the addition of commercial mycorrhizal inoculum as a biocontrol agent rely on the assumption that soil AMF communities mediate plant-root endophytic fungal feedback. It is expected that AMF species from commercial inoculum will compete with indigenous fungi (mycorrhizal and non mycorrhizal) for the root colonization interfering in the outcome of plant-root endophytic fungal interaction.

As part of these objectives, specific hypotheses were developed based on previous scientific work. It was proposed, based on the negative feedback model (Bever *et al.*, 2002), that plants growing in conspecific soil microbial communities would probably grow less than plants growing in heterospecific soil microbial communities. Additionally, the plant growth response to the heterospecific soil microbial communities would be differential, depending on the plant species used to pre-culture the soil (first generation), due to species-specific fungal pathogens. This means that each plant species would cause differences in soil rhizospheric microbial community and, therefore, those changes would reflect differences in the growth of plants from the next generation. A disturbed soil fungal community is expected to have less spore density and species diversity (mycorrhizal and non mycorrhizal fungi) than a soil community from an undisturbed area (Johnson *et al.*, 1992). The effect of disturbance, due to long term soil stockpiling, on the AMF communities, reducing their abundance and diversity, may also create a selective pressure effect. The early AMF species then selected may not be so effective for late successional woody plants (Hart *et al.,* 2001). Moreover, due to the absence of plant host species in the stockpiled soil, a reduction of the species-specific pathogens is expected. Consequently, for our experimental assay it was hypothesized that plants growing with the disturbed soil microbial community would benefit from a positive plant-fungal feedback, while plants growing in the undisturbed soil community would establish a negative plant-fungal feedback.

The same response variables were also used to test if commercial inoculum addition (*G. intraradices* spores) to the disturbed soil from stockpile would change AMF soil community, mediating the plant-root endophytic fungal interaction (Fig. 4.1). Based on studies from Graham and Abbott (2000), and Hart and Reader (2002b), it was expected that such an aggressive species such as *G. intraradices* would compete for plant roots with indigenous AMF species in the soil, as well as with non mycorrhizal fungal species. Therefore, it was expected that the diversity and root colonization of non mycorrhizal endophytic fungi would be lower in the presence of *G. intraradices*, leading to a possible plant-root endophytic fungal positive feedback.

2. MATERIALS AND METHODS

Study site

The study site is the same as the one described in the previous chapters. The quarry is located in a Mediterranean Natural Park (Outão, Serra da Arrábida;). The reference undisturbed area was adjacent to the quarry and it is representative of a middle-late seral native Mediterranean plant community (for further details of the site, see *Chapter 1*).

Soil collection and analyses

The two soils for the study were collected as described in the previous chapters: the undisturbed soil was collected from a well-preserved Mediterranean *maquis*, in the vicinity of the quarry, while the disturbed soil was collected from stockpiled soil within the quarry area. A detailed soil analysis is displayed in Table 2.1 (*Chapter 2*). The soils were sterilized following the procedure described in the previous chapter, in order to even nutritional discrepancies among soils that could induce differences in plant growth, and simultaneously to keep the AMF community from each soil intact.

Experimental design and set-up

A randomized factorial experimental design with two independent variables was devised: soil AMF communities (from the undisturbed quarry surrounding area, from stockpiled soil, and from stockpiled soil inoculated with AMF commercial inoculum), and soil community pre-cultured by host plant species rhizosphere: *Ceratonia siliqua* L. (carob tree), *Myrtus communis* (myrtle), and *Lavandula stoechas* L. (lavender).

The soil from the undisturbed area represented the natural species diversity and abundance of AMF community in this type of Mediterranean ecosystem. The stockpiled soil contained only the disturbed fungal community. The sterilized soil with the AMF commercial inoculum represented a model system of what may occur in a soil community when commercial AMF inoculum is applied, namely in the production of plants that are going to be used in revegetation trials. Therefore, this treatment enables the evaluation of the interaction of indigenous AMF species with one selected aggressive mycorrhizal species, and also with the non mycorrhizal soil community. Commercial inoculum was purchased from PremierTech, Canada; 4 000 spores of *Glomus intraradices* Schenck & Smith were added per plant.

In January 2001, seedlings of the three different host plant species were individually planted in 6 L pots with different soil AMF communities, as explained below. Plants grew under nursery conditions, were watered as necessary, and the pots were spatially arranged in a completely randomized design. During two years, each host plant built up its own soil community, conditioning the original soils and originating three different fungal communities per each initial soil treatment.

Field soil inoculum was used in order to preserve the full spectrum of AMF species (regardless of each species ability to initiate new infections from spores and/or colonized root pieces) in the relative proportions present in the tested soils (procedure similar to Burrows and Pfleger, 2002). The whole soil inoculum from each soil origin was not used, to avoid the soil “black box” effect. A new experimental set up varying only in the natural AMF abundance and diversity from each soil community treatment was proposed. For the disturbed and undisturbed soil community treatments, both soils were mixed to homogenize the nutritional levels. One of the soils was previously sterilized to eliminate the AMF community, contributing only to the mineral component of the mixture, and subsequently the microbial wash from the corresponding sterilized soil was added (see Fig. 3.2). Soil sterilization was obtained through autoclaving at 1 atm for 1 h and two consecutive days. Although the addition of microbial wash did not replace non mycorrhizal soil microorganisms abundance, it allowed to equalize their diversity. Microbial wash corresponded to the addition of 250 ml of soil filtrate to each plant replicate (pot). The filtrate was prepared by suspending 200 g from each soil in 1000 mL sterile water for 60 min. After shaking and decanting, the suspension was filtered twice (<20 mm). For the soil inoculated with commercial AMF, the same procedure was followed as in the disturbed soil treatment. The undisturbed soil was sterilized, and microbial wash from this soil was added. In conclusion, all soil community treatments had the same nutrient levels and the same diversity of non mycorrhizal communities, varying only in the AMF communities of each soil origin. Therefore, the differences in soil fungal communities resulted from the combination of each one of the three soil origins driven by the three host plant species rhizospheres, resulting in nine different soil community treatments (Fig.4. 2).

In January of 2003, the shoots of each plant species were cut and, under the same growing conditions, a second generation of seedlings (myrtle) were immediately planted (one per pot; six replicates per treatment) with the nine soil fungal community treatments; three of them were conspecific soil community (“pre-cultured” by its own species), and six were heterospecific (“pre-cultured” by lavender and carob tree plants) (Fig. 4.2). With this procedure it was probable that external AMF mycelium from the previous host plant would infect the new seedling roots and, therefore, AMF diversity would be maintained.

Harvest and material analyses

The second generation of myrtle plants were harvested in January 2004, after one year of growth. The dependent variables analysed were: plant biomass; AMF and non-AMF percent root colonization; AMF species diversity and abundance; and species diversity and abundance of non mycorrhizal endophytic fungi. For all variables, six plants (replicates) *per* treatment were collected, except to assess soil AMF species abundance and diversity where 100 g of fresh weight soil were collected from the rhizosphere of three plants (replicates) *per* treatment.

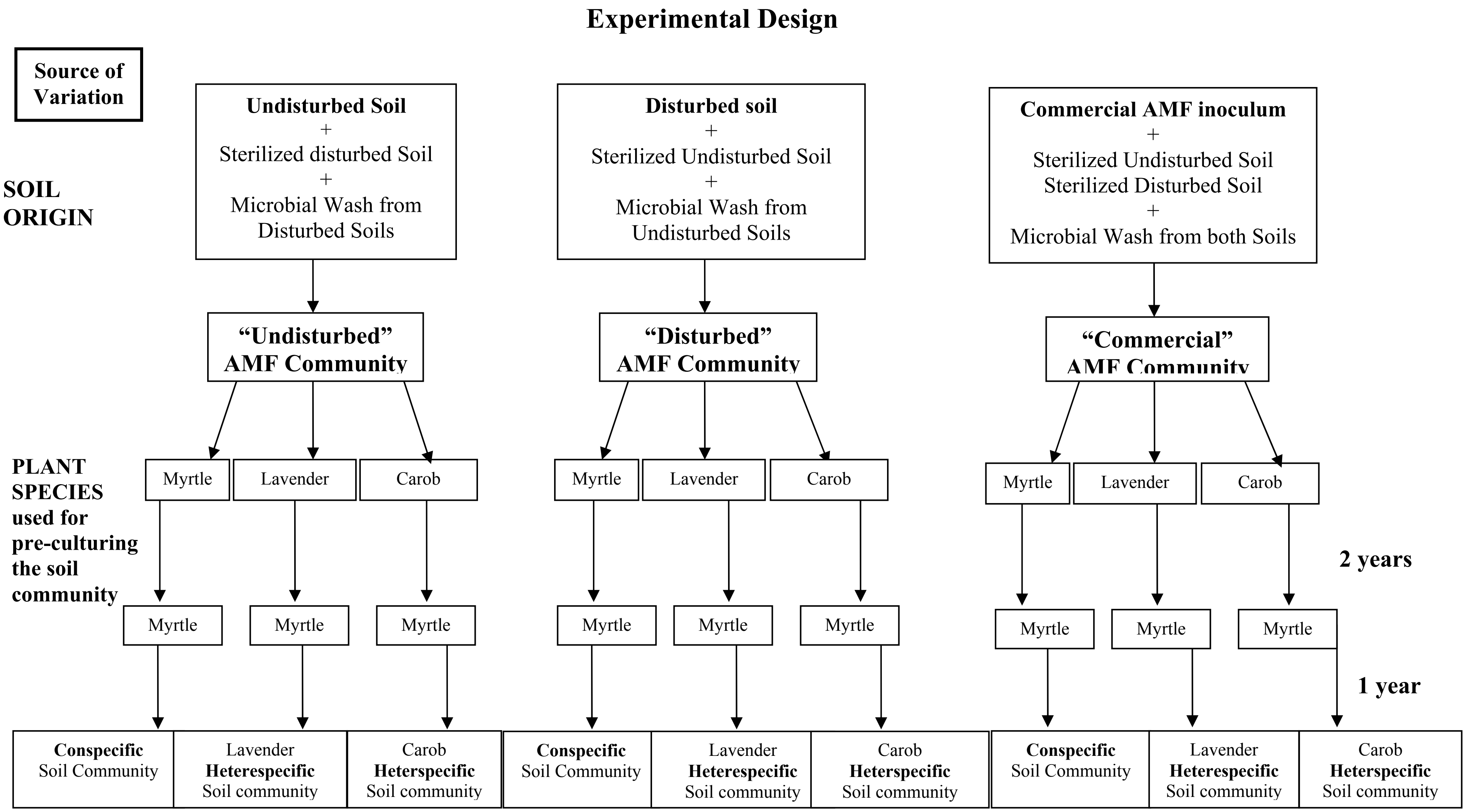

Figure 4.2. Schematic representation of the experimental set-up.

Plant biomass and root colonization

Plant material was cleaned and fresh weight determined. Two root sub-samples (0.5-1 g each) were removed from each root system. One sub-sample was immediately used to assess non mycorrhizal abundance and diversity, and the other sub-sample was stored in 50% ethanol for later determination of AM colonization. The latter samples were stratified, only the youngest part of the roots (the first 3-5 cm above the root tip) was collected and not the entire root length. This was done since the woody root characteristics would make the application of the methodologies difficult or would even prevent the root microscopical observation. Total plant dry weight was determined after drying at 60ºC for 3 days. Fresh weight ratio of the roots was used to calculate the dry weight of the root sub-sample and then of the entire plant biomass. Roots were cleared and stained for analysis of AMF and non mycorrhizal root endophytic colonization using an adaptation of Phillips and Hayman (1970) method (as described in *Chapter 2*). Lavender and myrtle roots were incubated for 50 min in KOH (10%) at 90ºC, while carob roots were incubated for 70 min. To remove the lignins from myrtle and carob roots they were immersed in H_2O_2 (10 vol.) for 30 min. After rinsing, the roots were placed in 10% HCl solution for 10 min and stained with glycerol-trypan blue solution (0.05%) at 70ºC for 30 min. Root colonization was estimated by the "magnified intersections" method (McGonigle *et al.*, 1990) at 200× magnification and expressed as percentage of root length colonization. For each plant roots, three slides were mounted with five root fragments mounted in parallel. AM were distinguished from non mycorrhizal root endophytes by the presence of arbuscules, vesicles, hyphal coils, and by asseptate hyphae with a characteristic "knobby" appearance. Each of these structures was also quantified.

Assessment of AMF abundance and diversity

Three rhizospheric soil samples (100g of soil fresh weight) from each soil treatment were collected from the second generation of myrtle to extract spores, and the soil samples were stored at 4ºC. AMF spores were isolated by wet sieving followed by sucrose gradient centrifugation (Daniels and Skipper, 1982). Each soil sample was sieved and the fraction collected in the last sieve (53 μm) was centrifuged in a 60% (w/v) sucrose solution for 2 min at 3 000 rpm. Spores were collected from the water-sucrose interface, poured through sieve, rinsed with distilled water and transferred to Petri dish (Carvalho *et al.*, 2003). Under a dissecting microscope, living spores were separated from dead parasited spores.

The spores selected for identification were kept in a solution (10% Glycerol, 10% ethyl alcohol, 80% water) (C. Walker; personal information) and sent for further classification by C. Walker.

In this work, instead of using total spore density for data analyses, AMF species abundance was used, calculated by dividing each AMF species spore density by the total spore number in that sample, and multiplied by 100.

AMF fungal spore density and community species composition was based on AMF spore morphological characteristics. Therefore, the characterization of AMF spore community results here reported concern only those AMF species that were present as spores in the soil at the end of the experiment, and therefore the diversity of the AMF spore community could have been underestimated (Sanders, 2004) since each AMF family has different life strategies and variable rates of sporulation (Hart and Reader, 2002b). Although it is not attempted to state that the total AMF species abundance and diversity in the soil was determined by the evaluation procedure, it was assumed that AMF spore community differences found in the soil treatments should be sufficiently sensitive to test whether host species affected the AMF community, and whether differences in the AMF community could be linked to differences in plant biomass and community composition of non mycorrhizal root endophytic fungi.

Assessment of non mycorrhizal endophytic fungal abundance and diversity

Non mycorrhizal endophytic fungal diversity was determined only at the final harvest of the second generation of myrtles. Eight pieces of roots (1 cm) were collected from the root system of each plant replicate. Each root sub-sample was washed with running tap water for a few minutes to remove soil particles, surface sterilized by immersion in 2% sodium hypochlorite solution for 15 min, and finally rinsed in autoclaved distilled water. Afterwards, roots were placed onto Rose Bengal Agar (RBA) medium supplemented with chloramphenicol (0.05 %). The plates were incubated at 28ºC for 1 month. Every three days the hyphae growing out from the root tissues were transferred to Potato Dextrose Agar (PDA), incubated at the same temperature, and periodically checked for culture purity.

Genus identification was based on macroscopic (texture, topography, surface and reverse colours and pigumentes diffusing into the medium) and microscopic (hyphae, fruiting structures and spores) colony characters. Final identification was performed by M.

Barata (University of Lisbon), according to Hanlin (1990, 1998), Ellis (1971, 1976), Sutton (1980) and Domsch *et al.* (1993).

The sterile mycelia that did not sporulate on PDA was sub-cultured on water agar (1.5%) medium to induce differentiation of reproductive structures for another month.

The relative frequency of non mycorrhizal endophytic fungi was calculated as:

$$\textit{Relative frequency (\%)} = \frac{\text{no. colonies from each fungal genus} \times 100}{\text{total no. colonies from each plant replicate}}$$

In this calculation, each Petri dish represented one plant replicate from each treatment. Some of the treatments did not have six replicates since a few Petri dishes were excluded because either they were contaminated or the media dried out due to prolonged incubation period.

Although the relative frequency was determined, in the present manuscript it will be denominated as abundance because at the treatment level it represents the abundance of those particular fungi.

Data and statistical analyses

Using the GLM procedure, a two-way ANOVA was separately processed for biomass, root:shoot ratio and AMF and percent colonization of non mycorrhizal root endophytic fungi, to evaluate the differences caused by each independent variable. Prior to statistical analysis, percent root colonization data were arcsin square root-transformed to satisfy the ANOVA assumptions. One-way ANOVA was also performed for myrtle plant biomass to search for differences in each soil origin community due to plant pre-cultured soil. To test for significant differences in root percent colonization within soil community treatments by mycorrhizal and non mycorrhizal endophytes, a one-way ANOVA was performed for the nine treatments, followed by the Tukey's *post hoc* test (P<0.05).

The community species similarities between treatments were evaluated by the *Renkonen index* (Zack and Willig, 2004):

$$P = \sum \text{minimum} (p_{i\,1}; p_{i\,2})$$

where p_i is the proportional representation of species (i) in site 1 and in site 2, respectively, and $\sum$ minimum is the minimum value of p_i detected from either site. The index equals one with complete similarity.

Both fungal communities diversity was quantitatively determined by the *Shannon index* (Zack and Willig, 2004). This index combines two diversity components, the numbers of species and the evenness of ratio of individuals among species:

$$H = -\sum p_i \ln p_i$$

where p_i is the proportional abundance of each species.

Total number of AMF spores extracted from soil was analysed by one-way ANOVA to compare the effect of the different host plant species in pre-culturing the soil communities. Since initially three distinct AMF communities existed, the AMF Shannon diversity index data were organized *per* each soil origin factor, and grouped into conspecific and heterospecific soil communities and further analyzed by Student t-test.

Two-way ANOVA was performed to compare differences in non mycorrhizal fungal diversity in soil communities, using the Shannon index data followed by one-way ANOVA to analyse significant results on main effect interaction. A student t-test was performed to compare conspecific and heterospecific soil communities within each soil AMF community or in all treatments. Another one-way ANOVA was performed with non mycorrhizal Shannon index data to compare the dependence of host plant pre-culturing the soil on AMF community.

To search for differences in AMF species spore density and the species abundance of root endophytic fungi among the soil fungal treatments, the data were arcsin square root-transformed or square root (x+1) transformed, however the data did not fulfil MANOVA assumptions. Instead, non-parametric methods were performed (Kruskal-Wallis tests) on non-transformed data. Since there is no *post hoc* test for non parametric methods, and the differences found only indicated an overall difference between the nine treatments, it was not possible to clarify which independent variable was inducing those differences.

Spearman rank correlation analyses were performed to analyse possible relationships between mycorrhizal and non mycorrhizal fungal community species abundance and diversity, root colonization and myrtle plant biomass.

Multiple regression analysis were performed using stepwise selection procedure to determine if soil spore densities of individuals AMF species and/or the abundance of non mycorrhizal fungal endophytes were significant predictors of myrtle plant biomass variability. Prior to analysis, data from single species abundance were log transformed. The fungal species abundance selection criteria for analysis was that each fungal species, independently of being mycorrhizal or non mycorrhizal, had to have an occurrence frequency superior to five. Residuals normality was tested through Kolmogrov-Sminorv test. In the model colinearity for each variable was also tested.

Statistical procedures were performed using the software SPSS (version 10) for Windows.

3. RESULTS

3.1. PLANT BIOMASS

Myrtle biomass was significantly smaller when grown in conspecific soil than in heterospecific soil community (pre-cultured by lavender and carob) (t-test T_{48}=3.49, P<0.001). Plant growth was significantly influenced by the plant species used to pre-culture (two-way ANOVA $F_{2,39}$=6.57, P<0.05), but was not affected by the soil AMF community ($F_{2,39}$=1.73, P=0.19) nor by the interaction between these two factors ($F_{4,39}$=0.65, P=0.63). The effect induced by conspecific soil community in plant biomass did not depend on the initial AMF community present in the soil, not even when it was inoculated with commercial *G. intraradices*. However, the degree of depression in myrtle plant biomass varied with the soil community, more evidently in the undisturbed soil, which was collected in the preserved area where myrtle was the most abundant plant species (one-way ANOVA $F_{8,41}$=2.38, P<0.05), and in inoculated soil with commercial AMF (one-way ANOVA $F_{2,15}$=5.12, P<0.05) (Fig. 4.3). The growth of myrtle plants in commercial inoculated soil treatment differed with the plant species used to pre-culture the heterospecific soil community, being larger in plants grown in lavender pre-cultured soils (Fig.4.3). The increase of AMF spore density in the soil did not promote a general myrtle growth, independently of the soil rhizospheric communities. The root/shoot ratio usually decreases when there is a positive AMF effect. In this study, and in agreement with the above results, no significant differences were found in this ratio for all treatments (two-way ANOVA: soil AMF community $F_{2,36}$=1.40, P=0.26; soil cultured by plant species $F_{2,36}$ 1.11, P=0.34; interaction effect $F_{4,36}$=0.57, P=0.68).

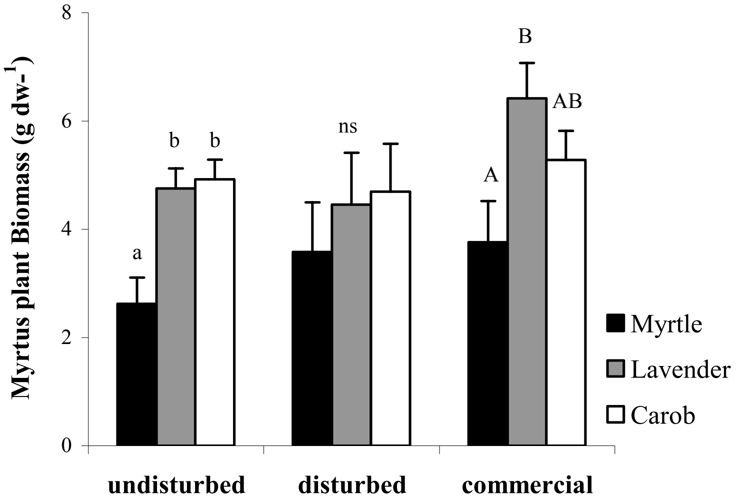

Figure 4.3. Total plant biomass of one-year-old myrtle plants grown in each of the nine soil community treatments (three different soil AMF communities × microbial soil communities pre-cultured by three plant species). The AMF communities were: the undisturbed community from the *maquis* area; the disturbed community from long-term stockpiled soil; and the commercial community that resulted from the addition of commercial inoculum to the stockpiled soil. Bars represent the average of myrtle plant biomass (± SE; n=6). For each soil AMF community, values sharing the same letters are not significantly different according to Tukey test (P>0.05) .

3.2. ROOT COLONIZATION

All myrtle roots from the different treatments were colonized by mycorrhizal and non mycorrhizal fungi. The percentage of root cortex colonized by the mycorrhizal fungi in all treatments was around the double of the non mycorrhizal fungi (Fig. 4.4).

AMF root colonization

Mycorrhizal root length colonization was not affected by any of the variable factors (two-way ANOVA: soil AMF community $F_{2,42}$=0.68, P=0.52; soil cultured by plant species $F_{2,42}$=0.22, P=0.80; interaction effect $F_{4,42}$=0.16, P=0.96). Myrtle plants greown in the soil inoculated with commercial AMF were intensely colonized by *G. intraradices*. Even though it was not possible to identify the different AMF species in the roots through direct observation, *G. intraradices* colonization could be identified in myrtle roots due to its characteristic features. Thus, it was confirmed that the commercial AMF inoculum treatment persisted in the soil and was able to successfully colonize plants from the second generation.

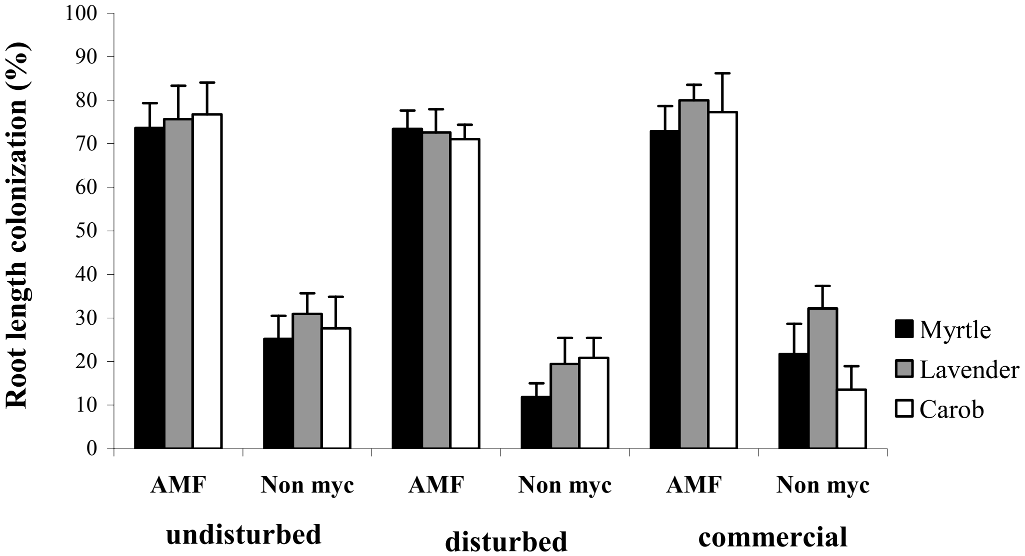

Figure 4.4. Root length colonization by mycorrhizal (AM) and non mycorrhizal endophytic fungi of myrtle plants grown in nine different soil communities - 3 AMF soil communities (undisturbed, disturbed and disturbed *plus* commercial inoculum) × 3 microbial soil communities pre-cultured by three plant species (myrtle, lavender and carob). Bars represent the average of myrtle root length colonization (± SE; n=6).

Despite the fact that the commercial soil community treatment comprised a large number of AMF propagules (4 000 spores *per* plant replica), it did not induce any significant differences in percent root colonization between the treatments. Nevertheless, the percent root colonization by vesicles was significantly different (two-way ANOVA: soil AMF communities $F_{2,42}$=0.68, P=0.10; soil cultured by plant species $F_{2,42}$=3.12, P=0.05; interaction effect $F_{4,42}$=0.65, P=0.63) probably due to *G. intraradices* abundant vesicles in commercial AMF community. This did not occur with other AMF structures evaluated: arbuscules (two-way ANOVA: soil AMF communities $F_{2,42}$=0.25, P=0.78; soil cultured by plant species $F_{2,42}$=0.69, P=0.51; interaction effect $F_{4,42}$=0.27, P=0.90) and hyphal coils (two-way ANOVA: soil AMF communities $F_{2,42}$=0.73, P=0.49; soil cultured by plant species $F_{2,42}$=0.88, P=0.42; interaction effect $F_{4,42}$=1.24, P=0.31).

Non mycorrhizal endophytic fungal colonization

The extent of non mycorrhizal root colonization was not affected by any variable factor (two-way ANOVA: soil AMF communities $F_{2,42}$=2.40, P=0.10; soil cultured by plant species $F_{2,42}$=2.13, P=0.13; interaction effect $F_{4,42}$=1.59, P=0.19). Myrtle plants grown in conspecific soil communities did not have significantly more non-mycorrhizal fungi colonizing the roots, as compared to those grown in heterospecific soil communities, for each soil origin community (for: undisturbed soil T_{15}=0.69, P=0.50; disturbed soil T_{15}=1.93, P=0.72; commercially inoculated soil T_{15}=0.54, P=0.60).

3.3. CHARACTERIZATION OF FUNGAL COMMUNITIES

3.3.1. Mycorrhizal communities (AMF)

AMF community diversity

AMF diversity from myrtle rhizosphere measured by the Shannon index was significantly lower when plants grew in conspecific comparatively to heterospecific soil communities previously inoculated by commercial AMF species in the first plant generation (t-test T_7= 3.15, P<0.05). However, no significant differences were found due to any factor effect, when the different soil communities were compared (two-way ANOVA: soil AMF community $F_{2,18}$=2.88, P=0.08; soil cultured by plant species $F_{2,18}$=1.89, P=0.18; interaction effect $F_{4,18}$=1.53, P=0.24) (Fig. 4.5).

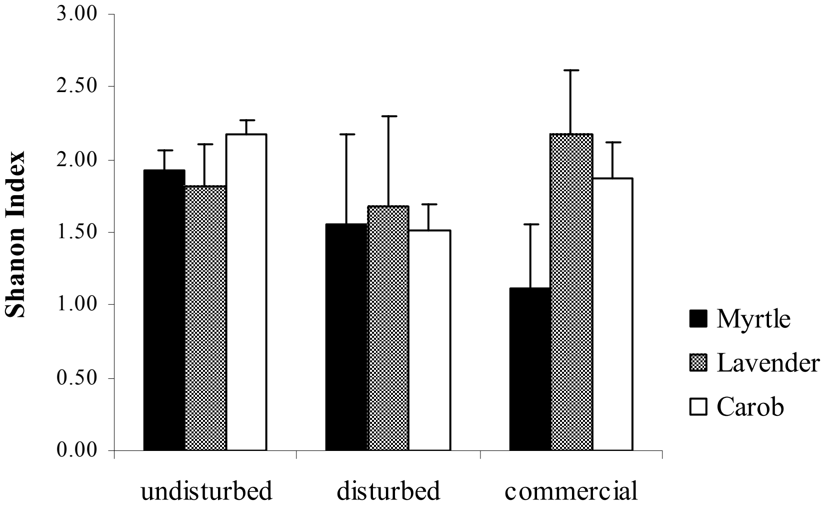

Figure 4.5. AMF species diversity at harvest of the second generation of myrtle plants in response to different soil communities - AMF soil communities (undisturbed, disturbed and disturbed *plus* commercial inoculum) × microbial soil communities pre-cultured by three plant species (myrtle, lavender and carob). Shannon index values (means ± SE; n=3) were not significantly different in a post-one way ANOVA Tukey test per each soil origin treatment (P>0.05).

AMF community composition

From all the nine soil treatments, 34 AMF species were identified belonging to six genera (*Acaulospora; Archaeospora; Glomus; Paraglomus; Scutellospora; Sclerocystis). Glomus* was the most frequent and diverse genus, comprising 26 species, but 15 species were not identified to the species level. The species composition of each AMF community differed significantly among treatments (Table 4.1). The total AMF spore number in nine soil communities analyzed ranged from 509 spores per 100 g of soil (fresh weight) in the undisturbed community pre-cultured by carob plants to 4 466 spores per 100 g of soil (fresh weight) in the commercial community pre-cultured by carob plants. The large amount of spores in the commercial treatment was due to the initial addition of a large amount of *G. intraradices* spores, which probably still remained in the soil after the second generation of myrtle plants. The host plant pre-cultured soil rhizosphere did not have a significant effect on total spore number of each soil community origin.

Interpreting the density differences showed in Table 4.1, *G. intraradices* species was significantly influenced by the soil community treatment, since one of the tested communities had been inoculated with commercial AMF inoculum that consisted of that single species. *Glomus luteum*, *Glomus* sp. (morphotypes 5 and 6) and *Acaulospora* sp. (morphotype 1) were significantly different because they only, or mainly existed in soil communities pre-cultured by lavender host plants. On the other hand, *G. clarum* was significantly influenced by myrtle plants rhizosphere, while *Glomus* sp. 11 was abundant exclusively in the carob rhizosphere. *Paraglomus occultum* had a very large abundance in soils pre-cultured by myrtle and carob from undisturbed and commercial AMF communities, being significantly different in all the data arrangement analyses.

The similarity of the overall AMF species composition was compared among the mycorrhizal community treatments using the Renkonen index. This metric index only measures the degree of similarity between pairs of communities, so the data from the nine soil treatments were organized by each factor of variance. The composition of AMF communities was different among the soils pre-cultured by different plant species, although the degree of difference was low (around 30 to 50%). Myrtle and lavender rhizospheres had the most different compositions (Table 4.2).

Table 4.1. List of AMF species and their relative density (%) in 100g of soil (fw) (n=3). AMF species with asterisks are significantly different among the nine soil community treatments (see Fig. 4.2 for experimental set-up details).

AMF SPECIES	UNDISTURBED			DISTURBED			COMMERCIAL		
	MYRTLE	LAVENDER	CAROB	MYRTLE	LAVENDER	CAROB	MYRTLE	LAVENDER	CAROB
Acaulospora cf. *foveata* Trappe & Janos	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acaulospora walkeri Kramadibrata & Hedger	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00
Acaulospora sp. 1*	0.00	0.88	0.00	0.00	0.08	0.00	0.00	0.00	0.00
Acaulospora sp. 2	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Archaeospora trappei (Ames & Linderman) Morton & Redecker*	14.15	5.12	8.52	3.69	2.22	0.91	2.62	5.41	3.06
Glomus clarum Nicol. & Schenck*	1.93	0.00	0.00	0.51	0.00	0.00	1.09	0.00	0.00
Glomus constrictum Trappe	11.25	9.19	8.08	2.77	4.89	4.25	2.81	19.83	1.57
Glomus coronatum Giovanetti	0.00	0.18	0.00	0.00	0.15	1.01	0.00	0.06	0.00
Glomus diaphanum Morton & Walker*	0.00	31.45	3.71	0.46	3.44	0.91	0.00	12.56	5.47
Glomus cf. *diaphanum*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	22.72	0.00
Glomus etunicatum Becker & Gerdemann	3.54	6.01	19.65	1.49	0.84	13.75	0.77	2.46	0.62
Glomus fasciculatum (Thaxter) Gerd. & Trappe emend. Walker & Koske	0.00	0.00	0.22	0.00	0.15	0.00	0.00	0.00	0.00
Glomus geosporum (Nicol. & Broome) Walker	3.54	0.18	2.18	2.31	0.08	0.81	0.26	0.00	0.32
Glomus intraradices Shenck & Smith*	1.93	1.41	4.15	2.82	0.00	0.00	7.61	4.87	7.74
Glomus luteum Kenn., Stutz & Morton*	0.00	1.24	0.00	0.00	0.46	0.00	0.00	0.96	0.02
Glomus mosseae (Nicol. & Gerd.) Gerd. & Trappe	4.82	1.06	1.31	0.92	1.30	2.22	10.10	0.24	2.26
Glomus sp.1	4.34	10.78	6.99	66.67	62.03	35.59	3.26	4.39	31.53
Glomus sp. 2	21.70	10.42	2.18	0.46	4.20	16.28	0.19	4.93	0.40
Glomus sp. 3	0.00	0.00	0.00	0.36	0.00	0.00	0.26	1.68	0.52
Glomus sp. 4	1.29	0.00	6.33	0.56	6.26	0.91	1.21	0.60	3.48
Glomus sp. 5*	0.00	4.06	0.00	0.00	0.46	0.00	0.00	0.00	0.00
Glomus sp. 6*	0.00	10.25	1.31	0.00	7.03	0.00	0.00	4.93	2.81
Glomus sp. 7	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glomus sp. 8	0.00	0.18	0.00	0.00	0.08	0.00	0.00	0.00	0.00
Glomus sp. 9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.54	0.00
Glomus sp. 10*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.07
Glomus sp. 11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	13.26
Glomus sp. 12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.74
Glomus sp. 13*	0.00	0.00	1.75	0.00	0.00	0.10	0.00	0.00	0.00
Glomus sp. 14**	0.00	0.00	3.71	0.00	1.38	0.91	0.00	6.13	5.47
Glomus sp. 15	3.54	0.53	2.40	2.31	1.15	1.92	0.26	0.96	0.40
Paraglomus occultum (Walker) Morton & Redecker*	24.92	4.42	24.02	13.85	1.22	19.11	68.29	5.35	16.40
Sclerocystis rubiformis Gerd. & Trappe	2.57	0.53	3.06	0.82	0.76	1.21	1.15	1.32	0.70
Scutellospora calospora (Nicol. & Gerd.) Walker & Sanders	0.48	1.59	0.44	0.00	1.68	0.10	0.13	0.06	0.15

* $P<0.05$; ** $P<0.005$

When comparing the AMF communities concerning the soil origin factor, large differences were found between them (Table 4.3). The main differences were found between the disturbed soil and the soil inoculated with commercial inoculum.

Table 4.2. Renkonen index values evaluating the similarity of AMF species compositions in myrtle plant rhizosphere between pairs of soil communities pre-cultured by three host plant species (myrtle, lavender and carob).

	LAVENDER SOIL	**CAROB SOIL**
MYRTLE SOIL	0.51	0.69
LAVENDER SOIL	—	0.68

Table 4.3. Renkonen index values evaluating the similarity of AMF species compositions in myrtle plant rhizosphere between pairs of soil origin (undisturbed, disturbed and disturbed soil *plus* commercial inoculum).

	DISTURBED SOIL	**SOIL WITH COMMERCIAL**
UNDISTURBED SOIL	0.49	0.56
DISTURBED SOIL	—	0.33

Variability of each AMF species abundance

This approach allowed searching for differences in abundance of each individual AMF species caused by the diverse soil community factors (soil origin and pre-cultured soil by different host plant species). Even though the differences in AMF species community composition along the treatments were not very large, there were some particularly important differences in terms of occurrence and abundance in some AMF species, not always significantly different by the statistical tests performed.

Glomus sp. 1 was the most abundant AMF species, present in all treatments with a relative abundance above 20%. It was particularly abundant in the disturbed soil, with a relative abundance of 58% (Table 4.2). *Paraglomus occultum* was also very abundant in soil pre-cultured by myrtle (36%) and carob (17%), and it was the most abundant in the commercial soil (25%). *Glomus* sp. 1, *Glomus* sp. 11 and *P. occultum* reached extraordinary high spore density in particular samples, mainly in the soil commercially inoculated treatment and pre-cultured by myrtle and carob (Table 4.2). *Glomus* *constrictum, G. diaphanum* and *G.* cf. *diaphanum* had a relative abundance between 10 and 20% in lavender pre-cultured soil.

Some other identified AMF species occurred primarily, or only at one of the nine soil communities tested, influenced by plant species rhizosphere or at particular soil AMF community. For instance, the four species of *Acaulospora*, as well as *G.* cf. *diaphanum* and four *Glomus* species (*Glomus* sp. 5, *Glomus* sp. 7, *Glomus* sp. 8 and *Glomus* sp. 9) only existed in lavender pre-cultured soils (Table 4.2). Four of the unidentified *Glomus* species (*Glomus* sp. 10, *Glomus* sp. 11, *Glomus* sp. 12 and *Glomus* sp. 13) only existed in carob rhizosphere pre-cultured soils. *Glomus clarum* was the only species that positively differentiated the myrtle rhizosphere cultured soil.

Lavender rhizosphere pre-cultured soil contained the highest diversity of AMF species and the largest number of “specific” species, while myrtle rhizosphere pre-cultured soil displayed the lowest ones.

The differences in AMF species composition also varied with the soil origin factor. For instance, the four species of *Acaulospora* never occurred in the commercially inoculated soil treatment. This treatment also contained several other AMF species besides *G. intraradices*, and some of them were exclusively present in this AMF community: *G.* cf. *diaphanum*; *Glomus* sp. 9; *Glomus* sp. 10; *Glomus* sp. 11; and *Glomus* sp. 12. In addition, *Glomus* sp. 8, *Acaulospora* cf. *foveata* and *Acaulospora* sp. 2 only occurred in the AMF community from the undisturbed soil.

3.3.2. Non-mycorrhizal endophytic fungal community

Non-mycorrhizal endophytic fungal community diversity

The diversity of communities of root non mycorrhizal endophytic fungal measured by the Shannon index was significantly affected by the interaction effect, as well as by the effect of the host plant species used to pre-culture the soil microbial communities (two-way ANOVA: soil AMF communities $F_{2,32}$=0.13, P=0. 88; soil cultured by plant species $F_{2,32}$=3.36, $P<0.05$; interaction effect $F_{4,32}$=5.32, $P<0.001$). That effect was caused by the AMF community diversity in disturbed soil pre-cultured by carob (one-way ANOVA: $F_{8,32}$=4.88, $P<0.001$) (Fig. 4.6).

The disturbed soil community displayed significant differences in the diversity of non mycorrhizal endophytes due to the effect of each host plant species used to pre-culture the soil AMF communities (one-way ANOVA $F_{2,10}$=14.09, $P<0.05$); the highest diversity was found in plants grown in soils pre-cultured by lavender, while the lowest was found in plants grown in soils pre-cultured by carob. However, the significant host plant species effect on the diversity of non mycorrizal endophytes in each soil origin was not related to be conspecific *vs.* heterospecific soil communities (t-test T_{11}=-0.17, P=0.87). Although this result is in agreement with what was previously obtained through two-way ANOVA, plant species played a main effect on soil fungal community by culturing the soil microbial communities that affected root endophytes of the next plant generation, regardless of belonging or not to the same plant species. Moreover, the pre-culturing effect by plant species on these root endophytic fungal diversity did not significantly vary with soil AMF community (one-way ANOVA per each plant species: myrtle $F_{2,9}$= 1.79, P=0.22; lavender $F_{2,13}$=2.88, P=0.92; carob $F_{2,10}$=2.39, P=0.14).

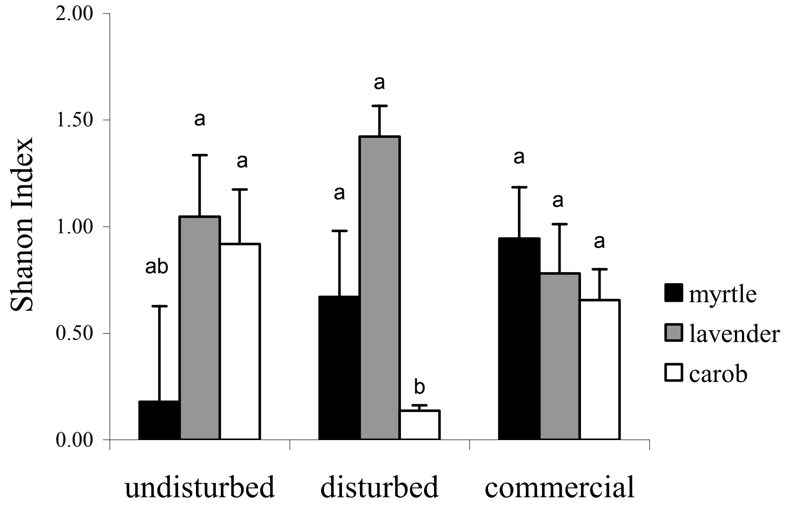

Figure 4.6. Shanon index diversity of non mycorrhizal root endophytic fungal species in response to different soil communities - AMF soil communities (undisturbed, disturbed and disturbed *plus* commercial inoculum) × microbial soil communities pre-cultured by three plant species (myrtle, lavender and carob). Values (means ± SE; n=3) sharing the same letters are not significantly different according to one way ANOVA as *post-hoc* test followed by Tukey test.

Non mycorrhizal endophytic fungal community composition

The isolated endophytic fungi comprised 36 morphotypes belonging to 14 genera (2 Ascomycetes and 12 Mitosporic fungi), three unidentified isolates and one group identified as the Dark Septate Endophytes (DSE) (Table 4.4). The latter functional group consisted of four morphotypes classified as sterile mycelia, presenting thick-walled dark pigmented septate hyphae in culture. This DSE group was very abundant and widespread through the different treatments. The list of the isolated fungi and their trophic characteristics is presented in Table 4.4. The three unidentified isolates were characterized and confirmed as distinct morphotypes. The genera richness was similar among the soils pre-cultured by the different plants species. Thirteen genera were found in conspecific root community, while in the heterospecific communities 15 genera were found in myrtle roots grown in soil pre-cultured by lavender and 12 genera in soil pre-cultured by carob.

Based on the literature (see *Materials and Methods*), fungi were characterized as saprobe, parasite, pathogenic or decomposer, according to their nutritional sources. Overall, the parasitic/pathogenic fungi were the most abundant groups, and the decomposers the most infrequent and specific to a particular treatment (Table 4.4).

Table 4.4. Ecological characteristics of the non mycorrhizal fungal genera found in second generation myrtle roots.

FUNGI	NUMBER OF MORPHOTYPES	NUMBER OF OCCURRENCES	TROPHIC CHARACTERISTICS
MITOSPORIC FUNGI			
Acremonium sp.	3	75	Saprotroph or parasite of living fungi or plant pathogen fungi
Broomella sp.	1	19	Weak parasite
Cylindrocarpon sp.	4	62	Very common in plant roots; some species can be pathogenic
DSE	4	68	Saprotroph or mutualistic symbionts; pathogens
Fusarium sp.	7	132	Saprotroph or parasite; it can be pathogenic for some plants
Gliocladium sp.	2	8	Saprotroph
Humicola sp.	1	9	Saprotroph; cellulose decomposer
Penicillium sp.	2	9	Saprotroph or parasite
Phailaphora sp.	1	34	Saprotroph
Phoma sp.	1	3	Saprotroph or parasite, some species can be pathogenic to plants
Pyrenochaeta sp.	1	95	Saprotroph or plant root parasite or leaves pathogens
Scopulariopsis sp.	3	17	Saprotroph
Trichoderma sp.	1	10	Saprotroph; cellulose decomposers
Unidentified isolates	3	10	
ASCOMYCETES			
Myrothecium sp.	1	7	Saprotroph; cellulose decomposers
Chaetomium sp.	1	1	Saprotroph; cellulose decomposers

The root non mycorrhizal endophytic communities were compared using the Renkonen index. The non-mycorrhizal community within the plant species pre-cultured differed from nearly similar (myrtle and lavender) to very little similarity (myrtle and carob) (Table 4.5).

The similarity values of non-mycorrhizal endophytic fungal communities among the soil origin treatments were exactly the same (only 37%). This indicates that myrtle plants growing in each soil AMF community had their own particular fungal community, inducing large differences in myrtle root non mycorrhizal fungi, which was unexpected since the diversity (Shannon index) was not affected by the soil AMF community factor. The non mycorrhizal fungal data were grouped by AMF community levels in order to be compared by the Renkonen index (Fig.4.6) So, it is possible that some "hiding" host plant effects were the responsible for the differences found.

Table 4.5. Renkonen index values evaluating the similarity of non mycorrhizal fungal endophytic communities in myrtle rhizosphere between pairs of soil communities pre-cultured by three host plant species (myrtle, lavender and carob).

	LAVENDER SOIL	**CAROB SOIL**
MYRTLE SOIL	0.71	0.23
LAVENDER SOIL	—	0.43

Table 4.6. Renkonen index values evaluating the similarity of non mycorrhizal fungal endophytic communities in myrtle rhizosphere between pairs of soil origin (undisturbed, disturbed and disturbed soil *plus* commercial inoculum).

	DISTURBED SOIL	**SOIL WITH COMMERCIAL**
UNDISTURBED SOIL	0.37	0.37
DISTURBED SOIL	—	0.37

Variability of each non mycorrhizal endophytic fungal genus

The abundance of individual root fungal endophytic species may have an effect on the plant growth independent of the community diversity effect when it is evaluated as a whole. Therefore, the variability of the most abundant fungi in the roots or the presence of particular genus-plant specific species were analysed.

In general, the occurrence frequency distribution of each fungal species is not associated with only one factor of variation. The same fungal species can range from highly frequent to scarce in the same soil origin due to host plant species effect on soil community. Therefore, it was not possible to demonstrate that there was a significant reduction of root non mycorrhizal endophytic fungi in the commercially inoculated soil regardless of the pre-cultured host plant species as it was initially hypothesized. When the occurrence frequency of myrtle root endophytic communities was compared among the three rhizospheric pre-cultured soils, only *Fusarium* genus (Kruskal-Wallis test=8.57, *P*<0.05), and *Gliocladium* genus (Kruskal-Wallis test=8.29, *P*<0.05) were significantly affected by the plant species used to pre-culture the soil.

According to Tan *et al.* (1989) the frequency of occurrence can be characterized in classes, the very frequent species being the ones that present an occurrence >20%, the frequent species an occurrence between 10-20%, and the infrequent ones <10%. In the present study, *Fusarium* sp. may therefore be classified as very frequent in myrtle roots grown in carob pre-cultured soils, and was particularly abundant in the disturbed soil treatment (78.3%) (Table 4.7). However, this species was infrequent when myrtle roots grew in the same soil source but pre-cultured by its own species. There was a significant reduction (Mann-Whitney test=203.50, *P*<0.05) in *Fusarium* sp. when conspecific were compared to heterospecific root fungal communities. This possibly indicates a defence mechanism of myrtle plant species that limited the growth of this fungal pathogen. On the other hand, it is also possible that carob and lavender were very vulnerable warranting the growth of *Fusarium* sp., and may have induced the sporulation of this endophyte in such a way that the next myrtle plant generation would have been affected in heterospecific soil communities (Table 4.7).

In the DSE group, soil origin was a significant source of variation (Kruskal-Wallis test=5.89, *P*=0.06). This group was more frequent in the undisturbed soil community than in any other community, regardless of the host plant pre-culturing effect (Table 4.7). Nevertheless, it was very frequent (66.7%) in myrtle roots grown in conspecific undisturbed soil communities.

Broomella sp. occurrence in myrtle root endophytic community was also significantly affected by the different soil fungal communities (Kruskal-Wallis test=7.91, *P*<0.05). This species was classified as infrequent in the disturbed soil community with or without commercial inoculation, occurring with higher frequency in myrtle roots grown in conspecific soil communities than in other pre-cultured soil communities, and it was absent in the undisturbed soil (Table 4.7).

Table 4.7. Relative frequency (%) of the occurrence of myrtle non mycorrhizal endophytic fungi grown in the three different soil communities (undisturbed, disturbed and disturbed *plus* commercial inoculum).

NON MYCORRHIZAL ROOT ENDOPHYTIC SPECIES	**UNDISTURBED**			**DISTURBED**			**COMMERCIAL**		
	MYRTLE	LAVENDER	CAROB	MYRTLE	LAVENDER	CAROB	MYRTLE	LAVENDER	CAROB
Acremonium sp.	0.00	1.79	13.16	6.59	11.49	21.74	2.20	0.00	42.47
Broomella sp.	0.00	0.00	0.00	6.59	6.90	0.00	5.49	1.79	1.37
Chaetomium sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.79	0,00
Cylindrocarpon sp.	22.22	19.64	5.26	16.48	22.99	4.35	0.00	12.50	0.00
DSE*	66.67	21.43	10.53	10.99	19.54	0.00	7.69	12.50	1.37
Fusarium sp	11.11	14.29	22.37	3.30	17.24	78.26	0.00	28.57	24.66
Gliocadium sp.	0.00	0.00	7.89	0.00	0.00	0.00	0.00	1.79	1.37
Humicola sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.33
Myrothecium sp.	0.00	1.79	1.32	0.00	1.15	0.00	1.10	0.00	4.11
Penicilium sp.	11.11	0.00	1.32	0.00	1.15	0.00	0.00	0.00	8.22
Philaphora sp.	0.00	5.36	9.21	9.89	10.34	0.00	3.30	0.00	4.11
Phoma sp.	0.00	1.79	0.00	1.10	0.00	0.00	1.10	0.00	0.00
Pyronochaeta sp.	0.00	21.43	2.63	35.16	12.64	0.00	36.26	8.93	0.00
Scopulariopsis sp.	0.00	1.79	0.00	2.20	5.75	0.00	8.79	1.79	0.00
Trichodermas sp.	0.00	3.57	0.00	1.10	8.05	0.00	0.00	0.00	0.00
Unidentified Isolate 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	14.29	0.00
Unidentified Isolate 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.37
Unidentified Isolate 3	0.00	0.00	0.00	1.10	0.00	0.00	0.00	0.00	0.00

* DSE = dark septate endophytes

Acremonium sp. occurred with higher frequencies in carob host plants, but it was not specific of this species, since it infected myrtle roots grown in soils pre-cultured by the other plant species (Table 4.7). However, *Acremonium* sp. certainly had some preference for carob plant species.

Pyrenochaeta sp. was as very frequent in myrtle roots grown in conspecific soil communities of the disturbed soil regardless AMF commercial inoculation effect, but inexistent in the undisturbed soil community (Table 4.7).

Other infrequent genera had their occurrence limited to some soil communities depending on very specific variable combinations (Table 4.7). For example, *Humicola* sp. was only present in commercially inoculated soil pre-cultured by carob plants, while *Chaetomiun* sp. only occurred in the same soil but when pre-cultured by lavender. However, *Humicola* sp. was the only non mycorrhizal fungus significantly different among the nine soil communities treatments were compared (Kruskal-Wallis test=15.96, $P<0.05$).

Each one of the unidentified isolates appeared exclusively in a single sample.

3.4. RELATION AMONG FUNGAL COMMUNITIES AND MYRTLE PLANT BIOMASS

Myrtle plant biomass was not significantly correlated neither with root colonization by AMF and non mycorrhizal endophytic fungi, nor with species diversity of AMF and non mycorrhizal endophytic fungal community. However, there was a significant positive correlation between root colonization of mycorrhizal and non mycorrhizal fungal endophytes (Table 4.8).

To test if there was a mediation effect between the abundance of mycorrhizal and non mycorrhizal root endophytic species, the Spearman correlation was also performed for all fungal species. However, no significant correlation was found (data not shown).

To examine if any identified species abundance from AMF soil communities or from non mycorrhizal root endophytic fungal communities, could be associated with myrtle plant biomass variation among the nine soils microbial communities tested, a multiple regression analysis was performed. Not all fungal species could be used (see Materials and Methods). Significant regression model (ANOVA $F_{4,15}$=8.84, P=0.001) could be fitted to myrtle plant biomass, and explained 62.3% (adjusted r^2=0.623) of plant biomass variation. The final model equation obtained was:

y= 8.17 -2.06 *G. geosporum* + 0.98 *Glomus* sp. 14 - 2.27 *G. constrictum* + 0.73 *G. etunicatum*

Table 4.8. Spearman rank correlations coefficients between myrtle plant biomass, root endophytic fungal colonization and fungal species diversity (mycorrhizal and non mycorrhizal) in nine different soil communities (see Fig. 4.2 for details of the experimental set-up).

SPEARMAN CORRELATIONS	MYRTLE BIOMASS	AMF ROOT COLONIZATION	NON MYCORRHIZAL ROOT COLONIZATION	AMF SPECIES DIVERSITY	NON MYCORRHIZAL SPECIES DIVERSITY
MYRTLE BIOMASS		0.29	0.08	-0.01	-0.03
AMF ROOT COLONIZATION	0.29		0.65*	0.34	0.15
NON MYCORRHIZAL ROOT COLONIZATION	0.08	0.65*		0.21	-0.19
AMF SPECIES DIVERSITY	-0.01	.34	0.21		0.21
NON MYCORRHIZAL SPECIES DIVERSITY	-0.03	0.15	-0.19	0.21	

*P<0.05

The AMF species were the only significant predictors for biomass variation for all the analysed fungal groups (mutualists, pathogens, saprobes and decomposers). Unexpectedly two of the most abundant AMF species in soil were negatively correlated with plant biomass. On the other hand, *Glomus* sp. 14 and *G. etunicatum* were positively correlated (Fig. 4.7, Table 4.9). Generally, in this model the AMF species had a major negative contribution to plant biomass. This type of data analysis does not establish a causation effect, so it cannot be concluded as a cause-and-effect relationship, but as an inverse strong association that can be caused by any other factor.

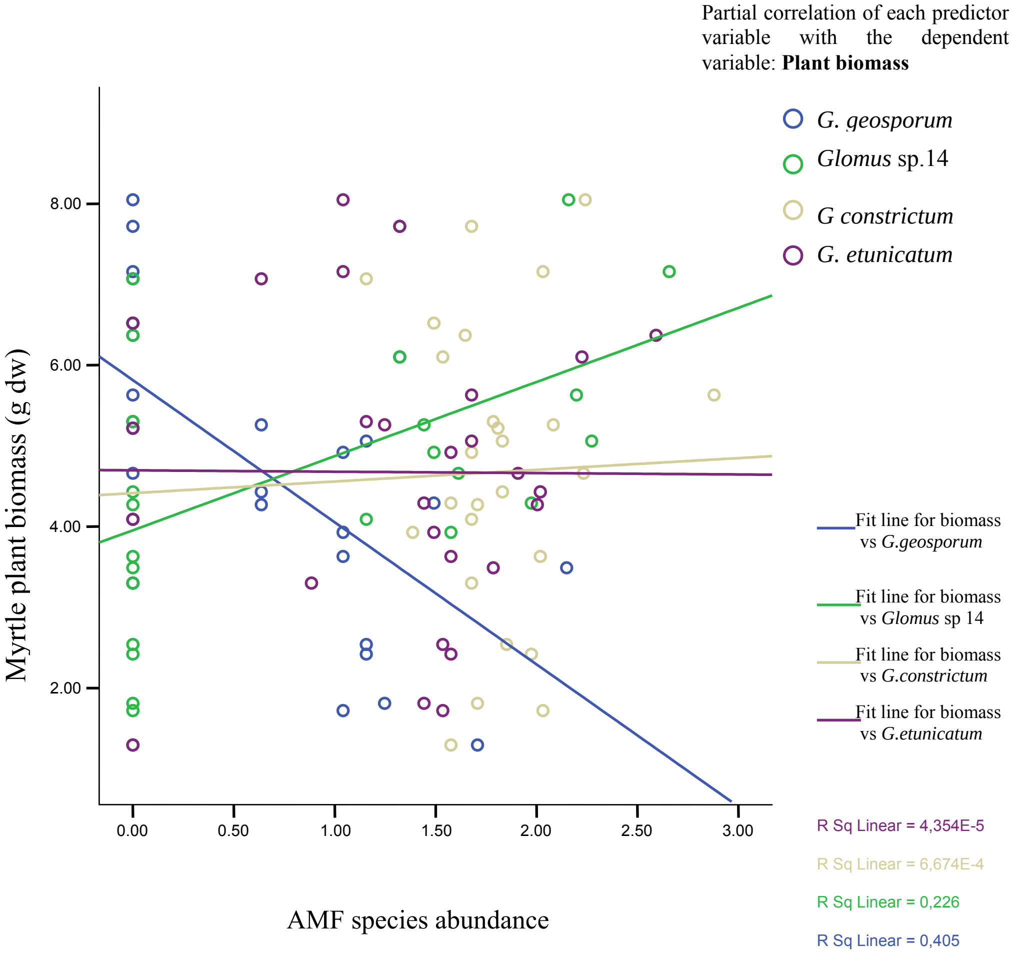

Figure 4.7. Representation of multiple linear regression of myrtle plant biomass with AMF predictor species abundance. The best fit lines illustrate the positive or negative contribution to plant biomass by each of the AMF species in the model. Previous to analysis, the AMF species abundance values were log-transformed.

Table 4.9. Summary of the Stepwise regression analysis concerning the variation of myrtle plant biomass. Coefficients concerning the predictor *Glomus* species are shown.

MODEL PREDICTORS	UNSTANDARDIZED COEFFICIENTS		T- TEST
	β	Std. error	(T values)
Constant (α)	8.17	1.14	5.79**
G.geosporum	-0.06	0.43	-4.76**
Glomus sp. 14	0.98	0.28	3.46**
G. constrictum	-2.28	0.79	-2.88*
G. etunicatum	0.73	0.34	2.14*

**P*<0.05; ***P*<0.005.

4. DISCUSSION

Several studies have shown that plant species can culture soil communities that reduce the growth of conspecifics (*e.g.*, van der Putten *et al.*, 1993; Klironomos, 2002; Callaway *et al.*, 2004), because differences in microbial colonization of the roots reflect differences in microbial indices found in the rhizospheres (Appuhn and Joergensen, 2006). Likewise, in the present study myrtle plants grown in soil communities pre-cultured by conspecifics were smaller than in soil pre-cultured by heterospecifics, indicating the occurrence of plant-soil community feedback. According to what was initially predicted, the growth response of myrtle plants to the heterospecific soil microbial communities varied depending on the first generation of plant species (lavender and carob) used to pre-culture the soil community. This means that each plant species cultured different soil rhizospheric microbial communities, and these differences were reflected in the variation found in the growth of the plants in the next generation. Moreover, this pattern occurred regardless of the AMF soil origin, and this dynamic feedback did not appear to be related to the root endophytic fungi as it was predicted. For the occurrence of this relation it would be expected that differences in the soil community composition should be closely followed by differences in the root fungal community, i.e., that the root colonization by non mycorrhizal endophytic fungi in myrtle plants grown on its own cultured soil would be larger than the root colonization by AMF, or even that the abundance of some non mycorrhizal fungal species, particularly of parasites or pathogens, would be higher than on heterospecific cultured soils. However, the results showed that root colonization by AMF and non mycorrhizal fungi did not differ among the nine soil community treatments. Despite the AMF colonization was two–fold higher than the non mycorrhizal colonization, even in the conspecific soil treatments, the benefit from the symbiosis to plant growth was not proportional. Other studies (Matthews and Clay, 2001; Gustafson and Casper, 2004) did not also find a relation between root fungal colonization and plant-soil community feedback. In the present study there was a positive relation between root length colonized by mycorrhizal and by non mycorrhizal endophytic fungi, probably indicating an absence of competition for root space.

Even though there were differences in the non mycorrhizal fungal species diversity in myrtle roots that were induced by the host plant species used to pre-culture the soil, they were not related with plant biomass variation. Contrarily to what was hypothesized, plants grown in the conspecific community, either from disturbed or undisturbed soil origin, had a decrease in the diversity of root non mycorhizal fungi, therefore the conspecific effect overcame the disturbance effect. The AMF soil inoculation had no effect on the diversity and root colonization extension of root non mycorhizal fungi. Contrarily to the hypothesis, *G. intraradices* did not mediate or compete with the non mycorrhizal fungi for myrtle roots. The net plant benefit from the soil commercial inoculation did not prevail over the effect of conspecific soil community. In the case of heterospecific community, the myrtle growth response commercial inoculation varied with the plant species used to pre-culture the soil community. Consequently, differences in soil microbial community on plant growth cannot be solved by increasing AMF spores species in the soil.

The hypothetical relation of soil AMF community that somehow regulates the root endophytic community composition, and that in turn may influence plant growth, was excluded by the present study. It can be concluded that, whatever microoganisms were shifting in this plant-soil feedback, they were neither related to root endophytic fungal community, nor mediated by soil AMF community, but likely related to other components in the soil community and their interactions. Nevertheless, the present results showed a close relationship between plant biomass variation and some AMF species present in the soil, as reported by others (*e.g.*, Lovelock *et al.*, 2003), but this relation varied from positive to negative regarding the AMF species. The mycorrhizal association has been generally described as a mutualistic symbiosis, but Klironomos (2003) clearly demonstrated that the range of plant growth response to mycorrhizae can vary from beneficial to detrimental. Based on previous studies (eg: Sanders and Fitter, 1992; Ruiz-Lozano and Azcon, 1993) Johnson *et al.* (1997) had already suggested that the mycorrhizal symbiosis can function along a parasitic to mutualistic continuum determined by biotic factors (host plant species and AMF species combination) or abiotic factors (soil nutrients). The results of the present study can be interpreted within this framework, since the abundance of *G. geosporum* and *G. constrictum* may have had a major negative influence on myrtle biomass. These results suggests that myrtle plant growth depression in conspecific soil was more related to an increase of certain AMF species spore abundance than to variation of root fungal endophytes species abundance, as predicted. However, on the basis of these results alone it is not possible to ascertain if some plant-AMF species interaction were the cause of the negative feedback mechanism observed in myrtle conspecific rhizospheric soil. It is possible that plants can select AMF that benefit them most (Klironomos, 2002), creating a temporary positive feedback between plants and certain AMF species. On the other hand, positive feedback in mycorrhizal symbiosis may be self-limiting because the enhancement of fungal biomass has carbon costs to the plant, whether due to the large intraradical mycelium described in Glomaceae family (Hart and Reader, 2002a,b) whether to an increased fungal sporulation (Graham, 2001). Therefore, in long-term pot experiments, like the present study, where nutrients can become depleted, particularly in pots where previous plants had the same nutritional demands (conspecific soil), the net cost-benefits for the plant can switch from a positive feedback to a parasitic relation at the end of the experiment.

The most common explanation suggested in the literature for negative plant-soil community feedback occurrence, is the effects of species-specific root fungal parasitic and/or pathogenic species (Mills and Bever, 1998; Packer and Clay, 2000; Callaway *et al.*, 2004). This was not supported by the results of this study. Since differences of non mycorrhizal endophytic fungal community were not associated with plant growth, there is no indication that these organisms were mediating the myrtle plant-soil community feedback. Instead of the expected increase in the abundance of particular myrtle-specific fungal species in plants growing in conspecific soil communities, the results showed that the most abundant species (*Fusarium* sp.) decreased, as compared to plants growing in heterospecific soil communities. This result suggests a shift in myrtle plant defences with soil microbial changes, which is larger in conspecific soil community than in heterospecific. Plants have the ability to control endophytic fungal growth through several mechanisms (Cordier *et al.*, 1998; Barea *et al.*, 2002; Bultman *et al.*, 2003). Graham (2001) explained that plant defence differences are induced, for certain levels of phosphorous, by mycorrhizal fungi present in the roots. The AMF mediate a decrease of sugar availability in root exudates by restoring membrane permeability of the root cells, with a subsequent reduction in pathogenic colonization and damage in roots. It is possible that some AMF species colonizing myrtle roots in conspecific soil indirectly stimulate the plant defences towards *Fusarium* sp. more than the other AMF species in heterospecific soil community. Additionally to changes in plant defences, the pathogenecity of some specific root pathogens could have changed during the experiment by watering the plants, because under natural conditions they are adapted to water stress (Reynolds *et al.*, 2003).

The data of this study did not provide an unquestionable explanation for the reduction of myrtle plant growth found in the conspecific soils. An evaluation of the root endophytic fungal community composition (mycorrhizal and non mycorrhizal) using recent molecular tools would provide more accurate data and probably further explanations of the myrtle plant-soil microbial community feedback. Some microbial or chemical alterations on myrtle rhizosphere undoubtedly occurred and were responsible for that detrimental effect on myrtle growth. One possible explanation is based on a direct negative effect of allelopathic compounds (Bush *et al.*, 1997), from root exudation of previous myrtles. Another explanation may be the indirect facilitation effect of other plant species rhizosphere, which is common in systems under abiotic stresses (Chanway *et al.*, 1991; Choler *et al.*, 2001). If lavender and carob changed the soil biotic or abiotic components in such a way that made it suitable for myrtle plants, then the growth of myrtles in the heterospecific soil would be higher than in the conspecific soil, which in fact occurred. These two mechanisms of direct antagonism or indirect facilitation effects could take place simultaneously, increasing the growth difference between myrtle plants from conspecific and heterospecific soil communities. Further research measuring different variables simultaneously are necessary to test these hypotheses.

Independently of any possible explanations for the plant-soil community feedback, it was not possible to increase myrtle plant size in the conspecific soil by inoculating the soil with commercially available AMF inoculum, even though the spores of AMF commercial inoculum were still present and able to colonize myrtle roots in the subsequent plant generation. These results alert to the over-generalization of commercial inoculum benefit in plant nursery production. Furthermore, the results point out to some possible future problems concerning the massive AMF species introduction in disturbed AMF communities by using single species commercial inoculum, since it was here demonstrated that these fungi will remain active for a long time in the soil.

Chapter 5 – GENERAL DISCUSSION

In this study, recent concepts of mycorrhizal ecology were used to interpret the results of mycorrhizal inoculum application in nursery plant production for restoration purposes. The contribution of this study to improve the understanding of the host plant-fungal association can be analyzed from different prespectives:

i) the effect of disturbance on the infectivity and effectivity of indigenous mycorrhizal community;

ii) the effect of commercial inoculum addition on native plant growth and on indigenous mycorrhizal community;

iii) the effect of woody Mediterranean plant species on AMF soil communities;

guidelines for decision-making concerning AMF management

i) The effect of disturbance on the infectivity and effectivity of indigenous mycorrhizal community

The results confirmed most of the disturbance related hypotheses initially proposed (Table 5.1). There was a major reduction in AMF propagule density, diversity and overall infectivity due to quarry soil stockpiling (*Chapters 2* and *3*). Similar results have been described for mining activities (Doerr *et al.*, 1984; Jasper *et al.*, 1987; Bell *et al.*, 2003).

Disturbance led to a decrease in AMF species diversity, in agreement with the initial hypothesis, and only *Glomus* species were isolated from the disturbed soil (*Chapter 3*). The same kind of reduction in AMF community diversity has been reported in other studies (*e.g.* Alves da Silva *et al.*, 2005). The observed reduction in AMF infectivity, measured through sorghum root colonization, was related with the reduction in viable propagules density (*Chapter 2*). However, that decrease was not followed by differences in the root colonization of native plant species. It was shown that the relation between propagule density and root colonization extension depends on the plant species (*Chapter 2*). The results are in agreement with other studies that also report an inconsistent effect of soil disturbance on root colonization; it can depend on the AMF inoculum level previous to disturbance (McGee *et al.*, 1999), on the interaction between the environment and the plant species (McGonigle and Miller, 2000), and on the AMF inoculum composition and distribution (Allen, 2001). Nevertheless, in the present study it was observed that AMF inoculum infectivity, measured by sorghum bioassay, increased with time (*Chapter 3*).

The negative effect of disturbance on soil inoculum infectivity, was not reflected on the growth differences of the target woody Mediterranean plant species (*Chapters 2* and *3*). This result does not support the initial hypothesis (Table 5.1) based in previous studies showing that the extent of host-plant benefit from mycorrhizae was density-dependent (Koide and Li, 1991; Facelli *et al.*, 1999). However, the absence of linear relations between soil inoculum density and mycorrhizal effect has also been reported by others (Gianinazzi-Pearson *et al.*, 1985; Clapperton and Reid, 1992; Karasawa *et al.*, 2001).

Abbott and Robson (1991) proposed that the degree to which AMF increase the plant growth and biomass is, in general, proportionally related to the rate and extent of the colonization. In agreement, there was a direct relation between root colonization and biomass of woody native plant species, except for myrtle plants (*Chapter 3*). Myrtle plants had similar root colonization in the AMF communities from the disturbed and undisturbed soils, but grew more with the AMF community from the disturbed soil, and this response pattern was constant along the time. On the other hand, carob and lavender presented similar biomass increments with both AMF communities (*Chapters 2* and *3*). Thus, it can be suggested that the response of woody Mediterranean plants to mycorrhizae is plant species-specific.

The fact that plants did not perform better when growing in the presence of AMF community from undisturbed soil (*Chapters 2* and *3*) did not support the initial hypotheses: (i) plants growing with the undisturbed soil community would perform better than the ones growing with the disturbed soil community; and (ii) middle-late successional Mediterranean plants would perform better when associated with AMF community from the same successional level (AMF community from the undisturbed area). Allen *et al.* (2003) found similar results with woody plants, which became taller when grown with early-seral AMF inoculum.

To explain the absence of an incremental plant growth like the one found in myrtle with the undisturbed AMF community, the occurrence of negative feedback due to plant species-specific pathogens interactions is usually suggested (*e.g.*, Mills and Bever, 1998; Packer and Clay, 2000). *Chapter 4* shows that this was not the case in this study. In fact, negative feedback interactions occurred, but apparently due to particular AMF species. These results cannot be also related to the effects of short term disturbance inherent to bulk soil collection, since in the experiment of *Chapter 2*, where the soils were collected as intact cores without disrupting the AMF soil community hyphal network, the plants grown in the two soils had also similar biomass increments.

In conclusion, the AMF community from disturbed soil had lower viable propagules density, diversity and overall infectivity as compared to the AMF community composition from the undisturbed soil, but these differences did not induce differences in root colonization and/or biomass of woody native plant species, except for myrtle plants. So, it is suggested that disturbance selected AMF species that are more mutualistic. This possible explanation is based by the work of Johnson (1993), who demonstrated that changes in soil properties can induce changes in mycorrhizal function and selection of AMF species. In fact, this work shows that mycorrhizal symbiosis can range from mutualism to parasitism depending on host plant-AMF species and the environmental conditions (Johnson *et al.*, 1997; Jones and Smith, 2004).

ii) The effect of commercial inoculum addition on plant growth and indigenous mycorrhizal community

The experiments presented in this thesis demonstrated that the AMF species introduced as commercial inoculum (*G. intraradices*) was able to adapt to the particular experimental soil abiotic conditions (*Chapters 3* and *4*), and persist viable in the indigenous AMF species community during the three years of study (*Chapter 4*). Palenzuela *et al.* (2002), in a study where plants were also inoculated with *G. intraradices* in the nursery, did not recover viable spores from the plant rhizosphere after plants were out-planted to the field. The addition of *G. intraradices* did not change the reestablishment of AMF community diversity from the disturbed soil (*Chapter 4*), which may indicate that no negative interactions with other indigenous AMF species took place, probably because no *G. intraradices* were found in the disturbed soil (*Chapter 3*), but had occured in that area prior to disturbance (undisturbed soil), observed through the evaluation of AMF communities from undisturbed soil.

Glomus intraradices extensively colonized native plant roots (*Chapters 3* and 4), as predicted, showing an ability to compete for root space with indigenous AMF species from the disturbed soil AMF community, because whenever *G. intraradices* was added to the soil it became the dominant AMF species in root colonization (data not shown). However, the total root length colonization by *G. intraradices* did not significantly differ from the indigenous AMF species in the disturbed soil, and this relation did not change along time (*Chapters 3* and *4*). The colonization extension ratio was plant species-specific. While myrtle and lavender plants showed broad root length colonization, carob showed less extensive colonization in the first six months, but after one year it increased to a level equivalent to the one found in the other native plants species. It is here suggested that carob has some control on root length colonization, as was also suggested by some authors (Smith and Smith, 1996; Brundrett, 2002). It has already been shown that carob plants are able to maintain arbuscules colonization in very specific zones of the root (Cruz *et al.*, 2004).

The increased soil AMF propagule density by the addition of commercial inoculum to the soil did not promote a significant growth benefit in any of the three tested Mediterranean woody plants (*Chapters 3* and *4*) These results do not support what was initially hypothesized: if AMF species diversity was redundant, as was the case in these experimental conditions, then the enhancement of AMF spore density in disturbed soils by the addition of commercial AMF inoculum would increase plant growth (Table 5.1). The results from *Chapters 2* and *3* confirm that indigenous AMF community from the disturbed soil was the most effective because it induced similar native plant growth results as the other AMF communities with higher diversity (undisturbed soil) and propagules density (inoculated AMF community). Therefore, it can be suggested that the differential effectivity of individual AMF species (*e.g.* Klironomos, 2003) can also be observed at AMF community functional level. Comparable results were found by Caravaca *et al.* (2005) that also inoculated Mediterranean woody shrubs with *G. intraradices* and a mix of indigenous AMF species, and observed that the allochthonous AMF species was more infective, although the indigenous AMF species were more effective in promoting plant growth. Furthermore, the commercial inoculum addition to the soil did not act as a mediator of the antagonic effect caused by any organisms that were affecting in myrtle plants growth on conspecific soil microbial community (*Chapter 4*).

Glomus intraradices asymptomatically colonized the native plant roots, without any observed plant benefit. One possible explanation to such a fact is given by Redman *et al.* (2001) who proposed theories of symbiotic plant-fungal interactions. These authors demonstrated that some pathogenic *Colletotrichum* spp. have also the ability to express other lifestyles as mutualists and/or commensalists, regarding plant growth enhancement or drought tolerance, based on the host genotypes differences. It is unlikely that this phenomenon would be specific of *Colletotrichum* spp. because many “pathogens” can be isolated from asymptomatic plant tissues (Schulz *et al.*, 1998). This suggests that, either these symbiotic fungi species have evolved to acquire maximum symbiotic flexibility, or that directional evolution has occurred in a host genotype-specific manner, instead of the prevailing view considering that selection pressure has imposed directionality on the symbiotic interactions evolution. This interpretation can be extended to all plant-fungal species interactions. Following this approach, *G. intraradices* in the presented experimental conditions may have displayed a commensal life strategy, but nothing can be concluded concerning its ability to establish truly mutualistic symbioses in other environmental conditions and different host plant genotypes. A similar framework was proposed by Jonhson *et al.* (1997) where mycorrhizal associations can function along a mutualism-parasitism continuum (see Fig. 1.2). The authors do not refer parasitism in the general microbiological perspective, but consider plant-AMF interactions as parasitic when the costs of association exceed the benefits. In addition, Klironomos (2003) demonstrated that a single AMF species can induce positive or negative effects on plants growth depending on the host species.

In conclusion, the differential plant growth responses are more dependent on the complexity of the plant-soil-microbe interactions than on the simple addition of commercial AMF inoculum by itself. The addition of commercial AMF inoculum is unavoidable for some restoration processes. However, for an accurate prediction of the outcomes in different plant soil microbial communities settings, the development of methodologies in such diverse areas as genomics, proteomics, microscopy, symbiosis physiology and new ecological modelling approaches in the field is still needed, to identify the underlying mechanisms involved in plant root-soil microbial associations.

iii) The effect of woody Mediterranean plant species on AMF soil communities

The build-up of the indigenous AMF community was promoted by the native host plants, reducing AMF communities composition differences along the time (*Chapters 3* and *4*). This ability to increase soil mycorrhizal infectivity was also found for other woody Mediterranean shrubs in other nursery and field studies (Palenzuela *et al.*, 2002; Azcón-Aguillar *et al.*, 2003; Caravaca *et al.*, 2005). In these studies, as in the present one, the target plant species differed in their capabilities to increase soil AMF inoculum infectivity. While carob plants did not promote a significant increment in inoculum infectivity in any AMF community after one year, myrtle and lavender increased the infectivity of the AMF community from undisturbed soil (*Chapter 3*). Nevertheless, after three years, AMF diversity in soil pre-cultured by carob had the same AMF species diversity as the other studied host plants (Chapter 4).

iv) Guidelines for decision-making concerning AMF management

Almost twenty years ago Hall (1988) stated that "whether AM technology will ever be used (…) will depend on the economics of employing it as compared with, for example stimulating growth with fertilizer." (cited in Miller et al., 1994).

Taking the results obtained in this thesis into account, some recommendations for the future use of commercial AMF inoculum in plant production for restoration purposes, as well as some alternative suggestions to increase the prediction capability in management strategies for restoration trials, are presented.

The most critical factors for quarry restoration success are the soil microbial quality to cover the slopes and a good plant production. Therefore, it is suggested to evaluate the indigenous AMF species infectivity and effectivity in the target plant species, but not through the evaluation of the viable propagules or through the use of other host plant species, besides the target plant species (*Chapter 2*). External inoculation for disturbed sites is not always required, as it was shown in this study and by Jenkin *et al.* (2004), who found that in a disturbed field sown plants were also colonized after the second growing season.

Concerning the nursery plant production, the results demonstrated that native plants enlarged indigenous AMF species inoculum, after three years in pots (*Chapters 3* and *4*). The occurrence of sporulation enhancement in pot culture environments is a common phenomenon, which was hypothesized by Stutz and Morton (1996) to be the result of more homogeneous and constant moisture conditions, of prolonged close contact among growing roots, and of more uniformly distributed fungal inoculum. When the native plants are transplanted to the field, their rhizosphere can act as "nurse plants" (Haselwandter and Bowen, 1996; Carrillo-Gracia *et al.*, 1999; Caravaca *et al.*, 2005), functioning as points of high biological activity, and promoting the dispersal of AMF propagules through the mycelium, which is important for the Mediterranean heterogeneous landscape (Caravaca *et al.*, 2005), where facilitation among plants may be highly fostered (Callaway, 1997). Another recommendation for plant growing in nurseries is to use pots with soil that is going to be used during quarry restoration (stockpiled soil or other), to increase future woody plant successful establishment in the restoration process, despite the low levels of spores found in Mediterranean soils (Azcón-Aguillar *et al.*, 2003) (*Chapters 3* and *4*). The advantage of this approach is to allow an evaluation of plant responses, not only to the indigenous mycorrhizal inoculum, but also to the overall soil abiotic (pH, soil texture and soil nutrient contents) and biotic variables (interactions with other non mycorrhizal fungi, bacteria, nematodes, etc.), similar to the ones found in restoration trials (*Chapter 4*). If plants grow in the bought enriched organic soils commonly used in the nurseries, then the disadvantages can be innumerous. For instance, when plants are planted there is a large possibility of simultaneously introducing an entirely new soil microbial community, including pathogens, nematodes and/or small animals; on the other hand plants do not develop adaptations to the natural poor soils of field conditions during nursery stages.

From the results of this thesis, the build-up of indigenous AMF community, oppositely to the introduction of commercial inoculum is greatly recommended in plant production for Mediterranean restorations (*Chapters 3* and *4*). However, the results cannot be generalized in terms of commercial inoculum used for plant production for quarries revegetation, since only one commercial AMF inoculum brand was tested, in very specific conditions. If it is necessary to apply commercial AMF inoculum in the target plants, it would be better to accomplish it in the nursery, as an alternative to a direct addition in the field. This would minimize the risk of an invasion effect by a novel AMF species inoculum (Friese and Allen, 1991; Schwartz *et al*., 2006), and reduce the inoculum acquisition costs, since a lower amount of inoculum is required. Comparative studies of commercial inoculum in nurseries (Carpio *et al.*, 2003; Corkidi *et al.*, 2003) are consistent: the investment on an increase of spore density is not met by plant growth increment. Other components present in the commercial AMF inoculum, as well as the plant growing medium, was also responsible to determine the success of mycorrhizal inoculation.

In this thesis, the importance of plant-AMF species combinations is emphasized. Since Mediterranean plant species show different life-history strategies to cope with drought stress characteristic of Mediterranean climate, those differences will act as sources of variation of the mycorrhizal symbiosis effectiveness. Therefore, the host plant species selection is determinant for the selection of methodologies for nurseries. Evergreen sclerophylls (trees or shrubs) grow intensively during a short period in spring. Semi-deciduous plants, such as lavender, grow all over the year except for a short period during summer when growth rates decrease. In this work, inoculum addition was also beneficial for plant height increment during the first three months of plant growth but afterwards this benefit was only expressed in evergreen sclerophyllous species. These results may also be explained by the plant life strategies of the target species. Sclerophylls, from the middle- and late-seral succession, are mycorrhizal facultative probably because they have an economic strategy of nutrient conservation, such as an efficient nutrient remobilization. Thus, mature plants are not dependent on mycorrhizal associations for nutrient uptake. However, at the early successional stages, these plants are either dependent on seed/cotyledons resources or on mycorrhizal associations or both. This means that during establishment they benefit from a short period of life from their AMF counterparts, particularly from the most ruderal and infective communities. On the contrary, semi-decidous plants are mycorrhizal dependent, depending on soil nutrients to grow all over the year (Cruz *et al*., 2003). Thus, the different interactions between plant-AMF soil organisms along a seral succession seem to be dependent on plant life strategies, particularly on the way plants use, explore and store nutrient availability.

The large variability in native host plant species response to each AMF community can be attributed to the large variability of host plant species genotype together with the combination of the different AMF species present in each community. Moreover, if plants show species-specific phenological, physiological and morphological adaptations to Mediterranean climate, it is most expected that those functional differences influence plant-AMF species relations. For example, leaf shedding is a response to summer drought in semi-deciduous species, like lavender; such biomass losses occurring over the sampling period are not due to any detrimental mycorrhizal effect, but to plant strategies to cope with summer drought.

In general, root length colonization in woody plants is similar between AMF communities, independently of AMF soil propagule density. In contrast, root colonization in sorghum, an herbaceous and fast growing species, differs according to AMF soil propagule (*Chapters 2* and *3*). This suggests an adaptation of Mediterranean plants to the low levels of inoculum in the Mediterranean soils (Requena *et al*., 2001). In agreement with this hypothesis, lavender initiates root colonization faster than myrtle and carob, and has increased growth in early plant development stages due to mycorrhizae in the undisturbed soil (*Chapter 2*). Both findings can be explained by lavender dependence on mycorrhizae, probably due to fast depletion of the seed resources, since lavander has the smallest seeds among the studied plant species.

In conclusion, the suggested guidelines to determine the need of commercial AMF inoculum for each specific restoration trial, in a preliminary nursery assay are mainly:

- to determine the indigenous AMF infectivity to restoration target plants (*Chapter 2*);
- to determine plant growth response to the soil microbial community (AMF and other organisms) (*Chapters 3* and *4*);
- to evaluate target plant species effect on the indigenous AMF soil inoculum build up (*Chapters 3* and *4*).

Table 5.1. Summary of the hypotheses proposed in *Chapter 1* for the effect of disturbance, commercial inoculum addition and native plants on AMF communities. It is indicated whether the results obtained supported the former hypotheses or not.

HYPOTHESES	**RESULTS**
Disturbance effect	
Reduction of AMF propagule density, diversity and infectivity	Accepted
Reduction of indigenous AMF effectivity for native woody plants	Rejected
Benefit extent of reduction is plant species-specific	Accepted
Effect on plant growth and AMF infectivity decrease along time	Accepted
Commercial inoculation effect	
If diversity is redundant: inoculation has a positive effect on plant growth	Rejected
If diversity is not redundant: increasing AMF community diversity increases plant growth benefit; the beneficial effect is plant species-specific	Rejected
Acts as a biocontrol agent (reducing other root endophytic fungal colonization)	Rejected
Plant effect on AMF community	
Woody Mediterranean plants are able to promote AMF inoculum build-up	Accepted
Inoculum build-up rate is plant species-specific	Accepted

Chapter 6 - FINAL CONCLUSIONS

As a general conclusion, and concerning the feedback relations between the studied woody Mediterranean plants and the AMF communities, in the tested experimental system, this thesis showed that AMF community effectiveness on plant growth, and that the reciprocal host plant species effect on AMF community composition, rely more on each symbiotic species interaction than on changes induced by disturbance or soil AMF inoculum addition. In particular, the major conclusions of this study are:

- Disturbance reduces AMF soil propagules, infectivity but not the effectivity to woody native plants.
- Soil inoculation had null effects on promoting target plants growth
- *Glomus intraradices* from commercial inoculum did not mediate plant-root endophytic fungal feedback.
- Myrtle had differential plant growth response to different soil microbial communities.

Chapter 7 - FUTURE PERSPECTIVES

Twenty years passed, since Fitter’s (1985) interpretation concerning the experimental field results: “….Several factors probably contribute to the low effectiveness of VA fungi as P uptake systems in field conditions, including incompetence of strains and species of fungi, grazing of hyphae….”. The mycorrhizal ecology knowledge evolved since, that but it is still mandatory to proceed with mycorrhizal research in field condition, taking in consideration all kinds of organisms that interdependently influence plant growth.

Progressively, it has been shown in the literature that each plant-AMF species pair combination produces a different outcome of the symbiosis effectivity, and the results change along with soil and edaphic characteristics. In agreement, Azcón-Aguillar and Barea (1997), suggested that the maximum benefit will only be obtained from inoculation with “efficient” AMF species (indigenous or commercially obtained) and with the selection of compatible host-fungus-substrate combinations, prior to field inoculation. However, information is still missing. A better understanding of the underlying mechanisms by which AMF differentially promote plant growth, would explain the possible functional compatibility or specificity of host plant responses to mycorrhizae, and therefore increase the ability to predict the success in future application of these fungi.

Manipulations of AMF inoculum in sustainable agriculture or restoration progress should be carefull, and plant benefits from mycorrhizal symbiosis should not be overestimated. It is likely that literature is biased in favor of positive results, as researchers often design mycorrhizal experiments only when they suspect mycorrhizal fungi to limit productivity (Lekberg and Koide, 2005). This represents a considerable challenge in terms for future experimental assays required to achieve reliable results concerning the commercial inoculum beneficial effects with a wide range of abiotic and biotic variables for each ecosystem. Moreover, these studies should include an evaluation of commercial AMF inoculum species effect on the indigenous AMF communities in the disturbed soils, like their life strategies in relation to persistence and competition abilities (*sensu* Hart *et al.*, 2001). Perhaps it would be important to study the ability for AMF natural dispersion from undisturbed surrounding areas (Tommerup and Carter 1982; Warner *et al.*, 1987) or from nursery inoculated plants out-planted to the field (Caravaca *et al.*, 2005).

After 13 years dealing with mycorrhizal issues, I allow myself to suggest for future "mycorrhizast" (name proposed by Harley for mycorrhizal symbiosis researchers, cited *in* Koide and Mosse, 2004) the selection different measurable variables besides plant growth and/or mycorrhizal root colonization.

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