DEPARTAMENTO DE CIÊNCIAS DA VIDA
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Arbuscular Mycorrhizal Fungal diversity and composition in pastures of the Azores: assessing the impact of management practices

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Tese apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para obtenção do grau de Doutor em Biologia, na especialidade de Ecologia, realizada sob a orientação científica da Professora Doutora Helena Freitas (Universidade de Coimbra) e do Professor Doutor Paulo A. V. Borges (Universidade dos Açores)

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Abstract

Arbuscular mycorrhizal fungi (AMF) are ubiquitous, underground, symbiotic associations involving a wide diversity of plants (approximately 80%) and obligate symbiotic fungi of the phylum Glomeromycota thought to have originated 400–500 million years ago. In this intimate association the obligate biotrophic fungi provide terrestrial plants with minerals, nutrients (particularly inorganic phosphate) and water increasing the host resistance to biotic and abiotic stresses, including pathogens, water limitation and environmental pollutants in return for photosynthates. Diversity of AMF is generally higher in natural and semi-natural systems than in more intensively managed agroecosystems, where it can be strongly reduced. The objective of this study was to investigate whether land use type can be characterized by their arbuscular mycorrhizal fungal (AMF) communities, and to test the potential effect of AMF indigenous as inoculants on crops productivity and host tolerance to root-knot *Meloidogyne incognita*.

AMF community structure associated to *Holcus lanatus* L. root was studied during two years in two land uses: semi-natural (Pico do Galhardo- PX; Terra Brava- TB) and intensive (Agualva 1- RP1; Agualva 2- RP2) pastures of Terceira Island (Azores). The spore community composition of AMF associated to *Holcus lanatus* L. root was determined by taxonomic identification of AMF spores. Thirthy-nine AMF species representing eight genera were detected in the land uses studied. The most representative genera of AMF spore community were *Glomus, Acaulospora* and *Scutellospora*. Land use type had no significant effect on AMF spore diversity and total abundance. This also was confirmed by molecular methods (PCR-DGGE) on roots of *H. lanatus*. In Azorean pastureland the input of fertilisers is lower than average inputs to pastures on the European mainland, which presumably did not contribute to occurrence of an evident pattern of AMF diversity. However, the reverse was observed for colonisation of different AMF structures (arbuscules, vesicles and hyphae) on *H. lanatus* root, where the highest of AMF colonisation was in semi-natural use. Colonisation of different AMF structures on *H. lanatus* root showed a seasonal pattern between sampling dates. The higher abundance of AMF structures in the summer than in autumn/winter could be related to nutrient exchanges, host metabolic pathway, phenology and climatic variations.

Striking differences were found in AMF spores composition between land uses. Species of *Glomus* were dominant in intensive land uses, while *Acaulospora* and *Scutellospora* predominated in semi-natural ones. Different strategies of colonisation among AMF and soil nutrient levels may be the most important factors influencing the AMF community. Members of Glomaceae have a highly infective extra-radical mycelium that could allow colonising immediately plant roots (early succession), while members of Gigasporaceae are only capable of propagation via spore dispersal, therefore colonize plant roots more slowly than members of Glomaceae. Both spore densities of *Glomus*, *Acaulospora* and *Scutellospora* genera, as well as root colonisation were related with soil nutrients (N, K, Mg, Ca, OM), but no correlation was found with soil phosphorus (P). Our results confirm that the degree of AMF benefit to a host plant depends on plant species. *Lolium perenne* L. was the most mycorrhizal dependent plant (DM= 62%), followed by *Holcus lanatus* L. (DM= 40%) which resulted in an increase of foliar biomass more than 30 % in both plant species caused by inoculation with native AMF. Conversely, *Lolium multiflorum* L. was the less mycorrhizal dependent (DM= 27%) plant and also the less colonised.

In more than 90% of the samples, inoculation with the native AMF conferred protection to *H. lanatus* from the root-knot *Meloidogyne incognita*. Among the mechanism proposed to explain the protective effect of AMF, we suggest that plant growth promotion by AMF, and direct competition between AMF and the nematode for host colonisation sites and photosynthates were involved.

We conclude that: either AMF spore diversity or AMF diversity on *H. lanatus* root were independent of land use intensity; However, in the intensive management regime, there was a trend to an increased incidence of certain AMF, especially from the *Glomus* indicating a potentially severe loss of ecosystem functions under this land use type; colonisation of different AMF structures was higher in semi-natural land use and showed a marked seasonal pattern, dependent of plant phenology and metabolic pathway and climatic conditions. Inoculation with indigenous AMF significantly increased plant productivity and tolerance to root-knot *M. incognita*, but degree of AMF benefit to a host plant varies in function of plant species. Thus, AMF may play an important role in ecosystems functioning, since their appropriate management can reduce the use of chemical and energy in agriculture and consequently lead to more economical and sustainable production systems.

Resumo

Os fungos micorrízicos arbusculares (FMA) são associações simbióticas envolvendo uma ampla diversidade de plantas (aproximadamente 80%), e fungos simbiontes obrigatórios do filo Glomeromycota cuja origem estima-se ter ocorrido há 400-500 milhões de anos. Nesta íntima associação o fungo biotrófico obrigatório supre a planta com minerais, nutrientes (particularmente fosfato inorgânico) e água, aumentando deste modo, a resistência da planta hospedeira a stresses bióticos e abióticos, incluindo agentes patogénicos, stress hidríco e poluentes ambientais em troca de produtos fotossíntetizados. A diversidade de FMA é geralmente mais elevada em sistemas naturais e semi-naturais do que em agro-ecossistemas mais intensivos, onde poderá ser fortemente reduzida. O objectivo deste estudo foi investigar se as comunidades de fungos micorrízicos poderão servir de ferramentas na caracterização do tipo de maneio do solo, e testar o potencial efeito da inoculação com fungos nativos na produtividade das culturas, bem como na sua tolerância a nemátodes formadores de galhas - *Meloidogyne incognita*.

A estrutura da comunidade micorrízica associada às raízes de *Holcus lanatus* L. foi estudada durante dois anos em dois sistemas de uso do solo: pastagens semi-naturais (Pico do Galhardo- PX; Terra Brava- TB) e intensivas (Agualva 1- RP1; Agualva 2- RP2) da ilha Terceira (Açores). A composição da comunidade de FMA associada a *H. lanatus* foi determinada tendo em conta as características morfológicas dos esporos de FMA. Nos dois sistemas de maneio do solo foram detectadas 39 espécies de FMA distribuídas por 8 géneros. Os géneros mais representativos foram *Glomus, Acaulospora* e *Scutellospora*. A diversidade de esporos de FMA, assim como, a sua abundância não foi afectada pelo tipo de maneio do solo. Este resultado também foi confirmado por via molecular (PCR-DGGE), em relação à diversidade de espécies da comunidade fúngica nas raízes de *H. lanatus*. Os níveis de fertilizantes aplicados nas pastagens açorianas são em média muito mais baixos, do que os aplicados em pastagens do continente Europeu, o que presumivelmente não terá contribuído para a obtenção de um evidente padrão de diversidade micorrízica. Contudo, a quantificação da colonização das diferentes estruturas micorrízicas (arbúsculos, vesículas e hifas) exibiu o padrão diferente, tendo sido mais elevada nas pastagens semi-naturais do que nas intensivas. Além disso, as diferentes estruturas micorrízicas exibiram um marcado padrão sazonal entre épocas

de amostragem, tendo sido mais abundantes durante o Verão do que no Outono/Inverno. Este facto, poderá estar relacionado com as trocas de nutrientes, a via metabólica do carbono da planta hospedeira, o estado fenológico e com as variações climáticas. Foram encontradas profundas diferenças na composição de espécies entre os dois sistemas de uso do solo. Nos sistemas semi-naturais os géneros mais representativos foram *Acaulospora* e *Scutellospora*, enquanto nos intensivos dominaram as espécies pertencentes ao género *Glomus.* O tipo de estratégia de colonização adoptado pelos diferentes FMA, bem como, os níveis de nutrientes do solo, poderão ser os principais factores responsáveis por essas diferenças na comunidade micorrízica. Os membros da família Glomeraceae possuem um micélio extra-radicular altamente infectivo que lhes permite uma rápida colonização radicular, enquanto os membros da família Gigasporaceae, visto serem apenas capazes de se propagarem via dispersão dos esporos apresentam uma taxa de colonização mais lenta. A densidade de esporos dos géneros *Glomus*, *Acaulospora* e *Scutellospora* assim como, a colonização radicular estiveram correlacionados com o nível de nutrientes do solo (N, K, Mg, Ca, MO), excepto com o fósforo (P). Os nossos resultados confirmam que, os benefícios resultantes da relação simbiótica dependem da espécie de planta hospedeira. *Lolium perenne* L. foi a planta que exibiu maior dependência micorrízica (DM= 62%) seguida de *Holcus lanatus* L. (DM= 40%), o que originou um incremento na biomassa foliar superior a 30 % em ambas as espécies de plantas. Contrariamente, *Lolium multiflorum* Lam. foi a espécie de planta com menor percentagem de colonização micorrizica, e por conseguinte a menos dependente da micorrização (DM= 27%).

A inoculação com FMA nativos aumentou a tolerância de *H. lanatus* à infecção por *Meloidogyne incognita* em mais de 90 % das amostras. Entre os vários mecanismos propostos para explicar o efeito protector dos FMA, destaca-se o aumento no vigor da planta resultante da colonização micorrízica, e a competição por espaço e compostos fotossíntetizados que se estabelece entre o FMA e o nemátode.

Com base neste estudo podemos concluir que: quer a diversidade de esporos de FMA, como a diversidade de FMA que colonizam as raízes de *H. lanatus* não dependem da intensidade do sistema de maneio; no entanto, nos sistemas intensivos há uma tendência para uma maior ocorrência de certos FMA, especialmente os pertencentes ao género *Glomus*, assistindo-se por conseguinte a uma potencial perda

da funcionalidade dos ecossistemas neste tipo de maneio do solo; a colonização das diferentes estruturas micorrízicas foi mais elevada nas pastagens semi-naturais, e exibiu uma marca sazonalidade relacionada com a fenologia e metabolismo de carbono da planta hospedeira, e com as condições climáticas. A inoculação com FMA nativos aumentou significativamente a produtividade das plantas e tolerância à infecção por nemátodos galhadores – *M. incógnita*, mas todavia depende da especificidade da planta hospedeira. Deste modo, os FMA poderão desempenhar um papel importante na funcionalidade dos ecossistemas, uma vez que o seu uso adequado poderá reduzir a aplicação de produtos químicos, bem como, os custos energéticos na agricultura e consequentemente conduzir a uma agricultura mais sustentável e económica.

CHAPTER I. GENERAL INTRODUCTION

General introduction

In almost all terrestrial ecosystems mutualistic interactions between plants and microorganisms in the soil play a major role for the structure and dynamics of plant communities (van der Heijden *et al*. 1998). An important group of mutualists are Arbuscular mycorrhizal fungi (AMF), an exceptional group of beneficial fungi. They are present in most soils and are capable of forming associations with the root systems of the great majority of plant species, where they act as bioregulators and protectors, and facilitate the uptake of mineral nutrients (Smith and Read, 1997).

1. The importance of soil microorganisms

Much of the terrestrial biosphere lives in the soil (Brussaard *et al*., 1997). Although soil provides a physical substrate for almost all human activities, the study of soil biota remains relatively uncommon, especially in systems such as conventional agriculture where soil is used exhaustively used (Whipps, 2001; Barea *et al.*, 2005). This is inattention is partly to a lack of recognition of the role of soil biota on determining soil's chemical and physical properties and potential productivity (Whipps, 2001; Barea *et al.*, 2005). In addition, knowledge of soil microbial diversity is partly limited by taxonomic and methodological challenges associated with studying these organisms (Torsvik and Øvreås, 2002; Kirk *et al*., 2004). The soil organisms are not only inhabitants of the soil, but they are also integral parts of it; they influence such diverse properties as hydrology, aeration and gas composition. These factors can have a major effect on primary production through the decomposition of organic residuals (Brussaard *et al*., 1997). The current shift toward sustainable land use, has contributed to an increasing awareness of soil biota among the planet's forms of life (Gianinazzi *et al*., 2008). Microorganisms' activities in the so-called "rhizosphere" promote the establishment, development, nutrition and health of plants, and are mutually, enabled by physical and nutritional support from the exudates of roots (Barea and Jeffries, 1995).

There are three main groups of beneficial microorganisms in soil-plant systems, and these groups are considered key to the sustainability of agro-ecosystems, namely: mycorrhizal fungi, N_2-fixing bacteria, and plant-growth-promoting rhizobacteria

(PGPR). N_2-fixing bacteria contribute to the symbiotic fixation of atmospheric N, which represent the essential access of nitrogen (N) in ecosystems (Barea and Olivares, 1998). Rhizobacteria which colonise the rhizosphere, are distinguished by their effects in promoting plant rooting and the biogeochemical cycling of plant nutrients (Barea, 1997). Mycorrhizal fungi are ubiquitous plant mutualists that comprise a large fraction of fungal biomass in the rhizosphere. These symbiotic organisms are found inside roots, in the rhizosphere itself and in the bulk soil, and they interact in all these zones with other organisms (Fitter and Garbaye, 1994; Azcón-Aguilar *et al*., 2002). The internal mycelium interacts principally with the root itself, though at the same time, it can interact with other organisms and even with pathogenic fungi. The AMF external mycelium developed in the soil improve plant's nutrient and water uptake and, interact with many organisms, such as bacteria, other fungi, protozoa, nematodes, arthropods and even large animal such as mammals (Fitter and Garbaye, 1994; Azcón-Aguilar *et al*., 2002). In this association, the obligate biotrophic fungi provides terrestrial plants with minerals, nutrients (particularly inorganic phosphate) and water, increasing the host resistance to biotic and abiotic stresses, including pathogens, water limitation and environmental pollutants in exchange for photosynthates (Smith and Read, 1997).

2. Mycorrhizal fungi

Among favourable plant-microbe interactions, the mycorrhizal symbiosis is now known to be an essential component of any plant community due to the beneficial role of fungi on plant growth and protection (Smith and Read, 1997). The term "mycorrhiza" was proposed for the first time in 1985 by the botanist A.B. Frank, to describe the peculiar association between tree roots and ectomycorrhizal fungi. It comes from the (Greek) *mycos-rhiza*, which means "fungus-root". Fossil records from the Devonian of the first vascular plants show that arbuscular mycorrhizal associations were present in underground rhizomatous structures, confirming the presence of this mutualistic association at least 400 million years ago (Remy *et al*., 1994). Evidence that the first land plants already possessed this association suggests that their aquatic ancestors also harboured these fungi. Redecker *et al*. (2000) have documented spores from the Ordovician period (460 Ma) that are similar to present-day spores of Glomalean fungi, indicating that earlier associations with non-vascular plants were likely.

Mycorrhizal fungi are present in almost all terrestrial habitats from aquatic (Nielsen *et al*., 2004; Wolfe *et al*., 2006) ecosystems to deserts (Díez *et al*., 2002; Shi *et al*., 2007) tropical forests (Husband *et al*., 2002; Mangan *et al*., 2004), at high latitudes and altitudes (Allen, 1991). It has been estimated that mycorrhizal fungi form symbiotic associations with more than 85 % of plant species (Smith and Read, 1997). Only a few botanic families as Crucifereae, Ciperaceae, Poligonaceae, Juncaceae and Chenopodiaceae have non-mycorrhizal plant species (Brundrett, 2002; Peterson *et al*., 2004). There is evidence that these nonmycotrophic groups may have mycorrhizal fungal invasions, though the physiological relationships are not well understood (Allen, 1991; Peterson *et al*., 2004).

Seven different categories of mycorrhizal symbiosis have been distinguished on the basis of their morphological characteristics and the fungal and plant species involved, namely: arbuscular mycorrhiza, ectomycorrhiza, ectendomycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ericoid mycorrhiza and orchid mycorrhizas(Smith and Read, 1997; Peterson *et al*., 2004; Finlay, 2008).

3. Arbuscular mycorrhizal fungi

Arbuscular mycorrhiza fungi (AMF) belong to the phylum Glomeromycota, and form association with most of land plants (about 80-90%) (Smith & Read, 1997). These fungi are obligate biotrophic symbionts, since they can only complete their life cycle in the presence of their host plant. The main morphological characteristic of these fungi is the repeated dichotomous branching as they penetrate the cortical cells of the root to form of arbuscules, highly branched tree-like structures typical of colonisation structures by these fungi. Initially, the fungus grows between the cortical cells, but it then rapidly penetrates the host cell wall of the host and develops inside the cortical cells.

In this type of association neither the cell walls of the fungus or the host cellular membrane are ruptured. As the fungus develops, the host cellular membrane invaginates and surrounds the fungus, forming a new compartment where material of high molecular complexity it is deposited (Bonfante & Perotto 1995).This apoplastic space prevents direct contact between the plant and the cytoplasm of the fungus, while allowing the transfer of nutrients between symbionts. Thus, arbuscules (Fig. 1)

are the main sites of metabolite exchange and the primary physiological structures of the symbiosis. They generally have a short life span (less than 15 days), after which they degenerate, returning the cell to normal activity (Smith and Smith, 1990).Other structures produced by some AMF include vesicles, auxiliary cells and asexual spores. Vesicles are globose, inter- or intracellular, between 30 to 100 mm in size and rich in lipids (Fig. 1). Their primary function is to reserve organs, however, they can also serve as reproductive propagules for the fungus.

All arbuscular mycorrhizal fungi form arbuscules, but some such as *Gigaspora* spp. or *Scutellospora* spp., do not form vesicles. This observation underlies, the argument to change the designation of "vesicular–arbuscular mycorrhiza" to "arbuscular mycorrhiza" (Walker, 1995). AMF can produce auxiliary cells in the extraradical mycelium (Peterson *et al.* 2004; Smith & Read 1997). Auxiliary cells are initiated as lateral branches that rapidly expand into globose structures of varying colour and with ornamented walls. They can occur singly or in clusters. Auxiliary cells formed in the soil may be “nodulated” or “coiled”, and their function is unknown (Peterson *et al*., 2004). Reproductive spores (Fig. 1) are produced on the extraradical hyphae, but they can also grow within the colonised roots, e.g. for *Glomus intraradices*, *Glomus clarum* and *Glomus fasciculatum* (Schenck & Smith 1982). Some AMF also form sporacarps or spore clusters (Redecker *et al.* 2007).

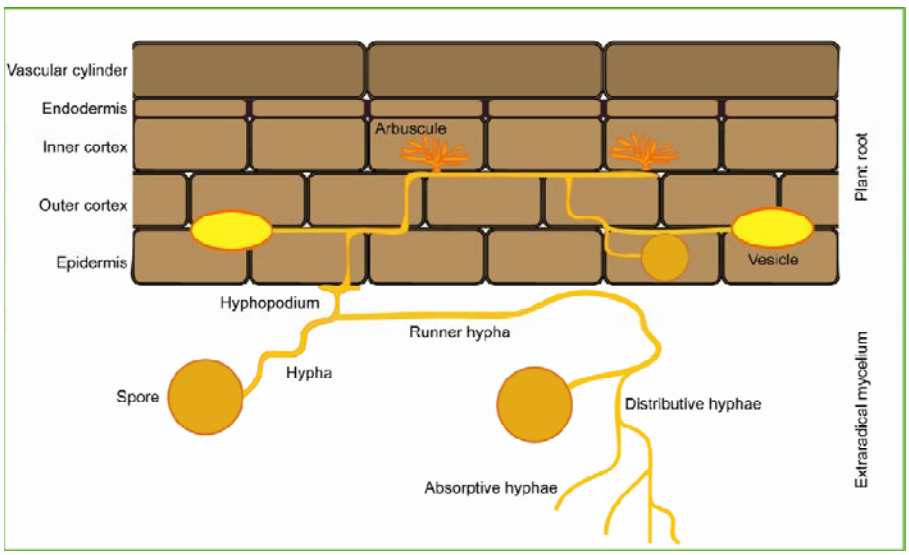

Figure 1. Structures of arbuscular mycorrhizal fungi outside and within the root of the phytobiont. (Adapted from Brundrett and Abbott, 2002).

3.1 The AMF life cycle

AMF are obligate biotrophs that live symbiotically in the roots of the host plants. For the establishment of the symbiosis, the key events in the life cycle of AMF are spore germination and the pre-symbiotic mycelial growth phase, differential hyphal branching, appressorium formation, root colonisation and arbuscule development (Giovannetti *et al*. 1994).

Under favourable environmental conditions, the AMF spores germinate and the hyphae colonise susceptible roots (Smith and Read, 1997). AMF are able to distinguish their hosts from other species, both non-mycorrhizal or non-arbuscular mycorrhiza through chemical of signals emitted by the roots to the fungus. These signals promote a specific morphogenesis, of profuse hyphal branching and proliferation (Giovannetti *et al*. 1994). Among the compounds released in root exudates, flavonoids have been suggested to be involved in the stimulation of pre-contact hypal growth and branching (Giovannetti and Sbrana, 1998). However, these compounds are not strictly necessary for the establishment of mycorrhizal symbiose (Bécard *et al*., 1995). Similar to the branching factors, which are produced by plants as a compatibility signal for the fungus, the branched fungal hyphae produced a diffusible signal to the roots (Kosuta *et al*. 2003). Thus, a “crosstalk” between the plant and the fungus is established.

After the recognising the host plant root, the hyphae form appressoria on the root epidermis (the root hair or other epidermal cell). AMF penetrate the root surface by enzymatic activity or mechanical force (Bonfante and Perotto 1995). The entry of the fungus is assisted by the plant by the establishment of a special cytoskeletal arrangement, the prepenetration apparatus (Genre *et al*. 2005). The appressorium formation is followed by hyphal penetration of the cell lumen and proliferation of the intraradical hyphae in the upper cell layers of the root cortex. Furthermore, the hyphae enter the middle and inner cortex of the root. However, the fungus never enters meristems or vascular cylinders, perhaps because of its inability to degrade suberin and lignin in the endodermal cell walls (Bonfante and Perotto 1995). When growing inside fungi are always surrounded by the intact plasma membrane of the plant host. In the deeper cortical layers, the fungal hyphae may form arbuscules. As part of this intracellular development, the periarbuscular membrane where nutrient exchange occurs between the plant and the wall of the fungus, is formed from the plasma

membrane (Gianinazzi-Pearson, 1996). Once fungal colonisation has been established in the root, extensive growth of the extraradical mycelium (ERM) begins. This is well adapted to the exploration of soil pores, mineral nutrient uptake and association with soil particles and, thus, stabilisation of soil aggregates. The ERM forms a complex network, that can link the roots of plants of the same or different species (Smith and Read 1997). The life cycle of the AMF is completed by the formation of spores or sporocarps on the ERM or rarely inside the roots.

3.2. *Paris*- and *Arum*- type colonisation

There are three important components of any mycorrhizal root systems: the root itself and two associated mycelial systems, one within the root apoplast and the other in the soil (Brundrett, 2004). The two mycelial systems grow and develop in diverse environments: the first it is very constant through root homeostasis, and the second is highly variable. According to Gallaud (1905), depending on the species of plant, mycorrhizal colonisation can fall into one of two general anatomical groups, the *Arum-type* and *Paris-type* (Figs. 2; 3).

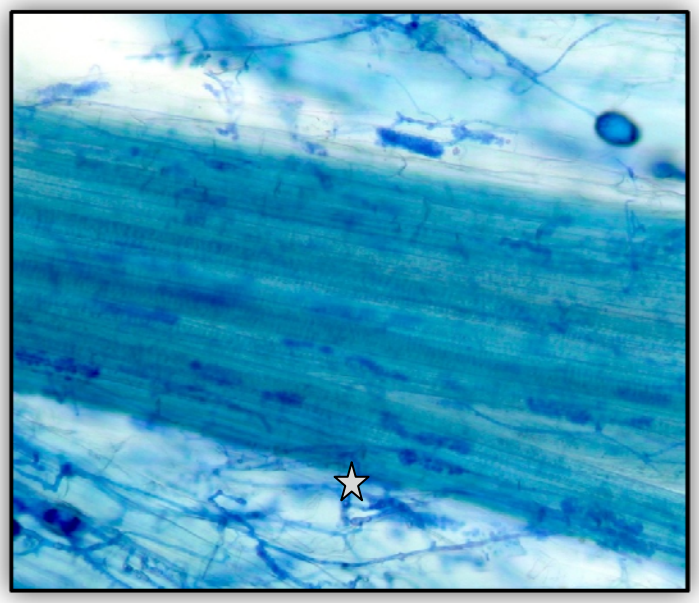

Figure 2. The *Arum*-type of AMF. Root of *Holcus lanatus* L. stained with ink, 40- X magnification. The start shows the intracellular hyphae with intercellular branches forming arbuscules.

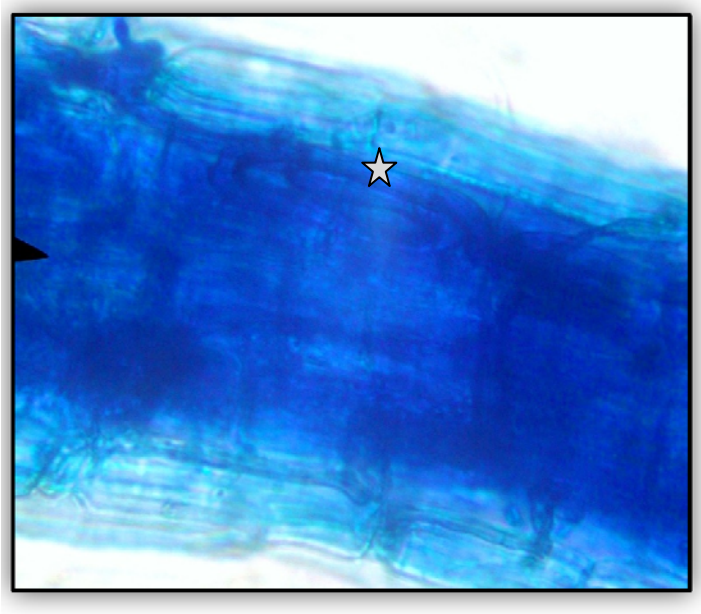

Figure 3. The *Paris*-type of AMF. Root of *Holcus lanatus* L. stained with ink, 40- X magnification. The start shows the intracellular hyphal coils.

In plants with *Arum* AMF associations (Fig. 2), the fungus spreads relatively rapidly in the root cortex via intercellular hyphae, which extend along well developed intercellular air spaces. Short side branches penetrate the cortical cells and branch dichotomously to produce characteristic arbuscules. Hyphal coils may be formed, particularly in the hypodermal (exodermal) cell layers of the root, but they are not usually a major component of the intraradical mycelium (Smith and Read, 1997; Brundrett, 2004). In contrast, in plants with *Paris* AMF associations (Fig. 3), colonisation of the roots is characterised by the extensive development of intracellular coiled hyphae that spread directly from cell to cell within the cortex. Arbuscules grow from these coils, and there is very little, if any, intercellular growth.

The two AMF types differ not only in their morphology but also in the kinetics of root colonisation. The development of *Paris*-type colonisation within the root is much slower than in the *Arum*-type organism (Azcon- Aguilar *et al*. 1994). However, the two types of colonisation have similar metabolic activity. Whereas arbuscules appear to be the main site of P transfer to Arum-type (Ezawa *et al*., 2002), in the Paris-type, transfer might occur in both hyphal and arbuscular coils (Van Aarle *et al*., 2005)

The type of AMF colonisation seems to be largely determined by the host plant genotype, as the same AMF species known to form the *Arum*-type in some host plant species, and produces the *Paris*-type in other (Demuth and Weber 1990; Sykorová *et al.* 2003; Ahulu *et al.* 2006).

3.3. Ecology of Arbuscular Mycorrhizae

3.3.1. Host specificity

The varying responses of plants to mycorrhizal infection have a profound impact on plant interactions and community structure. Depending on their response to mycorrhizal colonisation, plant species have been classified in three categories: obligate mycorrhizal, facultatively mycorrhizal or non-mycorrhizal, if they have high, intermediate or variable or no level of mycorrhizal association, respectively (Brundrett and Abbott, 2002; Brundrett, 2004). Obligate mycorrhizal plants are defined as those that will not survive to reproductive maturity in their natural habitats without their mycorrhizal fungi, while facultatively mycorrhizal plants have intermediate associations, where the plant's benefits are conditional on soil fertility.

Experiments have shown that these plants benefit from AMF only when soil P levels are relatively low. These plants typically have relatively long, narrow and highly branched roots with long root hairs compared to obligately mycorrhizal species. Facultatively mycorrhizal plants apparently have the capacity to limit the extent of their associations to reduce costs in cases where fungi provide little benefit (Koide & Schreiner, 1992). Non-mycorrhizal plants have roots that are highly resistant to colonisation by mycorrhizal fungi and do not form functional associations (Brundrett, 2002; Brundrett, 2004).

The responsiveness of plants to mycorrhizal colonisation further varies between species and cultivars and is markedly influenced by nutrient supply. This responsiveness is frequently referred to as mycorrhizal dependency and expressed as the percentage difference in dry matter between mycorrhizal and non-mycorrhizal plants grown in the same soil (Gerdemann, 1975). Responsiveness is strongly affected by both the capacity of the plant to absorb nutrients independently and its capacity to support a heterotrophic symbiont with 'excess' photosynthate are important and may be genetically and environmentally influenced (Read and Smith, 1997). Variation in mycorrhizal dependency among species, have potentially important applications to agroecosystem functioning and management.

3.3.2. Plant succession and mycorrhizal colonisation

In natural ecosystems, unrelated plant species with different nutrient uptake strategies and different AMF responsiveness occur together. Experimental evidence suggests that warm-season perennial C_4 grasses and perennial forbs are highly responsive to AMF colonisation (in terms of dry weight accumulation) and some even obligate mycorrhizal plants. Conversely, cool-season perennial C_3 grasses and annual and biennial forbs are both less responsive and somewhat less colonised (Wilson and Hartnett, 1998). The responsive warm-season grasses normally have less fibrous root systems, lower specific root length and a more plastic architecture than cool-season grasses, and their degree of branching is reduced when they become colonised. All of these characters are regularly associated with high responsiveness. Annual and biennial species with lower responsiveness are often associated with disturbed patches where interspecific competition is relatively low. In these environments investment in finer roots with high turnover rates appears to be a more advantageous strategy. Plant with facultative AMF status can presumably overcome occasional nutrient deficiencies and competitive stresses (Smith and Read, 1997).

AMF can thus influence the dominant plants in a community by mediating competition between plants of differing mycotrophy (Grime *et al*. 1987; Wilson and Hartnett 1998; van der Heijden *et al*. 1998; Hartnett and Wilson 1999; Eom *et al*. 2000; Hart *et al*., 2003). This interaction can be extended to a model of succession where the mycotrophy of dominant plants changes as the community develops (Johnson *et al.* 1991).

During primary succession only non-mycorrhizal pioneer plants can colonise, due to the lack of AMF propagules which gives the advantage to these plants (Janos, 1980). During this time of dominance by non-mycorrhizal plants, succession may be slowed or completely arrested until the establishment of facultatively mycorrhizal plants begins to build a community of AMF. This period paves the way for the establishment of obligately mycorrhizal plants, which eventually dominate the community (Gemma and Koske1997). In this way, the mycotrophy of the dominant plants can be used to indicate the successional trajectory of a community (Reeves *et al*., 1979).

There is evidence that AMF mediated sharing of nutrients (e.g., nitrogen and phosphorus) allows the survival of subordinate seedlings that act as nutrient sinks to

the dominant source plants (Grime *et al*. 1987). It has also been suggested that invasive plants can act as parasites, using the web of AMF to draw resources from native vegetation (Marler *et al*. 1999). Whatever the case, it is clear that AMF affect competition and thereby influence the level of diversity found in plant communities. Plant diversity can either be increased or decreased by the presence of AMF depending on the mycotrophy of the dominant species. Smith *et al*. (1999) reported that removal of the dominant warm-season C_4 grass species in a tall prairie, which exhibits high mycorrhizal responsiveness, increased plant diversity once the abundance of the dominant species declined in number, and subdominant cool-season C_3 grass and forb species increased in abundance. To generalize, increased diversity and spatial heterogeneity of AMF will increase the diversity of plants when a variation in host plant mycotrophy is present (van der Heijden et al. 1998; Hartnett and Wilson 2002). The diversity of AMF is affected by the diversity of the plant community (Eom *et al*. 2000). Monocultures of mycorrhizal plants may have lower AMF diversity than communities dominated by nonmycorrhizal plants (Johnson *et al*. 2004). While this pattern may seem counterintuitive at first, it is analogous to the situation where AMF increase the competitive dominance of mycorrhizal plants in tall grass prairies and, thereby reduce the overall diversity of the plant community. The successional status of the plant community also dramatically impacts AMF communities, with AMF diversity increasing along the successional gradient (Koske and Gemma 1997). Following the change in the level of diversity, there is a change in species composition, with a different group of AMF species represented in early and late successional communities (Johnson *et al*. 1991). The endomycorrhizal relationship is truly a two-way street; it is still unclear whether the AMF is the driver or the passenger in this relationship, or whether this distinction can be made (Hart *et al.* 2001).

3.4. Genetics of AMF

Arbuscular mycorrhizal fungi are obligate symbionts, and then only complete its life when associated to a compatible host. These fungi have an aseptate network (cenotytic) that enables them a quick drive cytoplasmic, facilitating the uptake of nutrients belonging to the depletion zone. Compared to others fungi, AMF have bigger spores, ranging between 22 and 1050 µm (Schüßler *et al.*, 1994).

Depending of the species, these spores may have hundreds or thousands of nuclei (Bever and Morton, 1999; Pawlowska and Taylor, 2004). Hijri and Sanders (2005) characterised the size, complexity and ploidy of the genome of a strain of *Glomus intraradices*. Their results indicate this AMF is haploid and has a small genome of approximately 16.54 Mb, which is at the lower limit of eukaryotic genomes. However, it is important to emphasise that other glomeromycota showed genome larger than that of *G. intraradices*; like *Gigaspora margarita* has three times higher more nuclear content than *Glomus versiforme* (Bianciotto and Bonfante, 1992). Such differences in genome size probably reflect differences in ploidy in addition to the, amount of repetitive sequences.

Glomeromycota probably follow an asexual reproductive cycle (i.e., clonal reproduction) by spore formation or by formation of a coenocytic (multinucleate) hypal network. Only one case of sexual reproduction has been reported in *Gigaspora decipiens* (Tommerup and Sivasitham, 1990), but never was truly confirmed. There is evidence of recombination among the Glomeromycota (Vandenkoornhuyse, 2001; LoBuglio and Taylor, 2002; Pawloska 2005). Molecular evidence suggests AMF is haploid but there is some controversy about whether their genome structures is homo- or heterokaryotic. Two basic organizational structures have been advocated. First, it is possible that all intra-cellular variation is present within individual nuclei and all of the nuclei within a cell are identical, i.e., homokaryosis (Pawlowska and Taylor 2004; Pawloska 2005). Alternatively, much of the genetic variation may be distributed between nuclei, with each cell containing multiple genomes, i.e., heterokaryosis (Bever and Morton 1999; Hijri and Sanders, 2005). These two scenarios have very different implications for our understanding of the inheritance and maintenance of genetic variation and maintenance of this genetic variation in AMF.

Pawloska (2005) argued that according to non- Mendelian genetic systems and, based on the heterokaryotic character of Glomeromycota (Sanders, 2002), these fungi must have developed a nuclear recombination process, identical to the first steps of the parasexual cycle. The parasexual cycle is characterised by occurrence of anastomoses followed by nuclear change among species or individual genetically different, but with vegetative compatibility. Initially, the parasexual cycle leads to heterokaryosis. However, heterokaryosis is an unstable condition; distinct nuclei fuse, resulting in a diploid nucleus that, may suffer chromosomal losses. These losses

cause a return to haploid status (Schardl and Craven, 2003), and consequent to the homokaryosis.

The genetic structure of AMF is currently a topic of intense discussion. Further investigation is necessary to understand the mechanisms by which different genetic features shape the ecology, evolution and symbiotic efficiency of the Glomeromycota.

4. Arbuscular mycorrhiza:

4.1. Nutritional benefits

Some of the most interesting and important questions about mycorrhizae concern the nutritional benefits that these symbioses confer to each participant. The symbiosis between AMF and autotrophic plants is generally regarded as mutualistic; the mutualism assumed as the bidirectional transfer of nutrients. With the exception of a few achlorophyllous species, AM host plants are autotrophic and, although normally colonised by mycorrhizal fungi in the field, they are usually capable of satisfactory growth in the absence of colonisation, if mineral nutrient supplies are adequate. In contrast, AMF do not complete their life cycle in the absence of symbiosis with a host root. This limitation gives some insight into the role of AMF and their contribution to nutrient absorption (Smith and Read, 1997). In the absence of a host root, AMF germ-tubes stop growing and do not produce new resting spores, even when large number of carbon (C) forms is added to the culture medium. One proposed explanation is that AMF experiences a failure or deficiency of the C metabolism in the asymbiotic state thus, understanding C metabolism in this stage of AMF development is of potential practical importance (Pfeffer *et al*., 1999; Bago *et al*., 2000). In the symbiotic interaction, the fungus enters the inner cortical root cells to form specialised haustoria called arbuscules. There is increasing evidence that phosphate, translocated from the soil through the fungus, is deposited at the arbuscular interface, where it is taken up by plant transporters. However, little is known about where the exchange of carbon takes place (Pfeffer *et al*., 2001; Requena *et al.*, 2003).

The assimilate transfer includes sucrose breakdown into glucose and fructose, their export across the plant plasma membrane and their active uptake by hexose transporters across the fungal plasma membrane, driven by an increased H^+ -ATPase

activity at the arbuscular membrane (Gianinazzi-Pearson *et al*. 2000). The function of H^+ -ATPase is to generate the proton electrochemical gradient across the plasma membrane that is required for effective cellular function. The, enzyme thus provides the driving force for the uptake and efflux of ions and metabolites through an interface otherwise impermeable to them and allows for the regulation of intracellular pH (Requena *et al*., 2003). The exchange of metabolites and ions across the plasma membrane is particularly important at the symbiotic mycorrhizal interface because it enables the control of exchanged nutrients between the soil, fungus and plant "compartments." This exchanges maintains the equilibrium that defines the association as mutualistic and not as parasitic. In plants, H^+ -ATPase is encoded by a large multigene family (Gianinazzi-Pearson *et al*. 2000; Ferrol *et al*., 2002; Ezawa *et al*., 2005) and may be recruited at different developmental stages to respond to the different requirements of symbiosis (Requena *et al*., 2003).

In the fungal cytosol, the hexoses are converted into triacylglycerides or, amino acids or incorporated into glycogen pools. The major storage forms of carbon in spores and hyphae are lipids, trehalose and glycogen (Pfeffer *et al*. 1999).

Hyphae from colonised roots extend into the soil and aid root hairs in absorbing water and mineral nutrients. AMF are recognised for their ability to stimulate plant growth via enhanced uptake of phosphate despite its low mobility and availability in soil, and they thus play important roles in the terrestrial phosphorus cycle (Ezawa *et al*., 2002). In the symbiotic phase, AMF take up inorganic phosphate (Pi) from the soil through fungal phosphate transporters, which in an extensive network of extraradical hyphae is rapidly converted into inorganic polyphosphate (polyP) and transported into the intraradical hyphae (Ezawa *et al*., 2004). In the arbuscules, polyphosphate is hydrolysed by enhanced phosphatase activity and released into the periarbuscular space (Ohtomo and Saito 2005). Phosphate is then taken up by the plant through a transmembrane transporter. As with carbon, the uptake of phosphate and other nutrients by arbuscular plant cells may be linked to the high plant and fungal H^+-ATPase activity observed at the periarbuscular membrane (Gianinazzi-Pearson *et al.* 2000). Although most of the nutrient transfer between the two symbiotic partners takes place in the arbuscules, they do not seem to be the sole site of nutrient exchange (Van Aarle *et al*., 2005).

4.2. Other beneficial features

This symbiosis is characterised by the bidirectional flow of nutrients. The plant through the compounds photosynthates promotes the growth and reproduction of the fungus. In turn, the fungus enhances the uptake of water and nutrients, especially immobile nutrients like phosphorus that are present beyond the depleted zones that develop around plant roots (Allen, 1991; Smith & Read, 1997).

However these fungi can also provide many other benefits to the host including: increased resistance to foliar-feeding insects through a reduction in the palatability of plant tissues, alteration in nutritional quality, or increase in the concentrations or types of defence compounds in the plant tissue (Gange and Weast, 1994; Master and Brown, 1997; Borowicz, 1997; Gange *et al*., 1999; Goverde *et al*., 2000; Wearn and Gange, 2007). AMF colonisation may also lead to an increased in overall plant fitness and resistance against nematodes (de la Peña *et al*., 2006; Rodriguez-Romero *et al*., 2005; Rodriguez-Echeverria *et al*., 2009) and pathogens (Smith *et al*., 1999; Klironomos *et al*., 2000; Idoia *et al*., 2004). These effects can, to a certain extent, reduce the need for pesticides and other synthetic chemical in agricultural practices. AMF also improves crops' tolerance of abiotic stresses, such as drought (Augé *et al*., 2001). The resistance to water stress is likely associated with the extraradical network of hyphae which are far more resistant to drought than fine roots and can resume growth immediately after drought conditions cease (Miller *et al*., 1995). AMF can also increase tolerance to salinity and heavy metals (Al-Karaki *et al*., 2001; Mohammad *et al*., 2003). Furthermore, AMF contributes to soil aggregation not only through the development of hyphal network builds into a macroporous structure that facilities circulation of water and air and prevents erosion but also through the action of glomalin, a glycoprotein produced AMF (Rilling *et al*., 2003; Piotrowski *et al*., 2004; Cavagnaro *et al.*, 2006; van der Heijden *et al*., 2006). Another important role of AMF is their contribution as drivers of plant community composition (Grime et al., 1997; Wilson and Hartnett, 1997; van der Heijden *et al*., 1998; Hartnett and Wilson, 1999).

5. Identification, systematic, phylogeny and diversity of the Glomeromycota

5.1. Systematics and phylogeny of the Glomeromycota

Before 1974, most of AMF were included in the genus *Endogone,* until Gerdemann and Trappe (1974) placed them in four different genera in the order Endogonales: *Glomus*, *Sclerocystic*, *Gigaspora* and *Acaulospora*. Previously, AMF had been assigned to the order Glomales, which included six genera and in the phylum Zygomycota (Morton and Benny 1990). The Zygomycota is no longer accepted as a monophyletic clade. However, based on their obligate symbiotic habit, the apparent lack of zygospores (i.e., spore produced through the conjugation of specialised hyphae during sexual reproduction) and the DNA ribosomal (rDNA) phylogeny, Schüβler *et al.* (2001) defined the phylum Glomeromycota as a sister clade of Basidiomycota and Ascomycota. Glomeromycota currently comprises approximately 200 described species distributed among ten genera, most of which were defined primarily based on the morphology of their spores or spore-bearing structures (http://www.lrz.de/~schuessler/amphylo/).

Recently, DNA sequences have also been used to describe some AMF taxa and their phylogenetic relationships (e.g. Schwarzott *et al.* 2001; Redecker and Raab 2006). However, the possible sequence variation within individuals makes it difficult to interpret molecular community data when sequences are obtained from colonised plant roots, especially when studies were made on organisms that were not well characterised and correctly identified. As a result, about half of the described species are not actually known from their molecular characters, and some are barely known from morphology because of very poor descriptions and lack of definable type material (C. Walker personal communication). Hence, a conservative approach in the evaluation of phylogenetic analyses is advisable; e.g. such as defining a monophyletic, well-supported sequence cluster as an AMF phylotype (i.e., sequence type). Presently, the phylogeny of all genera of AMF is largely based on analyses of small subunit of the ribosomal DNA gene (SSU rDNA). Although additional genes have begun to be sequenced from some taxa (e.g. Helgason *et al*. 2003; Corradi *et al.* 2004; Redecker and Raab 2006), phylogenetic hypotheses based on multilocus DNA sequence data have yet to be incorporated into the AMF classification. Four orders, containing nine families and fourteen genera have been described within the Glomeromycota (Figs. 4).

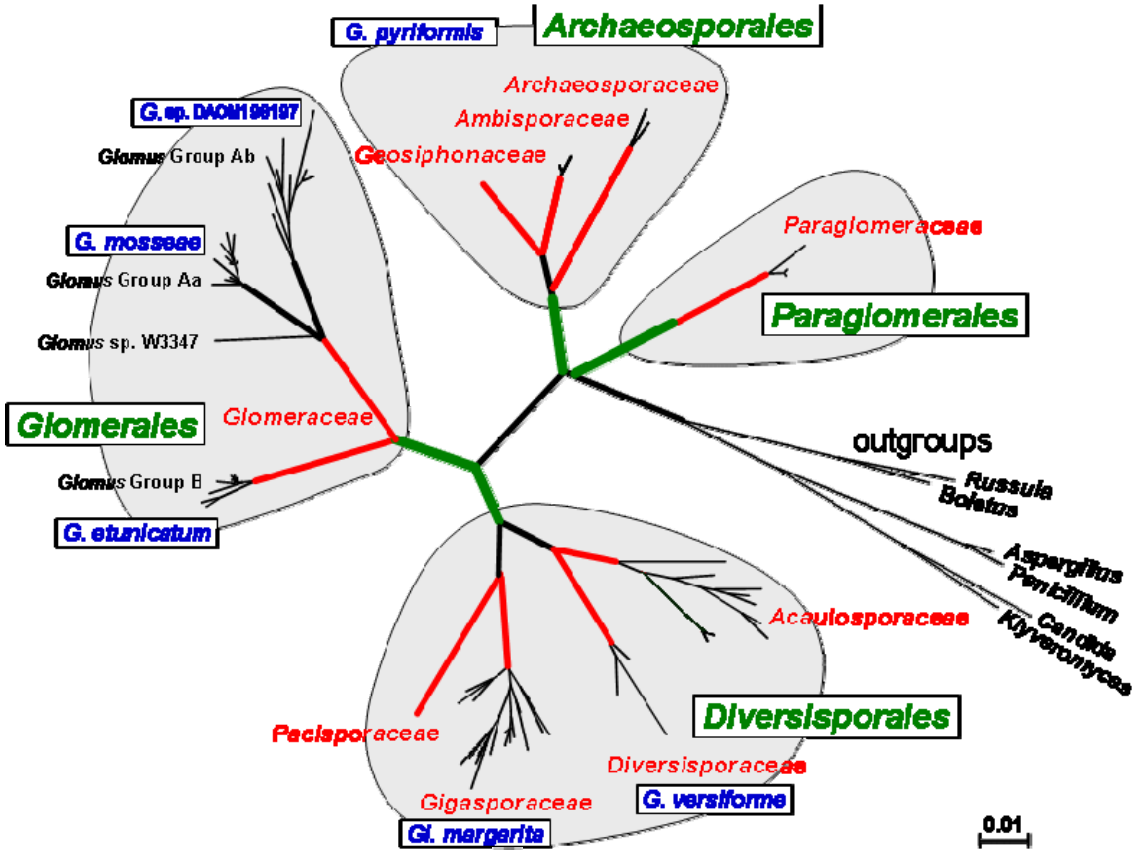

Fig. 4. Phylogenetic tree reflecting recent changes in the taxonomy of the Glomeromycota. The tree is based on SSU rDNA sequences. Some often used 'model species' are shown in blue. From http://www.lrz-muenchen.de/~schuessler/amphylo/.

The largest genus within this phylum, *Glomus, w*as originally defined by spore morphology and formation and was later revealed by molecular data to be polyphyletic (Schwarzott *et al*. 2001). Therefore, this genus was divided into the following five genera: *Glomus, Paraglomus, Archaeospora* (Morton and Redecker 2001), *Pacispora* (Oehl and Sieverding 2004) and *Diversispora* (Walker and Schübler. 2004).

Schwarzott *et al.* (2001) further analysed this largest genus of the AMF and established a new family structure by dividing the remaining Glomeraceae into the Diversisporaceae and Glomeraceae. The Glomeraceae family contain the subgroups "*Glomus* group A" (including e.g., *G. intraradices*, *G. proliferum*, *G. clarum* and *G. mosseae*; Fig. 5) whose members are the dominant and most diverse AMF in many field sites (Öpik *et al.* 2006), and "*Glomus* group B" (including e.g., *G. claroideum*, *G. etunicatum G. lamellosum*; see Fig. 5) whose members are difficult to distinguish

(Rodriguez *et al.* 2005). These two subgroups form two clades, that are separated phylogenetically by a distance equal to that found between others families (Fig.5).

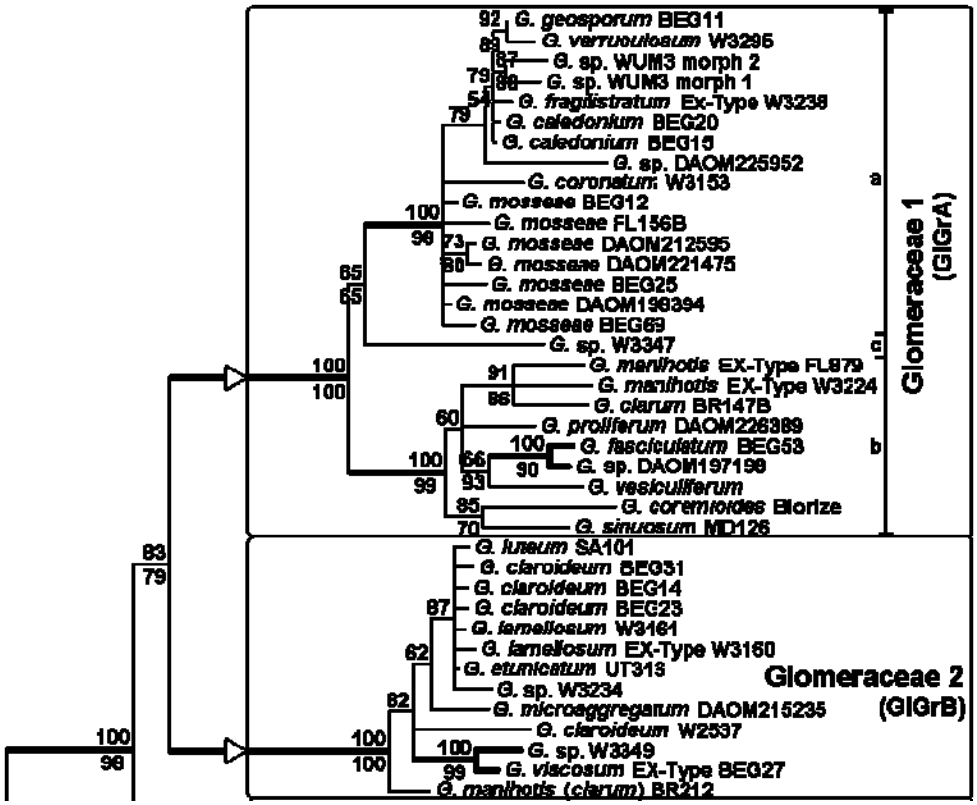

Figure 5. Phylogenetic tree (based on SSU rDNA sequences) of *Glomus* group A (GlGrA) and *Glomus* group B of the Glomeracecae. Modified from Schwarzott *et al.* (2001).

A further change was the establishment of the Ambisporaceae within the paraphyletic genus *Archaeospora*, while the family Archaeosporaceae was maintained with its type species (Walker *et al.* 2007). A new family Entrophosporaceae, was established (Sieverding and Oehl 2006), but its phylogenetic position is unclear. It is not included in the phylogenetic tree implementing the most recent phylogenetic analysis of the Glomeromycota (Fig. 4). Other recently erected families and genera have arisen from the by splitting of the Gigasporaceae (Oehl *et al.* 2008); their status is strongly debated in the scientific community (Morton & Msiska in press) and they are not included Fig. 4.

Several AMF genera share morphological spore features, which are easy to observe but might represent plesiomorphic characters (e.g., the mode of spore formation; Redecker & Raab 2006). Therefore, detailed microscopic analyses are required for the identification of anatomical subtleties, such as spore wall layers.

Spore formation has been an important criterion for the identification of genera. Members of *Glomus* (Berch and Fortin 1984), *Pacispora* (Oehl and Sieverding 2004), *Diversispora* (Walker and Schüßler 2004), *Ambispora* (Walker *et al.* 2007) and *Paraglomus* (Morton and Redecker 2001) develop spores by blastic expansion of a hyphal tip (glomoid spores). However in *Ambispora,* a second spore type occurs (even within the same "dimorphic" species) that is typical of *Acaulospora* (Gerdemann and Trappe 1974) and *Archaeospora* (*A. trappei*; Morton and Redecker 2001); a sporiferous saccule forms blastically at the hyphal tip, and the acaulosporoid spore is develops at the "saccule neck" (the side of the subtending hypha). *Entrophospora* (Ames & Schneider 1979) develops spores directly from the saccule neck, not laterally but within the subtending hypha (the entrophosporoid mode of spore formation). The fourth, gigasporoid, type of spore formation characterises both *Gigaspora* (Gerdemann and Trappe 1974) and *Scutellospora* (Walker and Sanders 1986). Spores are formed from a morphologically specialised bulbous base (25-50 µm in size) that is continuous with the structural spore wall.

Other distinguishing characteristics besides of spores are the presence or absence of flexible inner walls and type of germination, which occurs through the hyphal attachment (e.g. *Glomus*) or through the spore wall. In the latter mode of germination, germ tubes can arise from i) a germination orb (*Pacispora*); ii) a germination shield (*Scutellospora*) a structure that is occurs before spores germinate by penetrating the spore wall; or iii) a warty layer, typical for *Gigaspora*. The mode in which the spore is formed on the hypha ("mode of spore formation") has been an important criteria for defining genera and families; the number of walls, the structure of their layers and ornamentation have been used to distinguish species (Walker, 1983). Although these characteristics may be specific, they can differ according to environmental conditions and by the stage of the spore. Aged, dry or decaying spores can look different, even if they belong to the same species. However, many of these characteristics are just generic or significant only at the higher level, such as family (C. Walker personal communication). Moreover, it is quite difficult and requires expert knowledge to

distinguish and classify spores by their morphology, and, there is no coherent systems that allows the non-taxonomist to determine species with accuracy (C. Walker, personal communication).

Geosiphon pyriformis is a glomeromycotan species belonging to the order Archaeosporales. It is the only fungus in the Glomeromycota currently known to form a symbiosis with a cyanobacterium: it produces bladders that harbor symbiotic *Nostoc punctiforme* (Schüβler *et al.* 1994). Despite its different morphology and life strategy, molecular phylogenetic analysis has shown that *Geosiphon* is a member of the *Glomeromycota* (Schwarzott *et al.* 2001).

6. Methods for AMF identification

6.1. Morphological methods

Breakthroughs in AMF taxonomy were obtained after the taxonomic reviewed of Endogonaceae by Gerdemann and Tappe (1974). These authors not only restructured the relationships of genus and family but also established a basis for species identification and description. Previously to identify AMF such characteristics as presence of a subtending hypha, colour, diameter, and the presence of inner walls were used. However, Walker (1983) introduced the concept of “murographs” to describe and compare the layered structure of spore walls more easily. Thereafter, the descriptions of new AMF followed this methodology, but when they did not easily fit the “murographs” concept, the new AMF species found were described by others authors (Morton 1986; Walker, 1986; Spain *et al*., 1989). The AMF identification is still based on the patterns described by Walker (1983). Morton (1988) extensively reviewed the concepts and characters used in AMF identification. The way the spore is formed on the hypha has been an important criterion with which to circumscribe genera and families, and the layered structure of the spore walls is used to distinguish species. However, despite the progress in AMF taxonomy, many species were described before the formulation of these traditional taxonomic concepts; thus, additional description of these taxa is necessary to decrease the challenges of found on identification (Walker, 1992).

A new model of AMF description based on spore ontogeny was proposed (Franke and Morton, 1994; Bentivenga and Morton, 1995; Morton, 1995; Stürmer and Morton, 1997; 1999). According to this model, the wall of the spore is divided into two main groups: the “structural wall” and “germinal wall”, and each group bis subdivided into layers. Despite its complexity, the ontogenic method is more coherent and logical. In addition, the morphology of the spore, and the vegetative structures, can be used on the family identification of some genera. However, traditional studies on AMF have showed methodological limitations. Taxonomic identification of AMF spores collected directly from the field is quite difficult because they are often unidentifiable due to degradation or parasitism by other organism. AMF are obligate symbionts, thus they cannot be cultivated in the absence of their host. Catch plants are often used to produce identifiable spores (Bever *et al*., 2001). Although useful for the isolation and propagation of some AMF species, this indirect culture strategy is time-consuming. It also introduces biases by plant preferences for particular AMF species, different growth conditions and other environmental factors. These limitation hinder its suitability in characterising AMF communities (Jansa *et al*., 2002; Oehl *et al*., 2003). Furthermore, mycorrhizal root colonisation and spore numbers (Clapp *et al*. 1995) might not reflect AMF populations in the soils, but only the sporulation stage of the AMF life cycle (a dormant phase) rather than activity within a symbiosis. This result of the fact that not all AMF present as spores form active mycorrhizas and many taxa present in the roots may not be sporulating. Moreover, fungal spore diversity differs seasonally, with some fungi sporulating in late spring and others at the end of summer. In most cases, taxonomic identification to the species level thus requires a very experienced investigator.

However, an advantage of analysing spore numbers and morphology is that they can indicate over longer time periods (e.g., over months) the structures of the AMF community (Douds and Millner,1999; Oehl *et al.* 2003) with the exception of putative non-sporulating AMF species. Additionally, extracting spores from the soil can be done rapidly, and it may reveal taxa not easily detected by molecular methods, and provided the user has reasonable taxonomic expertise. Intensive soil sampling, including the use of successive trap culturing, typically takes much longer but reveals significantly greater diversity than ‘one-off’ methods of spore extraction (Oehl *et al*., 2004).

6.2. Molecular methods

Molecular methods allow the AMF to be identified in the host plant roots or directly in the soil. However, because Glomeromycota are obligate symbionts, it is difficult or even impossible to obtain their pure biomass, which is necessary for the development of molecular markers. Only some AMF can be cultivated in sterile conditions using transformed plant roots (Fortin *et al*. 2002), while others must be cultivated in non-sterile greenhouse pot cultures (Oehl *et al*. 2005) or are probably completely non-cultivable or non-sporulating. Furthermore, it has been demonstrated that AMF spores or sporocarps from filed or trap cultures can host numerous non-Glomeromycotans including other fungi and bacteria (Hijri *et al*., 2002).

Molecular markers have been employed to characterise AMF communities genetically. Molecular markers include: i) analysis of isoenzymes (Sander *et al*., 1992); ii) and; analysis of the genomic profile of the rDNA (ribosomal DNA) (White *et al*., 1990; Lanfranco *et al*., 2001) and mtDNA (mitochondrial DNA) (Bruns *et al*., 1991). Most molecular research investigating AMF identification and detection at species and isolate level has used techniques based on PCR (Polymerase Chain Reaction), which allow the characterisation of nucleic acids from small amounts of fungal DNA (White *et al.*, 1990). These molecular techniques include: RAPD (Random Amplified Polymorphic DNA) (Wyss and Bonfante, 1993), RFLP (Restriction Fragment Length Polymorphism) (Sanders *et al*., 1995; Helgason *et al*., 1998; Redecker, 2000; Appoloni *et al*., 2008), DGGE (Denaturing Gradient Gel Electrophoresis) (Kowalchuk *et al*., 2002; Ma *et al*., 2005; Santos *et al*., 2006; Wu et al., 2007) and qRT-PCR (Quantitative real-time PCR) (Alkan *et al*., 2004; 2006; Jansa *et al*., 2008).

All molecular tools described above have proven to be useful tools for characterizing the AMF community, and in the future they could be combined with avaible morphological tools to yield a better understanding of AMF community structure (Redecker *et al*., 2003). However, these molecular tools are costly and time-consuming, and they lack sufficient sensitivity to quantify AMF (Oehl *et al*., 2004; Egerton-Warburton *et al*., 2007). Hijri *et al*. (2006) noted that not all AMF taxa present as spores were detected in roots by PCR, probably because not all taxa were symbiotically active at the time or because they colonised the roots at levels below the detection threshold. There is, still uncertainty as to whether there are other AMF present that remain undetected because they are occur infrequently in intraradical

structures, or if there are other reasons why techniques still partly fail (Oehl *et al*., 2004; 2010).

6.2.1. Marker genes for AMF community studies

Since Clapp *et al*. (1995) initiated field studies using molecular tools, several different molecular markers have been developed; improvements are still nede and in development. Target genes are the nuclear-encoded small subunit (SSU) ribosomal RNA sequences (Helgason *et al*. 1998), and the large subunit (LSU) ribosomal RNA sequences (Van Tuinen *et al*., 1998; Kjøller and Rosendahl 2000; Wu *et al*. 2007). The polymorphism of the latter is higher and, LSU is therefore more suitable for the development of group-specific primers (Rosendahl 2008; Robinson-Boyer *et al*. 2009). The first designed AMF-specific-primer, the VANS1 primer for the 5' end of the SSU (Simon *et al,.* 1992), does not amplify all glomeromycotan lineages (Clapp *et al.,* 1995). Helgason *et al*. (1998) designed the AM1 primer, which in combination with the universal primer NS31 amplifies the variable central region of the SSU. However, it does not amplify this region in Archaeosporaceae, Paraglomeraceae or *Glomus* group B.

In another approach, Lee *et al*. (2008) developed the primer pair AML1/AML2 which targets the SSU and claimed that this marker detects all published AMF sequences in this gene region except for Archaeospora trappei. Generally, the resolution of phylotypes from this gene region is lower than in approaches using internal transcribed spacers (ITS). Several markers targeting the ITS have been developed and applied in field studies. For example, Renker *et al.* (2003) used a nested PCR for the ITS with an intermediate restriction digest, designed to prevent the amplification of other fungi than AMF that contain the restriction site. However, in a later publication, these authors clarified that the restriction digest misses certain AMF species in the detection procedure (Hempel *et al.* 2007).

Furthermore, this method amplified yeast species relatively frequently, which paradoxically allowed investigation of the biodiversity of basidiomycete yeasts inhabiting AMF-colonised roots or AMF spores (Renker *et al.* 2004). Other authors have constructed primers targeting the LSU of rDNA (Kjoller and Rosendahl 2000;

Gollotte *et al.* 2004). Similar to the AM1/NS31 primer pair, these primers also amplify only a subset of the Glomeromycotan taxa.

To reduce the risk of missing AMF species in plant roots, Redecker (2000) developed group-specific nested PCR approaches for the SSU-ITS, covering all families of the Glomeromycota. Like the approach of Renker *et al*. (2003), this method was applied in several studies (Wubet *et al.,* 2003; Hijri *et al*. 2006; Appoloni *et al*., 2008). Öpik *et al*. (2006) and Robinson-Boyer *et al*. (2009) summarised several of these studies using different target genes mentioned so far. It is obvious that all of these approaches have their advantages and disadvantages. The best approach depends on the scientific aim, e.g., if a resolution on the genus or species level is required.

Recently, Krüger *et al.* (2009) developed a nested PCR approach to overcome several of these problems. To avoid the need for several PCR reactions to detect all members of the Glomeromycota, mixed primer sets were used in the same reaction and successfully tested against several members of AMF families spanning the whole phylum. The target fragment includes all ribosomal gene regions that have been broadly used in other studies, resulting in approximately 1800 bp in the first and 1500 bp in the second PCR. Therefore, this approach permits comparison with previous studies on the same taxonomic level. In particular, the SSU fragment allows broad phylogenetic analyses, as this gene region exists in the majority of different AMF taxa (Rosendahl 2008). The ITS region allows resolution to the species level. However, his approach has still to be tested intensively under field conditions as possible primer matches have been found in other fungi and plants, even though no misamplifications were observed in few initially tested environmental samples (Krüger *et al.,* 2009).

The approach of Krüger *et al.* (2009) might lead to considerable sequence information about many unknown AMF taxa from various environments.

7. AMF and Agriculture

Agricultural practices have dramatic impacts on soil and soil organisms, and AMF are no exception. Several studies have shown that agriculture reduces the diversity of the AMF community (Helgason *et al.* 1998; Daniell *et al.* 2001; Oehl *et al*. 2003). This reduction has been attributed to physical disturbance from tilling (Kabir *et al.,* 1997;

Jansa *et al*., 2003), the effects of supplemental fertilisers (Linderman and Davis 2004) and the use of fungicides and soil fumigants (Menge 1982).Tillage has been shown to have a significant influence on AMF colonisation (Douds and Miller, 2000; Jansa *et al*., 2003; Schalamuck *et al*., 2006; Garcia *et al*., 2007). The extra-radical network of AMF mycelium functions as both the nutrient absorbing organ of mycorrhizae and as inocula for the colonisation of new roots. Thus, disruption caused by tillage can result in delayed or reduced root colonisation, and a decrease in the soil volume exploited by the AMF which may affect nutrient uptake by mycorrhizae (Evans and Miller, 1990; Boddington and Dodd, 2000). Moreover, tilling transports hyphae and colonised root fragments to the upper soil layer, decreasing and diluting their activity as viable propagules for subsequent crops. A potentially opposing effect is that phosphorus (P) uptake is generally increased with tillage, due partly to an improved distribution of P and increased P availability (Borie *et al*., 2006; Garcia *et al*., 2007). Not only agricultural practices such as tilling and fertilising cause a decline in AMF diversity; but there is also substantial evidence that they produce a shift in the composition AMF community (Boddington and Dodd 2000; Egerton-Warburton and Allen 2000; Jansa *et al*., 2003). Evidences suggests a homogenisation of AMF community structures under high-input fertiliser systems, which tend to be dominated by generalist AMF species such as *Glomus*, due to their large ecological plasticity to support disturbed systems. This plasticity could be related to differences in propagative units between the glomalean families (Hart and Reader, 2002; Jansa *et al*., 2003; Castillo *et al*., 2006). Conversely, the non-*Glomus* species have a tendency to inhabit niches characterised as specialist as they are only present in specific soil types preferentially organic soils (Oehl *et al*., 2010).

However, these agriculturally adapted AMF have been shown to be slower to infect, faster to sporulate, and to produces fewer arbuscules (Johnson 1993; Scullion *et al*., 1998; Oehl *et al*., 2003). These findings all suggest that the altered AMF community is draw more resources from the plant while providing less in return. The ramifications of these altered AMF communities may not be especially great as long as the field is kept under agricultural production.

Soil types differ in their soil chemical and physical characteristics. It has been well documented that pH (Ezawa *et al*., 20001; Oehl *et al*., 2010), nitrogen (N) (Jonhson *et al*., 2003; Egerton-Warburton *et al*., 2007) organic matter (OM) (Boddington and Dodd

2000) and soil phosphorus (P) availability (Oehl *et al*., 2004) influence the composition of AMF communities. An extreme pH (Clark *et al*., 1999; Ezawa *et al*., 2001; Khanam *et al*., 2006) may also create conditions in which AMF confers for little or no benefit to the host plant. Some AMF species (e.g., *Glomus*) prefer alkaline and neutral soil, while others (e.g., *Acaulospora*) sporulate more abundant in acid soils (Gai and Li, 2003). Also Oehl *et al*. (2010) reported that pH had a strong influence on AMF species genus and on the relative density of *Glomus* species. In addition to affecting plant performance, these factors also influence the host's plant dependency on AM symbiosis and the level of root colonisation.

Grazing has also been found to dramatically reduce AMF colonisation and spore density (Gange *et al*., 1993; Gehring *et al*., 1994; Lugo and Cabello, 2002; Dhillion and Gardsjord, 2004). This trend may be due to a decrease in leaf area and an increase in root/shoot ratio resulting in a decreased source capacity to satisfy root and AM fungi sink demands.

8. Study areas

8.1.1. Island overview

This study was conducted in the Terceira Island, the third island of the Azorean archipelago in terms of size (402 km^2). Terceira is a roundish island formed by four main volcanic complexes (Serra de Santa Bárbara, Serra do Morião, Pico Alto and Serra do Cume) (Zbyszewski *et al.*, 1971). The highest point (Serra de S. Bárbara, 1023 m) is simultaneously the most recent of the three major island's complexes of 0.025 Ma B.P. (Martins, 1993). The eastern flatted part of the island is its older part of about 3.52 My (Borges *et al*., 2009). Recent historical volcanic activity is evident in the western part of the island, where several well preserved lava tubes and volcanic chimneys are present (Borges *et al.*, 1993). The Azores archipelago have a temperate oceanic climate characterised by high levels of relative atmospheric humidity that could reach 95% at high altitude native forests and ensures slight thermal variations throughout the year (Azevedo *et al*., 1999). The average temperature is 17.5° C in low altitudes, while the maximum temperature is in August and minimum in February (Azevedo, 1996). The pluviometric regime reaches its peak in January-February and minimum and July (Azevedo, 1996): Angra do Heroísmo (47 m): 969 mm year (140

mm in January and 40 mm in July) and Serra de S. Bárbara (1,023 m): 3,000 mm year (Borges, 1997).

8.1.2. Studied pastures

The sampling areas were cattle-grazed upland pastures of two different types: semi-natural pastures (managed for more than 50 years, with a higher diversity of grasses and forbs; see Borges and Brown 2001) and intensive pastures (managed for more than 20 years, characterised also by a poor vascular flora of five or less dominant species).The semi-natural pastures are included in a site of communitarian interest (SIC- Serra de Santa Barbara e Pico Alto) inside of Rede Natura 2000: Pico Galhardo (PX) (Latitude 38º 41' 51.71'' N; Longitude 27º 13' 25.34'' W) and Terra Brava (TB) (Latitude 38º 41' 59.74'' N; Longitude 27º 12' 41.37'' W) (Fig. 6).

These pastures show a high floristic diversity, and they are dominated by the grasses *Holcus lanatus* and *Agrostis castellana*, perennial grasses that covers around 100% of sites (Dias, 1996; Borges, 1997). Frequently present in these pastures are also other grasses like *Anthoxanthum odoratum*, *Lolium multflorum, Holcus rigidus* and *Poa trivialis* and spontaneous species without forage value as: *Lotus uliginosus*, *Rumex acetosella* ssp. *angiocarpus*, *Potentilla anglica*, *Hydrocotyle vulgaris*, *Plantago lanceolata*, *Lobelia urens, Cerastium fontanum, Conyza bonariensis, Anagallis arvensis, Hypochoeris radicata, Ranunculus repens, Pteridium aquilinum, Juncus effusus* (Dias, 1996; Borges, 1997).

The intensive pastures are included on exotic forest of Eucalyptus. Agualva 1(RP1) is located at: Latitude 38º 45' 27.18'' N; Longitude 27º 11' 41.55'' W; and Agualva 2 (RP2) at: Latitude 38º 45' 24.44'' N; Longitude 27º 11' 42.24'' W (Fig. 6). In these pastures the dominant grasses are usually *Holcus lanatus* and *Lolium perenne* (plant cover of 100%). Another species with high cover is *Trifolium repens* (Dias, 1996; Borges, 1997). In these pastures, other common species are: *Plantago lanceolata*, *Cyperus esculentus, Mentha suaveolens, Cerastium fontanum, Rumex conglomeratus,* etc. (Dias, 1996; Borges, 1997).

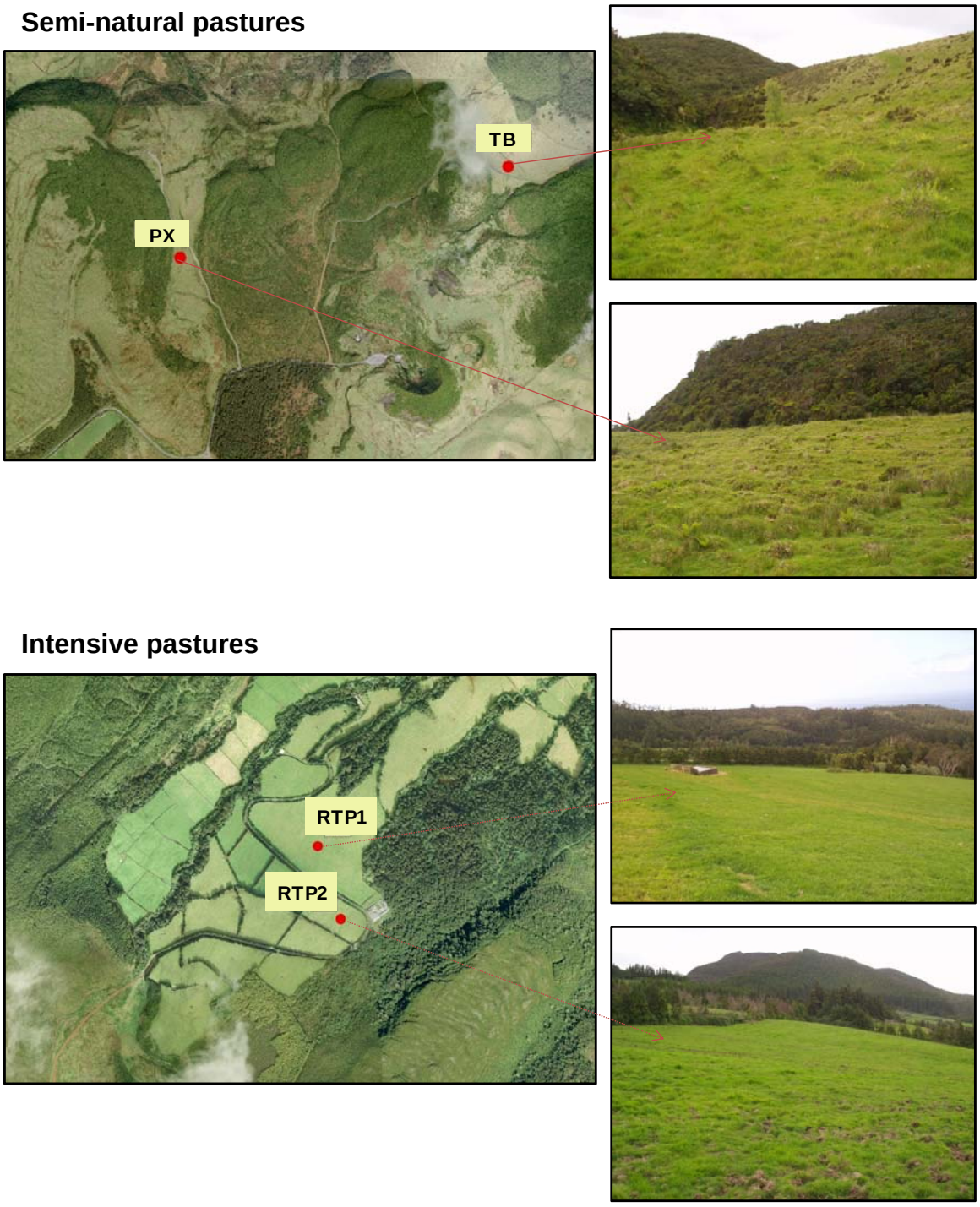

Figure 6. Aerial photograph of the study sites at Terceira Island. Semi-natural (PX – Pico Galhardo; TB- Terra Brava) and intensive (RP1- Agualva 1; RP2- Agualva 2) pastures.

8.2. Land use history and soil management

8.2.1. Land use in past

At the time of islands Human settlement, early colonizers have developed subsistence crops, namely wheat and vegetables were produced, as well as, the cattle that, in most cases, were released in native forest. The development of the islands Human population took place with these new European crops: sugar cane, pastel, wheat, wine and citrus fruits (Brito & Poeira, 1991). Sugar cane cultivation was also tested in this period, but, unlike Madeira, the climatic conditions in the Azores, mild temperatures and high cloudiness, did not help to develop this culture and so, the agricultural tendency of the Azores was itself divided between the area sown to cereals (wheat, rye, barley) and pastures (Brito & Poeira, 1991). Due to the small size of the islands and the high humidity levels of the cereal stubble, the livestock sector has been gradually adapting to the island environment (Armas, 1982).The cycle of cattle breeding, milk production, dairy and meat persists since the 19th century.

The cultural system then was based on livestock and agricultural production in the recent mobilized soils, so it can be considered as an agro-pastoral system, with a focus on export and subsistence in areas of low to medium altitude. In higher altitudes, the system consists in a silvopastoral, where the livestock left free in the middle of natural vegetation in the forest, called the "criações" (creations) or "serra aberta" (open mountain) as is known in Pico, Faial and Sao Jorge. On the island of Terceira, in these areas of open field, has been always created the wild cattle and goat cattle.

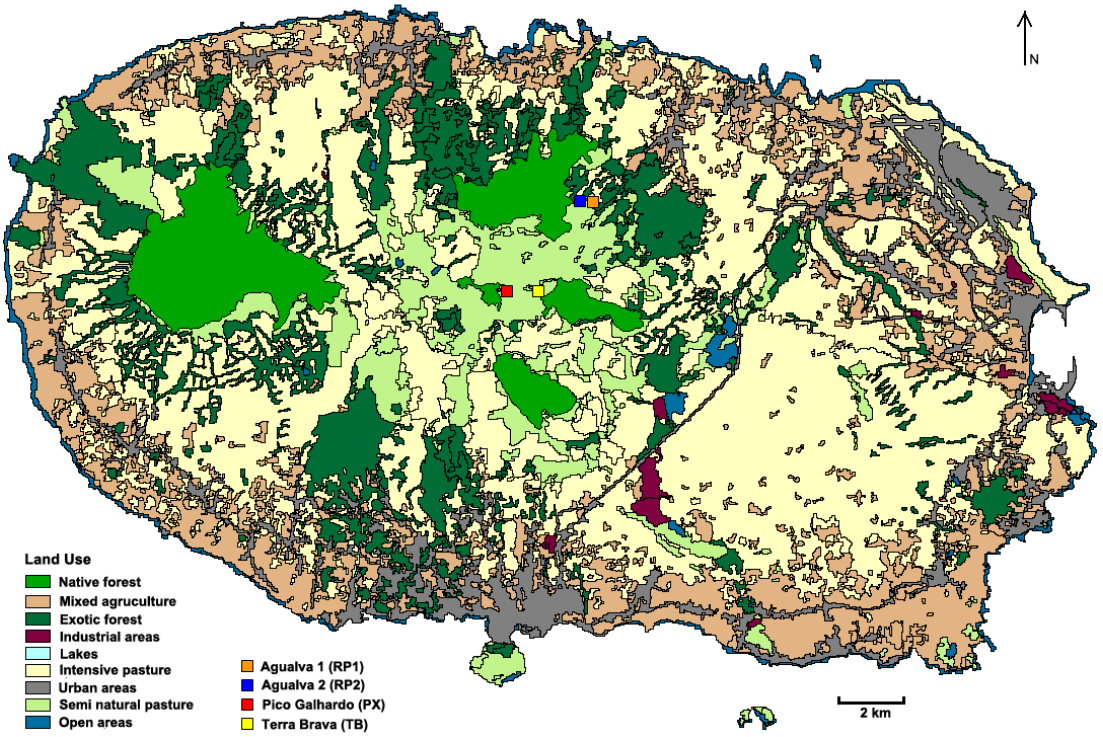

Figure 7. Location of the semi-natural (PX- Pico Gallhardo; TB- Terra Brava) and intensive (Agualva 1- RP1; Agualva 2- RP2) pastures in the Terceira land-use (extracted from DROTRH 2008)

The Azorean agriculture was based in the family system of land uses, relatively balanced with the natural environment, which modulate a landscape of fenced fields divided by stone walls, mostly in the lowland and medium altitude where soils are more productive. The stone-walls are the first building structure of the organization of agrarian space, which consist in limited fields. In the higher altitudes, the permanent pastures are dominant, characterized by closed fields in large dimensions with wooded fencerows of *Cryptomeria japonica*, whose suitability of the soil is agro-forestry. In the case of Terceira, this stratification is very clear: in the lowlands, from the coast to the mean average elevation of 200 to 300 m, the pastures are dominant in rotation with corn; in the middle altitudes, the multi-sown and the spontaneous pastures (Gomes, 2010); at higher altitudes (> 400 m), major permanent pasture fields ("bacia leiteira") with wooded Cryptomeria japonica fencerow, which is a communal lands, designated as "baldios" under Forest Service administration (Gabriel, 1994).

In lowland from the coastline up to 200 m, a period of dormancy of two months occurs during the summer. Between 200 and 400 m, the grasslands do not present a cycle of vegetative dormancy, but an annual production of grass for 9 to 10 months. From 400

m to 600 m, the pastures have a winter dormant period of 3 months. In uplands (> 600 m) grass production is limited by a dormant period of at least 5 months, restricting it only in summer (Oliveira, 1989).

8.2.2. Land use today

The current Azorean farming system is based on a Ley farming ruled by rotations of sown grasses and leguminous pastures and corn crops for silage (Altieri, 1998). This system is based on multi-annual pasture (grasses and leguminous intercropping) with a rotation of annual crops - corn for fodder during spring-summer and autumn such as *Lollium multiflorum* Lam. and *Avena sativa* L., according to altitudinal zoning of land altitude and the season of the year. This farming system creates serious problems of soil conservation, since it is unprotected during the winter, when the erosion is more intense (PRA, 2001). However, this system permits increase production levels with little annual and seasonal fluctuation in rotational grazing system with pasture, corn silage, grass silage, bales and concentrates to ensure economic stability and comfort of farmers.

About 83 % of utilized agricultural area of the Azores is occupied by pasture (Lopes, 1999). According to North- American Land Use Land Classification, that was adapted to the Azores, and according to Pinheiro *et al*. (1987), there are seven classes, whose limit of intensity increases from I to VII, and the first four classes correspond to arable land and the others to non-arable land. In the case of Terceira Island, 45% of its soils are arable land (classes I to IV).

When Portugal joined the European Union Market, in 1986, there was a gradual specialization of the farming system from the traditional Azorean to the Ley farming system in detriment of other arable cultures, caused a deep impact on the economy and land use. In these islands with favorable soil and with weather conditions optimal for the production of milk and meat, farmers faced a new and major challenge to optimize production to levels to reach the level of large agricultural or farming regions of northern Europe. The quota system of milk production imposed by EU as membership served as a driving mechanism for the modernization of farms.

The agricultural policy then, encouraged farmers to intensify production with the first Community Support Framework. With the second and third EU framework in action,

demands to reverses the situation of intensification and advances to an extensive production, and diverse cultures implementation and agro ecosystems balance in order to implement a sustainable agriculture.

In the dairy production, the certification was implemented and denomination of geographical protected origin of cheeses was created, especially for São Jorge cheese. Meat production is also regulated and classified as a protected geographical origin.

Nowadays, the implementation of a sustainable agriculture is associated with plant cultivation. So, the first explorations of organic farming emerge together with a growing local market demand. On the other hand, the conventional agriculture is dominant, heavily dependent on imported inputs, such as fossil fuels, imported seeds of pasture and maize, fertilizers and specialized agricultural machinery, with increasing water consumption by livestock due to increased stocking. Agri-livestock farms have been declining in number, but have been increasing its utilized arable superficies to meet the technical requirements of the farming system and market. The closed fields tend to be resized with the removal of stone walls between parcels and have increased the number of contiguous parcels.

The stocking rate average is 2.1 animals per hectare in the Azores. Terceira has a higher stocking rate of 2.5, but under the current common agricultural policy the island aim at reducing to the value of 2.0 animals per ha in the coming years (PRA, 2001).

However, the cultural landscape of the Azores is moving to a uniform system of land use patterns, characterized by pastures, planted forests and wooded fencerows of *Cryptomeria japonica* (PROTA, 2000), associated with losses of biodiversity, of natural resources, environmental quality and aesthetic and cultural values.

9. Aims of this dissertation

The aims were to:

1) Study the AMF species composition and diversity using the classic characteristics of spore morphology, to highlight both qualitative and quantitative differences in semi-natural and intensive pastures on Terceira Island in the Azores archipelago;

2) Study the effect of land use type on AMF diversity using molecular tools, and analyse the occurrence of seasonal patterns in colonisation of AMF associated with the host plant *Holcus lanatus* L. in the two land use systems. *Holcus lanatus* L. was the selected for its commonness in the two land use systems and the Azores;

3) Assess the control mechanisms of AMF in protecting *Holcus lanatus* L. from the root-knot nematode;

4) Address the question of the preferences of these root-colonising AMF for the three most abundant grasses used in pastures of the Azores: *Holcus lanatus* L., *Lolium multiflorum* Lam. and *Lolium. perenne* L.; and identify the implications for plant productivity.

CHAPTER II. SPECIES COMPOSITION OF ARBUSCULAR MYCORRHIZAL FUNGI IN SEMI-NATURAL AND INTENSIVE PASTURES FROM TERCEIRA ISLAND (AZORES)

1. Introduction

AMF are ubiquitous, underground, symbiotic associations involving a wide diversity of plants (approximately 80%) and obligate symbiotic fungi of the phylum Glomeromycota thought to have originated 400–500 million years ago (Schülbler *et al*., 2001). In this intimate association the obligate biotrophic fungi provide terrestrial plants with minerals, nutrients (particularly inorganic phosphate) and water increasing the host resistance to biotic and abiotic stresses, including pathogens, water limitation and environmental pollutants in return for photosynthates (Smith and Read, 1997).

These beneficial effects of AMF are important in natural ecosystems, although they may be unimportant in high-input agriculture (Barea and Jeffries 1995; Galvez *et al.* 2001). Clearly, agricultural practices such as the intensity of cultivation, the quality and quantity of fertilisers applied and the plant protection strategies used may have dramatic impacts on soil and soil organism, and the AMF community structure is not an exception (Sieverding, 1989; Douds and Millner, 1999; Oehl *et al*., 2003; Li *et al*., 2007).

There have been some recent reports comparing the AMF community structures within farming systems differing in management practices such as tillage, fertiliser input, pesticide use and crop rotation (reviewed by Douds and Millner, 1999). Some agricultures practices, such as tillage and fertilisation, can affect the structure of communities causing a decline in AMF diversity and producing a shift in the AMF composition (Boddington and Dodd, 2000; Egerton-Warburton and Allen, 2000; Jansa *et al*., 2002; 2003).

The extra-radical network of AMF mycelium functions as both the nutrient absorbing organ of mycorrhizae and as inocula for the colonisation of new roots. Thus disruption caused by tillage can result in delayed or reduce root colonisation, and a decrease in the soil volume exploited by AMF which may affect nutrient uptake by mycorrhizae (Miller *et al*., 1995).

In addition, Jansa *et al*. (2002) reported that tillage affected significantly the community structure of AMF, decreasing the sporulation of some non-*Glomus* like *Gigaspora*, *Scutellospora* and *Entrophospora* in tilled treatment.

Also Oehl *et al*. (2004) found that differences between land use systems (conventional *versus* organic) did not only reflect in species richness but also in species composition.

Although they have detected no change in abundance of *Glomus* species between land uses, the same pattern was not observed in relation to spore abundances of *Acaulospora* and *Scutellospora* species which were more abundant in organic systems. These findings suggest that in high-input fertilisers systems tend to occur a homogenisation of AMF community composition, almost dominated by AMF generalist species, since the non-*Glomus* species have a tendency to inhabit determinate niches being characterised as specialist as they are only present in specific soil types, preferentially organic soils (Oehl *et al*., 2010). It have been shown these agriculturally adapted AMF have slower rates of infection, are faster to sporulate and to produce fewer arbuscules, the site of nutrients transfer to the host (Johnson, 1993; Scullion *et al*. 1998; Oehl *et al*. 2003). These findings suggest that the altered AMF community is draw more resources from the plant while providing less in return. Thus, we could assist to a loss in AMF species diversity during agricultural use which may result in a diminished biodiversity of the reestablishing plant community (Van der Heijden *et al*. 1998).

Soil types differ in soil chemical and physical characteristics. Recent works have showed that pH (Ezawa *et al*., 2001; Oehl *et al*., 2010), nitrogen (N) (Jonhson *et al*., 2003; Egerton-Warburton *et al*., 2007) organic matter (OM) (Boddington and Dodd 2000) and phosphorous (P) availability (Oehl *et al*., 2004) influence the composition of AMF communities. It has been showed in several studies that pH it is an important factor influencing the AMF species composition. Some AMF species (e.g., *Glomus*) prefer alkaline and neutral soil, while other (e.g., *Acaulospora*) sporulate more abundantly in acid soils (Gai and Li, 2003). Also Oehl *et al*. (2010) reported that pH had strongly influence in AMF species genus and on relative density of *Glomus* species. Nitrogen (N) has also proved to be a preponderant factor in determining AMF composition via carbon (C) allocation. This way, N enrichment generally decreases allocation to AMF structure at the field site with ample soil, but it increases allocation to mycorrhizae at sites with P-deficient soils (Johnson *et al*., 2003). Extraradical structures are more responsive to N enrichment than are intraradical. Hence, one time that Gigasporaceae produce more extraradical hyphae than Glomacaeae and Acaulosporaceae, both Gigasporaceae spores and extraradical hyphae decrease with N-enrichment in high-P soil and the opposite occurred in low-P soils (Johnson *et al*. 2003).

Organic matter (OM) is also susceptible to influence AMF composition via increase or decrease the species abundance of some AMF. Zhang *et al*. (1999) reported that frequency of occurrence of *Glomus mosseae* decreased with increasing OM content, while *Glomus sinuosum* and *Glomus taiwanense* were found only when soil organic matter content was less than 1.5%.

Some authors have suggested that differences in AMF composition between conventional and organic systems can be explained by balance of nutrients applied which combined produce variables levels of soil P (Eason *et al*., 1999; Oehl *et al*., 2004). The largest effect of AMF is on P nutrition. It is well know that higher soil P can reduce AMF formation and the inhibition may be due to a direct effect on external hyphal growth or be indirect associated with host P status (Sanders, 1975). *Glomus* due to its large ecological plasticity is generally the genus with greater number of species found in intensively management (Oeh *et al*., 2003; 2004). Johnson (1993) detected an increase on relative abundance of *Glomus intraradices* in response in fertilisation witch classify it in P-tolerant, as well as, a decrease in relative abundance of *Gigaspora gigantea*, *Gigaspora margarita*, *Scutellospora calospora* and *Paraglomus occultum*. Also Bhadalung *et al*. (2005) showed that long-term fertilisation caused decreases in AMF total spore numbers and also relative spore abundance varied with species and sampling time. They found a decreased in absolute number of species *Intraspora scheckii*, *Glomus mosseae*, *Glomus* sp.1, *Glomus geosporum*-like and *Scutellospora fulgida* with fertilisation, which were classified as AMF species insensitive to fertilisation.

Despite ecological importance of AMF community, little is known about their population biology and diversity in natural ecosystems. Due to methological limitations, only about 200 morphospecies were described. Inoculum of AMF in soil appears in three forms: spores, soilborne hyphae and colonised roots. Traditional studies on AMF diversity are based mainly on spore morphology (Walker, 1992). However, taxonomic identification of AMF spores collected directly from the field is quite difficult because they are often unidentifiable due to degradation or parasitism by other organism, and thus do not necessarily reflect the AMF populations in soil (Clapp *et al*., 1995). Adding, fungal spore diversity differs seasonally, with some fungi sporulating in late spring and others at the end of summer.

However, an advantage is that spore numbers are indicators over a longer time period, i.e. over months, which allow us to described AMF community structures based on

spore morphology (Douds and Millner,1999; Oehl *et al*., 2003), with the exception of putative non-sporulating AMF species. Providing the user has reasonable taxonomic expertise, the extraction of spores from soil is a rapid method, and it may reveal taxa not easily detected by molecular methods. Moreover, intensive soil sampling, including the use of successive trap culturing, takes much longer but reveals significantly greater diversity than a 'one-off' spore extraction (Oehl *et al*., 2004). Recently, alternative molecular methods have been developed (Clapp *et al*., 1995; Helgason *et al*., 1998; Redecker, 2000). These molecular tools have proved to be useful tools for characterising of the AMF community, and in the future could be combined with the morphological tools available to a better understanding of AMF community structure. However, the molecular tools are costly and time-consuming, and AMF populations cannot as yet be quantified by these techniques, because they lack sufficient sensitivity detecting only the most abundant taxa (Oehl *et al*., 2004; Egerton-Warburton *et al*., 2007). Hijri *et al*. (2006) noted that neither all AMF taxa present as spores were detected in roots by PCR, probably because they were not symbiotically active at the respective time or because they colonise the roots at levels below the detection threshold. Then, it is still uncertain whether there are other AMF that remain undetected because their percentage of intraradical structures are low, or if there are other reasons for these techniques still partly to fail (Oehl *et al*., 2004).

The Azores archipelago has an extended area of pastures (Martins, 1993) including natural, semi-natural and intensive pastures (see Borges *et al.*, 2008). Trough time, native forests have been destroyed by human activities and replaced by agricultural land. These disturbances can significantly alter the proportion of mycotrophic versus nonmycotrophic plant species in a community, and consequently affect the dynamics of the AMF community and its sporulation patterns (Violi *et al*., 2008). After several economical cycles, and some catastrophic experiences with different types of monocultures (e.g. wheat *Triticum* spp.; "Pastel" *Isatis tinctoria* L.; orange-tree *Citrus* spp.) (Martins, 1993), during the second half of the 20th century cattle milk production has grown in importance (Martins, 1993; Borges, 1997). In order to respond to the high density of cattle milk, pastures became the dominant landscape in the Azores archipelago, covering 67.7 % of the arable land, and there are also semi-natural pasture comprising 11.2 % of the total area (Garcia and Furtado, 1991). Most semi-natural pastures are located at high altitude in recent infertile acid volcanic soils (e.g.

Terceira and Pico), or in more mature but still poor soils (e.g. S. Maria) (Borges, 1997). The floristic composition of pastures is dominated by introduced grasses and leguminous forbs, while native forbs (e.g. *Lotus uliginosus*), grasses, rushes, sedges and ferns are more frequent in the poorly managed old semi-natural pasture (Borges, 1997).

To date, little is known about the diversity and composition of arbuscular mycorrhizal fungal (AMF) communities in this environment as affected by changing land use. Therefore, in this study we assessed the AMF community structure based on the classic spore morphology characteristics in order to highlight both qualitative and quantitative differences in semi-natural and intensive pastures in Terceira Island (Azores).

2. Materials and methods

2.1. Study sites

This study was conducted in the Terceira Island, the third island of the Azorean archipelago in terms of size (402 km^2). Terceira is a roundish island formed by four main volcanic complexes (Serra de Santa Bárbara, Serra do Morião, Pico Alto and Serra do Cume) (Zbyszewski *et al.*, 1971). The highest point (Serra de S. Bárbara, 1023 m) is simultaneously the most recent of the three major island's complexes of 0.025 Ma B.P. (Martins, 1993). The eastern flatted part of the island is its older part of about 3.52 My (Borges *et al*., 2009). Recent historical volcanic activity is evident in the western part of the island, where several well preserved lava tubes and volcanic chimneys are present (Borges *et al.*, 1993). The Azores archipelago have a temperate oceanic climate characterised by high levels of relative atmospheric humidity that could reach 95% at high altitude native forests and ensures slight thermal variations throughout the year (Azevedo *et al*., 1999). The average temperature is 17.5° C in low altitudes, while the maximum temperature is in August and minimum in February (Azevedo, 1996). The pluviometric regime reaches its peak in January-February and minimum and July (Azevedo, 1996): Angra do Heroísmo (47 m): 969 mm year (140 mm in January and 40 mm in July) and Serra de S. Bárbara (1,023 m): 3,000 mm year (Borges, 1997).

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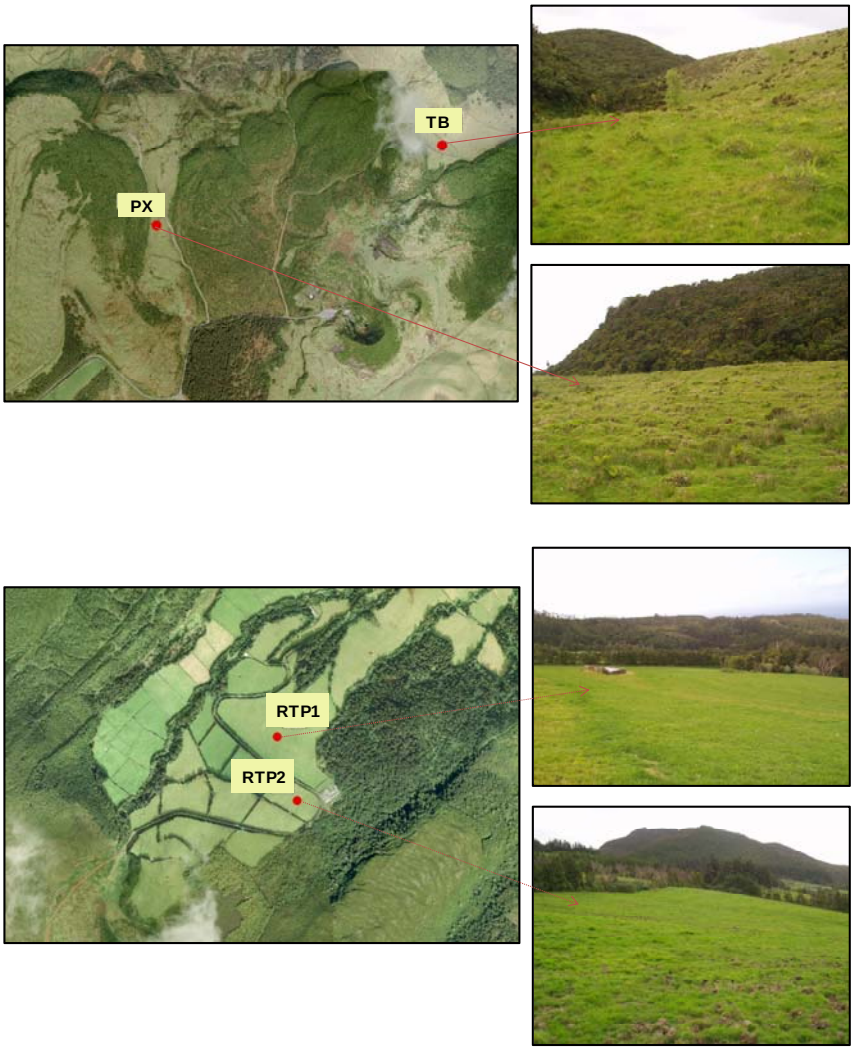

Figure 1. Location of the study sites at Terceira Island

2.2. Sample collection

Soil sample were collected in August 2007 from the root zones of the dominant and common plant species *Holcus lanatus* L., to a depth of 20 cm at 10 different locations in each sampling area (PX, TB, RP1 and RP2).

2.3. Study plant

The Azorean pastures are dominated by *Holcus lanatus* L. a non-indigenous plant with a frequency more than 50% (Dias, 1996; Silva and Smith, 2006; Kueffer *et al*., 2010). In Terceira Island in a total of 30 exotic vascular plants, *H. lanatus* L. was the third more frequent (Silva and Smith, 2006). This plant is a C_3 perennial, 20–110 cm tall, tufted grass (Ross *et al.*, 2003). It is very common, growing in all kinds of soil but the

optimal growth occurs in moist conditions (Franco, 1984). *H. lanatus* grows well in very wet conditions and can survive moderate periods of drought (Franco, 1984).

2.4. Soil analyses

The soils of semi-natural and intensive pastures are andossols and sandy-loam (Pinheiro, 1990). The soil analyses were performed only with soil samples collected in August 2007. The 10 samples of rhizospheric soil from each site were pooled and analysed as a single sample. Chemical soil parameters for each soil are presented in Table 1. They were measured in the University of Azores Soil Laboratory (CITA-A), according to standard methods. Potassium, calcium and magnesium were extracted with sodium acetate (1/5) at pH 7, and were determined using a Varian ICP atomic emission spectrophotometer. Soil pH was determined on a soil/water paste (1/2.5) and available phosphorus as described by Olsen and Sommers (1982) by extraction with a 0.5 M $NaHCO_3$ solution at pH 8.5, using AAS. Total soil nitrogen content was estimated using the Kjehldahl method (Allen, 1989). The organic carbon (OC) and organic matter (OM) present in the soil were estimated using the dry ashing method.

Table 1. Chemical soil parameters at field sites differing in land use in August 2007. PX – Galhardo; TB – Terra Brava; RP1 – Agualva 1; RP2 – Agualva 2.

Land use	field site	pH	P (Olsen) (mg kg^{-1})	K (mg kg^{-1})	N (%)	Ca (mg kg^{-1})	Mg (mg kg^{-1})	OM (%)	OC (%)
Semi-natural									
	PX	5.7	7	143	0.55	93	84	24	13.92
	TB	5.7	11	190	0.40	89	73	21	12.18
Intensive									
	RP1	5.7	4	102	0.11	54	17	14	8.12
	RP2	5.7	10	80	0.26	45	13	16	9.28

2.5. Establishment of a trap culture

Trap cultures were established from fresh soil samples mixed with autoclaved sand and “bagacina” (volcanic soil type) in a ratio of 2:1:1. A 1.5-kg aliquot of the mixture was placed in each pot to produce trap culture from each sample (Fig. 2). Corn (*Zea mays* L.) was used as host plant. Seeds of corn were surface sterilised by immersion in alcohol (96 %) for 30 sec, 4 % bleach for 2 min, and rinsed twice in sterile distilled

water. Then, they were sown in pots on sterilised sand soil and left to germinated in the greenhouse. After one week of growth, seedlings were transplanted to pots containing the fresh soil samples mix. A total of 4 seedlings were used per plot. Trap cultures in 40 plots were grown in glasshouse for 5 months and then harvested. All pots were watered every 2 days with distilled water, and no nutrient solution was added to the pots. One month before harvested, we left watering the pots to stimulate the sporulation.

Figure 2. Trap cultures with *Zea may* host, using original soil from the semi-natural (PX; TB) and intensive (RP1; RP2) pastures.

2.6. AMF spore extraction and identification

Spores were extracted from each soil sample using a centrifugation technique modified from that used for extracting nematodes from soil (Jenkins, 1964). A subsample of about 50 cm^3 was weighed and sieved (2 mm) to remove some debris and lumps. Next, the sample was placed in an erlemeyer (1000 mL) containing a solution of sodium polyphosphate $(NaPO_3)_n$, stirred vigorously for 4 min on a stirrer and allowed to settle for 15 sec. Then, the supernatant was gently poured to a stack of 1000-, 500-, 250-, 100-, and 45 µm sieves. This operation was repeated more than 2 times but it was used only distilled water as a solution. Then, we proceeded to the collection of the material found in each sieve to two 50-mL plastic centrifuge tubes, and centrifuged for 5 min at 4000 r.p.m. on horizontal centrifuge. The supernatant was discarded and the tubes were filled with sucrose solution at a concentration of 480 g/ L (specific gravity, 1.18). Their contents were then stirred thoroughly and centrifuged for 3 min at 3000 r.p.m. The supernatant was quickly poured onto a 38 µm sieve and

sluiced with tap water to remove the sugar. The material retained on this last sieve was transferred to a 50-mL plastic centrifuge tube and stored at 4 ˚C in autoclaved distilled water until count could be made. After, tubes containing the spores were discharged into a glass Petri plate (9 cm diameter), all spores were counted into at a 2-mL plastic tube and stored at 4 ˚C in autoclaved distilled. Next each plastic tube containing all spore extracted from each sample (a total of 40 samples) were sent to a specialist, AMF spore in identification, Dr. Christopher Walker which made its morphological identification.

Species description was based on recommendations of Dr. C. Walker, as well as, on current species descriptions and identification manuals (International Culture Collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal Fungi [http://invam.caf.wvu.edu/Myc_Info/Taxonomy/species.htm]).

This work was a preliminary study of AMF diversity and composition only based on species extracted from trap culture, caused by low spore number, parasitism of spores, and age and environmental alteration of spores (e.g., discoloration) in field which could hinder accurate identification, Hence, it's probable that has occurred some bias from the use of this technique.

Discrepancies between trap culture roots and field-collected roots are likely to be the result of the conditions in the pot cultures, which may favor certain AMF taxa. Consequently, trap culture may provide a different picture of AMF communities than analysis of field-collected roots. Such a difference in AMF species composition between trapping and direct spore extraction had been report in several studies (Brundrett *et al*. 1999; Oehl *et al*. 2003). Brundrett *et al*. (1999) found that most species of *Scutellospora*, *Acaulospora* and *Gigaspora* were obtained primarily from field-collected spores, but only 50% of these culture attempts were successful. With *Glomus*, they found the opposite pattern. Several *Glomus* spp. rarely detected in field soil were dominant in trap cultures. Oehl *et al.,* (2004) reported three out of 35 species recorded at a field site failing to produce spores in traps. Hijri *et al*. (2006) noted that *Paraglomus* was never detected in trap cultures, although it occurred frequently in the field site experiment. However, the opposite was observed by them in relation to *Archaeospora trappei* which only be detected in trap cultures.

Also Wang *et al*., 2008 found a distinct difference in AMF diversity in the two sets of intensively managed agricultural soil in Sichuan Province of SE China. Only five

species out of thirty were detected both by direct extraction (soil set 2) from soil and by trap culturing (soil set 1).

However, despite all limitations of trap culture it's know that identification of species at a site and quantification of diversity, dominance, etc., actually it's limited to the sporulating species, and that non-sporulating species only can be detected via trap culture. The differences between isolation methods often is pronounced with prolonged (Oehl *et al*., 2003) or repeated trapping (Stutz and Morton, 1996). However, it is clear that several isolation methods should be used simultaneously to obtain a more complete picture of the AMF species composition in soil. Brundrett *et al*. (1999) studied four different techniques for isolating AMF, i.e. (1) spores separated from soil, (2) soil trap cultures, (3) root samples or (4) transplanted seedlings. They found that all these techniques complemented each other because they often produced cultures dominated by different species from the same soil sample.

Furthermore, although morphological identification of AMF is the most widespread method and in many cases still unavoidable, it should be carried out concomitantly with other (e.g. molecular) methods for mutual confirmation.

2.7. Data analysis

Spore density, richness, occurrence and relative abundance were based on spore identification extracted from the trap culture. The ecological parameters were calculated as follows: i) spore density was expressed as number of fungal spores in 50 g dried soil; ii) relative of abundance was defined as the number of spore of a given specie or genus divided by the total number of spores in each soil type; iii) frequency of occurrence was calculated as the number of samples from which particular species or genus were isolated; iv) species richness was defined as the number of AMF taxa found in particular field site.

To analyse the relatedness of sampling sites in respect of AMF species composition a hierarchical cluster analysis (HCA) using Ward's minimum variance method was performance in CAP 4 (Community Analysis Package 4, CAP4), to determine the similarity (more precisely: dissimilarity) with respect to AMF species composition between land use, based on the χ^2 distance. We also used Principal Correspondence in CAP 4, to study the contribution of each sampled site to the total variation based on

their AMF species composition, as well as the contribution from each AMF species based on their abundance along sampled sites.

Pearson correlations coefficients was calculated between chemical proprieties of each soil type and the total AMF density, as well as between AMF spore density of the more representative genera (*Acaulospora*, *Glomus* and *Scutellospora*).

The sampling design was nested hierarchical, with field sites nested within land use type. Then, to investigate the variation on species richness between land uses and between field sites within each land use we used Nested ANOVA (Minitab, version 13.31, 2000). The same proceed was made to estimated the variation on total AMF spore density, and the variation on AMF spore density of the more representative genera (*Acaulospora*, *Glomus* and *Scutellospora*) between land uses and between field sites within each land use. All data were tested for normality when required to fulfil assumptions of the Nested ANOVA.

3. Results

3.1. AMF species recorded

Thirthy-nine AMF species representing eight genera were detected in two land uses studied (Table 2). Nineteen species could not clearly be named at the species level, the majority of them bellowed to the genus *Glomus* (13), *Paraglomus* (2) following by genera *Acaulospora*, *Ambispora*, *Gigaspora* and *Scutellospora* each with just one species not morphologically identified to the species level (Table 2).

The two most representative genera were *Glomus* and *Acaulospora* with 19 and 10 species respectively, following from the genera *Paraglomus* and *Scutellospora* with 3 species each. The other AMF species detected were two from the genus one species (Table 2).

Table 2. Occurrence of glomeromycotan species in semi-natural (PX and TB) and intensive (RP1 and RP2) pastures.

Fungal species recorded	PX	TB	RP1	RP2
Acaulospora delicata Walker, Pfeiffer & Bloss	x			
Acaulospora elegans Trappe & Gerd.			x	
Acaulospora excavata Ingleby & Walker		x		
Acaulospora cf *koskei* Blaszk.	x		x	x
Acaulospora laevis Gerd. & Trappe	x	x	x	x
Acaulospora myriocarpa Spain, Sieverd. & Schenck	x	x	x	x
Acaulospora cf *paulinae* Blaszk.	x	x	x	x
Acaulospora cf *thomii* Blaszk.	x			
Acaulospora tuberculata Janos & Trappe	x			x
Acaulospora sp.	x	x		
Ambispora sp.	x	x		
Entrophospora infrequens (Hall) Ames and Schneid.				x
Gigaspora sp.	x			
Glomus clarum Nicolson & Schenk	x	x		
Glomus cf *etunicatum* Becker & Gerd.		x	x	x
Glomus globiferum Koske & Walker			x	x
Glomus intraradices Schenck & Sm.	x			
Glomus cf *lamellosum* Dalpé, Koske & Tews				x
Glomus rubiforme (Gerd. &Trappe) Almeida & Schenck	x	x	x	x
Glomus sp. 1	x		x	
Glomus sp. 2	x			x
Glomus sp. 3		x		
Glomus sp. 4		x		
Glomus sp. 5			x	
Glomus sp. 6			x	
Glomus sp. 7			x	
Glomus sp. 8			x	
Glomus sp. 9				x
Glomus sp. 10				x
Glomus sp. 11				x
Glomus sp. 12				x
Glomus sp. 13				x
Intraspora schenckii (Sieverd. &Toro) Oehl & Sieverd.	x			
Paraglomus brasilianum (Spain & Miranda) Morton & Redecker			x	
Paraglomus sp. 1	x	x	x	x
Paraglomus sp. 2				x
Scutellospora calospora (Nicolson & Gerd.) Walker & Sanders	x	x	x	x
Scutellospora pellucida (Nicolson & Schenck) Walker and Sanders	x		x	x
Scutellospora sp.	x	x	x	x

3.2. Species descriptions

***Acaulospora delicate* Walker, Pfeiff. & Bloss**

Spores subglobose, 80 x 73 µm, subhyaline to pale yellow. Wall structure of 4 components in 2 groups (A and B). Wall group A of 2 components and wall of group B of 2 thin, hyaline flexible inner components which react to became purple in Melzer's reagent.

Fig. 3.1. a) Spore reacting in PVLG; **b)** crushed spore in PVLG; **c)** spore reacting in PVLG with Melzer's reagent after crushing; **d)** spores in water showing variation in shape.

***Acaulospora elegans* Trappe & Gerd.**

Spores subglobose, 150 x 130 µm, ochre to dark brown with a dull surface due to light scattering from surface ornamentation. Wall structure 4 components in 2 groups (A and B). Wall group A of 2 components and wall of group B of 2 hyaline, flexible components. **Fig. 3. 1. e)** Spore reacting in PVLG.

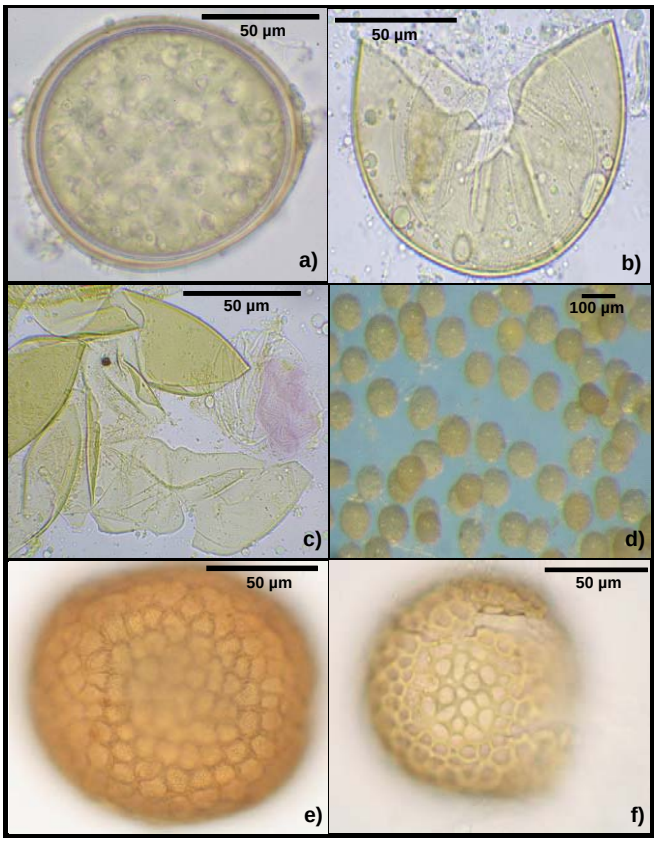

Figure 3.1. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Acaulospora excavata* Ingleby & Walker**

Spores broadly ellipsoid, 110 x 90 µm, pale ochre to orange. Wall structure of 3 components in 3 groups (A, B and C). Wall group A of a unit component, ornamented by circular to subcircular to elliptical pits. Wall groups B and C both of a single component. **Fig. 3.1. f)** Spore reacting in PVLG.

Acaulospora koskei Blaszk.

Spores globose, 180 x 180 µm, pale yellow-brown to orange brown. Wall structure of 6 components arranged in 2 groups. Wall group A of 3 components and wall group B of 2 flexible hyaline inner components which reacted to became pale pinkish purple in Melzer's. **Fig. 3.2. a)** Spore reacting in PVLG with Melzer's reagent after crushing; **b)** spores in water showing variation in shape.

***Acaulospora laevis* Gerd. & Trappe**

Spores globose, 260 x 260 µm, yellow-brown to red-brown. Spores formed laterally in a neck of a hyaline single terminal sporiferous saccule. Wall structure composed of 5 components in 2 groups. Wall Group A of 3 components. Wall group B of a 2 flexible hyaline inner components, very thin. **Fig. 3.2. c)** Spore in PVLG with Melzer's; **d)** spore invaded by non-AM fungi in PVLG with Melzer's reagent; **e)** spore reacting in PVLG with Melzer's reagent after crushing.

***Acaulospora myriocarpa* Spain, Sieverd. & Schenck**

Spores broadly ellipsoid, 70 x 60 µm in size, hyaline to pale yellow. Wall structure composed of 3 components in one group. **Fig. 3.2. f)** Spore reacting in PVLG.

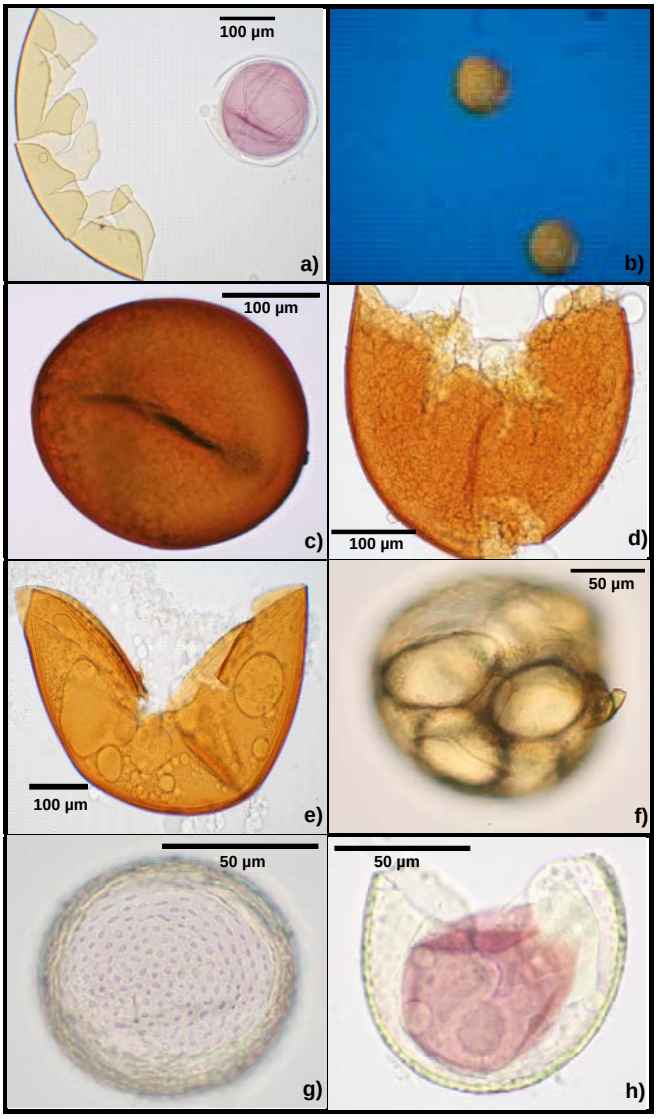

Figure 3.2. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Acaulospora paulinae* Blaszk**

Spores globose, 75 x 75 µm, hyaline to pale yellow. Wall structure composed 3 components in two groups. Wall group A of 2 components, and wall group B of a unit component which reacted to became purple in the Melzer 's reagent. **Fig. 3.2. g)**

Spore reacting in PVLG with Melzer's reagent; **h)** spore reacting in PVLG with Melzer's reagent after crushing.

***Acaulospora thomii* Blaszk**

Spores globose to subglobose, 280 x 280 µm brownish orange to brown and shiny some with a thick outer hyaline wall component. Wall structure of 5 components arranged in 2 groups (A and B). Wall group A of 3 components and wall group B of 2 hyaline components.

Fig. 3.3. a) Spore cluster reacting in PVLG; **b)** crushed spore in PVLG; **c)** spore reacting in PVLG with Melzer's reagent after crushing; **d)** spores in water showing variation in shape.

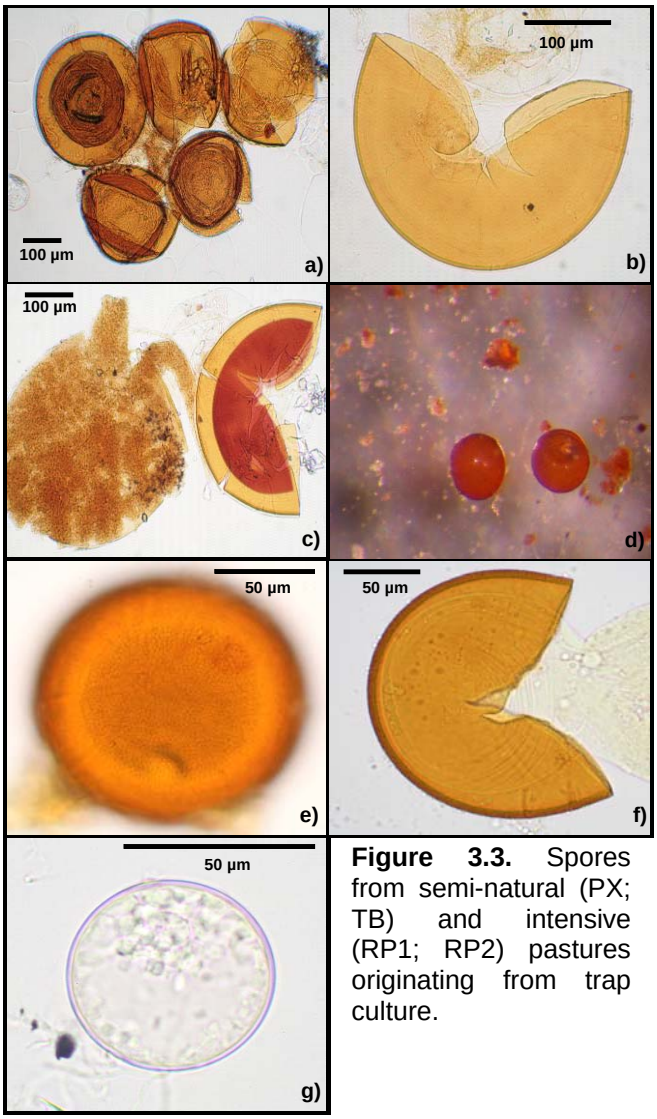

Figure 3.3. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Acaulospora tuberculata* Janos & Trappe**

Spores globose to subglobose, 120 x 110 µm, pale orange-brown to orange-brown. Wall structure of 3 components in
a single group.

Fig. 3.3. e) Spore reacting in PVLG with Melzer's reagent; **f)** spore reacting in PVLG with Melzer's reagent after crushing.

***Acaulospora* sp.**

Spore globose, 60 x 60 µm, colourless to white. Wall structure composed 3 components in two groups.

Wall group A of 2 components, and wall group B of a unit component which reacted to became slight pink in the Melzer's reagent. **Fig. 3.3. g)** Spore reacting in PVLG with Melzer's reagent.

***Ambispora* sp.**

Spores singly in the soil, globose, 70 x 70 µm, pale pinkish cream, with a roughened appearance due to degradation of outer wall component. Spore wall structure of 3 groups. Group A of two components: component 1 evanescent, becoming roughened through degradation; component 2 laminated. Group 2 composed of a single flexible component. Group 3 of two tightly adherent flexible components. This appears to be an undescribed species in the genus *Ambispora* with some similarity to *A. fennica*, the type species of the genus, but the spores are much smaller and as yet no link has been made with a glomoid morphotype. Further work will be required to verify its identity though isolation followed by molecular and morphological study.

Fig. 3.4. a) Uncrushed spore in PVLG, showing aggregated spore contents, and debris adhering to the degrading evanescent wall component; **b)** crushed spore showing wall structure; **c)** spores extracted from the soil in water.

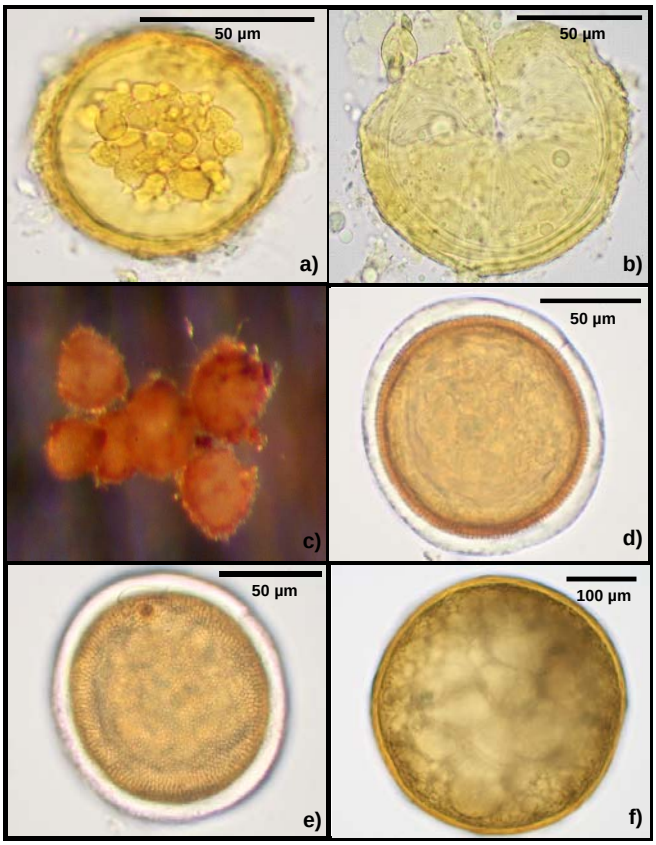

Figure 3.4. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Entrophospora infrequens* (Hall) Ames & Schneid.**

Spores globose to subglobose, 130 x 120 µm, orange-brown to dark orange-brown. Wall structure of 7 components arranged in two groups (A and B).

Wall of group A of 4 components and wall of group B of 3 hyaline and flexible.components. **Fig. 3.4. d; e)**; Uncrushed spore in PVLG.

***Gigaspora* sp.**

The spore subglobose, 370 x 400 µm, pale to brownish yellow. The bulbous subtending hypha formed terminally or laterally detached (presumably during extraction from the soil or substrate).

Wall structure of 2 components in a single group, a thin outer unit component and a main structural laminated component. **Fig. 3.4. f)** Uncrushed spore in PVLG.

***Glomus clarum* Nicolson & Schenck**

Spores globose to subglobose, 70 x 70 µm, hyaline to yellow brown, sometimes pale yellow to pale yellow brown. Wall structures of 3 components arranged in a single group. **Fig. 3.5. a)** uncrushed spore in PVLG.

***Glomus etunicatum* Becker & Gerd.**

Spores globose to subglobose, 120 x 110 µm, orange to red brown. Wall structure of 2 components in group. **Fig. 3.5. b)** Spore uncrushed spore in PVLG.

***Glomus globiferum* Koske & Walker**

Spores globose, 80 x 73 µm, orange brown to brown red. Wall structure of 3 components arranged in a single group. **Fig. 3.5. c)** Spore uncrushed spore in PVLG.

***Glomus intraradices* Schenck & Sm.**

Spores broadly ellipsoid to ellipsoid, pale yellow (ochre) to yellow brown, 64 x 109 µm. Wall structure of 2 components in a single group. The outer component reacts in Melzer's to become pink, and the innermost surface reacts similarly. **Fig. 3.5. d)** Spores in PVLG in root; **e)** spores clusters reacting in PVLG with Melzer; **f)** spores extracted from the soil in water.

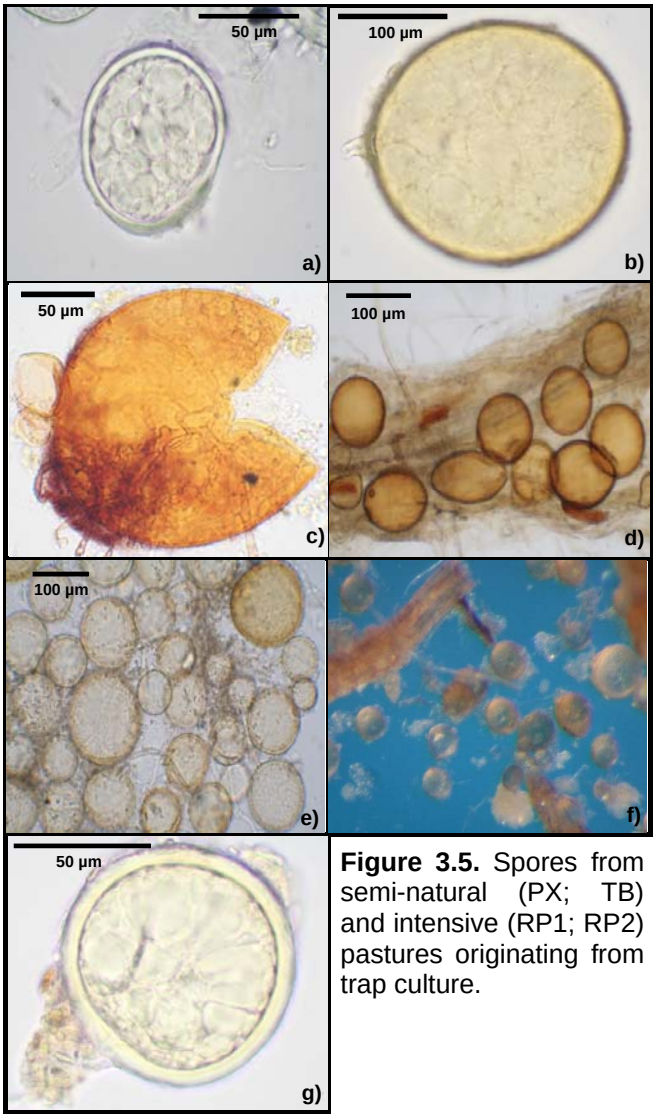

Figure 3.5. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Glomus lamellosum* Dalpé, Koske & Tews**

Spores globose to subglobose, 85 x 80 µm, cream to pale yellow. Wall structure of 3 components arranged in a single group. **Fig. 3.5. g)** Uncrushed spore in PVLG.

***Glomus rubiforme* (Gerd. & Trappe) Almeida & Schenck**

Spores globose to subglobose, 80 µm x 70 µm, light brown to dark brown. This species showed hypogeous sporocarps in dense cluster. Wall structure of a single group. **Fig. 3.6. a)** Uncrushed spore in PVLG; **b)** Sporocarps in dense cluster extracted from soil in water.

***Glomus* sp. 1**

Spores more or less globose, brown, 77-93 x 77-85 µm. Spores dead and much degraded. These darkly pigmented spore types are very common worldwide, and most often are found only as dead spores. They are commonly known as 'red brown laminate spores. **Fig. 3.6. c)** Uncrushed spore in PVLG.

***Glomus* sp. 2**

Spores globose, 150 x 150 µm, brown to red brown. Wall structure of 2 components in a single. **Fig. 3.6. d)** Uncrushed spore in PVLG.

***Glomus* sp. 3**

Spores broadly ellipsoid, 85 x 75 µm, pale yellow to yellow. Wall structure of 3 components arranged in one group. **Fig. 3.6. e)** Spore reacting in PVLG with Melzer's reagent.

***Glomus* sp. 4**

Spores were globose to broadly ellipsoid, 20 x 25 µm in size hyaline. Wall structure of 3 components arranged in one group. **Fig. 3.6. f)** Uncrushed spore in PVLG.

***Glomus* sp. 5**

Spores globose to subglobose, 120 x 110 µm, colourless. Wall structure of 2 thin components in only one group. **Fig. 3.6. g)** Crushed spore in PVLG.

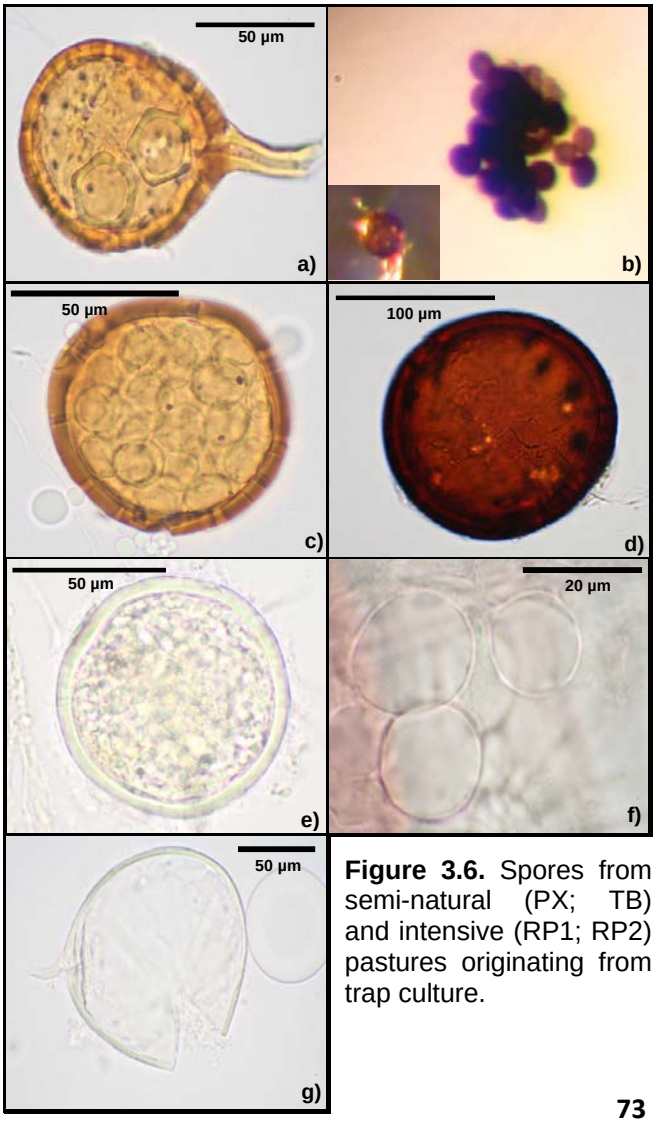

Figure 3.6. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Glomus* sp. 6**

Spores globose, 50 x 50 µm, colourless to pale yellow. Wall structure of 2 rough components in one groups which reacted to became purple in the Melzer‘s reagent. **Fig. 3.7. a)** Uncrushed spore in PVLG.

***Glomus* sp. 7**

Spores globose to subglobose, 80 x 80 µm, colourless. Wall structure of 2 components in one group which reacting to become pink in Melzer’s reagent. **Fig. 3.7. b)** Spore reacting in PVLG with Melzer’s reagent.

***Glomus* sp. 8**

Spores subglobose, 100 x 85 µm, yellow to greyish yellow. Wall structure of 3 components arranged in a single group. **Fig. 3.7. c)** Uncrushed spore in PVLG.

***Glomus* sp. 9**

Spores globose, 90 x 90 µm, orange to dark red. Wall structure of 3 components arranged in a single group. **Fig. 3.7. d)** Uncrushed spore in PVLG.

***Glomus* sp. 10**

Spores subglobose, 130 x 125 µm, yellow to brownish yellow. Wall structure of 2 components in one group. **Fig. 3.7. e)** Uncrushed spore in PVLG.

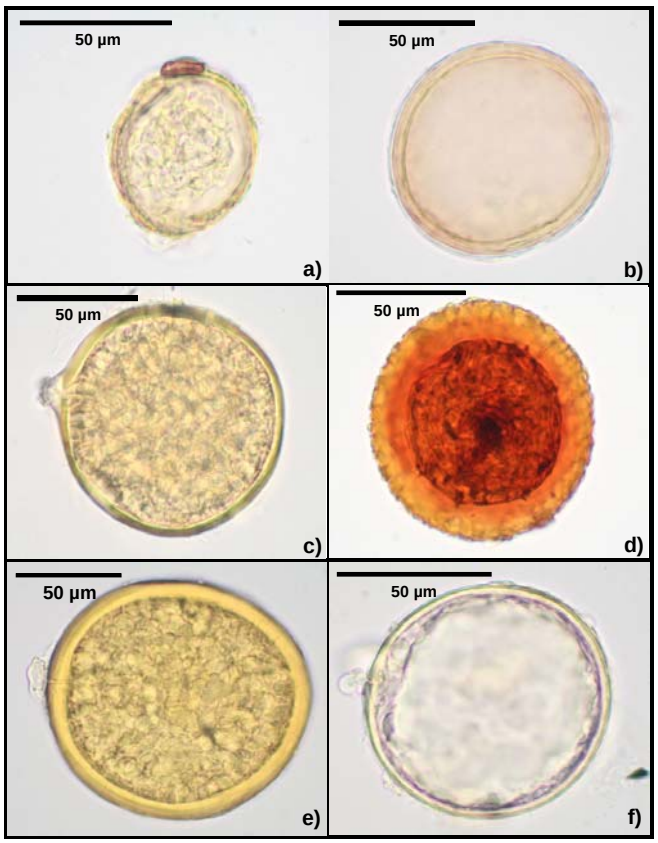

Figure 3.7. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Glomus* sp. 11**

Spores globose, 80 x 80 µm, hyaline. Wall structure of 2 components arranged in a single group which reacting to become pink in Melzer’s reagent. **Fig. 3.7. f)** Spore reacting in PVLG with Melzer’s reagent.

***Glomus* sp. 12**

Spores were globose, 250 x 250 µm in size, yellow to brownish yellow. Wall structure of 3 components in a single . **Fig. 3.8. a)** Crushed spore in PVLG.

***Glomus* sp. 13**

Spores globose to subglobose, 120 x 110 µm, yellow to yellow pale. Wall structure of 2 components in a single. **Fig. 3.8. b)** Uncrushed spore in PVLG.

Intraspora schenckii
(Sieverd. & S. Toro) Oehl & Sieverd

Spores globose to subglobose, ellipsoid to ovoid, hyaline, 37-99 x 27-72 µm. Wall structure of 3 components arranged in one group. **Fig. 3.8. c)** Spore reacting in PVLG with Melzer's reagent; **d)** uncrushed spore in PVLG; **e)** spores in water showing variation in shape.

***Paraglomus brasilianum* (Spain & Miranda) Morton & Redecker**

Spores globose to subglobose, 80 x 60 µm in size, subhyaline to cream. Wall structure of 3 components in a single. **Fig. 3.8. f)** uncrushed spore in PVLG

***Paraglomus* sp 1.**

Spores broadly ellipsoid, 69 x 50 µm, hyaline to pale cream. Wall structure of 3 components in a single. **Fig. 3.8. g)** Uncrushed spore in PVLG.

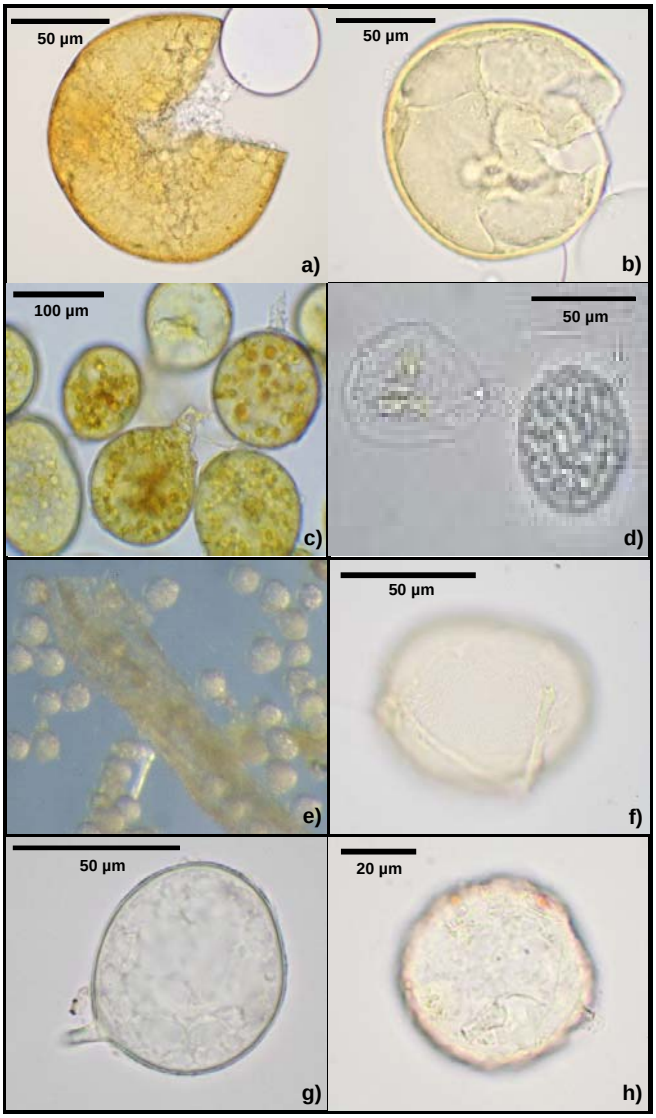

Figure 3.8. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

***Paraglomus* sp. 2**

Spores globose to ellipsoid, 60 x 60 µm, pale yellow to colourless. Wall structure of one group composed of 2 components wich reacting to become pink in Melzer's reagent. .**Fig. 3.8. h)** uncrushed spore in PVLG.

***Scutellospora calospora* (Nicolson and Gerd.) Walker & Sanders**

Spores broadly ellipsoid to ellipsoid, 250 x 210 µm, pastel yellow, formed terminally on a bulbous subtending hypha. Wall structure of 4 components (A and B). Wall group A of two components, an outermost unit component and a main structural laminated component. Wall group B of two colourless flexible components, reacting to become purple in Melzer's reagent. Bulbous base ovoid to clavate, pastel yellow to dark orange. Germination shield formed by wall group B, ovoid to oblong, hyaline to pale brown. **Fig. 3.9. a)** Crushed spore in PVLG, showing signs of invasion by (presumed) pathogen; **b)** spore reacting in PVLG with Melzer's reagent after crushing; **c)** spores in water showing variation in shape.

***Scutellospora pellucida* (Nicolson & Schenck) Walker & Sanders**

Spores globose to subglobose, 230 X 210 µm, colourless to white, yellow-brown, formed terminally on a colourless to yellow bulbous base. Wall structure of 4 components in 2 groups (A and B). Wall group A of 2 components (unit and laminated), and wall of 2 (perhaps 3) inner flexible and colourless components that formed a kind of 'endospore'. The laminated structural component reacted to Melzer's reagent to became red (more or less dextrinoid) whereas the innermost component reacted to that reagent to became purple (more or less dextrinoid).

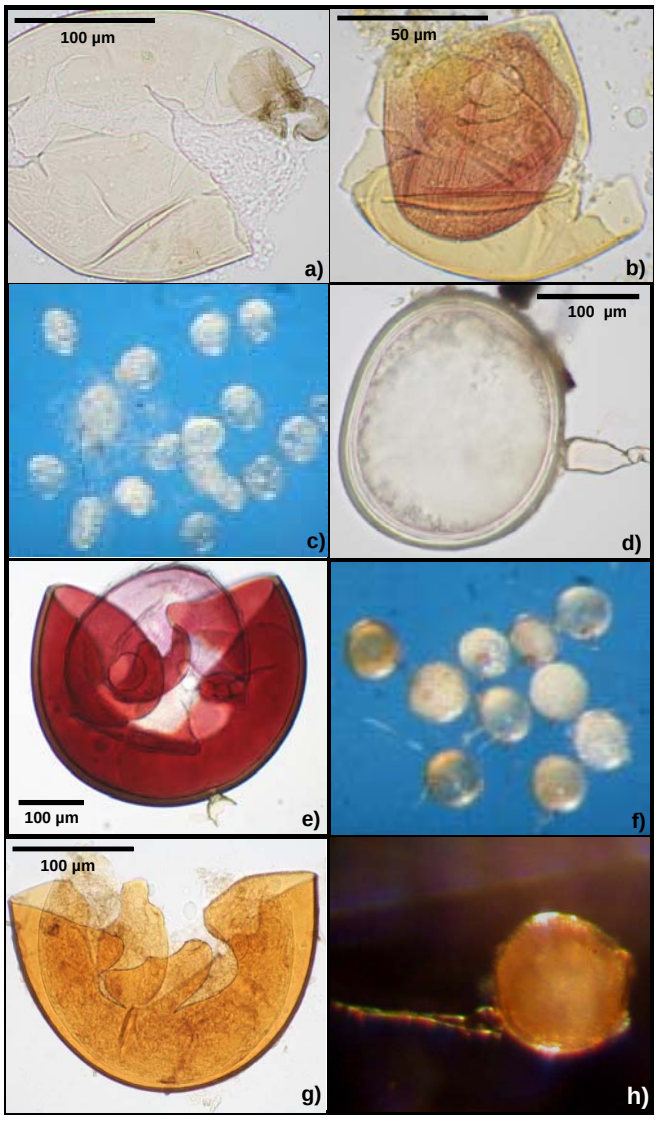

Figure 3.9. Spores from semi-natural (PX; TB) and intensive (RP1; RP2) pastures originating from trap culture.

These characteristics fit with the species description and with recent interpretations (C. Walker pers. comm). **Fig. 3.9. d)** Uncrushed spore in PVLG; **e)** spore reacting in PVLG with Melzer's reagent after crushing; **f)** spores in water showing variation in shape.

***Scutellospora* sp.**

Spores broadly ellipsoid to ellipsoid, 270 x 170 µm, pastel yellow, formed terminally on a bulbous subtending hypha. Wall structure of 4 components in 2 groups (A and B). Wall groups A of 2 components, an outermost unit component and a main structural laminated component. Wall group B of 2 colourless flexible components. **Fig. 3.9. g)** Spore crushed in PVLG, showing contents degraded by parasitism or by saprobic organisms; **h)** intact spore showing bulbous base (lower left).

3.3. AMF species composition

The number of species found at both land uses types was almost identical, as the semi-natural and intensive pastures contained 24 and 28 AMF species respectively. Then, no significant differences was found between land uses (Nested Anova: F= 2.44; d.f= 1. 2; p= 0.13) neither between field sites within each land uses (Nested Anova: F= 1.31; d.f= 1. 36; p= 0.28).

However, the AMF species composition appeared to differ between both land uses.

The hierarchical cluster analyses showed that land use intensity was the dominating factors which separated the similarities in AMF species composition between field sites (Fig. 4). The semi-natural sites with lower levels of land use intensity (PX; TB), are clearly separated clearly from intensives sites (RP1; RP2).

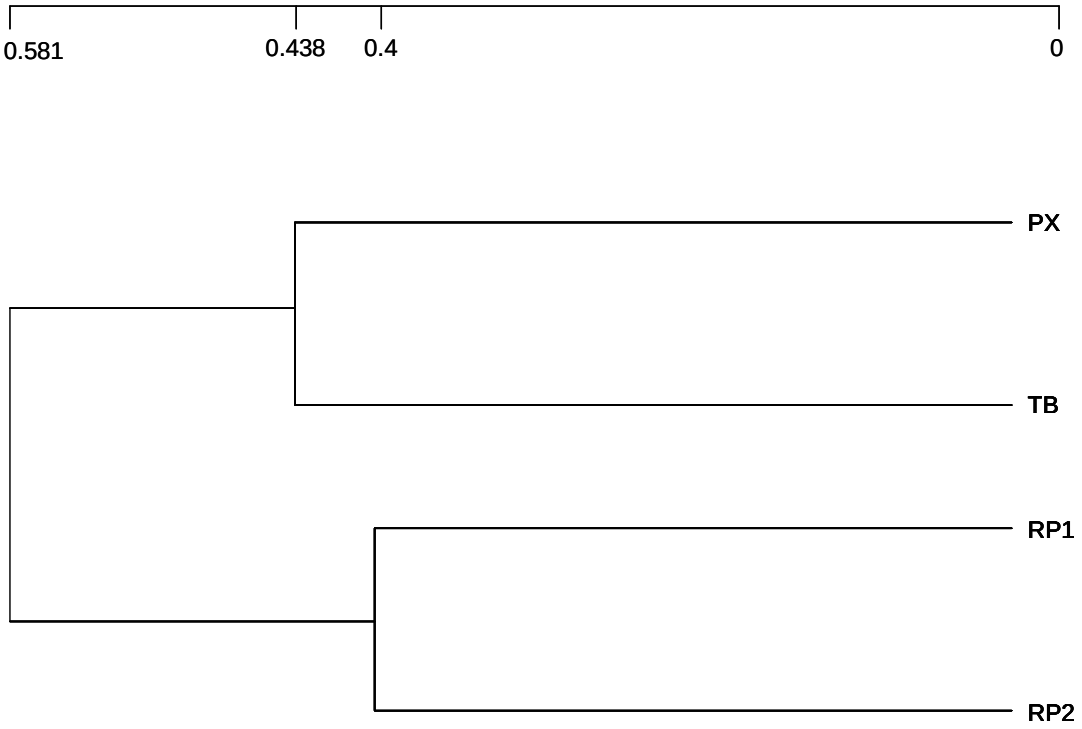

Figure 4. Hierarchical, agglomerative cluster analysis (Ward's linkage, 1-sorensen dissimilarity measure) for the AMF species in the semi-natural (PX and TB) and intensive (RP1 and RP2) land uses.

The contribution of AMF spore abundance and land use type to the total variation in species composition was analysed via Principal Component Analyses (PCA) (Fig. 5). Figure 5 presents the biplot for the first and second PCA axes, which explained 76.2% of total variation. The first axis which contributed with 46.50 % to the variance in species composition separated clearly the 2 land use types. In fact, only 18% of AMF species had "a generalist" behaviour, having been detected in both land uses, like *Acaulospora paulinae*, *Acaulospora laevis*, *Acaulospora myriocarpa*, *Scutellospora calospora*, *Scutellospora pellucida*, *Glomus clarum* and *Glomus rubiformis*.

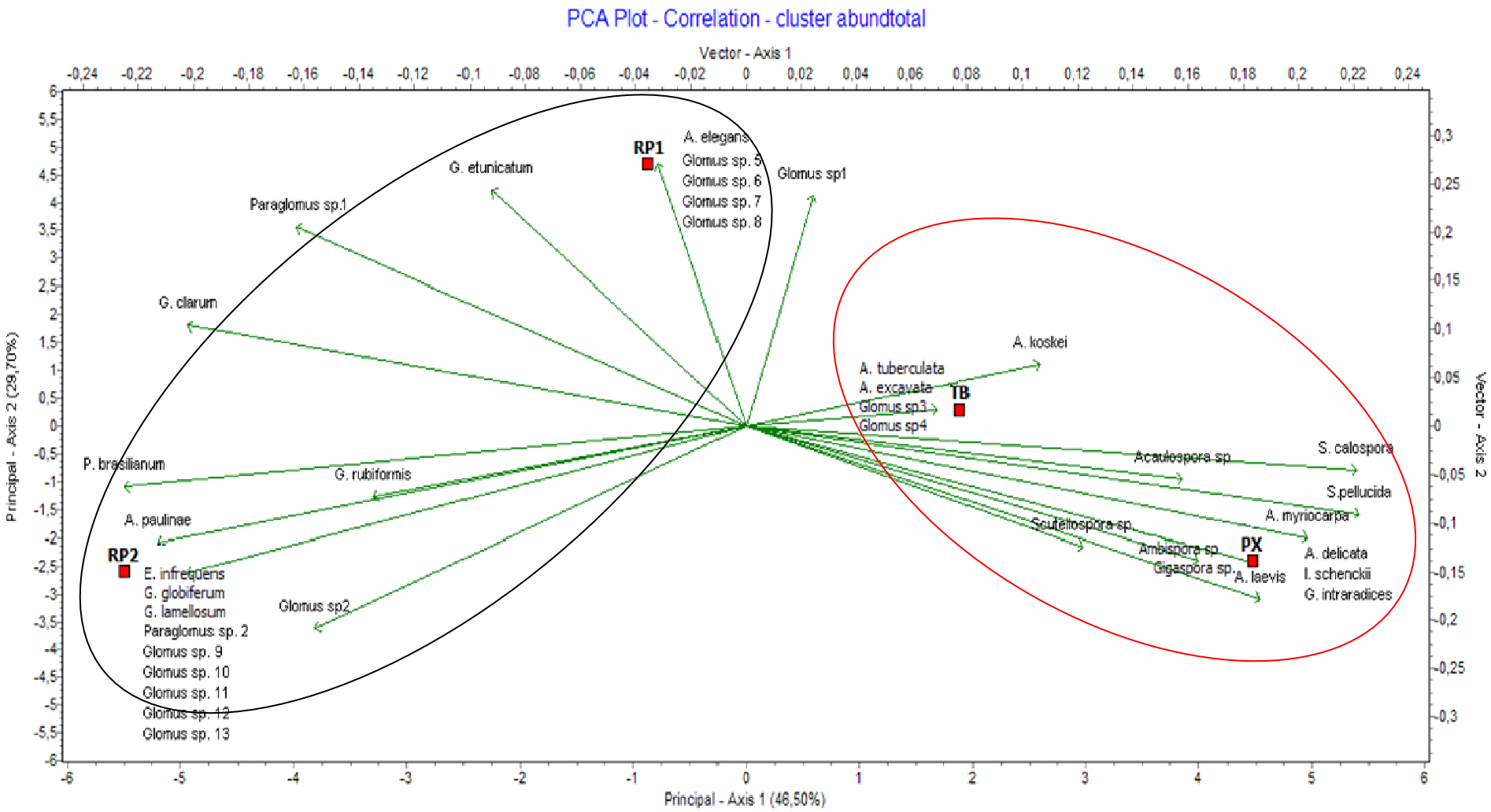

Figure 5. First and second axis in the Principal Correspondence Analysis for the abundance of AMF spores in the four field sites studied (PX and TB - semi-natural pastures; RP1 and RP2 – intensive pastures). AMF species are presented as vectors.

We detected that some AMF species occurred exclusively in just one land use type or in just one field site. In this way, *Acaulospora* sp. and *Ambispora* sp. were only observed in the semi-natural land use (Fig. 5) while *G. etunicatum* and *Paraglomus brasilianum* was only found in the intensive ones (Fig. 5). In fact, *Acaulospora* and *Scutellospora* were not only the two genera with higher frequency of occurrence (Fig. 6 a; 6 b) but also the most abundant (Fig. 7 a; 7 b) in semi-natural sites, having been *S. calospora* and *A. myriocarpa* the dominant species of these genera (Fig. 5). On the other hand, in intensive land use *Glomus* was the more frequent genus having been present in more than 90 % of the samples (Fig. 6 c; 6 d). *Glomus* was the most abundant genus (Fig. 7 c; 7 d), having contributed with about 40% of the total AMF spores density of intensive land use field sites. Between the various species of *Glomus*, *G. etunicatum* was the most representative species of this genus (Fig. 5). *Paraglomus* also was more frequent in intensive field sites than in semi-natural ones (Fig. 6 c; 6 d), as well as more abundant (Fig. 7 c; 7 d).

Interesting was the frequency of occurrence of *Acaulospora*, having been present in almost all samples of intensive land use (Fig. 6 c; 6 d), especially the relative density of *A. paulinae*, that made it in the dominant species of this genus in RP2 (Fig. 6 d).

Furthermore, other species were more or less restricted to specific field sites which contributed to enhance, the role of management systems as key factor in species composition between semi-natural and intensive land uses. Thus, in relation to the field sites of semi-natural land use we observed that *Acaulospora delicata*, *Acaulospora thomii*, *Glomus intraradices*, *Intraspora schenckii* and *Gigaspora* sp. only occurred in PX, whereas *Acaulospora excavata*, *Acaulospora tuberculata*, *Glomus* sp. 3 and *Glomus* sp. 4 were detected only in TB (Fig. 5). On the other hand, we also noted that some species exclusively occurred in RP1, like *Acaulospora elegans*, *Glomus* sp. 5, *Glomus* sp. 6, *Glomus* sp. 8 and *Glomus* sp. 9 (Fig. 5). Finally we observed that 9 AMF were only detected in RP1, most of them belong to genus *Glomus*, such *Glomus lamellosum*, *Glomus globiferum*, *Glomus* sp. 9, *Glomus* sp. 10, *Glomus sp*. 11, *Glomus* sp. 12, *Glomus* sp. 13. Also only presented in RP2 field site were *Paraglomus* sp. 2 and *Entrophospora infrequens* (Fig. 5).

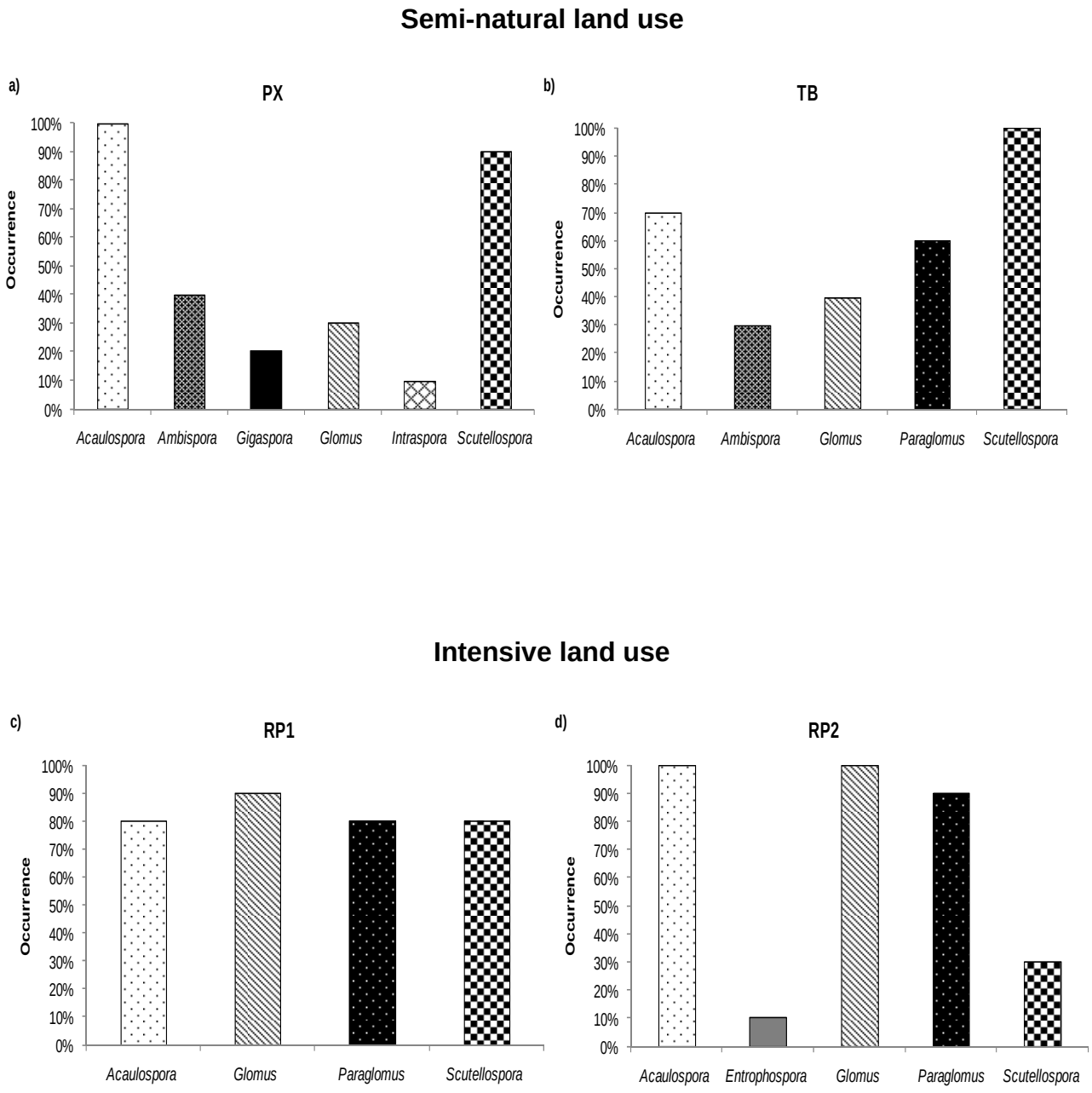

Figure 6. Frequency of occurrence of each AMF genus from each field site in semi-natural (a; b) and intensive (c; d) pastures.

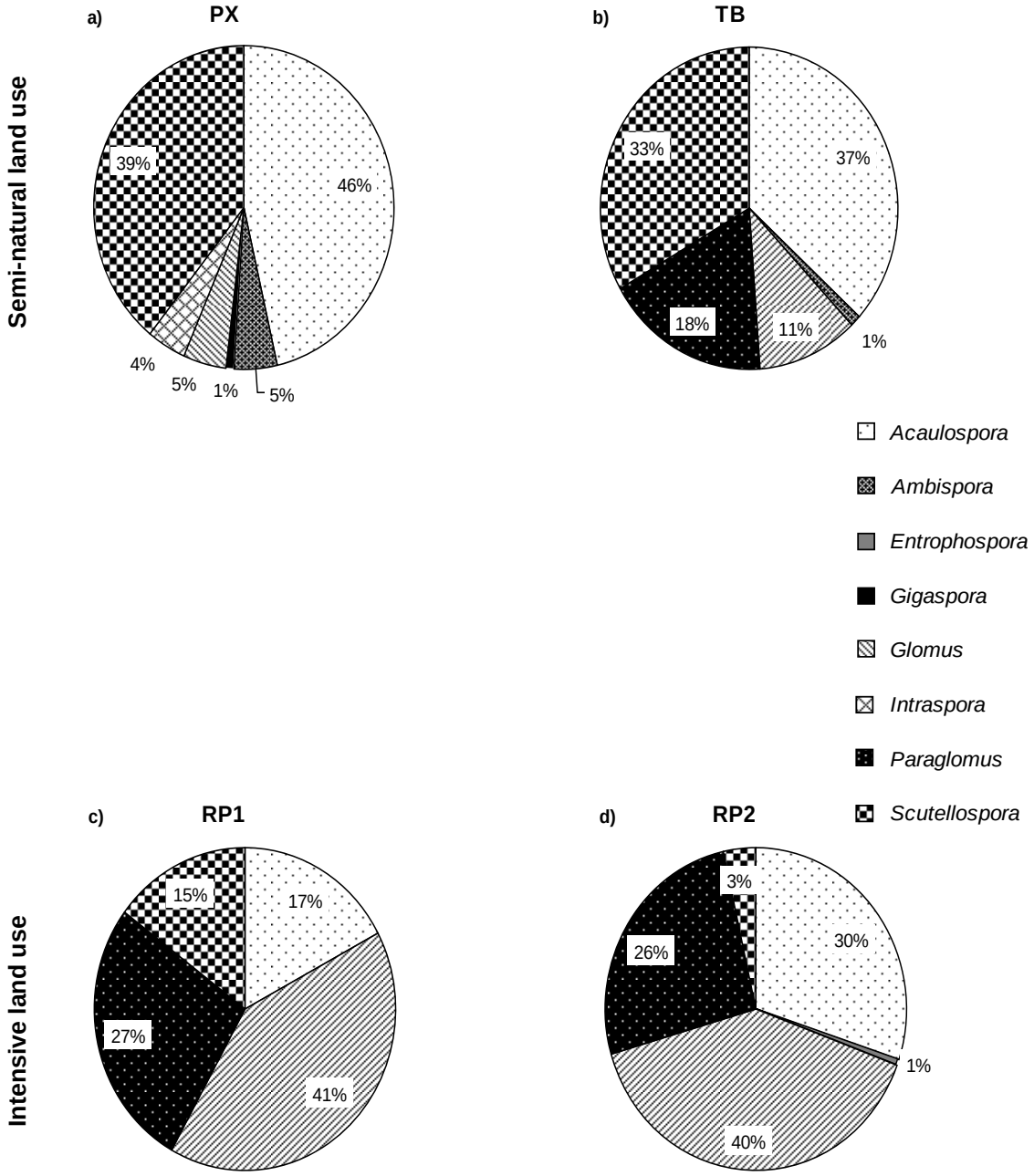

Figure 7. Contribution of each AMF genus to total AMF spores density of each field in semi-natural (a.PX; b.TB) and intensive (c.RP1; d.RP2).

3.4. Soil nutrient parameters

The correlation coefficient of soil nutrient parameters which influenced the total AMF spore density as well as the AMF spore densities belong to the genera: *Acaulospora*, *Glomus* and *Scutellospora* is present in the Table 3. The total AMF spore density was not significantly correlated with any soil nutrients. However, a different scenario was obtained when we analysed the AMF spore densities of the 3 more representative genera (*Acaulospora*, *Glomus* and *Scutellospora*).

Table 3. Correlation coefficients of Total AMF spore density and AMF spore densities belong to the genera: *Acaulospora*, *Glomus* and *Scutellospora* in semi-natural and intensives field sites and soil nutrient levels.

	Spore density			
	Total	***Acaulospora***	***Glomus***	***Scutellospora***
N	0.084	0.446**	-0.502**	0.415**
P	-0.12	0.058	-0.282	0.001
K	0.009	0.215	-0.404*	0.337*
Mg	0.087	0.406**	-0.494**	0.461**
Ca	0.086	0.383*	-0.474**	0.460**
OM	0.09	0.443**	-0.508**	0.444**

The AMF spore density belong to the *Acaulospora* genus was positively correlated with N (*r*= 0.446), Mg (*r*= 0.406) and OM (*r*= 0.443) at p< 0.01. Significant and positive correlations were also observed between *Acaulospora* spores density and Ca (*r*= 0.383, p< 0.05). Negative and significant correlations existed between *Glomus* spores density and all soil nutrients analyzed except with P, with which had also a negative but statistically insignificant correlation. Thus, *Glomus* spore density was negatively correlated with N (*r*= -0.502), Mg (*r*= -0.494), Ca (*r*= 0.474) and OM (*r*= -0.508) at p< 0.01 as well as with K (*r*= -0.404, p< 0.05). Contrary to *Glomus* spore density, AMF spore density belong to *Scutellospora* genus was positively correlated with all soil nutrients, except with P, with which expressed a positive but insignificant correlation. This way, *Scutellospora* spore density was positive and significantly correlated with N (*r*= 0.415), Mg (*r*= 0.461), Ca (*r*= 0.460) and OM (*r*= 0.444) at p< 0.01. *Scutellospora* spore density also had a positive and significant correlation with K (*r*= 0.337, p< 0.05).

3.5. AMF spore density

The AMF spore densities did not differ among the two land use in question (Nested Anova: F= 0.16; d.f= 1. 2; p= 0.696), neither between field sites within each land use (Nested Anova: F= 0.59; d.f= 2. 36; p= 0.558). Nevertheless, when we analysis the genus which had more weight to the spore density of each field site the result was interestingly different (Table. 4). The spore density of *Acaulospora* genus, *Glomus* genus and *Scutellospora* genus differed significantly among the various soil types and depended on land use. Then, the AMF spore density belong to *Acaulospora* genus varied significantly between management systems (Nested Anova: F= 6.70; d.f= 1. 2; p= 0.014) (Table 4). However, there were no significant differences among field sites within each land use (Nested Anova: F= 2.13; d.f= 2. 36; p= 0.134) (Table 4).

Table 4. F and p values from Nested ANOVA of dependent variables (AMF spore densities belong to the genera: *Acaulospora*, *Glomus* and *Scutellospora*) to significance between land use (semi-natural and intensive) and field site within each land use (PX, TB, RP1 and RP2). * P< 0.05; P< 0.01; P< 0.001; n.s= not significant.

Source	F	*p*
Genera		
a) *Acaulospora*		
Land use	6.70	*
Field site within land use	2.13	n.s
b) *Glomus*		
Land use	9.73	**
Field site within land use	1.53	n.s
c) *Scutellospora*		
Land use	9.06	**
Field site within land use	1.23	n.s

In fact, the relative abundance of *Acaulospora* genus was higher in the semi-natural land use than in intensive ones, having been the dominant genus in both semi-natural field sites, especially in PX with a relative abundance of 46% (Fig. 8). The same patterns of results was observed for the AMF spore density belong to the genus *Glomus*, which also varied significantly between the two managements systems (Nested Anova: F= 9.73; d.f= 1. 2; p= 0.004) (Table 4). However, we did not observe significant differences among the field sites within each land use (Nested Anova: F= 1.53; d.f= 2. 36; p= 0.231) (Table 4). *Glomus* was the most abundant genus in intensive land use, having contributed with more than 40 % to the AMF spore density of each intensive field sites (Fig. 8).

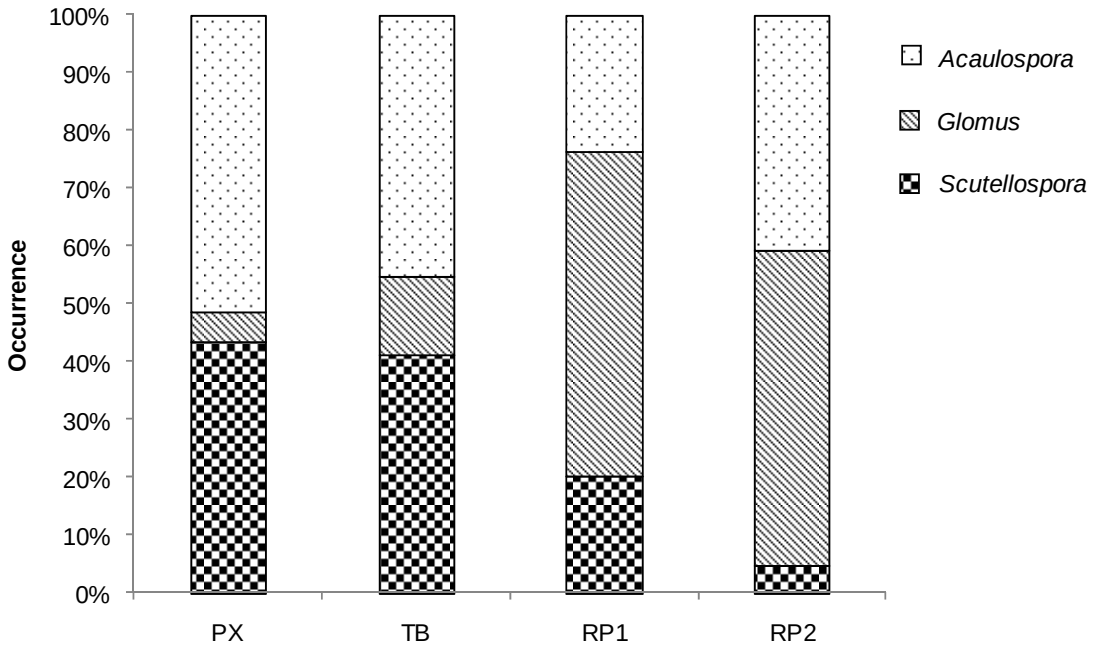

Figure 8. Contribution of *Acaulospora* genus, *Glomus* genus and *Scutellospora* genus to AMF spores density of each field site in semi-natural (PX; TB) and intensive (RP1; RP2) pastures.

Finally the AMF spore density belong to the *Scutellospora* genus, also was significantly influenced by the land use type (Nested Anova: F= 9.06; d.f= 2. 36; p= 0.005) (Table 4). But, there were no significant differences between field site within each land use (Nested Anova: F= 1.23; d.f= 2. 36; p= 0.303) (Table 4). Like *Acaulospora* genus, *Scutellospora* also was one of the most abundant genera in semi-natural land use, contributing with approximately 40% in the AMF spore density of each semi-natural field site (Fig. 8).

4. Discussion

AMF species diversity

Pasture management can affect the structure of AMF communities causing a decline in AMF diversity and producing a shift in the AMF community composition (Boddington and Dodd, 2000; Jansa *et al*., 2003). In the current study we found that based on spore morphology there was no differences in AMF diversity in semi-natural and intensives pastures. A similar result was obtained in relation to AMF spore density, which although it was slightly higher in semi-natural systems was not statistically

significant. Lack of consistent differences across the two managements practices investigated are opposite to previous reports of lower diversity and abundance of AMF spores in disturbed than in more pristine soils (Boddington and Dodd 2000; Oehl, *et al*., 2003; Gai *et al*., 2006). Among others, Eason *et al*. (1999) detected root colonisation and AMF spore density was significantly lower in conventional grassland than in organic ones. Oehl *et al*. (2004) noted that AMF spore abundance and species diversity was significantly higher in the organic than conventional systems. Also Castillo *et al*. (2006) observed that the number of AMF spore was affected by the tillage systems, have been higher under no-tillage than under no-tillage conditions.

Gai *et al*. (2009) found that the lowest species richness, spore density and Shannon index of AMF in the montane scrub could be explained by the intensity of land use, once these grasslands are severely disturbed as result of agriculture practices.

However our result was consistent with found by some authors. Among others, Jansa *et al*. (2002) found that spore abundance was not significantly affected by soil tillage. Also Sjoberg *et al*. (2004) did not find significant differences in spore densities between semi-natural grassland and ploughed fields. Schalamuk *et al*. (2006) found spores of at least 24 species belonging to 6 genera of AMF in non-tilled and tilled wheat soils in Argentina. In this study, tilling or fertilisation did not seem to affect the diversity of spores present, instead spore communities were affected by other factors particular wheat phenology.

More recently, Jefwa *et al*. (2009) found along a land use gradient, that land use type had not significant effect on AMF spore abundance.

AMF spore composition

However, striking differences were found in species composition between land uses. Species in *Glomus* were dominant in intensive land uses, indicating adaptability in adjusting patterns of sporulation to environmental conditions as evidenced by its global distribution (Gai *et al*., 2009). Prevalence of *Glomus* species in agricultural soil is widely reported (Jansa *et al*., 2002; Oehl *et al*., 2003; Mathimaran *et al*., 2005; Wang and Vestberg, 2008).

Oehl *et al*. (2003) found that some species that had a “generalist” behavior, most of them belong to *Glomus* genus, were prevalent in intensively managed systems. The same author pointed out not only a decreased in AMF species richness with increase of land use intensity, but also a preferential selection of species that colonised roots

slowly but formed spores rapidly. Also Jansa *et al*. (2003) found that in conventional tilled soils, almost exclusively AMF belonging to *Glomus* spp. were present, and that this could be explain by a stimulatory effect of tillage on hyphae growth and/or by the lower completion for root colonization cause by the absence of other AMF species. Lately, Castillo *et al*. (2006) in same field site conditions also noted that *Glomus* spp. constituted about 65 % of total spores in both cultivation systems. Mathimaran *et al*. (2005) only detected Glomus genus in Swiss agricultural soil.

Colonisation strategies of AMF

The dominance of *Glomus* genus in such disturbed systems could be related to differences in propagative units between the glomalean families (Jansa *et al*., 2003; Castillo *et al*., 2006). Members of Glomaceae have a highly infective extra-radical mycelium that could allow colonising immediately plant roots (Hart and Reader, 2002). Adding, *Glomus* forms anastomose between mycelia and possibility therefore has the ability to re-establish an interconnected network after mechanical disturbance (Daniell *et al*., 2001). Contrary members of Gigasporaceae, those are only capable of propagation via spore dispersal or infection from an intact mycelium would colonise plant roots more slowly than members of Glomaceae. Spore dormancy and specific environmental conditions for spore germination probably slow the rate at which regenerating AMF can colonise plant roots (Hart and Reader, 2002). Then, these differences can explain the dominance of the Glomaceae over members of the Gigasporaceae in an environment with repetitive severe physical disturbance, for example ploughing between crop cycles.

The most striking effect of different managements systems concerned the spore abundance of AMF species not belonging to the genus *Glomus*. Many of these species appeared to prefer or even to be restricted to the semi-natural field sites. This was the case for most species of *Acaulospora*, *Scutellospora* as well as *Entrophospora.* Jansa *et al*. (2002) argued that tillage had a significant influence on sporulation of some species and non-*Glomus* AMF tend to be more abundant in the no-tilled soil. Then they have reported, although not significant, a slight increased in the incidence of *Gigaspora*, *Scutellospora* and *Entrophospora* in the no-tilled soil. The absence or lower abundance of non-*Glomus* AMF under tillage condition might be attributed to the yearly disruption of the extraradical hyphae of these fungi induced by this management practice (Jansa *et al.*, 2003).

This trend towards an increase in AMF belong to genera *Acaulospora*, *Scutellospora* as well as *Entrophospora* under long-term reduced tillage managements was also observed by Oehl *et al*. (2004) in organic farming. These authors argue that some species, especially *Acaulospora*, *Scutellospora*, *Cetrapora* and *Gigaspora* have specific and rather narrow, inhabitating preferably organic farming systems, which could be probably related with lower nutrient level (especially available P) in these agroecosystems (Oehl *et al*., 2010).

Also Börstler *et al*. (2006) found in a study development in an extensively/ intensively farmed meadows that *Acaulospora* was only detected at the extensively ones.

However, despite *Acaulospora* has been not only the most abundant but also the more frequent genus in semi-natural land use, it is interesting to note that in intensive ones *Acaulospora* also was one of the most frequent genus and together with *Glomus* genus formed the highest proportion of spores in intensive field sites. A similar result was found by Castillo *et al.* (2006) who reported a tendency for a slightly higher relative presence of spore of *Acaulospora* spp. under conventional-tillage than under no-tillage sytems. Also, Jefwa *et al*. (2009) point out that AMF species spore abundance was a response to stress with Glomaceae and Acaulosporaceae responding to harsh conditions by producing spores, which making them to persist and dominate disturbed landscapes longer.

Soil nutrient availability

Nutrient availability affects the composition of AMF communities in the soil (Ezawa *et al.*, 2001; Titus and Leps, 2000). In fact, the density of 3 genera most representative *Acaulospora*, *Glomus* and *Scutellospora* was correlated with almost soil nutrients. Boddington and Dodd (2000) observed the development of indigenous AMF by adding organic matter. Khanam *et al*. (2006) also found in important available agricultural crops in Bangladesh a positive correlation between soil nutrients levels (N, K, OM) and spore numbers. Nevertheless, the opposite was observed in relation to total AMF spore density, which corroborated the generalisation that inorganic fertilisers always have a lowering effect on all glomale species (Jefwa *et al*., 2006).

Among soil nutrients phosphorus is widely known to inhibit the composition and diversity of AMF communities as well as spore and mycelium densities in temperate and tropical systems (Agwa and Al-Sodany, 2003; Jansa *et al*., 2005; Khanam *et al*., 2006). Kahiluoto *et al.* (2001) demonstrated reduced AMF colonisation of roots and

AMF spore density in soil with increasing P fertilisation for several crops on two soils with low and intermediate concentrations of available P.

However in our study no correlation statically significant was obtained between AMF spore density and this soil nutrient. A similar result was reported by Mathimaran *et al*. (2005) who not found an effect of P fertilisation on spore density in field site and in trap culture, either by Rodriguez-Echeverria *et al*. (2007) who also not detected a correlation between AMF abundance and soil fertility. It is interesting to note that the P level in the two land use under study was low and below to the level considered prejudicial to AMF, this may have been reflect in the way how other soil nutrients, particularly nitrogen, may affect AMF community. Under P-limited conditions, the investment of a host plant in AMF genus or species should be strengthened to maintain the uptake of-limiting nutrients (Johnson *et al*., 2003). So as predicted by the functional equilibrium model, in low-P soil, relative allocation to arbuscules, coils and extraradical hyphae is generally increased by N enrichment and vice-versa (Johnson *et al*., 2003). Consequently, we can observe an increase in AMF productivity, species richness and diversity with N fertilisation, being in these conditions the most responsive AMF taxa *Acaulospora*, *Scutellospora* and *Gigaspora*. The opposite occur in P-rich with N fertilisation application, observing an intensification of AMF community due to the loss of rare AMF species and the increase on abundance of *Glomus* species (Egerton-Warburton *et al*., 2007). Then, changes in AMF community may be explained by changes in the soil N:P ratio.

Our results were consistent with found by Johnson *et al*. (2003) and Egerton-Warburton *et al*. (2007). In fact, the spore densities of *Acaulospora* and *Scutellospora* were positively correlated with N soil nutrient, but the opposite occurred with spore density of *Glomus* genus, which was reflected in a greater abundance of genera *Acaulospora* and *Scutellospor*a in semi-natural land use, as well as, in higher abundance of *Glomus* genus in intensive ones.

We conclude that land use type had no significant effect on AMF spore diversity and total density. However clear differences were found in AMF spores composition between land uses. Species of *Glomus* were dominant in intensive pastures, while *Acaulospora* and *Scutellospora* predominated in semi-natural pastures. This could be explained through different strategies of colonisation adopted by AMF and by soil nutrient levels. Members of Glomaceae have a highly infective extra-radical mycelium

that could allow colonising immediately plant roots, while members of Gigasporaceae are only capable of propagation via spore dispersal, colonising plant roots more slowly than members of Glomaceae. Moreover, *Glomus* forms anastomose between mycelia therefore has the ability to re-establish an interconnected network after mechanical disturbance.

On the other hand, the spore densities of the three most representative genera – *Glomus*, *Acaulospora* and *Scutellospora* were correlated with soil nutrients.Thus, the improvement of soil fertility, especially the availability of K, N, Ca and Mg, provided favourable environment for mycorrhizal formation and function in these systems. However, a correlation between spore density and soil P content was not found. The low level of soil P content and its implication on absorption of the other soil nutrients, particularly N, could explained some change in the AMF spore composition.

CHAPTER III. SEASONAL DYNAMICS OF ARBUSCULAR MYCORRHIZAL FUNGAL COMMUNITIES IN ROOTS OF *HOLCUS LANATUS* L. IN SEMI-NATURAL AND INTENSIVE PASTURES IN TERCEIRA ISLAND (AZORES)

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate mutualistic symbionts that colonise more than 85% of terrestrial plants (Smith and Read, 1997; Smith *et al*., 1999; Ronsheim and Anderson, 2001; Wilkinson, 2001). In addition AMF are essential components of soil microbiota in most agroecosystems (Smith and Read, 1997; Boddington and Dodd, 2000). By forming an extended hyphal network, AMF can improve the growth of different plant species due to enhanced nutrient uptake, particularly immobile nutrients like phosphorus, because the hyphae can reach farther that the nutrient depleted zones that build up around plant roots (Smith and Read, 1997; Clark and Zeto, 2000). As symbionts of plants, AMF fungi can indirectly affect herbivores by reducing acceptability of the plant tissue, altering the nutritional quality, or increasing concentrations or types of defence compounds in the plant tissue, which can alter host resistance to these enemies (Gange and Weast, 1994; Master and Brown, 1997; Borowicz, 1997; Gange *et al*., 1999; Goverde *et al*., 2000; Wearn and Gange, 2007). AMF colonisation commonly improves tolerance of crops to other forms of biotic stress, such as nematodes (de la Peña *et al*., 2006; Rodriguez-Romero *et al.*, 2005, Rodriguez-Echeverria *et al*., 2009) and root pathogens (Smith *et al*., 1999; Klironomos *et al*., 2000; Idoia *et al*., 2004) as well as to a abiotic stresses, such as drought and metal toxicity (Gonzalez-Chavez *et al*., 2002). Furthermore, AMF contribute to soil aggregation because the hyphal network builds up of a macroporous structure that facilities penetration of water and air and prevents erosion (Rilling *et al*., 2003; Piotrowski *et al*., 2004; Cavagnaro *et al.*, 2006; Van Der Heijden *et al*., 2006). Another important role of AMF is their contribution as drivers of plant community composition (Van der Heijden *et al*., 1998; Wolfe *et al*., 2006).

AMF are therefore beneficial for plant performance, playing a crucial role for the sustainability of natural and agriculture ecosystems (Bareae *et al*., 2005). However, the distribution and colonisation by AMF are affected by various physico-chemical factors of soil such as pH, moisture and temperature (Abbott and Robson, 1981). Furthermore, agricultural practices like tillage, crop sequence and the composition and application of chemicals alter AMF populations, species composition and root colonisation (Eason *et al*., 1999; Boddington and Dodd, 2000; Jansan *et al*., 2002; Oehl *et al*., 2003; Purin *et al*., 2006). Tillage has been shown to have a significant influence on AMF colonisation (Douds and Miller, 1999; Jansa *et al*., 2003; Schalamuck *et al*., 2006; Garcia *et al*.,

2007). The AMF hyphal network is the main source of AMF inoculum in soils, and tillage causes severe disruption to the mycorrhizal network resulting in delayed or reduced root colonisation and a reduction in the volume of soil that is exploited by AMF (Evans and Miller, 1990; Boddington and Dodd, 2000). In the same way, hyphae and colonised root fragments are transported to the upper soil layer, decreasing and diluting their activity as viable propagules for subsequent crops. On the other hand, phosphorus (P) uptake by plants is generally increased with tillage, due partly to an improved distribution of P and increased P availability (Borie *et al*., 2006; Garcia *et al*., 2007). Castillo *et al*. (2006) reported that, under conditions of no tillage AMF root colonisation rates as well as the species richness and number of spores were higher than in tillage conditions. However, Jansa *et al*. (2002) found no significant effect of tillage on diversity indices of the AMF community, and Schalamuk *et al*. (2006) observed a higher number of AMF species in conventional tilled soils than in non-tilled soils cropped with wheat, suggesting that is not always detrimental to AM biodiversity.

Mycorrhizal benefits are usually reduced when soil nutrients are abundant. For instance, an increase in soil P availability negatively affects mycorrhizal colonisation of plant roots (Al-Karaki and Clark, 1999; Eason *et al*., 1999; Bhadalung *et al*., 2005; Li *et al*., 2006). Other edaphic factors such as an excess of nitrogen (N) (Blanke *et al*., 2005; Bradley *et al*., 2006; Porras-Alfaro *et al*., 2007) and extreme pH (Clark *et al*., 1999; Ezawa *et al*., 2001; Khanam *et al*., 2006) may also create conditions in which there is little or no benefit to the host plant from AMF. In addition to affecting plant performance, these factors also influence host plant dependency on AMF symbiosis and the level of root colonisation.

Grazing has also been found to dramatically reduce AMF colonisation and spores densities (Gange *et al*., 1993; Gehring *et al*., 1994; Lugo and Cabello, 2002; Dhillion and Gardsjord, 2004). This may be due to a decrease in leaf area and an increase in the root/shoot ratio that results in a decreased source capacity that is insufficient to satisfy root and AMF sink demands. Indeed, Dhillion and Gardsjord (2004) found a significantly higher level of AMF colonisation in ungrazed grasslands than in grazed ones.

Nevertheless, Eom *et al*. (2000) found that root colonisation by AMF was greater under moderate and intense grazing in a tallgrass prairie when compared to ungrazed sites. Frank *et al*. (2003) also observed that mycorrhizal communities differed markedly in grazed and fenced grassland. The spore abundance, species richness and diversity of

AMF were all higher in grazed soil compared to ungrazed soil as result of grazing stimulating a greater allocation of energy belowground increasing the investment in the mycorrhizal symbiosis. Similarly, Wearn and Gange (2007) also found that moderate grazing by rabbits had a rapid and persistent positive effect on the mycorrhizal colonisation of roots of three grasses studied in two grasslands.

In many terrestrial ecosystems, temporal changes in AMF colonisation show a seasonal pattern correlated with several factors such as plant phenology, with the highest rates of colonisation occurring during flowering and fruiting (Titus and Leps, 2000; Lugo and Cabello, 2002; Böhrer *et al*., 2004) when demand for phosphorus is high (Sanders and Fitter, 1992). Likewise, AMF colonisation is also influenced by the host metabolic pathway (Wilson and Hartnett, 1997; Lugo *et al*., 2003; Busso *et al*., 2008; Collins and Foster, 2009), because the growth responses of AMF fungal species depend on the particular host/fungus combination, i.e. whether the host species is a facultative (C_3) or an obligate (C_4) mycotroph. So, some symbiotic relationships are more favoured than others. Nutrient exchanges (Eom *et al*., 2000; Titus and Leps, 2000) and climatic variations (Kachi and Rorison, 1990; Heinemeyer and Fitter, 2004; Gavito *et al*., 2005) also have been reported to affect temporal changes in AMF colonisation.

The Azores archipelago has an extended area of grasslands (Martins, 1993), including natural grasslands, semi-natural pastures and intensive pastures (see Borges *et al.*, 2008). Through time, native forests have been destroyed by human activities and replaced by agricultural land. After several economic cycles and some catastrophic experiences with different types of monocultures (e.g. wheat *Triticum* spp.; "Pastel" *Isatis tinctoria* L. and; orange-tree *Citrus* spp.) (Martins, 1993) during the second half of the 20th century cattle milk production has grown in importance (Martins, 1993; Borges, 1997). In order to respond to the high density of cattle milk farms, pastures became the dominant landscape in the Azores archipelago, covering 67.7 % of the arable land, and there are also semi-natural pastures that comprise 11.2 % of the total area (Garcia and Furtado, 1991). Most semi-natural pasture are located at high altitude in recent infertile acidic volcanic soils (e.g. Terceira and Pico), or in more mature but still poor soils (e.g. S. Maria) (Borges, 1997). The floristic composition of pastures is dominated by introduced grasses and leguminous forbs, while native forbs (e.g. *Lotus uliginosus*), grasses, rushes, sedges and ferns are more frequent in the poorly managed, old, semi-natural pastures (Borges, 1997). Relatively little is known about the AMF community structure in these agroecosystems and their role in maintaining pasture productivity.

Whether there are differences in the AMF community between the semi-natural and intensively managed pastures is also unknown. Therefore, in order to better understand the basic biology of AMF and their role in these agroecosystems, it is necessary to analyse the AMF diversity and seasonal patterns of the AMF communities in these agroecosystems.

Several approaches have been developed to study AMF community structure. Traditional studies on AMF diversity are based mainly on spore morphology (Walker, 1992), but taxonomic identification of AMF spores collected directly from the field is quite difficult because they are often unidentifiable due to degradation or parasitisation by other organisms. Furthermore, as they are obligate symbionts, they cannot be cultivated in the absence of their host. Catch plants are often used to produce identifiable spores (Bever *et al*., 2001). Although useful for the isolation and propagation of some AMF species, this indirect culture strategy is time-consuming, and biases are often introduced by plant preferences for AMF species, different growth conditions, and other environmental factors, which hinder its suitability for the characterisation of AMF communities (Jansa *et al*., 2002; Oehl *et al*., 2003). Furthermore, mycorrhizal root colonisation results, as well as spore numbers (Clapp *et al*. 1995), do not necessarily reflect the AMF populations present in soils but only the sporulation stage of the AMF life cycle (a dormant phase) rather than the activity within a symbiosis. This results of fact, that not all AMF present as spores form active mycorrhizae and that many taxa present in roots may not be sporulating.

Recently molecular methods based on the amplification of DNA from soil or roots by polymerase chain reaction (PCR) in combination with denaturing gradient gel electrophoresis (DGGE) have proved to be useful tools for characterising of AMF community (Kowalchuk *et al*. 2002; Ma *et al*. 2005; Santos *et al*. 2006). Kowalchuk *et al*. 2002 reported discrepancies between the AMF-like groups detected in spore population versus direct 18S rDNA (small subunit of ribosomal DNA) analyses of root material by DGGE, corroborating previous suggestions that spore inspection alone may poorly represent actual AMF population structure. Similarly, Zhu *et al*. (2007) found that PCR-DGGE profiles clearly revealed the difference between the native AMF community structures in roots at two different pH levels (5.0 and 6.0). They detected more bands in the samples from pH 5.0 pots than from pH 6.0 pots (Fig. 1), indicating higher diversity of the native AMF at pH 5.0, i.e. the pH value similar to their original environmental pH.

In this molecular approach, PCR amplicons sharing the same length are separated electrophoretically in a sequence-dependent manner based on their GC-content (Rodriguez-Echeverria *et al*. 2009). The increasing gradient of denaturing components along the gel causes the double-stranded amplicons to denature into single stranded DNA through melting domains that decrease their mobility (and thus their position in the gel). A GC-clamp attached to the 5' end of one of the PCR primers prevents the amplicons from completely denaturing. Different sequences have different origins of melting domains and, consequently different positions in the gel where DNA fragments halt (Rodriguez-Echeverria *et al*. 2009). This strategy provides a robust means of detecting and identifying AMF-like species without the use of trap plant cultivation methods (Kowalchuk *et al*. 2002).

The main objective of this study was to determine the effects of land use and the sampling time on the community structure of arbuscular mycorrhizal fungi associated with *Holcus lanatus* L. (Poaceae) in two land use systems: semi-natural and intensive pastures.

2. Materials and methods

2.1. Experimental design

AMF community structure was studied during two years (2006, 2007) in two land uses: semi-natural and intensive pastures. Two sites were selected in each land use type: Pico do Galhardo (PX) and Terra Brava (TB) in the semi-natural pastures; Agualva 1 (RP1) and Agualva 2 (RP2) in the intensive pastures. In the first year of study, the study of AMF community structure was performed in two sampling dates (July and September), while in the second year of study was performed in August and November. Then, we have the fowling nested hierarchical design: 2 land uses x 2 sites x 2 sampling dates.

2.2. Site description

This study was conducted in the Terceira Island, the third island of the Azorean archipelago in terms of size (402 km^2). Terceira is a roundish island formed by four main volcanic complexes (Serra de Santa Bárbara, Serra do Morião, Pico Alto and Serra do Cume) (Zbyszewski *et al.*, 1971). The highest point (Serra de S. Bárbara, 1023 m) is simultaneously the most recent of the three major island's complexes of 0.025 Ma B.P. (Martins, 1993). The eastern flatted part of the island is its older part of about 3.52

My (Borges *et al*., 2009). Recent historical volcanic activity is evident in the western part of the island, where several well preserved lava tubes and volcanic chimneys are present (Borges *et al.*, 1993). The Azores archipelago have a temperate oceanic climate characterised by high levels of relative atmospheric humidity that could reach 95% at high altitude native forests and ensures slight thermal variations throughout the year (Azevedo *et al*., 1999). The average temperature is 17.5° C in low altitudes, while the maximum temperature is in August and minimum in February (Azevedo, 1996). The pluviometric regime reaches its peak in January-February and minimum and July (Azevedo, 1996): Angra do Heroísmo (47 m): 969 mm year (140 mm in January and 40 mm in July) and Serra de S. Bárbara (1,023 m): 3,000 mm year (Borges, 1997).

The sampling areas were cattle-grazed upland pastures of two different types: semi-natural pastures (managed for more than 50 years, with a higher diversity of grasses and forbs; see Borges and Brown 2001) and intensive pastures (managed for more than 20 years, characterised also by a poor vascular flora of five or less dominant species).The semi-natural pastures are included in a site of communitarian interest (SIC- Serra de Santa Barbara e Pico Alto) inside of Rede Natura 2000: Pico Galhardo (PX) (Latitude 38º 41' 51.71'' N; Longitude 27º 13' 25.34'' W) and Terra Brava (TB) (Latitude 38º 41' 59.74'' N; Longitude 27º 12' 41.37'' W) (Fig. 1).

These pastures show a high floristic diversity, and they are dominated by the grasses *Holcus lanatus* and *Agrostis castellana*, perennial grasses that covers around 100% of sites (Dias, 1996; Borges, 1997). Frequently present in these pastures are also other grasses like *Anthoxanthum odoratum*, *Lolium multflorum, Holcus rigidus* and *Poa trivialis* and spontaneous species without forage value as: *Lotus uliginosus*, *Rumex acetosella* ssp. *angiocarpus*, *Potentilla anglica*, *Hydrocotyle vulgaris*, *Plantago lanceolata*, *Lobelia urens, Cerastium fontanum, Conyza bonariensis, Anagallis arvensis, Hypochoeris radicata, Ranunculus repens, Pteridium aquilinum, Juncus effusus* (Dias, 1996; Borges, 1997).

The intensive pastures are included on exotic forest of *Eucalyptus* sp.. Agualva 1(RP1) is located at: Latitude 38º 45' 27.18'' N; Longitude 27º 11' 41.55'' W; and Agualva 2 (RP2) at: Latitude 38º 45' 24.44'' N; Longitude 27º 11' 42.24'' W (Fig. 1). In these pastures the dominant grasses are usually *Holcus lanatus* and *Lolium perenne* (plant cover of 100%). Another species with high cover is *Trifolium repens* (Dias, 1996; Borges, 1997). In these pastures, other common species are: *Plantago lanceolata*,

Cyperus esculentus, Mentha suaveolens, Cerastium fontanum, Rumex conglomeratus, etc. (Dias, 1996; Borges, 1997).

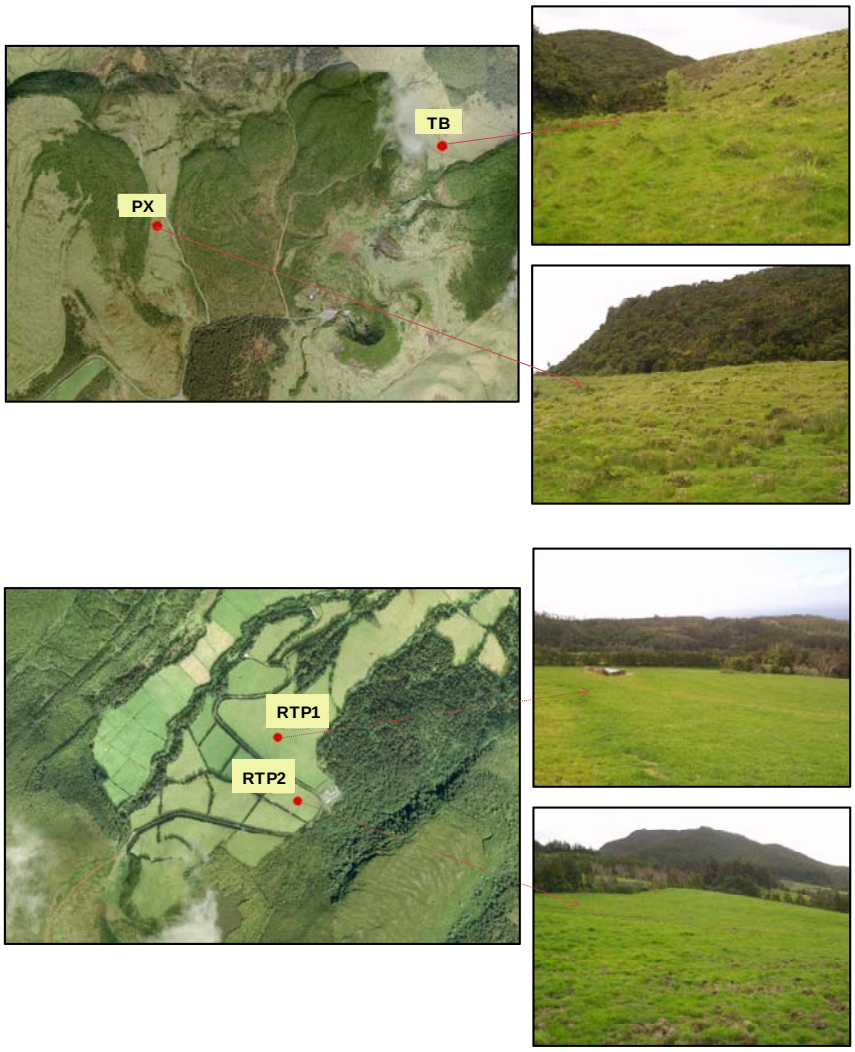

Figure 1. Location of the study sites at Terceira Island

2.3. Study plant

The Azorean pastures are dominated by *Holcus lanatus* L. a non-indigenous plant with a frequency more than 50% (Dias, 1996; Silva and Smith, 2006; Kueffer *et al*., 2010). In Terceira island in a total of 30 exotic vascular plants, *H. lanatus* L. was the third more frequent (Silva and Smith, 2006). This plant is a C_3 perennial, 20–110 cm tall, tufted grass (Ross *et al.*, 2003). It is very common, growing in all kinds of soil but the optimal growth occurs in moist conditions (Franco, 1984). *H. lanatus* grows well in very wet conditions and can survive moderate periods of drought (Franco, 1984).

2.4. Rhizosphere extraction

Samples were collected in July 2006, September 2006, August 2007 and November 2007. *Holcus lanatus* rhizospheric samples (including soil and root fragments) were collected at the depth of 20 cm. The distance between samples was approximately of 15 meters approximately.

2.5. Soil analyses

The soils of semi-natural and intensive pastures are andossols and sandy-loam (Pinheiro, 1990). The soil analyses were performed only with soil samples collected in August 2007. The 10 samples of rhizospheric soil from each site were pooled and analysed as a single sample. Chemical soil parameters for each soil are presented in Table 1. They were measured in the University of Azores Soil Laboratory (CITA-A), according to standard methods. Potassium, calcium and magnesium were extracted with sodium acetate (1/5) at pH 7, and were determined using a Varian ICP atomic emission spectrophotometer. Soil pH was determined on a soil/water paste (1/2.5) and available phosphorus as described by Olsen and Sommers (1982) by extraction with a 0.5 M $NaHCO_3$ solution at pH 8.5, using AAS. Total soil nitrogen content was estimated using the Kjehldahl method (Allen, 1989). The organic carbon (OC) and organic matter (OM) present in the soil were estimated using the dry ashing method.

Table 1. Chemical soil parameters at field sites differing in land use in August 2007. PX – Galhardo; TB – Terra Brava; RP1 – Agualva 1; RP2 – Agualva 2.

Land use	field site	pH	P (Olsen) (mg kg^{-1})	K (mg kg^{-1})	N (%)	Ca (mg kg^{-1})	Mg (mg kg^{-1})	OM (%)	OC (%)
Semi-natural									
	PX	5.7	7	143	0.55	93	84	24	13.92
	TB	5.7	11	190	0.40	89	73	21	12.18
Intensive									
	RP1	5.7	4	102	0.11	54	17	14	8.12
	RP2	5.7	10	80	0.26	45	13	16	9.28

2.6. AMF root colonisation assessment

The collected roots were stored in 50 % ethanol until staining. Roots were cleared in 2.5% KOH for 1h at 90 ˚C. Subsequently they were left to acidify overnight in 1% HCl. Staining was done with blue ink (Parker Quink) for 30 min at 60 ˚C, followed by distaining in lactoglycerol. The amount of colonisation was estimated using a grid-intersect method with examination of 100 intersects under a compound microscope at 200x magnification (McGonigle *et al*., 1990). Root-intersects that contained vesicles, arbuscules or hyphae were scored as mycorrhizal. The decision to score hyphae as mycorrhizal was based on the absence of septate hyphae, and to presence of vesicles, arbuscules and spores associated. In total, 80 samples were examined to score 100 intersections in each (McGonigle *et al*., 1990).

2.7. Diversity of AMF communities

2.7.1. Root sampling

In each sampling date (July-September/2006; August-November/2007) five out of ten samples of *Holcus. lanatus* roots collected from field were randomly selected. A total of 80 sampled roots were used for the molecular analysis. About 1g of fine roots per sample were stained with ink (see above) to access AMF colonisation and the remaining preserved in alcohol 48%, until to be use to DNA extraction.

2.7.2. DNA extraction from roots

DNA was extracted from 80 samples of *H. lanatus* roots using the REDExtract-N-Amp Plant PCR KIT (Sigma). Aliquots (1 g) of fresh roots sampled in each sample were firstly carefully cleaned with tap water and washed several times with sterile water to remove the alcohol, and then finely crushed, macerated and transferred into a 1,5-ml Eppendorf tube. After adding an equal volume of glass beads, the roots were macerated again, and stirred in a vortex for 4 min. Afterwards, 200 µl of extraction solution was added to the extraction mix and the roots were incubated at 60 ˚C for 30 min. Then a dilution solution was added (200 µl) to the roots, and the mix was stirred in a vortex for 30 sec. The supernatant was separated by root fragments by centrifugation (8000 r.p.m) and a 4 µl aliquot was used for PCR amplification of 28 S ribosomal subunit gene.

2.7.3. PCR and DGGE analysis

A nested-PCR was used to selectively amplify fungal DNA from the extracts. The DNA purified was used to the first PCR amplification of the 5' end of Large subunit of rDNA (LSU rDNA) sequences. We used the primers LR1 (Trouvelot *et al*. 1999) specific of eukaryotic and FLR2 (van Tuinen *et al*. 1998) specific of fungi. A 25 µl reaction mix contained 12.5 µl of Red Extract solution, 1.25 µl each primer and 5 µl of PCR-grade water. PCR was performed on a Gene Amp. PCR system 2700 Thermo Cycler (Applied Biosystems, Faster City, California) and the program was a followed: 93 ˚C for 1 min, 58 ˚C for 1 min and 72 ˚C for 10 min (35 cycles), followed by 10 min at 72 ˚C. The PCR products were used as templates for the second PCR with the primer FLR3-GC (FLR3 plus a GC at the 5' end) and FLR4 under the same PCR condition. FLR3 and FLR4 were described by Gollote *et al*.(2004) as specific for Glomeromycota fungi. The PCR products were run on 2 % (W/V) agarose gel in 1 X TAE buffer and visualised under UV light after staining with ethidium bromide. About 0.4 µg of DNA from each sample was used for DGGE analysis (Rodriguez-Echeverria *et al*. 2009). Gels contained 8% (w/v) polyacrylamide (37:1 acrylamide/bis-acrylamide) and 1 X TAE. The linear gradient used was from 27% to 43% denaturant, where 100% denaturing is defined as containing 7M urea and 40% (v/v) formamide (Muyzer & Smalla, 1998). Electrophoresis was run at 20V for 15 min, and afterwards at 200V for 330 min with a constant temperature of 60 ˚C in a DGGE-2401 system from CBS Scientific (CA, USA). Afterwards, gels were stained with ethidium bromide and visualized in a UV transilluminator. Digital images were captured using a Vilber Loumat system and analyzed with GelCompar II version 4.0 (Applie dMaths, Kortrijk, Belgium).

2.8. Detection of dominant AMF species (Development of molecular markers)

2.8.1. Establishment of trap culture

Trap culture were established from fresh soil samples collected from each field site, mixed with sterilised sand and volcanic soil ("bagacina") in a ratio of 2:1:1. A 1.5 Kg aliquot of the mixture was placed in each pot to produce culture from each sample. *Zea mays* L. was used as host plant. Seeds of *Zea mays* L. were surface sterilised by immersion in 96% alcohol for 30 sec, 0.4% sodium hypochlorite for 2 min and rinsed twice in sterile distilled water. They were then sown in pots on a sterilised sand soil and left to germinate in greenhouse. After 1 week of growth, seedlings were transplanted to

the pots. Trap cultures in 40 pots (10/field site) were grown in a glasshouse for 5 month and then harvested. All pots were watered every 2 days with distilled water, and no nutrient solution was added to the pots. After 4 month of sowing we left watering the pots to stimulate the sporulation.

2.8.2. Spore assessment

AMF spores produced in the trap culture were extracted by sieving and decanting (Gerdemann and Nicolson, 1963). The procedure included the passage of 50 cm^3 of harvested trap culture substrate from each pot through 1000-, 500-, 250-, 100-, and 45 µm sieves. The material retained in 250 µm, 100 µm and 45 µm was collected to different petri dish and AMF spores were counted under a stereoscopic microscope (Leica MZ 12).

2.8.3. Culture of AMF

Seeds of *H. lanatus* were surface sterilised using the same procedure applied to the *Z. mays* seeds (see above). Then, they were sown in boards on a sterilised sand soil and left to germinate in greenhouse. After 1 week of growth, seedlings were transplanted to pots containing 1.5 Kg of sterilised soil. Their roots were then carefully exposed and 250 spores and 1g colonised root pieces obtained from trapping or sieved materials were pipetted on to them. Sterilised sand was added to the surface of each pot to reduce the risk of cross-contamination. All pots were watered every 2 days and fertilized with Hoaglands-billeason nutrient solution monthly. Plants were harvested 5-6 months after sowing.

2.8.4. DNA extraction

DNA from 14 samples of *Holcus lanatus* roots collected from a trap culture was extracted using the REDExtract-N-Amp Plant PCR KIT (Sigma) following the protocol described above.

2.8.5. PCR amplification

The DNA isolated from the roots of *Holcus lanatus* collected from a trap culture was subject to a first PCR using the primer pair LR1/FLR2. A 20 µl reaction mix contained 10 µl of Red Extract solution, 1 µl each primer and 4 µl of PCR-grade water. PCR was performed on a Gene Amp. PCR system 2700 Thermo Cycler (Applied Biosystems, Faster City, California) and the program was a followed: 93 ˚C for 1 min, 58 ˚C for 1 min and 72 ˚C for 10 min (35 cycles), followed by 10 min at 72 ˚C. The PCR products were used as templates for the second PCR with the primer pair FLR3/FLR4 under the same PCR condition. The PCR products were run on 2 % agarose gel in 1 X TAE buffer and visualised under UV light after staining with ethidium bromide.

2.8.6. DNA purification and sequencing

The PCR products generated from roots of *Holcus lanatus* using the primers FLR3 and FLR4 were purified using GenElute PCR Clean-up Kit (Sigma) with a final elution volume of 30 µl, and then sequenced on MegaBace using the DYEnamic ET Dye Terminator Cycle Sequencing Kit following the manufacturer's instructions. Sequences were compared to known sequences using BLASTN.

Using the sequence information we designed using Citing Primer3 software the primer pair to specifically amplify five disjunctive groups of AMF (Table 2). The primers were synthesized from Sigma-Genosys Ltd

Table 2. Specificity and length of the expected fragment with primers developed for identification of AMF in plant roots.

Primer name	DNA sequence in 5' - end to 3' - end orientation	Targeted specificity	Product length (base pairs)
GetuR	ACG GTC TGG TGC ACT TTT GT	*Glomus etunicatum*	253
GetuL	CAG GTG GAA CAG CCC TAA AG		
GintR	ACA CCG TTC ATG TTT TGC AT	*Glomus intraradices*	339
GintL	ATC CGT TGC AAT CCT CAA TC		
GmosL	GGA TTG GGA TCT CTC GGT GT	*Glomus mosseae*	268
GmosR	CTT TCA ACA TCA AGG CAA CG		
GclaR	TGA CAA CTC CCG TCT GTG AC	*Glomus claroideum*	329
GclaL	TTC AAT CTT TTC CCC TCA CG		
GmicL	ATG ATC GGA GGA ATG TAC GC	*Glomus microaggregatum*	267
GmicR	TCA TGG ATG ACC GTA AGC AA		

2.8.7. Molecular identification of AMF in the roots

DNA extracted from the 80 *H. lanatus* samples roots collected from field sites, was first used in the same nested PCR protocol as previously described to confirm the AMF presence on roots. After that, five separated PCR reactions were performed with five different primer pairs (Table 2) on each DNA sample. Presence/absence of reaction product was scored after electrophoresis of a 5 µl aliquot on 2% agarose gel in 1 x buffer and visualised under UV light after staining with ethidium bromide.

2.9. Data analysis

The sampling design was nested hierarchical design, with sampling date nested within field sites and field sites nested within land use. Then, to investigate the variation on percentage of colonisation of different AMF structures between land uses, between field sites within each land use and between sampling dates within each sites we used Nested ANOVA (Minitab, version 13.31, 2000). AMF diversity was estimated via the number of bands with different positions in the acrylamide gel per sample and subsequently per field site and land use. Then, a Nested ANOVA was performed to estimate de variation on species richness between land uses, between field sites within each land use and between sampling dates within each field site. The same proceed was made to investigate the variation on occurrence of five AMF species (*Glomus etunicatum*, G*lomus intraradices*, *Glomus mosseae*, *Glomus claroideum* and G*lomus microaggregatum)* between land uses, between field sites within each land use and between sampling dates within each field site. All data were tested for normality, and data of percentage of colonisation of different AMF structures were arcsine-transformed when required to fulfil assumptions of the Nested ANOVA. Since only complete abiotic data were available for the year 2007, the correlation analyses between the abiotic factors and the proportion of colonisation of different AM structures and between occurrence of five AMF species (*G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. claroideum* and *G. microaggregatum*) were only performed for the 2007 data using the Pearson correlation coefficient.

3. Results

3.1. Root colonisation

Over this study the percentage of AMF colonisation was strongly influenced by the land use and the month/season in which the harvest took place within each local (Table 3). Thus, for the first year of study (2006), we found that the percentage of AMF colonisation varied significantly between land use (Nested Anova: F= 31.43; d.f= 1. 2; p< 0.001), with the sampling time (Nested Anova: F= 5.72; d.f= 4. 72; p< 0.001) as well as between field sites with each land use (Nested Anova: F= 4.43; d.f=2. 4; p= 0.01) (Table 3). The average percentage of AMF colonisation was higher in the first sampling date (July, 2006) than the second (September, 2006) in the semi-natural land use especially in TB field site (Fig. 2 a; 2 b). The same result was obtained to percentage of AM hyphal colonisation, which also varied significantly between land use (Nested Anova: F= 33.61; d.f= 1. 2; p< 0.001), with the sampling time (Nested Anova: F= 5.86; d.f= 4. 72; p< 0.001) as well as between field sites with each land use (Nested Anova: F= 4.53; d.f=2. 4; p= 0.01) (Table 3). The average percentage of AMF colonisation was higher in the first sampling date (July, 2006) than the second (September, 2006) in the semi-natural land use especially in TB field site (Fig. 2 a; 2 b).

The percentage of AMF vesicle colonisation varied significantly between land use (Nested Anova: F= 39.10; d.f= 1. 2; p< 0.001) and between field sites within each land uses (Nested Anova: F=5.07; d.f= 2. 4; p= 0.009) (Table 3). The average percentage of AMF vesicle colonisation was higher in the semi-natural than in the intensive land use particularly in PX field site (Fig. 2 a; 2 b).

Table 3. F - and p values from nested ANOVA of dependent variables (% AMF colonisation, % AMF hyphal colonisation, % AMF vesicle colonisation and % AMF arbuscule colonisation) to significance between land use (semi-natural and intensive), field site within land use (PX, TB, RP1 and RP2) and time sampling within field site (July/September; August/November 2007). * P< 0.05; * P< 0.01;*** P< 0.001; n.s= not significant.

Source	F	*p*
a) Year 2006		
Percent AMF colonisation		
Land use	31.43	***
Field site within land use	4.43	*
Time sampling within field site	5.72	***
Percent AMF hyphal colonisation		
Land use	33.61	***
Field site within land use	4.53	*
Time sampling within field site	5.86	***
Percent AMF vesicle colonisation		
Land use	39.10	***
Field site within land use	5.07	**
Time sampling within field site	0.79	n.s
Percent AMF arbuscule colonisation		
Land use	5.89	*
Field site within land use	7.93	**
Time sampling within field site	0.18	n.s
b) Year 2007		
Percent AMF colonisation		
Land use	151.18	***
Field site within land use	4.68	*
Time sampling within field site	4.97	**
Percent AMF hyphal colonisation		
Land use	95.67	***
Field site within land use	3.68	*
Time sampling within field site	5.46	**
Percent AMF vesicle colonisation		
Land use	26.53	***
Field site within land use	3.35	*
Time sampling within field site	4.29	**
Percent AMF arbuscule colonisation		
Land use	13.36	***
Field site within land use	2.94	n.s
Time sampling within field site	5.64	*

However, there were no significant differences between sampling dates in each field sites (Nested Anova: F= 0.79; d.f= 4. 72; p= 0.54).The same pattern of results was observed for the percentage of AMF arbuscule colonisation, which varied significantly between field sites within each land use (Nested Anova: F= 7.93; d.f= 2, 4; p= 0.001) and between land uses (Nested Anova: F= 5.89; d.f= 1. 2; p= 0.02) (Table 3). The average percentage of AMF arbuscule colonisation was higher in the semi-natural than in the intensive land use especially in TB field site (Fig. 2a, b). However, there were no significant differences between sampling dates in each field sites (Nested Anova: F= 0.18; d.f= 4. 72; p= 0.95).

For the second year of harvesting (2007) the AMF colonisation also varied significantly between land uses (Nested Anova: F= 151.18; d.f= 1. 2; p< 0.001), with the sampling date in each field site (Nested Anova: F= 4.97; d.f= 4. 72; p= 0.001) (Table 3), and between field sites with each land use (Nested Anova: F= 4.68; d.f= 2. 4; p= 0.01) (Table 3).

In fact, the average percentage of AMF colonisation in the first sampling date (August, 2007) was higher than in the second (November, 2007) in all field sites, and was also higher in sites of semi-natural pasture compared to the sites of intensive pasture (Fig. 2c; 2d). The same result was observed in the percentage of AMF hyphal colonisation, which varied significantly between land use (Nested Anova: F= 95.67; d.f= 1. 2; p< 0.001), with the harvest date in each field site (Nested Anova: F= 5.46; d.f= 4. 72; p= 0.001) and between field sites within each land use (Nested Anova: F= 3.68; d.f= 2. 4; p= 0.03) (Table 3). The average percentage of AMF hyphal colonisation in the semi-natural land use was higher than in intensive particularly in the summer harvest (August, 2007) in all field sites (Fig. 2c, 2d). The percentage of AMF vesicle varied significantly between land uses (Nested Anova: F= 26.53; d.f= 1. 2; p< 0.001), with the sampling time in each field site (Nested Anova: F= 4.29; d.f= 4. 72; p= 0.004) and between field sites within each land use (Nested Anova: F=3.35; d.f= 2. 4; p= 0.04) (Table 3).

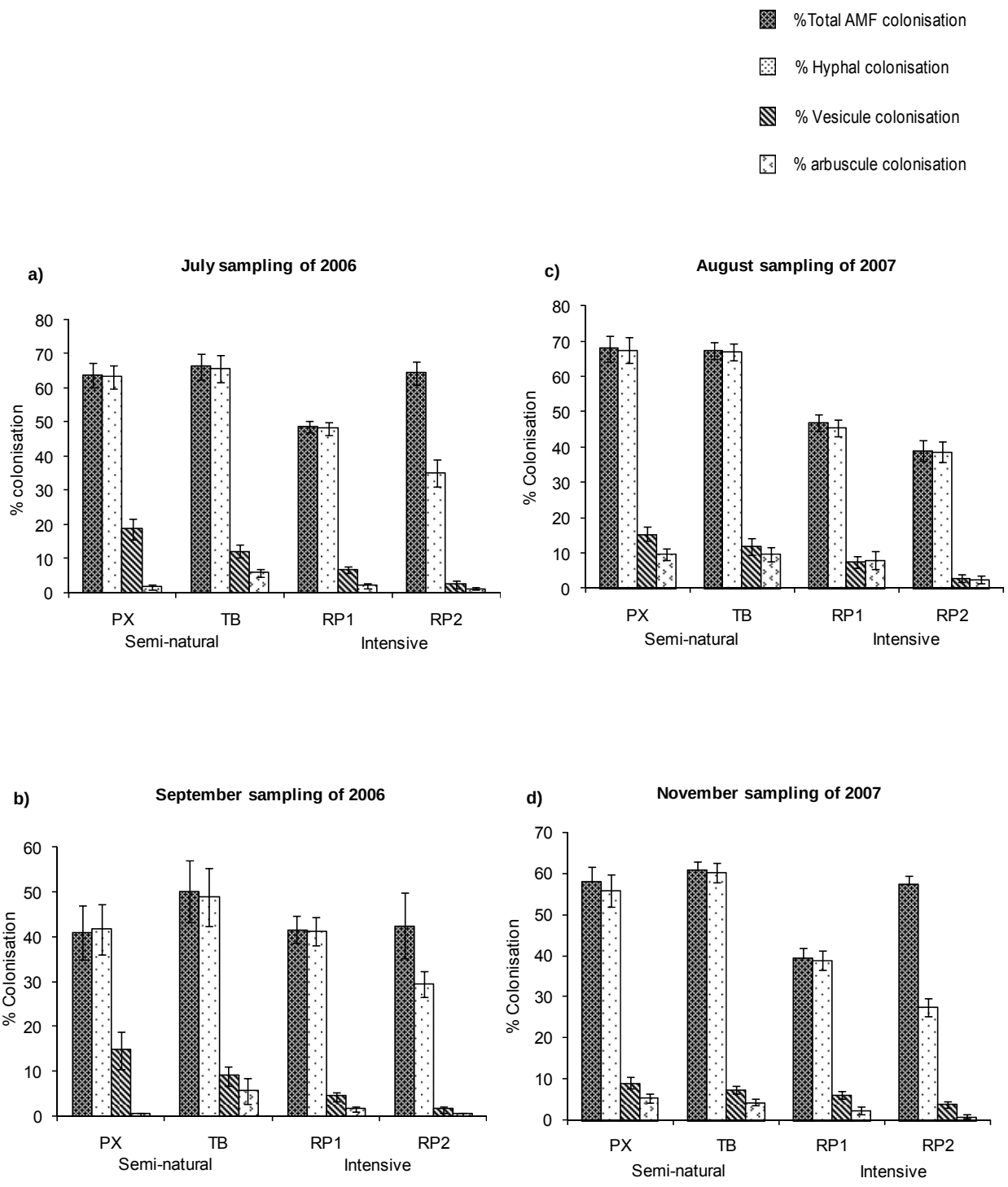

Figure 2. Average percent of total AMF, hyphae, vesicles and arbuscules colonisation during 4 sampling times (a, b, c, and d) from two different pasture management regimes (semi-natural and intensive). Standard errors of the mean (n= 10) are shown. Values of percent are arcsine-transformed. Nested ANOVA *p*-values are presented in Table 3.

The average percentage of AMF vesicle colonisation in the semi-natural land use was higher than in the intensive in the first harvest (August, 2007) in all field sites, especially in PX (Fig. 2 c). Finally, the percentage of AMF arbuscule colonisation also varied significantly between land uses (Nested Anova: F= 13.36; d.f= 1. 2; p< 0.001) and with the time of sampling within each field site (Nested Anova: F= 5.64; d.f= 4. 72; p= 0.001) (Table 3).

The average percentage of AMF arbuscule colonisation in the semi-natural land use was higher than in the intensive especially in the first harvest (August, 2007). However, there were no significant differences between field sites within each land use (Nested Anova: F= 2.94; d.f= 2. 4; p= 0.06) (Table 3).

3.2. Species richness

A total of 80 root collected in 2006/2007 was subjected to DNA extraction. PCR products of the expected size were obtained from a total of 66 samples (82.5% of roots). The 80 root samples produced a total of 214 bands of AMF, 58% of them in 2007.

Table 4. F - and p values from nested ANOVA of Species Richness to significance between land use (semi-natural and intensive), field site within land use (PX, TB, RP1 and RP2) and time sampling within field site (July/September; August/November 2007) * P< 0,05; * P< 0,01;*** P< 0,001; n.s= not significant.

Source	F	*p*
a) Year 2006		
Percent AMF colonisation		
Land use	1.51	n.s
Field site within land use	7.47	**
Time sampling within field site	5.00	**
b) Year 2007		
Percent AMF colonisation		
Land use	0.36	n.s
Field site within land use	10.10	***
Time sampling within field site	4.31	**

Over the two years of study the species richness exhibited the same pattern of result. Thus, in the first year (2006) the species number varied significantly between field sites within each land use (Nested Anova: F= 7.47; d.f= 2. 4; p= 0.002) and with the sampling time in each field site (Nested Anova: F= 4.30; d.f= 4. 29; p= 0.003) (Table 4). The average of species richness was higher in PX and RP1, especially in the second sampling time (September). Indeed, bands of AMF were detected in 100 % of September samples, but only in 35 % of the samples taken in July.

Although the number of detected AMF bands having been higher in semi-natural (51 bands) than in intensive (42 bands), we did not found significant differences between land uses (Nested Anova: F= 1.51; d.f= 1. 2; p= 0.23) (Table 4). However, the number of bands/sample was higher in the semi-natural land use than in the intensive (Fig. 3 a).

In the second year (2007) the species number also varied significantly between field sites within each land use (Nested Anova: F= 10.10; d.f= 2. 4; p< 0.001) and with the sampling time in each field site (Nested Anova: F= 4.31; d.f= 4. 32; p= 0.007) (Table 4). The average of species richness was higher in TB and in RP1, especially in the first sampling time (August). Indeed, in August we detected 83 bands of AMF against 47 detected in November, i.e. about the double of the bands were detected in summer.

Again, there were no significant differences between land uses (Nested Anova: F= 0.36; d.f= 1. 2; p= 0.55) (Table 4). In fact the number of bands per land use was very similar, a total of 69 and 61 bands of AMF were detected in semi-natural and intensive land uses respectively. Nevertheless, the number of bands/sample was in the semi-natural land use than in intensive Fig. 3 b). Thus, seems that in the intensive land use the plants are colonised by dominant AMF species.

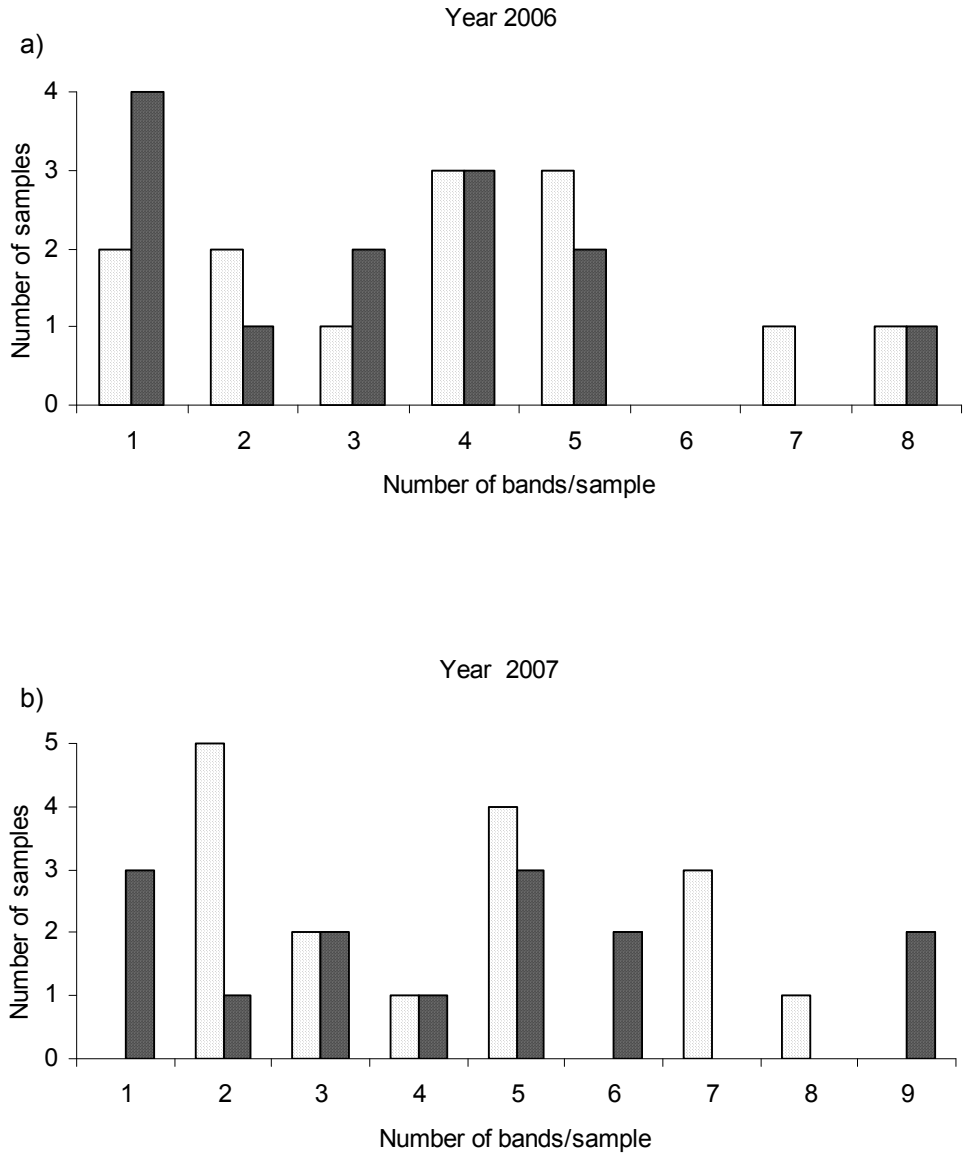

Figure 3. Number of bands/sample during two sampling times of 2006 (a) and two sampling time of 2007 (b) from two different pasture management regimes (semi-natural and intensive).

3.3. Incidence of *Glomus etunicatum*, *Glomus. intraradices*, *Glomus mosseae*, *Glomus claroideum* and *Glomus microaggregatum.*

In the first year of study, the presence of the 5 AMF species analysed (Fig. 4 a; 4 b) did not vary significantly between field sites within each habitat (Nested Anova: F= 2.83; d.f= 2, 4; p= 0.07) (Table 5), or with sampling time in each field site (Nested Anova: F=

1.07; d.f= 4, 32; p= 0.39) (Table 5), as well as between land uses (Nested Anova: F= 0.20; d.f= 1. 2; p= 0.66) (Table 5).

However, when we separately analysed the presence of each species there were significant differences between sampling dates. In the first year of our study the presence of *G. etunicatum* varied significantly with the harvest date within each field site (Nested Anova: F= 2.67; d.f= 4. 32; p= 0.05) (Table 5). The occurrence of *G. etunicatum* in the first sampling date (July, 2006) was higher than the in second (September, 2006) in all field sites (Fig 5 a). *G. etunicatum* was one of the dominant species, present in 100% of all samples in July, and only in 70% in September. However, there were no significant differences between land uses (Nested Anova: F= 0.89; d.f=1. 2; p= 0.35) (Table 5) or between field sites within each land use (Nested Anova: F= 0.89; d.f= 2. 4; p= 0.42). The occurrence of *G. intraradices* varied significantly with the sampling time in each field site (Nested Anova: F= 4.50; d.f= 4. 32; p= 0.005), between land uses (Nested Anova: F= 4.17; d.f=1. 2; p= 0.05) as between field sites within each land use (Nested Anova: F= 4.17; d.f= 2. 4; p= 0.02) (Table 5). In fact, this species was mostly found in the second month of sample collection particularly in the intensive land use (Fig. 5 b).

The presence of *G. intraradices* in all the samples increased from 15% in July to 60% in September.

Moreover, the occurrence of *G. mosseae* (Fig. 5 c) did not vary with the sampling date within each field site (Nested Anova: F= 1.00; d.f= 4. 32; p= 0.42) (Table 5), or between field sites within each land use (Nested Anova: F= 1.00; d.f= 2. 4; p= 0.38) (Table 5), as well as between land uses (Nested Anova: F= 0; d.f= 1. 2; p= 1.00) (Table 5). Like *G. etunicatum*, this species was one of the dominants, being present in the same frequency of occurrence (95%) in two sampling dates. *G. claroideum* (Fig. 5 d) presence varied significantly between land uses (Nested Anova: F= 5.33; d.f=1. 2; p= 0.03) and with the date of harvest within each site (Nested Anova: F= 2.67; d.f= 4. 32; p= 0.05) (Table 5).

Table 5. F - and p values from nested ANOVA of dependent variables (Total species occurrence, occurrence of *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. claroideum* and *G. microaggregatum*) to significance between land use (semi-natural and intensive), field site within land use (PX, TB, RP1 and RP2) and time sampling within field site (July/September 2006). * P < 0,05; ** P < 0,01; *** P < 0,001; n.s= not significant.

Source	F	p
Year 2006		
Total species occurrence		
Land use	0.20	n.s
Field site within land use	2.83	n.s
Time sampling within field site	1.07	n.s
Occurrence *of G. etunicatum*		
Land use	0.89	n.s
Field site within land use	0.89	n.s
Time sampling within field site	2.67	*
Occurrence of *G. intraradices*		
Land use	4.17	*
Field site within land use	4.17	*
Time sampling within field site	4.50	**
Occurrence of *G. mosseae*		
Land use	0.00	n.s
Field site within land use	1.00	n.s
Time sampling within field site	1.00	n.s
Occurrence of *G. claroideum*		
Land use	5.33	*
Field site within land use	0,00	n.s
Time sampling within field site	2.67	*
Occurrence of *G. microaggregatum*		
Land use	0.25	n.s
Field site within land use	1.25	n.s
Time sampling within field site	2.25	n.s

In fact, this species only occurred in semi-natural land use in the first year of study, and its frequency of occurrence was higher in the second sampling date (10% in September) than in the first (5 % in July). The presence of *G. microaggregatum* (Fig. 5 e) did not vary significantly with the sampling date in each field site (Nested Anova: F= 2.25; d.f= 4. 32; p= 0.09) (Table 5) or between field sites within each land use (Nested Anova: F= 1.25; d.f= 2. 4; p= 0.30) (Table 5), as well as between land uses (Nested Anova: F= 0.25; d.f=1. 2; p= 0.62) (Table 5).

In the second year of study (2007) the seasonal effect was more evident. In this year the total occurrence of the five AMF species analysed varied significantly with the

harvesting date in each field site (Nested Anova: F= 11.65; d.f= 4. 32; p< 0.001) and between field sites within each habitat (Nested Anova: F= 7.85; d.f=2. 4; p= 0.002) (Table 6). The total species occurrence in first month of sampling (August, 2007) was higher than in the second in all field sites, especially in the semi-natural field sites (Fig. 4 c; 4 d) However, there were no significant differences in total species occurrence between land uses (Nested Anova: F= 2.45; d.f= 1. 2; p= 0.13) (Table 6).

Table 6. F - and p values from nested ANOVA of dependent variables (Total species occurrence, Presence of *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. claroideum* and *G. microaggregatum*) to significance between land use (semi-natural and intensive), field site within land use (PX, TB, RP1 and RP2) and time sampling within field site (August/November 2007). * P < 0,05; ** P < 0,01; *** P < 0,001; n.s= not significant.

Source	F	*p*
Year 2007		
a) Total species occurrence		
Land use	2.45	n.s
Field site within land use	7.85	**
Time sampling within field site	11.65	***
b) Occurrence *of G. etunicatum*		
Land use	0.67	n.s
Field site within land use	2.67	n.s
Time sampling within field site	3.00	*
c) Occurrence of *G. intraradices*		
Land use	0,00	n.s
Field site within land use	2.86	n.s
Time sampling within field site	2.57	*
d) Occurrence of *G. mosseae*		
Land use	0.13	n.s
Field site within land use	0.67	n.s
Time sampling within field site	0.93	n.s
e) Occurrence of *G. claroideum*		
Land use	0.33	n.s
Field site within land use	0.33	n.s
Time sampling within field site	1.00	n.s
f) Occurrence of *G. microaggregatum*		
Land use	32.67	***
Field site within land use	6.00	**
Time sampling within field site	19.33	***

Regarding the presence of our 5 species, we verified that the occurrence of *G. etunicatum* varied significantly with the sampling date in each field site (Nested Anova: F= 3,00; d.f= 4. 32; p< 0.03) (Table 6). The occurrence of this species in the first date of harvest was higher than in the second in TB and RP2, but the opposite was found in sites PX and RP1 (Fig. 6 a). *G. etunicatum* remained the dominant species, with a frequency of occurrence of 80% in August and 70% in November.

However, no significant differences were found in the presence of *G. etunicatum* between field sites within each land use (Nested Anova: F= 2.67; d.f= 2. 4; p= 0.09) (Table 6), or between land uses (Nested Anova: F= 0.67; d.f= 1. 2; p= 0.42) (Table 6).

A similar result was found to *G. intraradices*; the presence of this species varied significantly with the sampling date in field each site (Nested Anova: F= 2.57; d.f= 4. 32; p< 0.05) (Table 6). The occurrence of this species in the first date of harvest was higher than the second in PX and RP1, but it was found the opposite in sites TB and RP2 (Fig. 6 b). However, no significant differences were detected in the presence of *G. intraradices* between field sites within each land use (Nested Anova: F= 2.86; d.f= 2. 4; p= 0.07) or between land uses (Nested Anova: F= 0; d.f= 1, 2; p= 1.00) (Table 6). The presence of *G. mosseae* (Fig. 6 c) did not vary significantly with the sampling date within each field site (Nested Anova: F= 0.93; d.f= 4. 32; p= 0.46) or between field sites within each land use (Nested Anova: F= 0.67; d.f= 2. 4; p= 0.52) as well as between land uses (Nested Anova: F= 0.13; d.f= 1. 2; p= 0.72) (Table 6). As in the first year of study, *G. mosseae* continued to be one of the dominant species, and showed little changes in its occurrence between the two harvesting dates (80% in August and 70% in November).

A similar result was found for *G. claroideum* (Fig. 6 d), which did not vary significantly with the harvest date in each field site (Nested Anova: F= 1.00; d.f= 4. 32; p= 0.42) or between field sites within each land use (Nested Anova: F= 0.33; d.f= 2. 4; p= 0.72) as well as between land uses (Nested Anova: F= 0.33; d.f= 1. 2; p= 0.57) (Table 6). Finally, the presence of *G. microaggregatum* varied significantly between land uses (Nested Anova: F=32.67; d.f= 1. 2; p<0.001) with the sampling date within each field site (Nested Anova: F= 19.33; d.f= 4. 32; p< 0.001) and between field sites within each land use (Nested Anova: F=6.00; d.f= 2. 4; p= 0.006) (Table 6). This species was only present in the first harvest date in semi-natural field sites, showing a frequency of occurrence of 35 % in PX and TB (Fig. 6 e).

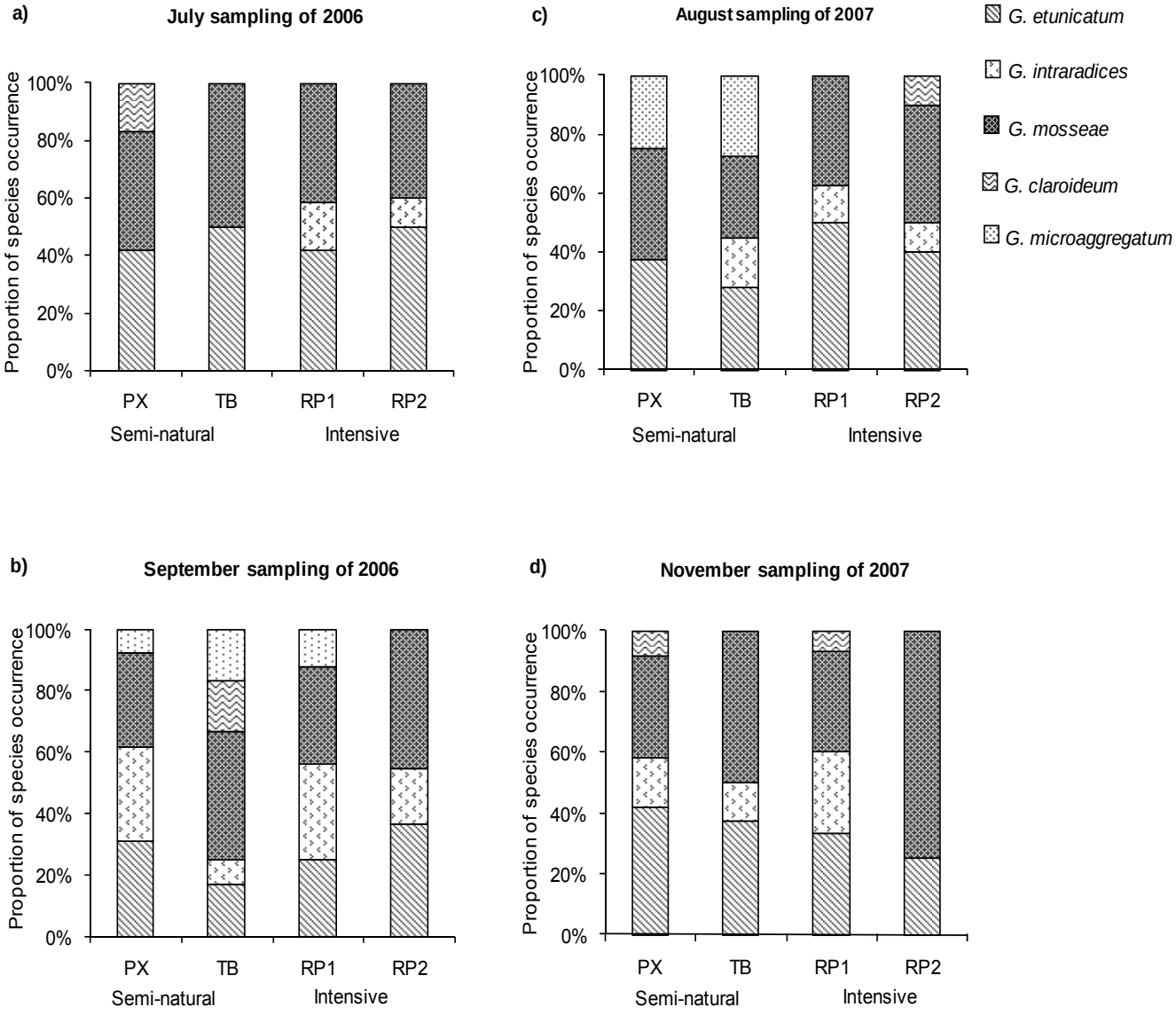

Figure 4. Proportion of occurrence of *G. etunicatum*, *G. intraradices, G. mosseae, G. claroideum* and *G. microagreggatum* present in each local (*n=5*): Pico Galhardo (PX), Terra Brava (TB), RP1 Agualva (RP1) and RP2 Agualva (RP2) during 4 sampling times (a, b, c, and d) from two different pastures management regimes (semi-natural and

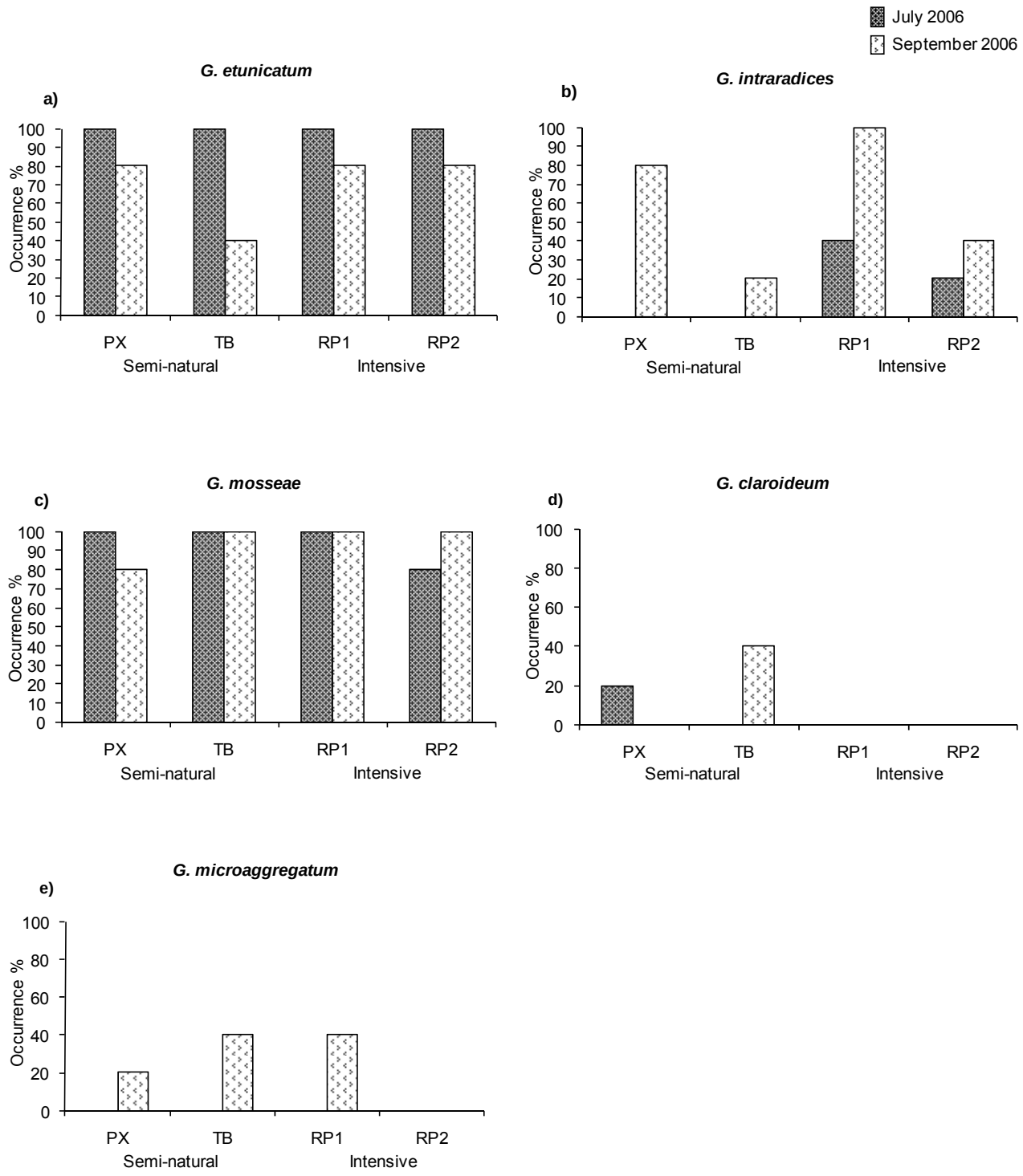

Figure 5. Frequency of occurrence of *G. etunicatum* (a), *G. intraradices* (b), *G. mosseae* (c), *G. claroideum* (d) and *G. microagreggatum* (e) present in each local (*n*= *5*) Pico Galhardo (PX), Terra Brava (TB), RP1 Agualva (RP1) and RP2 Agualva (RP2) during 2 sampling times (July and September 2006) from two different pastures management regimes (semi-natural and intensive).

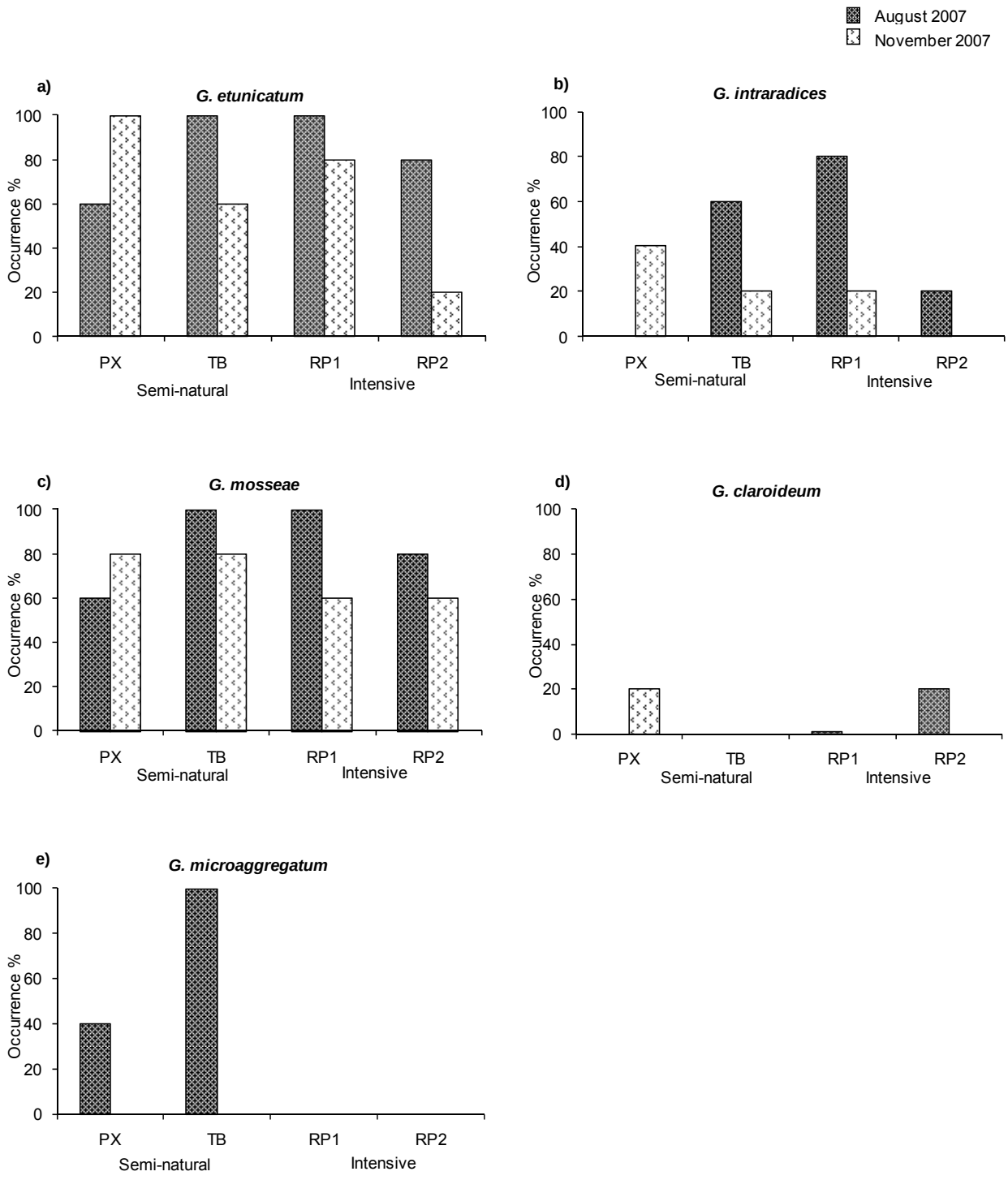

Figure 6. Frequency of occurrence of *G. etunicatum* (a), *G. intraradices* (b), *G. mosseae* (c), *G. claroideum* (d) and *G. microagreggatum* (e) present in each local (*n*= *5*) Pico Galhardo (PX), Terra Brava (TB), RP1 Agualva (RP1) and RP2 Agualva (RP2) during 2 sampling times (July and September 2007) from two different pastures management regimes (semi-natural and intensive).

3.4. Soil nutrient parameters

The correlation coefficient of soil nutrient parameters which influenced the percentage of AMF colonisation during the first sampling date of 2007 (August) is present in Table 7. The percentage of AMF colonisation was significantly correlated with all soil nutrients except phosphorus. The percentage of AMF colonisation was significantly positive correlated with K (*r*= 0.793), N (*r*= 0.624), OM (*r*= 0.708), Ca (*r*= 0.836), Mg (*r*= 0.805) and OC (*r*= 0.699) at p< 0.001. A similar result was obtained with the percentage of hypal colonisation, which was significantly correlated with K (*r*= 0.713, p< 0.001), N (*r*= 0.449, p< 0.01), OM (*r*= 0.532, p< 0.001), Ca (*r*= 0.698, p< 0.001), Mg (*r*= 0.805, p< 0.001) and OC (*r*= 0.711, p< 0.001). The percentage of vesicle colonisation also exhibited significant correlation with K (*r*= 0.344, p< 0.05), OM (*r*= 0.307, p< 0.05), Ca (*r*= 0.380, p< 0.05), Mg (*r*= 0.366, p< 0.05) and OC (*r*= 0.314, p< 0.05). At the same time, percentage of arbuscule colonisation was significantly positive correlated with K (*r*= 0.468, p< 0.01), N (*r*= 0.385, p< 0.05), OM (*r*= 0.438, p< 0.01), Ca (*r*= 0.509, p< 0.01), Mg (*r*= 0.496, p< 0.01) and OC (*r*= 0.442, p< 0.01).

Table 7. Correlation coefficient of percentage of different AMF structures and soil nutrient levels

	Microbial				Soil nutrients					
	% AMCol	% HypCol	% VesCol	% ArbCol	P	K	N	OM	Ca	Mg
P	0.143	0.294	-0.059	0.041						
K	0.793***	0.713***	0.344*	0.468**	0.389*					
N	0.624***	0.449**	0.257	0.385*	0.408**	0.610***				
OM	0.708***	0.532***	0.307*	0.438**	0.346*	0.712***	0.986***			
Ca	0.836***	0.698***	0.380*	0.509**	0.278	0.942***	0.805***	0.889***		
Mg	0.805***	0.640***	0.366*	0.496**	0.265	0.852***	0.906***	0.963***	0.976***	
OC	0.711***	0.531***	0.314*	0.442**	0.306*	0.704***	0.982***	0.999***	0.888***	0.965***

*. Correlation is significant at the 0,05 level (2 tailed) **. Correlation is significant at the 0,01 level (2 tailed). Correlation is significant at the 0,001 level (2 tailed) ***.

% AMCol- Percentage of AMF colonisation; % HypCol- percentage of Hyphal colonisation; % VesCol- Percentage of Vesicle colonisation; % ArbCol- Percentage of Arbuscule colonisation; P- Phosphorus; K- Potassium; N- Nitrogen; OM- Organic matter; Ca- Calcium; Mg- Magnesium; OC- Organic carbon.

The phosphorus content of soil expressed significant positive correlation with K (*r*= 0.389, p< 0.05), N (*r*= 0.408, p< 0.01) OM (*r*= 0.346, p< 0.05) and OC (*r*= 0.439, p< 0.01). The potassium content also showed positive and significant correlation with N (*r*= 0.610), M.O (*r*= 0.712), Ca, Mg (*r*= 0.906) as well as with OC (*r*= 0.733) all at p< 0.001. At the same time nitrogen content was significant correlated with OM (*r*= 0.986, p< 0,001), Ca (*r*= 0.805, p< 0.001), Mg (*r*= 0.906, p< 0.001) and OC (*r*= 0.986, p< 0.001). Soil organic matter was significant correlated with Ca (*r*= 0.889, p< 0.001), Mg (*r*= 0.963, p< 0.001), and OC (*r*= 0.995, p< 0.001). The calcium content also had a positive and significant correlation with Mg (*r*= 0.976) and OC (*r*= 0.887) at p< 0.001. Finally, the magnesium content of soil was significantly correlated with OC (*r*= 0.906, p< 0.001).

For the same sampling period, we also analysed the correlation between soil nutrients level and the total species occurrence of *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. claroideum* and *G. microaggregatum* present in each site within each land use (Table 6). The total species occurrence was significantly correlated with N (*r*= 0.444); M.O (*r*= 0.505), Ca (*r*= 0.523), Mg (*r*= 0.554) and C.O (*r*= 0.456) at p< 0.05. However when we analysed each species separately, only the presence of two species seemed to be affected by soil nutrients, especially phosphorus (Table 8). This way, the occurrence of *G. etunicatum* was significantly and negative related with P (*r*= -0.558, p< 0.05), and also the occurrence of *G. intraradices* had a negative and significant correlation with P (*r*= -0.574, p< 0.01).

The phosphorus content of soil expressed significant positive correlation with OC (*r*= 0.439, p< 0.05). The potassium content was positively correlated with N (*r*= 0.610, p< 0.01), OM (*r*= 0.712, p< 0.001), Ca (*r*= 0.942, p< 0.001), Mg (*r*= 0.852, p< 0.001) as well as with OC (*r*= 0.986, p< 0.001). At the same time N level was significantly positive correlated with OM (*r*= 0.986), Ca (*r*= 0.805) Mg (*r*= 0.906) and OC (*r*= 0.955) at p< 0.001. The organic matter of soil had positive and significant correlation with Ca (*r*= 0.889, p< 0.001), Mg (*r*= 0.963, p< 0.001) and OC (*r*= 0.995, p< 0.001). The calcium content was significantly positive correlated with Mg (*r*= 0.976, p< 0.001) and with OC (*r*= 0.887, p< 0.001). Finally Mg content was significantly positive correlated with OC (*r*= 0.954, p< 0.001).

Table 8. Correlation coefficient of total species occurrence and occurrence of five AMF species with soil nutrient levels

	Microbial					Soil nutrients					
	Total number of species	Presence of species									
	AMF	Getunic	Gintrar	Gmoss	Gclaroid	P	K	N	OM	Ca	Mg
P	0.243	-0.558*	-0.574**	-0.274	-0.304						
K	0.375	0.188	-0.043	0.066	-0.05	0.389*					
N	0.444*	0.094	-0.238	-0.115	0,000	0.408**	0.610***				
OM	0.505*	0.179	-0.165	-0.063	0.021	0.346*	0.712***	0.986***			
Ca	0.523*	0.288	-0.024	-0.053	0.02	0.278	0.942***	0.805***	0.889***		
Mg	0.554*	0.281	-0.06	0.016	0.039	0.265	0.852***	0.906***	0.963***	0.976***	
OC	0.524*	0.206	-0.548	-0.052	0.035	0.306*	0.704***	0.982***	0.999***	0.888***	0.965***

*. Correlation is significant at the 0,05 level (2 tailed) **. Correlation is significant at the 0,01 level (2 tailed). Correlation is significant at the 0,001 level (2 tailed) ***.

Getunic- *Glomus etunicatum*; Gintrar- *Glomus intraradices*, Gmoss- *Glomus mosseae*; *Gomus claroideum*; P- Phosphorus; K- Potassium; N- Nitrogen; OM- Organic matter; Ca- Calcium; Mg- Magnesium; OC- Organic carbon.

4. Discussion

Fungal intraradical structures

For the first time in the Azores we provided key insights into patterns of root colonisation by AMF, which was strongly constrained by land use type. Over the two years of study, root colonisation was always higher in semi-natural land use than in intensively management pastures. This result is in agreement with those found by several authors. Among others, Eason *et al*. (1999) reported that AMF root colonisation was lower in grassland soils with a history of high-input and, conventional management than in soil with a low-input or organic management. Kahiluoto *et al*. (2001) demonstrated reduced AMF colonisation of roots and reduced AMF spore density in soil with increasing P fertilization for several crops on two soils with low and intermediate concentrations of available P. Similarly, Oehl *et al*. (2003) found higher colonisation levels in plants grown on organic soils compared to ones grown conventionally. Also Gryndler *et al*. (2006) found that long-term mineral fertilisation and manure addition had a negative effect on AMF root colonisation, measured by AMF hyphal length in soil.

The abundance of fungal structure also showed a marked seasonality, particularly in the second year of study. This could have been related to nutrient exchanges, host plant phenology, host metabolic pathways and climatic variations (Titus and Leps, 2000; Lugo

and Cabello, 2002; Lugo *et al*., 2003; Bohrer *et al*., 2004; Heinemeyer and Fitter, 2004; Rodriguez-Echeverria *et al*., 2007). In the studied semi-natural and intensive pastures *H. lanatus* was the dominant species. This species is a weakly mycotrophic species as it is a C_3 plant (Cornelissen *et al*., 2001). Allen and Allen (1988) postulated that the presence of facultative mycorrhizal plant species can result in increased mycorrhizal inoculum levels in disturbed soils, which may help obligatory mycorrhizal plant species to become established later in the succession. Egerton-Warburton *et al*. (2007) found a higher hyphal length in C_3 plants than in C_4 plants, and they suggested that this could be related with the greater energetic investment for carbon (C) and P acquisition from soil by C_3 plants. Another possibility is the that phenological differences between C_3 and C_4 plants enhance the preemption of space; in other words, AMF in C_3 plants take over any soil space vacated by AMF associated with C_4 plants.

All fungal structures (hyphae, vesicles and arbuscules) were more abundant in summer harvests. This result is consistent with Titus and Leps (2000) and Lugo *et al*. (2003). These authors found that in *B. subaristata* (C_3) root colonisation measured as the number of arbuscules and vesicles changed with season and reached at maximum in summer.

The abundance of intraradical structures is usually closely related to host growth, flowering and fruit production (Smith and Read, 1997). Thus the high percentage of AMF root colonisation in our summer sampling periods matches the start of the reproductive phase of *Holcus lanatus* at the sites. During flowering and/or fruiting the phosphorus demand is high and thus AMF colonisation levels were also high. This can also be demonstrated by the higher percentage of AMF arbuscule colonisation during this season. Likar *et al*. (2009) also found another peak in arbuscule formation during flowering, in *Cruciata laevipes* and *Mentha piperata* and argue that the rate of arbuscule formation can be enhanced during periods of extensive productivity in plants, such flowering. Arbuscules are the places where nutrients are exchanged between a plant and fungus, and a high percentage of AMF arbuscule colonisation indicates intense symbiotic activity (Titus and Leps, 2000; Greipsson and DiTommaso, 2006).

Molecular diversity

We found that AMF diversity in semi-natural pastures had a marked seasonal pattern. Over the two years of the study larger number of DGGE bands was found during the

summer, coinciding with the flowering and /fruiting phase of *H. lanatus*, i.e., the phenological stage during each nutrient demands are highest.

However, among the two managements systems established there was a higher AMF diversity in semi-natural pastures than in intensively managed pastures, though the difference was not significant. Several authors reported larger AMF diversity for organically managed fields compared to conventionally managed fields. Among these, Oehl *et al*. (2003; 2004) found that the intensification of land use and conventional farming practices, as opposed to organic farming practices cause a reduction in AMF spore abundance and AMF species richness in agroecosystems of Central Europe. Rodriguez-Echeverria and Freitas (2006) also reported that the diversity associated with *Ammophila arenaria* ssp. *arundinacea* was higher in the least disturbed sites. However, our result is consistent with the work by Börstler *et al*. (2006) that found a similar diversity in intensively farmed fields (with 11 species) compared with the non-intensively farmed ones (with 10 species). Similarly, Galván *et al*. (2009) also found that AMF diversity of organically grown onions did not differ from that those conventionally grown.

The similarity in species richness between land uses could be related to: a) the fact that the AMF communities are highly generalist; b) the levels of intensification of the well-managed intensive pastures are low enough to allow similar patterns to semi-natural pastures. In Terceira, the influence of human activities on the simplification of ecosystems is quite dramatic with very few endemic arthropods occurring in intensive pastures (Cardoso *et al*., 2009). In fact, it is known that the input of fertilisers and pesticides and several other management practices (e.g., grazing intensity) are important in defining the quality of the pasturelands for AMF (e.g. Gange *et al*., 1993; Dhillion and Gardsjord, 2004). However, the input of fertilisers to Azorean pastureland is lower than average inputs to pastures on the European mainland (Garcia & Furtado, 1991). This fact gives support to our second hypothesis, which means that at Terceira, both semi-natural and intensive pastures are reasonably benign habitats for AMF.

On the other hand, certain lanes in some gels produced double bands. This is a well-known and common problem when using DGGE, in which two bands with the same sequence can appear very close to each other (Ma *et al*., 2005; Santos *et al*., 2006). This observation may complicate the interpretation of field DGGE profiles, because any AMF isolate may represented by two or more bands. Therefore, the actual number of AMF in a field DGGE profile may be less than half of what is visually detected by DGGE.

Moreover, the molecular identification system used in this study is known to miss certain groups of AMF (Krüger *et al*., 2009). In general, the LSU rDNA region allows for species-level resolution, and thus, the LSU primer pair FLR3–FLR4 (Gollotte *et al*., 2004) was used for species level community analyses. However, FLR4 in particular is not phylogenetically inclusive (Gamper *et al*., 2009) and it does not amplify many lineages, including *Diversisporales*, *Archaeosporales* and *Paraglomerales*, which results in a strong bias in community analyses towards the *Glomeraceae*. The primer FLR3 binds to the DNA of many non-target fungi. It has no mismatch to more than 1300 basidiomycete sequences and some ascomycete sequences in the public databases (Krüger *et al*., 2009). Therefore, it is very likely that this study has missed a considerable portion of the AMF diversity.

Although AMF diversity did not differ between management systems, it varied between field sites within each land use. Our data unequivocally show that some arable soils show an unexpectedly high diversity of AMF (especially RP1), which is in strong contrast to the studies by Helgason *et al*. (1998) and Daniell *et al*. (2001). Hijri *et al*. (2006) also reported variation in AMF diversity within management systems. Despite having checked for differences between organic and conventional field sites, they also reported differences between two organic field sites. At lower AMF diversity was observed in conventional field sites and in one organic field site. These authors argue that, in some agroecosystems, the farming systems do not influence AMF diversity, but rather the specific management practices (e.g., crop rotation, tillage) or environmental conditions (pH, P) may contribute to the maintenance of a more diverse AMF community. In fact, RP1 had the lower level of phosphate in the soil and the two semi-natural field sites had the higher levels. Thus, as high phosphate concentration is known to reduce AMF root colonisation, it is very likely that AMF diversity is concurrently reduced in these field sites. Moreover, in RP1, *Trifolium repens* is one of the most abundant species of plants, and it is know that the continuous cultivation of this plant might favour the presence of more AMF species in the long term (Gosling *et al*. 2006; Hijri *et al*. 2006).

Occurrence of *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. claroideum* and *G. microaggregatum*.

In the present study, we only found isolates from the Glomaceae in *H. lanatus* roots. Previous studies have also showed that the genus *Glomus* is usually a dominant group in AMF communities in agricultural soil ecosystems (Helgason *et al*., 1998; Daniell *et al*., 2001; Jansan *et al*., 2002; Oehl *et al*., 2003, 2004; Mathimaran *et al*., 2005). Some *Glomus* species have high plasticity to a range of habitats and are present even in the most intensively managed fields. These species are typically called "AMF generalists" (Oehl *et al*., 2003).

Arbuscular mycorrhizal fungi have distinct colonisation strategies that are related to taxonomic differences at the family level (Daniell *et al*., 2001). Hart and Reader (2002) showed that isolates of Glomaceae were faster colonisers than Acaulosporaceae and Gigasporaceae because they regenerate primarily from hyphal fragments and produce an extensive mycelium in roots rather than in soil. Furthermore, Giovannetti *et al*. (1999) have shown that *Glomus* (but not *Gigaspora* or *Scutellospora*) readily form anastomoses between mycelia and might therefore have the ability to re-establish an interconnected network after mechanical disruption. Such differences can explain the dominance of the Glomaceae over members of the Gigasporaceae in disturbed environments. On the other hand, the Glomales have traditionally been thought to be non-specific, but there is evidence suggesting that, while there may not be host specificity per se, partners may show preferences (van der Heijden *et al*., 1998).

Finally, the absence of other AMF species in this study could be related also to other factors, like the number of cycles of trap culture, sampling times, local environmental factors or host plant regulation of carbon expenditure, that enable the species to produce spores and persist as infective hyphae in roots or soil (Douds and Millner, 1999).

The total species occurrence (of the detected *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. claroideum* and *G. microaggregatum*) *H. lanatus* roots only varied with sampling date in the second year of study. Probably, the proximity of sampling periods in the first year (July and September) contributed to the lack of an evident seasonal pattern. Species occurrence was highest in August, coinciding with the reproductive phase of *H. lanatus* (Smith and Read, 1997; Titus and Leps, 2000; Lugo *et al*., 2003) and with the highest colonisation levels.

The occurrences of five AMF species varied with sampling time over the two years of study. *Glomus mosseae* was the most abundant and constant species, and it was the only species for which the frequency did not change with sampling time. Helgason *et al*. (1998) also found that in arable field sites the dominant arbuscular mycorrhizal fungal type was a putative *G. mosseae*. The same authors argue that the absence of this species in the woodland studied was related to differences in propagative units between glomalean families. In fact, this species sporulates abundantly and colonises readily from spores, which may be more important in a field that is ploughed annually than in a woodland.

G. claroideum and *G. microaggregatum* were less abundant, both spatially and temporally, and can be considered as rare species in some of the sites sampled. The symbiotic performance of AMF isolates depends on two main parameters; colonisation ability and efficiency. The rate of colonisation is influenced by the ability of AMF to spread rapidly and extensively across plants and is affected by factors linked to spore germination, presymbiotic mycelial growth and appressorium formation (Avio *et al*., 2006). In fact, *G. mosseae* and *G. etunicatum* were the two dominant species at all sampling dates; this may be related to competitive advantages for carbon uptake during early root colonisation by these species (Jansa *et al*., 2008).

Jansa *et al*. (2008) observed that *G. mosseae* was the most effective competitor out of three AMF species (*G. mosseae*, *G. intraradices*, and *G. claroideum*) due to its fast rate of colonisation and early peak in colonisation. According to this same study, *G. claroideum* was the weakest competitor, and *G. intraradices* was intermediate between the two of them.

Farmer *et al*., (2007) noted that in a study done under field conditions, *G. etunicatum* introduced either as a pure inoculum or mixed with *G. mosseae* and *G. intraradices* had great success and was, detected in the roots of nearly all samples. However, our results suggest that the high efficiency of *G. etunicatum* in establishing a mycorrhizal symbiosis could explain the cross contamination of *G. mosseae* during inoculation production.

Soil parameters and root colonisation

AMF root colonisation levels have been associated with soil nutrient acquisition in perennial grasses (Jackson and Caldwell, 1996). In fact, the percentage of all fungal structure and the total species occurrence (considering *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. claroideum* and *G. microaggregatum*) were positively correlated with

soil nutrient levels. Thus, the improvement of soil fertility, especially the availability of K, N, Ca and Mg, provided a favourable environment for mycorrhizal formation and function in these systems. A similar result was found by Oliveira and Oliveira (2005). These authors observed a strong influence of edaphic factors, such as soil moisture, pH, Mg, K and Mn on symbiotic associations in the acidic soils and nutrient-limited soils of Central Amazonia. Khanam *et al*. (2006) also noted a positive correlation between root colonisation and soil moisture, OM, total N and K. However, as shown in other studies (Mathimaran *et al*., 2005; Ezawa *et al*., 2005; Rodriguez-Echeverria *et al*., 2009), a correlation between the percentage of AMF colonisation and soil P content was not found. The two investigated land uses (semi-natural and intensive pastures) had a soil P content clearly above the P concentrations that limit AMF colonisation and within the P concentrations that limit plant growth (P< 10 ppm). Miller (2000) has speculated that the narrow ranges of P (0–35.8 ppm and 2.5–15.7 ppm, respectively) in their study sites caused the lack of a significant correlation between AMF colonisation and soil P. Johnson *et al*. (2003) reported an increased allocation to AMF structures with nitrogen enrichment in mesic grasslands where ambient soil nitrogen was highest and phosphorus availability lowest. These same authors point out that AMF responses to N and P enrichment are mediated by ambient N and P availability, and this response could be caused by allocation plasticity within individual AM fungal taxa, changes in the species composition of the AMF community, or both.

Because the soil P levels are so low, and the range in soil P is so small in our study, it is not possible to speculate on whether or not large fluxes of P in other pastures habitats would significantly influence the extent of AMF colonisation at any given time.

Nevertheless, the presence of two AMF species (*G. etunicatum* and *G. intraradices*) was negatively correlated with soil P content. This result contrasts with that of Johnson (1993), who showed that fertilisation of soils tended to decrease the abundance of AMF species such as *Scutellospora calospora*, and increase others like G. *intraradices*.

In summary, this study shows that AMF diversity did not differ between semi-natural and intensively managed pastures, and that AMF communities associated with both pasture types are mainly dominated by five *Glomus* species. All of these species, except *G. mosseae*, show seasonal variations that are reflected in the variation of fungal structures found at different sampling times. These temporal changes in fungal structures are also related with plant phenology, showing that flowering is a time of high nutrient demand for the plant, which translates into a higher proportion of arbuscules

and a greater hyphal colonisation. This study also shows that the poorer soils were limiting for the development mycorrhizal fungi. Three of the five species found (*G. mosseae, G. intraradices* and *G. etunicatum*) were quite abundant in the studied systems, independent of soil management, therefore behaving as AMF generalists. The structure of mycorrhizal communities is complex even in recent habitats such as islands. Thus, to obtain a better understanding of the seasonal dynamics of AMF communities, more intensive sampling distributed throughout the season, with measures of both colonisation and species composition, as well as other ecological factors, would be necessary.

CHAPTER IV. CONTRIBUTION OF ARBUSCULAR MYCORRHIZAL FUNGI TO CONTROL ROOT-FEEDING NEMATODES IN THE PERENNIAL GRASS *HOLCUS LANATUS* L. IN TERCEIRA ISLAND (AZORES)

1. Introduction

Nematodes are a major component of all soil food webs and thus comparisons of abundance, biomass and community structure can be made across ecosystems (Brussaard *et al*., 1997). However, it's well know the damage that they cause to agriculture crops, which can be traduced into a decrease of plant productivity, disruption of plant nutrient and water transfer, as well as, a decrease of fruit and tuber quality and size (Sipes and Schmitt, 1994; Cofcewicz *et al*., 2001). Some studies, also have pointed to changes in composition of plant communities as consequence of feeding herbivores (de la Peña *et al*., 2006; Piśkiewicz *et al*., 2008; Rodriguez-Echeverria *et al*., 2009).

De la Peña *et al*. (2006) showed that root feeding herbivores affected not only the plant performance of *Ammophila arenaria* in coastal dunes, but at same time altered the composition of plant communities. The same authors reported that mycorrhizal communities associated with the roots of *A*. *arenaria* were almost represented by *Glomus* sp. i.e. the genus with great plasticity to environmental disturb, and probably it represents the fraction of soil that can survive and grow to effect of root feeding herbivores. Thus, it's seems that root feeding herbivores may have a selective pressure in AMF communities, benefiting the species considered "generalist" at the expense of the species classified as "rare" (Oehl *et al*., 2004).

An identical result was also reported by Rodriguez-Echneverria *et al*. (2009) who found that root-herbivores can alter mycorrhizal community associated to *A*. *arenaria*, although the total percentage of root colonisation is not affected by its presence. Since the degree of sensitive to herbivory varies among AMF species, the herbivore effect could benefit some AMF species in detriment of the other in terms of root colonisation.

Various physical, chemical, and agronomic measures have been proposed to control root-nematode disease (van der Putten *et al*., 2006). Chemical nematicides are most effective in inhibiting nematode infestations but most of the active ingredients are prohibited due to their detrimental effects on human health and the environment. Agronomic practices including crop rotations, fallow, and cover crops can also reduce disease incidence but these methods are difficult to practice under Chinese conditions due to the limited land area and intensive cultivation systems. Biological control

techniques are preferable because of their limited effects on the environment and the land can be used continuously for economic production.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous plant mutualists which represent a large fraction of fungal biomass of the rhizosphere. These symbiotic organisms are found inside roots, in the rhizosphere itself and in the bulk soil, interacting in all these zones with other organism (Fitter and Garbaye, 1994; Azcón-Aguilar *et al*., 2002). The internal mycelium interacts principally with the root itself, but at the same time with other organism even pathogens fungi. The AMF external mycelium developed in the soil helping plant nutrient and water uptake, interacts with many organisms such bacterial, other fungi, protozoa, nematodes, arthropods and even large animal as mammals (Fitter and Garbaye, 1994; Azcón-Aguilar *et al*., 2002). Then, it's well evident the several benefits that AMF may provide to their host plants, including better phosphorus (P) nutrition (Jaizme-Vega and Azcón-Aguilar, 1995; Guissou, 2009), increased abiotic stress (Augé, 2004; Smith *et al*., 2009) and increased disease resistance (Jaizme-Vega *et al.*, 1998; Idoia *et al*., 2004; Al-Askar and Rashad, 2010). Due to these beneficial effects of AMF on host plants, they are suggested to have the potential to serve as both biological fertilisers and biological control agents (Barea *et al*., 2005; Mukhopadhyay and Maiti, 2009).

Both arbuscular mycorrhizal fungi (AMF) and root knot nematodes are indigenous soil organisms and share plant roots as resources of food. Consequently, there is interest in AM-nematode interactions because of the possibility of enhanced resistance or tolerance of AMF- infected plants to nematodes (Whipps, 2001; Hol and Cook, 2005). The mechanism proposed to explain this protective effect includes: direct competition; mechanism mediated by alteration in plant growth, nutrition and morphology; biochemical and molecular changes in mycorrhizal plants that induce pathogen resistance, and alterations in the soil microbiota and development of pathogens antagonism (Azcón-Aguilar *et al*., 2002; Vierheilig *et al*., 2008).

However the control mechanisms of root-feeding nematodes appear to be dependent on the feeding type of the nematode i.e. if they are ectoparasites, sedentary endoparasites or migratory endoparasites (Borowicz, 2001; Hol and Cook, 2005; Wehner *et al*., 2010), as well as, on the species of nematode (de la Peña *et al*. 2006; Piśkiewicz *et al.* 2008). In general interactions between AMF and ectoparasitic nematodes are less strong than those endoparasites, because ectoparasites feed on

superficial cells, and do not reach the cortical cells colonised by AMF. Thus, ectoparasitc nematodes are more likely to be affected indirectly by AMF-induced changes in plant physiology than directly by competition with the fungus (Hol and Cook, 2005). Sedentary endoparasites include two nematodes groups – root-knot and cyst nematodes, both induce changes in plant cells and feed on modified cells. The feeding cells induced within the vascular cylinder by these endoparasites may invade the endodermis and proliferate into the cortex, where we can find the cells colonised by AMF, occurring competition by space between these two organisms (Hol and Cook, 2005).

Finally de migratory endoparasites, such as root-lesion and burrowing nematodes feed and move within the cortex, thus they can feed on cells appropriate for AMF colonisation (Hol and Cook, 2005). On the other hand, AMF can change root morphology with consequences for penetration and movement of migratory nematodes. AMF will colonize much faster than nematode resulting in alteration of root physiology of host like increasing wall thickness and changing the chemical composition of root exudates.

Much work has been done on AMF-nematode interactions especially on specific groups on nematodes, i.e. root-knot (*Meloidogyne* spp.) and root lesion (*Pratylenchus* spp.) nematodes given its economic importance. AMF have interactions with root nematodes of many crop plant species including fruit tree (Pinochet *et al*., 1996; Jaizme-Vega *et al*., 2005) banana (Jaizme-Vega *et al*., 1997; 2005), tomato (Cofcewicz *et al*., 2001; Shreenivasa *et al*., 2007) and cucumber plants (Zhang *et al*., 2009).

Jaizme-Vega *et al*. (1997) noted that inoculation with *Glomus mosseae* favours growth of banana plants by enhancing plant nutrition and by suppressing *M. incognita* reproduction and galling during the early stages of plant development. Shreenivasa *et al*. (2007) also reported that decrease on plant height, dry biomass weight as well as fruit weight in tomato plants infected with root-knot *Meloidogyne incognita* was minimized in presence of AMF.

Jaizme-Vega and Pinochet (1997) point out that mycorrhizal inoculation on the first stages of banana's development could increase host tolerance to *P. goodeyi* by enhancing plant nutrition and by reducing the nematode induced lesions in the root.

Also Elsen *et al*. (2008) noted that AMF have the ability to induce systemic resistance against plant parasitic nematodes in banana root system, since AMF reduced more than 50% the populations of two nematode *Radopholus similis* and *P. coffeae*. Likewise, Rodriguez-Echeverria *et al*. (2009) showed in inoculation experiment that *Ammophila arenaria* roots infection by *P. penetrans* and *P. dunensis* was reduced in all mycorrhizal plants when compared to no-mycorrhizal plants.

The Azores archipelago has an extensive area of pastures, in these agroecosystems, herbivorous nematodes take up as much as one quarter of the net primary production (Stanton 1988). Rosa *et al.* (1996) in a study aiming to harvest and genetic characterization of entomopathogenic agents in the Azores, namely in Terceira, reported that 33.7 % of soil samples showed nematodes, most of them of free living and phytoparasitic, and 20% of which had been collected in pastures. In Azores evident effects of endoparasite nematodes, especially root-knot, have been detected in some horticulture crops such banana (*Musa* sp.), cucumber plants (*Cuccumis melo* L.) and tomato (*Solanum lycopersicum* L.), but there is no peer-reviewed on subject. The first record of *M. incognita* in Azores islands, especially in S. Miguel, was made by Vovlas *et al*. (2004) in tabaco (*Nicotiana tabacum* L.). Little is known about how the variation of these plants along their range affects their relationship with root-feeding nematodes, as well as, with their symbiotic organism like AMF. This way, would be interesting to evaluate the potential contribute of indigenous AMF not only on nematode control but also in increasing productivity of these crops. Thus, a study was developed to assess the contribution of AMF in reducing nematode infection and reproduction by using *Holcus lanatus*, due its extensive occupation in Azorean lands (Dias, 1996; Silva and Smith, 2006; Kueffer *et al*., 2010).

2. Material and methods

2.1. Arbuscular mycorrhizal fungi

In July of 2006, soil was collected from the rhizosphere of ten different *H. lanatus* plants in Pico Galhardo (PX), Terra Brava (TB), Agualva RP1 (RP1) and Agualva (RP2) and used to set up trap cultures of the AMF community. The fresh soil samples were mixed with autoclaved sand and “bagacina” (volcanic soil type) in a ratio of 2:1:1. A 1.5-kg aliquot of the mixture was placed in each pot to produce trap culture from

each sample. Corn (*Zea mays* L.) was used as host plant. Seeds of corn were surface sterilised by immersion in alcohol (96 %) for 30 sec, 4 % bleach for 2 min, and rinsed twice in sterile distilled water. Then, they were sown in pots on sterilized sand soil and left to germinated in the greenhouse. After one week of growth, seedlings were transplanted to pots containing the fresh soil samples mix. A total of 4 seedlings were used per plot. Trap cultures in 40 plots were grown in glasshouse for 5 months and then harvested. All pots were watered every 2 days with distilled water, and no nutrient solution was added to the pots. After 4 month of sowing we left watering the pots to stimulate the sporulation.

2.2. AMF root colonisation assessment

After 5 months, plants were harvested and root examined to confirm AMF colonisation. A portion of roots were cleared in 2.5% KOH for 1h at 90 ˚C. Subsequently they were left to acidify overnight in 1% HCl. Staining was done with blue ink (Parker Quink) for 30 min at 60 ˚C, followed by distaining in lactoglycerol. The amount of colonisation was estimated using a grid-intersect method with examination of 100 intersects under a compound microscope at 200x magnification (McGonigle *et al*., 1990). Root-intersects that contained vesicles, arbuscules or hyphae were scored as mycorrhizal. The remaining corn roots were cut into 2-cm pieces, and disinfected by immersion in 2 % chloramines T for 3 min and in an antibiotic solution (streptomycin 200 mg l^{-1} + penicillin 100 mg l^{-1}) for 3 h. After this time root were rinsed in autoclaved water and dried (de la Peña *et al*., 2006).

2.3. Spore assessment

AMF spores produced in the trap culture were extracted by sieving and decanting (Gerdemann and Nicolson, 1963). The procedure included the passage of 50 cm^3 of harvested trap culture substrate from each pot through 1000-, 500-, 250-, 100-, and 45 µm sieves. The material retained in 250 µm, 100 µm and 45 µm was collected to different petri dish and AMF spores were counted under a stereoscopic microscope (Leica MZ 12). Spores from 4 genera were detected in trap culture, namely *Acaulospora*, *Ambispora*, *Glomus* and *Scutellospora.*

2.4. Nematode inoculation procedure

The nematode inoculum consisted of a population of *Meloidogyne incognita* isolated from a culture of tomato originally collected in the North-East of Tenerife (Valle de Guerra). The nematode inoculum was prepared by macerating infected roots in a blender for 15 s at 14 500 rpm in a 0.12 - 0.15% NaClO solution (Hussey and Barker, 1973). Eggs and juveniles (J2) were collected using a 25-µm-pore sieve and rinsed with tap water. The inoculum was adjusted to deliver a suspension of 1150 nematodes per plot through two 3 cm deep holes located at 5 cm distance from the base of the plant in the nematode treatments.

2.5. Experiment

Seeds of *H. lanatus* were surface sterilised by immersion in 96% alcohol for 30 sec, 0.4% sodium hypochlorite for 2 min and rinsed twice in sterile distilled water. They were then sown in boards on a sterilised sand soil and left to germinate in greenhouse. After 2 week of growth, seedlings were transplanted to the pots. Four seedlings of *H. lanatus* were planted in 2-l pots filled with a pasteurized substrate contained, soil, sand and “bagagina” (volcanic soil type) in a ratio of 2:1:1 soil.

The experiment include four treatments with 14 replicates per treatment: an uninoculated control (C), inoculation with arbuscular mycorrhizal fungi (AMF), inoculation with *M. incognita* (Mi), simultaneous inoculation with AMF and *M. incognita* (AMF + Mi). At the time of *H. lanatus* seedling transplantation’s each pot for inoculation with AMF, as well as, for combined inoculation with AMF and *M. incognita* received 250 spores and 1g of dried corn roots from trap cultures, which was used as AMF inoculum. We also inoculated four additional plots with AMF inoculum, to assessment the mycorrhizal colonisation after 2 weeks and 4 weeks after inoculation. Plots were disposed in the greenhouse in a completely randomised design. Every 2 days pots were watered and one time per month they were fertilized with 100 ml of half-strength modified (P-free) Hoagland’s solution. Afterwards, the *H. lanatus* seedlings were left to growth for 6 additional weeks to obtain roots enough to split.

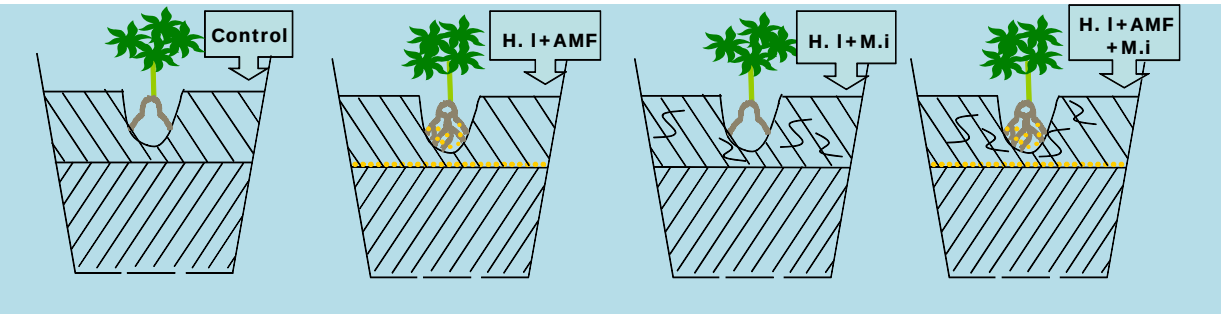

Figure 1. Experimental design in which: Control- non-mycorrhizal plants; HL+AMF- mycorrhizal plants; H.l+M.i – plants inoculated with *M. incognita*; H.l+AMF+m.i- plants inoculated with *M. incognita* and AMF.

Then, after the AMF colonisation has been confirmed the roots of each plant were placed into a new plot with a sterilised mixture composed by sand, "bagacina" and soil in a ratio of 3:1:1. The substrate surface was then covered by a volcanic ash layer in order to keep the substrate humid and avoid the contamination. Ten days later we proceed to the inoculation with *M. incognita*, which consisted in adding 10 ml of nematode suspension to each plot. The experiment was conducted from June 2007 until October 2007.

2.6. Harvest and data collection

Plants from experiment were harvested 14 weeks after nematode inoculation. The following physical parameters were measured: fresh root weight, aerial fresh root weight, total fresh root weight (aerial and root) and aerial dry weight. After taking the root fraction for assessing AMF colonisation (5% in fresh weight), the remaining plant material was dried at 72 ºC for 48 h to estimate plant biomass. AMF colonisation was determined by the same method used for assess mycorrhizal infection in *H. lanatus* roots from trap culture (see above).

For nematode were estimated the following parameters: the number of nematode per gram of root, number of nematode per root and the reproduction rate (final population/initial population). The nematode extraction method from roots was similar to that used for inoculums preparation. Nematodes were concentrated using 150-, 74- and 25-µm-pore sieves. The suspension of 25- µm sieve was collected and concentrated in order to determine number of nematodes per ml by using a Hawksley

slide under a light microscope. Nematodes in any developmental stage were taken as a positive count.

2.7. Statistical analysis

The statistical analysis was performed with ANOVA General Linear Models and Tukey's multiple range tests for overall comparison (Minitab, version 13.31, 2000). All data were tested for normality when required to fulfil assumptions of the ANOVA. AMF colonisation and nematode reproduction data were arcsine (x/100) transformed to reduce the variance in the data.

3. Results

3.1. Effect of AMF on plant development

The importance of shoot growth and aerial biomass production on *H. lanatus* seedlings inoculated with native AMF compared to the non-inoculated control plants and plants inoculated with *M. incognita* are displayed in Figures 1 and 2, respectively.

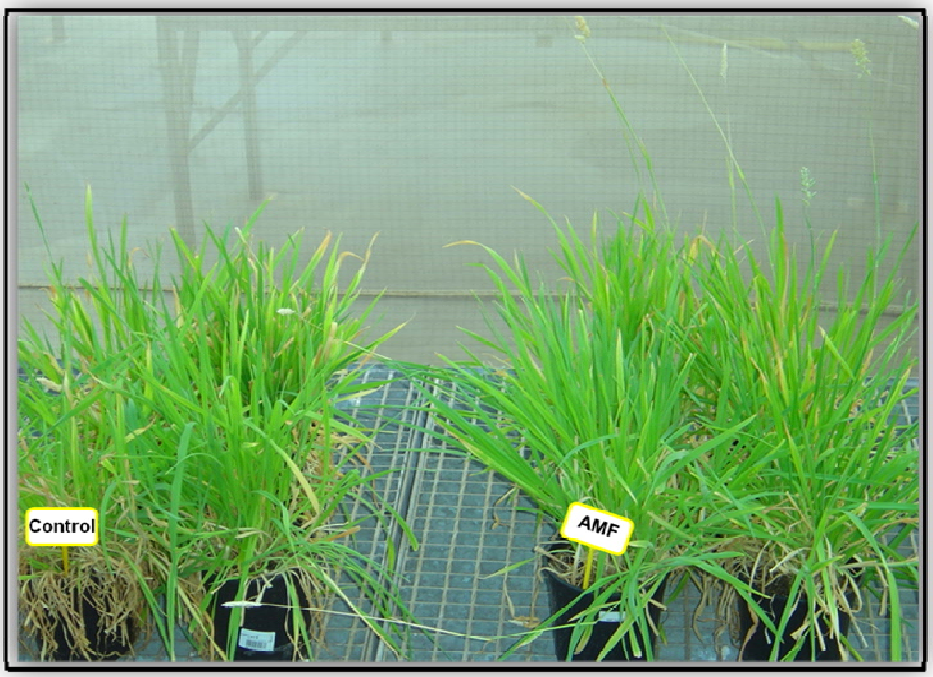

Figure 1. Effect of inoculation with an indigenous mixture of AMF or not on aerial biomass production in nursery-grow *H. Lanatus* seedlings grow for 6 months after inoculation.

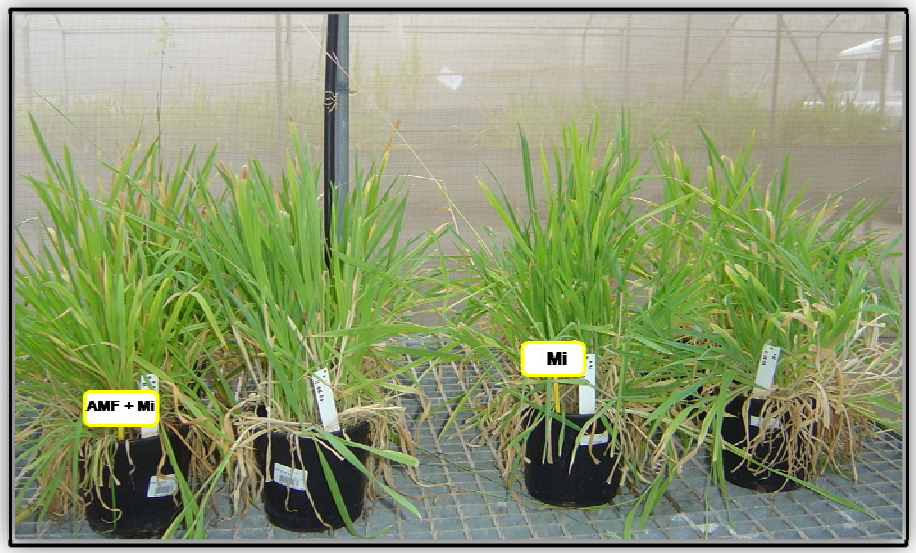

Figure 2. Effect of inoculation with an indigenous mixture of AMF or not on aerial biomass production in nursery-grow *H. Lanatus* seedlings infected with *M. incognita* for 6 months after inoculation with AMF.

There were differences highly significant between treatments on fresh root weight of *H. lanatus* (Anova: F= 11.52; d.f.= 3, 52; p< 0.001) (Table 1). The Tukey HSD *post hoc* test showed that average of fresh root weight was significantly greater in plants inoculated with the combination AMF and nematode ($\bar{x}$ = 71.25 ± 4.44) (Fig. 3 a), but root infection only by nematode had significantly the lowest weight (43.86 ± 1.31) (Fig. 3 a).

The fresh weight of *H. lanatus* plants also varied significantly between treatments (Anova: F= 22.80; d.f.= 3, 52; p< 0.001) (Table 1). Take in account the Tukey HSD *post hoc* test, we can observed that the mycorrhizal plants had significantly the largest average of aerial fresh weight ($\bar{x}$ = 86.92 ± 5.05) (Fig. 3 b), and the nematode presence caused a decreased of about 50% on average of aerial fresh weigh of *H. lanatus* plants. Furthermore, in treatment with inoculation of nematode and AMF, although average of aerial fresh weight of plants ($\bar{x}$ = 67.16 ± 3.24) had been lower than only in presence of AMF, did not differ significantly from the weight of non-mycorrhizal plants (Fig. 3 b).

Table 1. F- and p values from one-way ANOVA of growth response of *H. lanatus* to inoculation with AMF and 1.150 *M. incognita* per plant 6 and 4 months after AMF and nematode exposure respectively. * P< 0.05; * P< 0.01;*** P< 0.001; n.s= not significant.

Treatments	F	*p*
Fresh root weight	11.53	***
Aerial fresh weight	22.80	***
Total fresh weight	18.07	***
Aerial dry weight	12.22	***
Nematodes/gr of root	321.54	***
Nematodes/root	211.40	***
Reproduction rate	207.29	***

The total fresh weigh was similar to previous, i.e. there were significant differences in average of total fresh weight of *H. lanatus* plants among treatments (Anova: F= 18.07; d.f.= 3, 52; p< 0.001) (Table 1). The Tukey HSD *post hoc* test showed a highly significant effect of AMF in fresh biomass of *H. lanatus* plants (Fig. 3 c). In fact, average of fresh biomass of mycorrhizal plants ($\bar{x}$ = 139.55 ± 7.82) was higher than in the other all, and the plants inoculated with nematode alone had the lowest average of total fresh weight ($\bar{x}$ = 86.96 ± 2.81). By other side, there were not significant differences between total fresh weight of plants simultaneously inoculated with AMF and nematode and non-mycorrhizal plants, as well as, between mycorrhizal plants.

The aerial dry weight of *H. lanatus* plants followed the same trend, had being significantly different between treatments (Anova: F= 10.06; d.f.= 3, 52; p< 0.001) (Table 1). The Tukey HSD *post hoc* test revealed a significantly great of AMF effect on average of aerial dry weight of *H. lanatus,* which was higher in mycorrhizal plants ($\bar{x}$ = 27.50 ± 2.58) than in the other treatments (Fig. 3 d). Adding, in the presence of nematode the average of aerial dry weight decreased about 40% (Fig. 3 d).

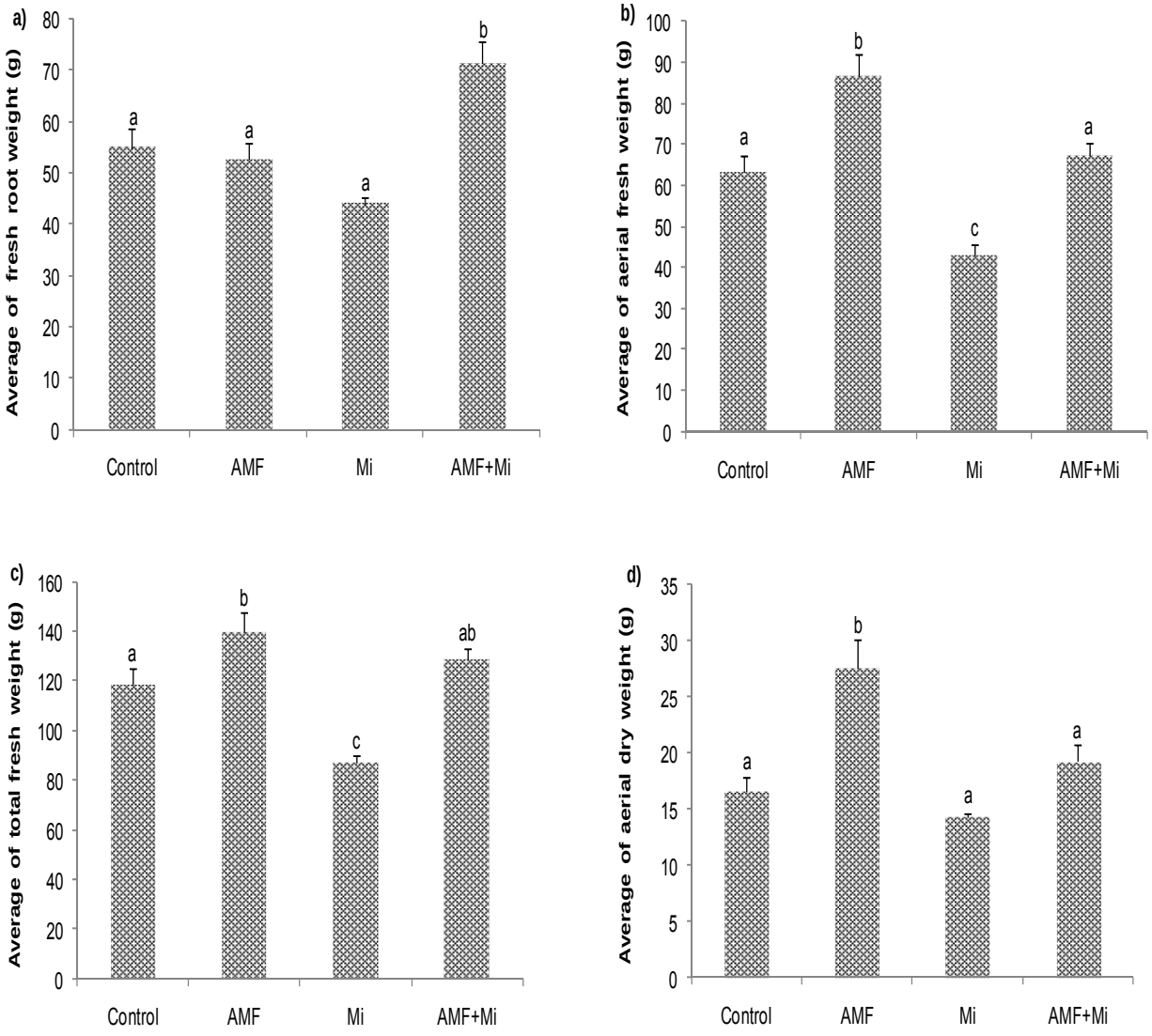

Figure 3. Experimental inoculation. Average of fresh root weight (a), aerial fresh weigh (b), total fresh weight (c) and aerial dry weight (d) of *H. lanatus* from four different treatments: non-mycorrizal plants (control); mycorrizal plants (AMF); inoculation with *M. incognita* (Mi) and simultaneous inoculation with AMF and nematode (AMF+Mi). Standard errors of the mean (n= 14) are shown. Values of percent are arcsine-transformed. For each parameter, different letters above the bars indicate significant differences between treatments after one-way ANOVA and Tukey's HSD test (P≤ 0.05).

3.2. Effect of AMF on root-feeders

Concerning to effect of AMF in the number of nematode per gram of root, there were significant differences between the mycorrhizal plants and the plants infected only by nematode (Anova: F= 321.54; d.f.= 1. 26; p< 0.001) (Table 1). In fact, the average of number of nematode per gram of root was drastically reduced by the presence of AMF than in its absence (Fig. 4 a), in other words, AMF cause a reduction of 323 individuals per gram of root in plants of *H. lanatus* infected by nematode.

The same result was obtained in relation to the number of nematode on *H. lanatus* root which also varied among the 2 treatments (Anova: F= 211.14; d.f.= 1. 26; p< 0.001) (Table 1) with the micorrhizal plants had the lower average number of nematode on root ($\bar{x}$ = 339 ± 339) than plants infected only by nematode ($\bar{x}$ = 14521 ± 915) (Fig. 4 b), i.e, the number of individuals was less than 13606 in plants infect by nematode and AMF.

These results were reflected in reproduction of nematode which also varied significantly among the 2 treatments (Anova: F= 207.29; d.f.= 1. 26; p< 0.001). Again, the AMF had a significant effect in reducing the reproduction rate of nematode which range between 0.3 % and 13% in presence and absence of AMF respectively (Fig. 4 c).

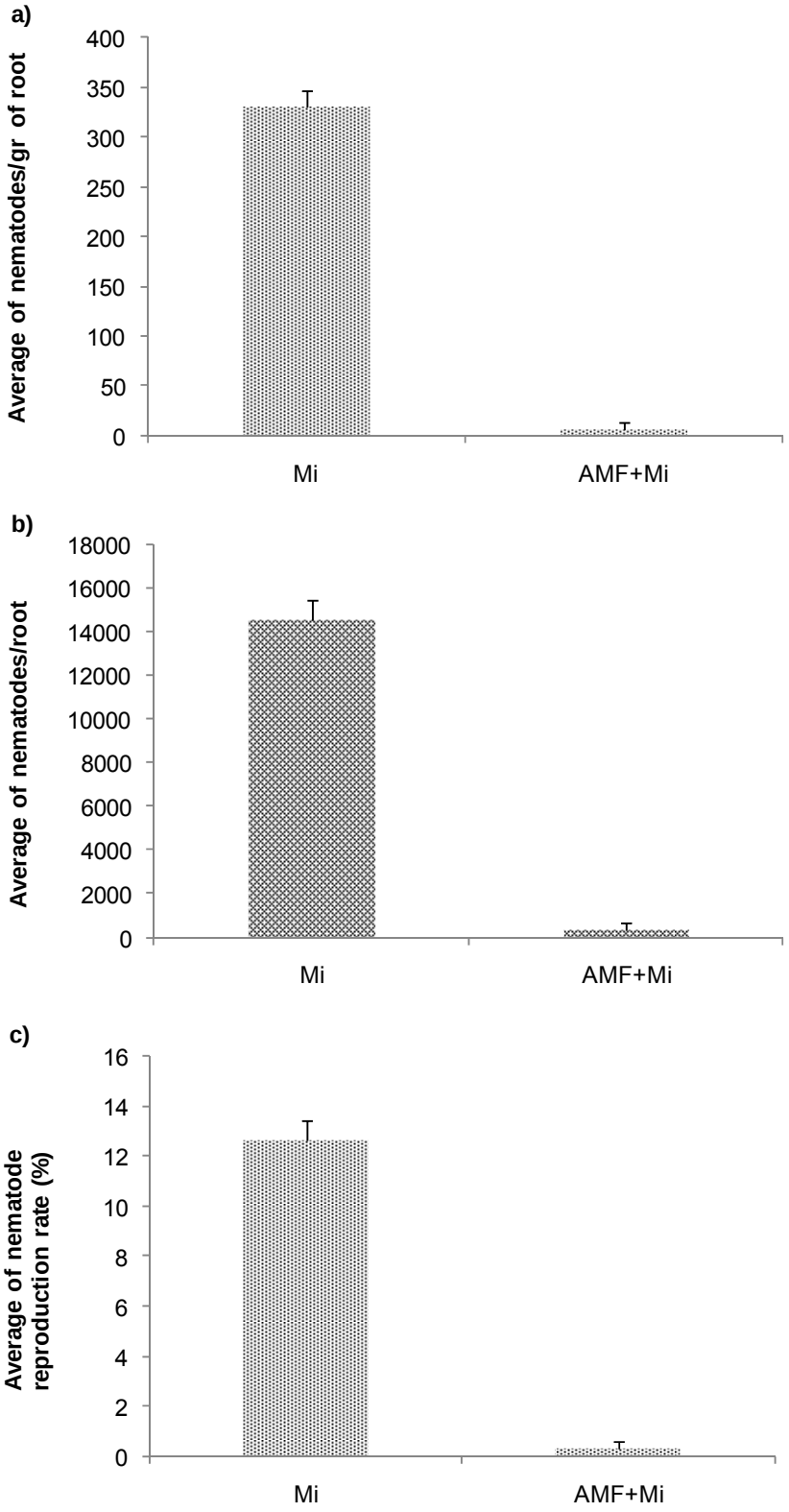

Figure 4. Experimental inoculation. Average of nematode/gr of root (a), nematodes/root and nematode reproduction rate of *H. lanatus* from two different treatments: inoculation with *M.incognita* (Mi) and simultaneous inoculation with AMF and nematode (AMF+Mi). Standard errors of the mean (n= 14) are shown. Values of percent are arcsine-transformed. One-way ANOVA *P*-values are presented in Table 1.

3.3. Mycorrhizal colonisation

Mycorrhizal colonisation was detected 4 weeks after inoculation with AMF. The average percentage of AMF colonisation ranged between 50 % and 60% at the end of experiments in treatments with simultaneous inoculation with AMF and nematode, and in treatments inoculated with AMF respectively (Fig.). However, although *H. lanatus* treated with nematode showed a lower mycorrhizal colonisation percentage, no significant differences of the presence of nematode were observed on percentage of root colonised by AMF (Anova: F= 3.81; d.f.= 1. 26; p= 0.062).

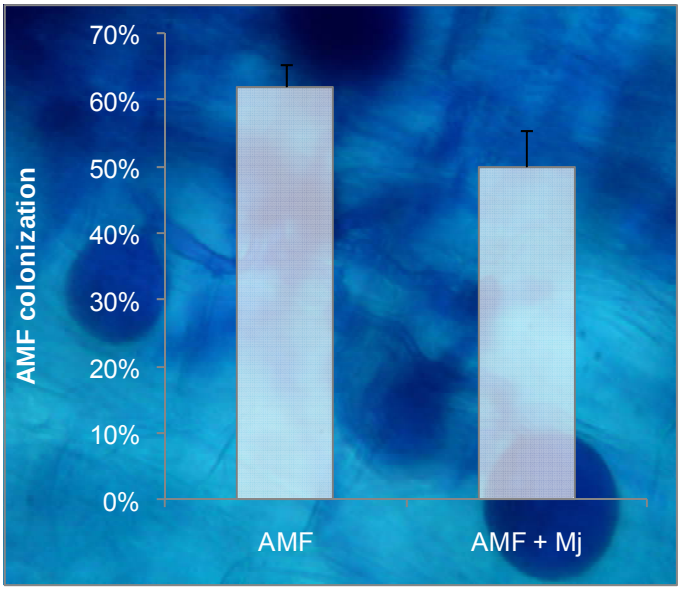

Figure 5. Percentage of *H. lanatus* roots infected by arbuscular mycorrhizal fungi (AMF) and percentage of of *H. lanatus* infected by arbuscular mycorrhizal fungi and *M. incognita* (AMF+Mi). Standard errors of the mean (n= 14) are shown. Values of percent are arcsine-transformed.

4. Discussion

In general native AMF reduced *M. incognita* population in *H. lanatus* plants. This confirmed previous works on a wide variety of crops. Among them, Jaizme-Vega *et al*. (1997) reported that the association between AMF and the banana plantlet increased the host tolerance to *M. incognita*, compensating for de damaged caused by nematode, mainly by enhancing plant nutrition.

Jaizme-Vega *et al*. (2005) in a study with papaya also showed that *M. incognita* infection was significantly reduced in mycorrhizal plants. Shreenivasa *et al.* (2007) found that application of *Glomus fasciculatum* increased yield of tomato in plants inoculated with *M. incognita*, whose population was reduced about half in the presence of AMF.

Zhang *et al*. (2009) also reported in a study to investigate the tolerance of cucumber plants (*Cucumis sativus* L.) to root-knot nematode *M. incognita*, that the number of root galls and the number of egg masses, eggs and J2 were significantly lower on plants inoculated with *Glomus intradices*, indicating that *G. intraradices* has the potential to confer enhanced tolerance to root-knot nematode infestation to cucumber.

Many hypotheses have been proposed on the mechanism of the AMF-induced resistance against plant pathogens (Azcón-Aguilar *et al*., 2002; Hol and Cook, 2005, Vierheilig *et al*., 2008). Based on our results we can suggest that increased capacity for nutrient uptake by the mycorrhizal association may allow host plant to be more vigorous and, consequently, more resistant or tolerant of pathogen attack (Azcón-Aguilar *et al*., 2002). In fact, root biomass of *H. lanatus* plants simultaneously inoculated with AMF and *M. incognita* was significantly higher than in the others treatments. The AMF presence may have promoted this increase in root biomass to compensate tissue damage and decay of root sections by the pathogen and consequently reduce significantly disease symptoms. This result was consistent with found by Cofcewicz *et al*. (2001) who found that the increase of number of galls in tomato roots simultaneously inoculated with AMF and *M. javanica* could be related with increase of root systems promote by AMF, which put at disposal of nematode a greater number of sites for infection.

Furthermore competition for host colonisation sites and photosynthates may also explain our results. Really, the lack of significant differences in AMF root colonisation

between mycorrhizal plants and plants with combined inoculation, suggest that there was a competitive interaction between *M. incognita* and AMF on *H. lanatus* plants by space. AMF might occupy infection sites on the root surface needed by *M. incognita* to penetrate root, or cells in the root already occupied by the AMF cannot be colonised any further by the root-knot. This result was consisted to found by others authors. Between them, de la Peña *et al.* (2006) reported that when both AMF and *P. penetrans* were together in the same root compartment of *A. arenaria* occurred competition between these organisms, although root colonisation by AMF was not affect the nematode. Also Rodriguez-Echeverria *et al*. (2009) showed that suppression of *P. penetrans* and *P. dunensis* could be explain trough direct competition, one time this is one of the mechanisms which regulated the relative abundance of nematodes in the rhizosphere of *A. arenaria*.

Adding like AMF, *M. incognita* growth also depends on carbon from photosynthesis. Since plants colonised by AMF much carbon is used by the symbiotic AMF, less carbon could be available for nematode colonisation (Azcón-Aguilar *et al*., 2002; Vierheilig *et al*., 2008). Our findings were similar to the results obtained by Osman *et al*. (1991), who associated the decreased of *M. incognita* population in *Phaseolus vulgaris* colonised by AMF, to a mechanism of competition for space and food supply in the root systems between the two organism. Also Jaizme-Vega *et al*. (1997) point out that the suppression of *M. incongnita* on micropropagated banana inoculated with *Glomus mosseae* could be associated with competition for space or physiological changes in the root that make it an unfavorable food source for the nematode.

A significant increase on plant growth was observed in mycorrhizal plants compared to the others treatments, which is in agreement as observed in other work (Jaizme-Vega and Azcón-Aguilar, 1995; Regvar *et al*., 2003; Al-Askar and Rashad, 2010).

Regvar *et al*.(2003) showed that an indigenous mixture of arbuscular mycorrhizal fungi (AMF) containing *Glomus mosseae*, *Glomus fasciculatum*, *Glomus etunicatum, Glomus intraradices* and *Scutellospora* sp. significantly increased the plant biomass parameters of pepper, and parsley and the root biomass of carrots.

The main AMF benefits to the plants it's the growth improvement due to enhanced nutrient uptake, particularly immobile nutrients like phosphorus, because the hyphae can reach farther that the nutrient depleted zones that build up around plant roots

(Smith and Read, 1997; Clark and Zeto, 2000). Furthermore, in mycorrhizal plants nematode presence did not appear to have affected plant development. In fact, either aerial fresh weight or total fresh weight and dry weight of *H. lanatus* plants simultaneously inoculated with AMF and nematode were higher than in non-mycorrhizal plants or in plants only inoculated with nematode. A same result was found by Shreenivasa *et al*. (2007) who reported that maximum plant height, dry shoot and root weighties, as well as, total dry weight and fruit weight per plant were higher in tomato plants simultaneously inoculated with *Glomus fasciculatum* and *M. incognita* than in control or in plant only inoculated by nematode.

This study concludes that inoculation with native AMF could be a new nematode management approach in Azores ecosystems by increasing plant resistance (a case of induced resistance) to the nematode where root galling and nematode reproduction is negatively affected and plant growth enhanced by the endophyte. However, due to the complexity of AMF-nematode interactions more nematodes species should be tested and the mechanism need to be further elucidated.

CHAPTER V. THE PRODUCTIVITY OF THE AZOREAN PASTURES BENEFITS FROM THE INDIGENOUS ARBUSCULAR MYCORRHIZAL FUNGI (AMF)

1. Introduction

Associations between plants and arbuscular mycorrhizal fungi (AMF) are common in natural and agricultural ecosystems (Smith and Read, 1997). In this intimate association the obligate biotrophic fungi supply inorganic nutrients to plants in return to photosynthates. Thus, the main benefit of AMF to their host is principally the increased uptake of relatively immobile phosphate ions, due to the ability of the hyphal network to grow beyond the phosphate depletion zone that quickly develops around the root (Smith and Read, 1997). However these fungi can also provide many other benefits to the host as: increased resistance to foliar-feeding insects (Wearn and Gange, 2007), improved drought resistance (Augé *et al.,* 2001), increased resistance to soil pathogens (Filion *et al.*, 1999; Borowicz, 2001; Idoia *et al*., 2004) and increased tolerance to salinity and heavy metals (Al-Karaki *et al*., 2001; Mohammad *et al*., 2003). Increased uptake of macronutrients other than P, including nitrogen (N) potassium (K) and magnesium (Mg) has also been measured (Smith and Read, 1997; Clark and Zeto, 2000) as well as increased uptake of some micronutrients (Ryand and Angus, 2003; Cho *et al*., 2009). Thus in face of their effects on plant fitness and soil quality, AMF may play an important role in ecosystems functioning, since their appropriate management can reduce the use of chemical and energy in agriculture and consequently lead to a more economical and sustainable production systems (Barea *et al*., 2005). Therefore, it's important to increase our understanding about the ecological functioning of these mutualistic microbial symbionts, in order to facilitate its manipulation as inoculants because not all plants species form AMF associations and not all AMF plant show an evident nutritional improvement from colonisation by AMF under all growth conditions (van der Heijden *et al*., 1998; Hart and Reader, 2002).

AMF show varying degrees of host specificity, even in colonisation ability or functioning, since the outcome of the symbiotic interaction is affected by factors related to host plant and fungal symbiont genotypes (van der Heijden *et al*., 1998; Hart and Reader, 2002).

The symbiotic performance of AMF isolates depends on two main parameters: colonisation ability and efficiency. The rate of colonisation is influenced by the ability of AMF to spread rapidly and extensively in plant roots, and is affected by factors linked

to spore germination, presymbiotic mycelial growth and appressorium formation (Hart and Reader, 2002). However, the ability of particular AMF genotype to infect and colonise root systems may depend on the presence of particular host plant species (Croll *et al*., 2008). Hart and Reader (2002) found that Acaulosporaceae isolates were superior only in presence of *Poa annua*, a plant species considered as unresponsive to AMF (Gange *et al*., 1999; Smith *et al*., 1999). This suggests that host genotypes influence the response to mycorrhizal symbiosis both in terms of plant growth or protections against pathogens (Azcón-Aguilar *et al*., 2002). Thus, depending on the species of plant two general anatomical groups can be distinguished in mycorrhizal colonisation, namely *Arum-type* and *Paris-type* (Smith and Read, 1997; Brundrett, 2004; Peterson *et al*., 2004). In the *Arum*-type arbuscular mycorrhiza, the hypha that penetrates into the epidermis generally forms a coil either in the epidermal cell or first cortical cell layer before it enters the intercellular spaces of the cortex. In this type, roots can become rapidly colonized along the root axis due to the free movement of hyphae in the intercellular spaces. In the *Paris*-type mycorrhiza, the intraradical hyphae pass from cell to cell, forming complex coils in both epidermal and cortical cells. Species of plants developing *Paris*-type mycorrhizas lack conspicuous intercellular spaces in the root cortex so that the growth of hyphae in the longitudinal direction of the root is slower than in the *Arum*-type (Smith and Read, 1997; Brundrett, 2004; Peterson *et al*., 2004).

Turn, efficiency of colonisation is correlated with the ability of different isolates to promote plant growth by improving mineral nutrition and increasing tolerance to biotic and abiotic stresses (Giovannetti and Avio, 2002; Jakobsen *et al*., 2002). There is evidence that intra-family variation in mycelium size and host specificity, may affect the relationship between mycelial size and host benefit, indicating that family with the largest internal mycelium (Glomaceae) confer the most host benefit. (Hart and Reader, 2002). Furthermore, Koch *et al*. (2004) obtain 16 different isolates of one AMF species, *Glomus intraradices*, from one site. They observed that these isolates (which were genetically different from each other) varied from each other in that they produced different amounts of hyphae. Therefore in face to exposed, plant communities may often include species with varying response to the presence of AMF. According to their response to mycorrhizal colonisation, they may be classified in 3

categories (Brundrett and Abbott, 2002), i.e. i) obligate mycorrhizal: are defined as those which will not survive to reproductive maturity without being associated with mycorrhizal fungi in their natural habitats; ii) facultative mycorrhizal: plants that benefit from mycorrhizas only in infertile soils or iii) non-mycorrhizal: plants with roots that are resistant to colonisation by mycorrhizal fungi.

Mycorrhizal dependency is defined by Gerdemann (1975) as 'the degree to which a plant is dependent on the mycorrhizal condition to produce its maximum growth on yield at a given level of soil fertility'. Growth responses to AMF associations are measured by comparing the growth of plants with and without mycorrhizas in a particular soil. (Brundrett and Abbott, 2002). The morphological as well as physiological properties of root system can influence the P absorption of plants in soil, and therefore the beneficial effects observed on mycorrhizal plants. Therefore, root architecture and morphology has been as one of the most sensitive indicators of plant response to mycorrhizal. Plants with facultative or non-mycorrhizal mycorrhizal associations normally have much finer roots and longer root hairs than obligate mycorrhizal species. Since relatively less energy is directed to root formation, mycorrhizal plants showed a greater shoot production, and consequently a lower root:shoot ratio in response to a greater increment of shoot mass relative to root mass (Azcón-Aguilar and Barea, 1997).

However, despite these discrepancies between different plant growth responses to AMF, there is some evidence that it may be temporally variable and age-dependent (Dodd *et al*., 2000; van der Heijden *et al*., 2006). Santo-González *et al*. (2007) noted in semi-natural grassland that AMF community not only varied significantly among *Prunella vulgaris* and *Antennaria dioica*, but also through growing season. *P. vulgaris* showed a high AMF diversity along the entire growth season while the level of AMF richness associated to *A. dioica* decreased dramatically in autumn. This pattern of host specificity has been observed in many systems, including agricultural fields (Douds and Miller, 1999). Accumulating evidence indicates that mycorrhizal associations can be important determinants of diversity in ecosystems and can modify the structure and functioning of plant communities in complex ways (Grime *et al*. 1987; Wilson and Hartnett, 1997; Van der Heijden *et al*. 1998; Hartnett and Wilson 1999; Eom *et al*. 2000; Hart *et al*., 2003).

Grime *et al.* (1987) found that community structure could be significantly altered by mycorrhizal activity in species-rich mixtures of plants in experimental microcosm. The presence of mycorrhizas can increase floristic diversity (Grime *et al.*, 1987) and species richness (Gange *et al*., 1990). However, this may depend on identity and mycorrhizal responsiveness of dominant plant species (Smith *et al*. 1999; O' Connor *et al*., 2002; Vogelsang *et al*., 2006; Jansa *et al*., 2008)). Consequently, host-specific changes in the AMF community could lead to increases in the relative growth rates of the most abundant plant species (i.e., positive feedback) or to decreases in the relative growth rates of the most abundant (i.e., negative feedback). These two dynamics lead to very different predictions for the community. Positive feedback causes strengthening of the mutualism between plant and fungal guilds, but a decline in species diversity. Negative feedback causes a weakening of the mutualism, but can contribute to the coexistence of competing plant species (Bever, 2002; Hart *et al*., 2003). Then, differential responses of AMF to host plant species may play an important role in regulation of plant intraspecific interactions as well as in the composition of plant communities (Hartnett and Wilson, 1999; Smith *et al.*, 1999). Smith *et al*. (1999) reported that removal of the dominant warm-season C_4 grass species in tall prairie, which exhibits high mycorrhizal responsiveness, increased plant diversity, once the dominant species declined in abundance, and number of subdominant cool-season C_3 grass and forb species increased in abundance. Thus AMF may affect plant community indirectly by influencing the pattern and strength of plant competitive interactions. Eom *et al*. (2000) also point out that the different level of responses of host plant species to mycorrhizas may result in differences in AMF community structure. The same authors report that four of the host plant species examined in their studies, *Solidago missouriensis* Nutt*., Baptisia bracteata* Muhl. ex Ell., *Panicum virgatum* L., and *Sporobolus heterolepis* (A. Gray) A Gray*,* are obligate mycotrophs and are highly responsive to mycorrhizal colonisation. By contrast, *Poa pratensis* L. is non-mycorrhiza-dependent and shows no significant growth response to mycorrhizal colonisation. O' Connor *et al*. (2002), also found that plant species diversity increased when mycorrhizal colonisation was suppressed because growth of the highly mycorrhiza-responsive dominant *Medicago minima* was reduced and subordinate plant species were released from competition. More recently, Karanika *et al*. (2008) argued that AMF suppression declined the growth of subordinate, more mycotrphic and P demanding perennial forbs and most legumes with compensatory

increases of the less P demanding *Doricnium herbaceum* L. and less mycotrophic and P demanding C_3 grass. Grasslands can be characterized by the composition of their plant community (Rodwell, 1992) with unimproved (typically *Agrostis*, *Festuca* dominated), semi-improved (*Holcus* dominated) and improved *(Lolium* dominated) grasslands often found adjacent to each and maintained through different levels of management (particularly inputs of lime and fertiliser). In the Azores, upland semi-natural and intensive pastures are usually dominated by the grass species *Holcus lanatus*. In contrast, *L. multiflorum* and *L. perenne* are characteristic of lowland improved pastures receiving regular imputs of fertilizer (Dias, 1996; Borges, 1999). These three plant species are C_3 and their mycorrhizal responses may range from facultative to non mycorrhizal plants (Wilson and Hartnett, 1998).

Mycorrhizal dependency among species has potentially important applications to agroecosystems functioning and management. The purpose of current study was to assess patterns of interspecific variation in host-plant benefit (i.e., growth response) from the symbiosis with AMF among the three most abundant grasses used in pastures of the Azores.

2. Material and methods

2.1. Study plants

The Azorean pastures are dominated by *Holcus lanatus* L. and *Lolium perenne* L. (Dias, 1996; Silva and Smith, 2006). These two grasses are among the thirty non-indigenous vascular plants with high importance value, whose frequencies in pastures are 50.5% and 54.2 % respectively to *L. perenne* and *H. lanatus* (Silva and Smith, 2006). Although less dominant, *Lolium multiflorum* Lam. is also widely distributed along the nine islands of the Azores archipelago (Silva *et al.,* 2005). *L. multiflorum* is usually used in crop rotation with *Zea mays* L. in lowland pastures during the winter given its high productivity and diseases resistance under adverse climatic conditions (Fernandes *et al*., 2006).

2.2. Arbuscular mycorrhizal fungi collection and culture

In July of 2006, soil was collected from the rhizosphere of ten different *H. lanatus* plants in Pico Galhardo (PX), Terra Brava (TB), Agualva RP1 (RP1) and Agualva

(RP2) and used to set up trap cultures of the AMF community. The fresh soil samples were mixed with autoclaved sand and "bagacina" (volcanic soil type) in a ratio of 2:1:1. A 1.5-kg aliquot of the mixture was placed in each pot to produce trap culture from each sample. Corn (*Zea mays* L.) was used as host plant. Seeds of corn were surface sterilised by immersion in alcohol (96 %) for 30 sec, 4 % bleach for 2 min, and rinsed twice in sterile distilled water. Then, they were sown in pots on sterilized sand soil and left to germinated in the greenhouse. After one week of growth, seedlings were transplanted to pots containing the fresh soil samples mix. A total of 4 seedlings were used per plot. Trap cultures in 40 plots were grown in glasshouse for 5 months and then harvested. All pots were watered every 2 days with distilled water, and no nutrient solution was added to the pots. One month before harvested, we left watering the pots to stimulate the sporulation.

2.3. AMF root colonisation assessment

After 5 months, plants were harvested and root examined to confirm AMF colonisation. A portion of roots was cleared in 2.5% KOH for 1h at 90 ˚C. Subsequently roots were left to acidify overnight in 1% HCl. Staining was done with blue ink (Parker Quink) for 30 min at 60 ˚C, followed by distaining in lactoglycerol. The amount of colonisation was estimated using a grid-intersect method with examination of 100 intersects under a compound microscope at 200x magnification (McGonigle *et al*., 1990). Root-intersects that contained vesicules, arbuscules or hyphae were scored as mycorrhizal. The remaining corn roots were cut into 2-cm pieces, and disinfected by immersion in 2 % chloramines T for 3 min and in an antibiotic solution (streptomycin 200 mg l^{-1} + penicillin 100 mg l^{-1}) for 3 h. Then, roots were rinsed in autoclaved water and dried (de la Peña *et al*., 2006).

2.4. Spores assessment

AMF spores produced in the trap culture were extracted by sieving and decanting (Gerdemann and Nicolson, 1963). The procedure included the passage of 50 cm^3 of harvested trap culture substrate from each pot through 1000-, 500-, 250-, 100-, and 45 µm sieves. The material retained in 250 µm, 100 µm and 45 µm was collected to different petri dishes and AMF spores were counted under a stereoscopic microscope

(Leica MZ 12). Spores from 4 genera were detected in the trap culture, namely *Acaulospora*, *Ambispora*, *Glomus* and *Scutellospora.*

2.5. Experimental design

The experiment was performed with the three grasses with the widest application in the pastures of the Azores, i.e. *H. lanatus*, *L. multiflorum* Lam and *L. perenne.* Seeds of these three plant species were surface sterilised by immersion in 96% alcohol for 30 sec, 0.4% sodium hypochlorite for 2 min and rinsed twice in sterile distilled water. They were then sown in boards on a sterilised sand soil and left to germinate in greenhouse. After 2 week of growth, seedlings were transplanted to the pots. Four seedlings of each plant species were planted in 2-l pots filled with a pasteurized substrate contained, soil, sand and “bagagina” (volcanic soil type) in a ratio of 2:1:1 soil. To each plant species was made two treatments with 14 replicates per treatment: an uninoculated control (C) and an inoculation with a mixture of native arbuscular mycorrhizal fungi (AMF) (Fig. 1). The experience included a total of 84 pots.

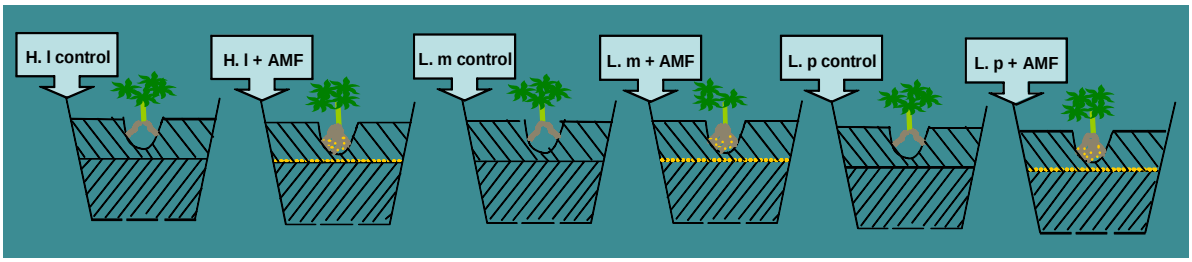

Figure 1. Experimental design in which: H.L – *H. lanatus*; L.m. - *L. multiflorum* and L.p. - *L. perenne;* AMF - arbuscular mycorrhizal fungi.

At the time of transplantation each pot containing 4 seedlings of each plant species for inoculation with AMF, received 250 spores and 1g of dried corn roots from trap cultures, which was used as AMF inoculum. Pots were covered by a volcanic ash layer in order to keep the substrate humid and avoid the contamination, and then arranged in the greenhouse in a completely randomised design. Every 2 days pots were watered and once per month they were fertilized with 50 ml of half-strength modified (P-free) Hoagland’s solution. The experiment was conducted from June 2007 until October 2007.

2.6. Harvest and data collection

Plants were harvested 16 weeks after mycorrhizal inoculation. The following physical parameters were measured: fresh root weight, aerial fresh root weight, total fresh root weight (aerial and root) and aerial dry weight. After taking the root fraction for assessing AMF colonisation (5% in fresh weight), the remaining plant material was dried at 72 ºC for 48 h to estimate plant biomass. AMF colonisation was determined by the same method used to assess mycorrhizal infection in *Z. mays* roots from trap culture (see above).

2.7. Data analysis

The statistical analysis was performed with one-way ANOVA (Minitab, version 13.31, 2000) to compare between the 3 plant species species (*H. lanatus*, *L. multiflorum* and *L. perenne*) for percentage colonisation of all mycorrhizal structures. These data were arcsine (x/100) transformed to reduce the variance in the data. We also used a two-way ANOVA to test for treatment (non-mycorrhizal plants - control and mycorrhizal plants -AMF) and plant species (*H. lanatus*, *L. multiflorum* and *L. perenne*) and all interactions on the fresh root weight, aerial fresh weight, total fresh weight and aerial dry weight. For *post hoc* comparison we used the Tukey's multiple range tests (Minitab, version 13.31, 2000). Mycorrhizal dependency (DM) was calculated for each plant species as follows. Percentage mycorrhizal responsiveness 5 [(dry mass mycorrhizal plant 2 dry mass nonmycorrhizal plant)/dry mass mycorrhizal plant] 3 100 (Hetrick *et al*.,1992).

Pearson correlations coefficients were calculated between mycorrhizal root colonisation and plant growth parameters (fresh root weight, aerial fresh weight, total fresh weight and aerial dry weight), as well as, between mycorrhizal dependency (DM) and root colonisation.

3. Results

3.1. Mycorrhizal colonisation

Non-mycorrhizal control plants showed no colonisation. The AMF root colonisation was well establish and ranged between 60 % and 30 % in *H. lanatus* and *L. multiflorum* respectively, and most of the observed fungal structures corresponded to hyphae and vesicles (Table 1; Fig. 1). Furthermore, percentage of root colonisation by AMF was strongly affected by the host plant. Thus, we found that AMF total colonisation varied significantly with plant spp. (Anova: F= 23.17; d.f.= 2, 39; p< 0.001). The Tukey HSD *post hoc* test showed that average percentage of AMF total colonisation was significantly higher in *H. lanatus* ($\bar{x}$ = 0.62 ± 0.035) than in *L. perenne* ($\bar{x}$ = 0.40 ± 0.041) and *L. multiflorum* ($\bar{x}$ = 0.31 ± 0.025). Moreover, there were no significant differences in average percentage of AMF total colonisation between *L. multiflorum* and *L. perenne* (Fig. 2 a).

Table 1. *F* - and *P*- values from ANOVA of dependent variables (AMF total colonisation, AMF hyphal colonisation, AMF vesicle colonization and AMF arbuscule colonisation) to significance between plant spp. (*H. lanatus*, *L. multiflorum* and *L. perenne*). * P < 0,05; ** P < 0,01; *** P < 0,001; n.s= not significant.

Source	*F*	*p*
AMF total colonisation	23.17	***
AMF hyphal colonisation	7.01	**
AMF vesicle colonisation	12.78	***
AMF arbuscule colonisation	6.79	**

The percentage of AMF hyphal colonisation also varied significantly among plant species. (Anova: F= 7.01; d.f.= 2, 39; p= 0.003) (Table1). The Tukey HSD *post hoc* test revealed significant differences in average of AMF hypal colonisation among the 3 plants inoculated, which was significantly higher in *H. lanatus* ($\bar{x}$ =0.43 ± 0,035) than in the *L. multiflorum* ($\bar{x}$ = 0.27 ± 0.021), but did not differ significantly from *L. perenne* ($\bar{x}$ = 0.39 ± 0,032) (Fig. 2 b).

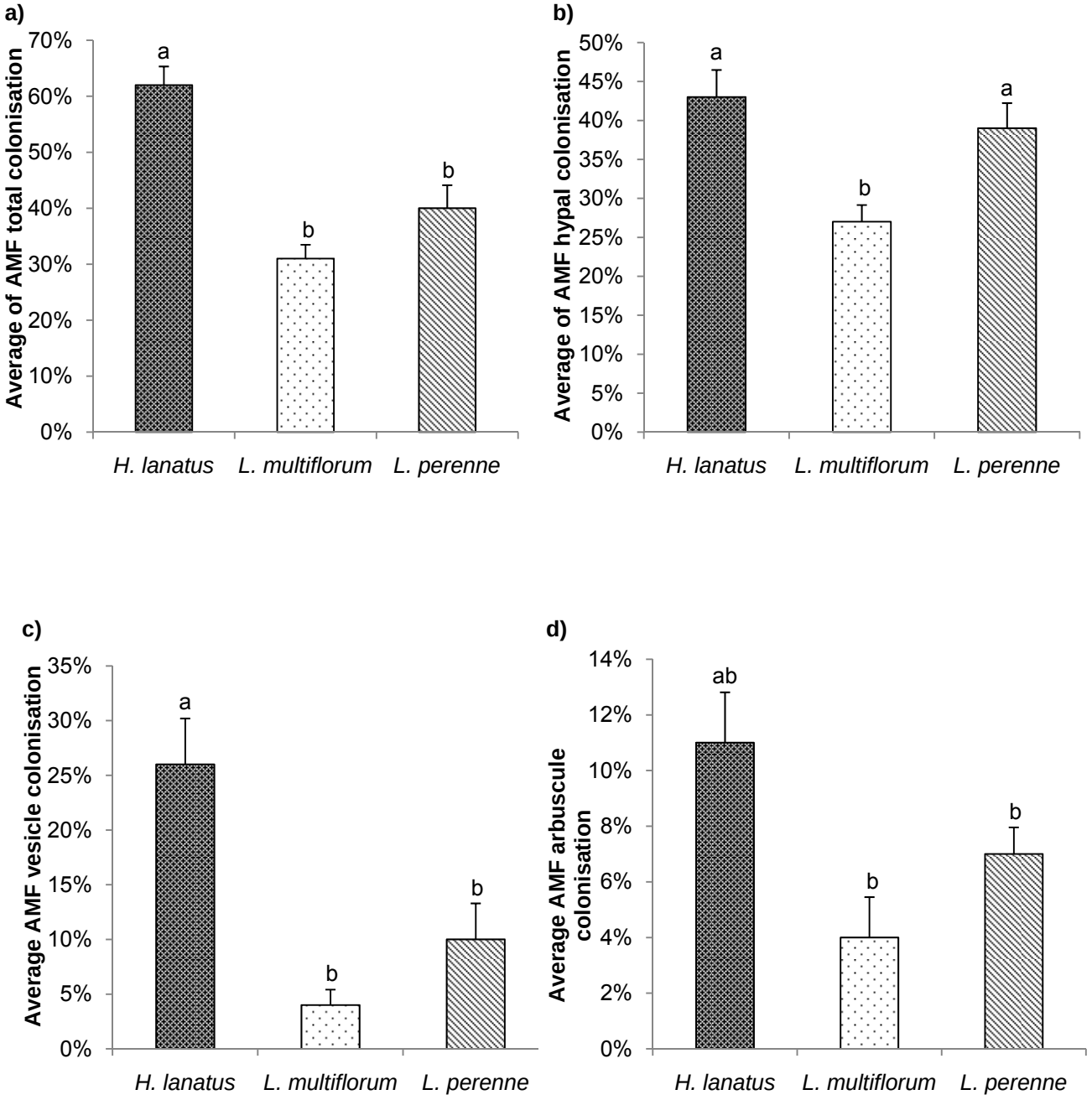

Figure 2. Average percent of total AMF, hyphae, vesicles and arbuscules colonisation from *H. lanatus*, *L. multiflorum* and *L. perenne*. Standard errors of the mean (n= 14) are shown. Values of percent are arcsine-transformed. For each parameter, different letters above the bars indicate significant differences between treatments after two-way ANOVA and Tukey's HSD test (P≤ 0.05). ANOVA *P*-values are presented in Table 1.

The same result was obtained with the percentage of AMF vesicles colonisation, which differed significantly with the host plant (Anova: F= 12.78; d.f.= 2, 39; p< 0.001) (Table 1). In fact, vesicles were present in approximately half of the micorrhizal colonisation of *H. lanatus*, hence this plant species had significantly greater average percentage of AMF vesicles colonisation ($\bar{x}$ =0.26 ± 0.042) than *L. perenne* ($\bar{x}$ =0.10 ± 0.033) or *L. multiflorum* ($\bar{x}$ = 0.04 ± 0.014) (Tukey multiple comparison test p< 0.05) (Fig. 1 c). However, no significant differences were found between *L. perenne* and *L. multiflorum* (Fig.2 c).

The percentage of arbuscule colonisation followed the same trend, i.e. varied significantly with the plant species (Anova: F= 6.79; d.f.= 2, 39; p= 0.003) (Table 1). The Tukey HSD *post hoc* test showed that average percentage of AMF arbuscule colonisation was significantly higher in *H. lanatus* ($\bar{x}$ = 0.11 ± 0.018) than in *L. multiflorum* ($\bar{x}$ = 0.04 ± 0.015), but did not differ significantly from *L. perenne* ($\bar{x}$ = 0.07 ± 0.010) (Fig. 2 d).

3.2. Mycorrhizal dependency

L. perenne showed the greater response to mycorrhizal colonisation compared with the remaining plant species. Mycorrhizal dependency (MD) values were about 62 %, 40 % and 27 %, respectively, in *L. Perenne*, *H. lanatus* and *L. multiflorum*. Moreover, the relationship between mycorrhizal dependency and root colonisation was significantly positive (*r= 0.306,* P< 0.05).

3.3. Effects of AMF species on plant growth

The importance of native AMF inoculation on aerial biomass production of *H. lanatus*, *L. multiflorum* and *L. perenne* seedlings are displayed on Figure 3.

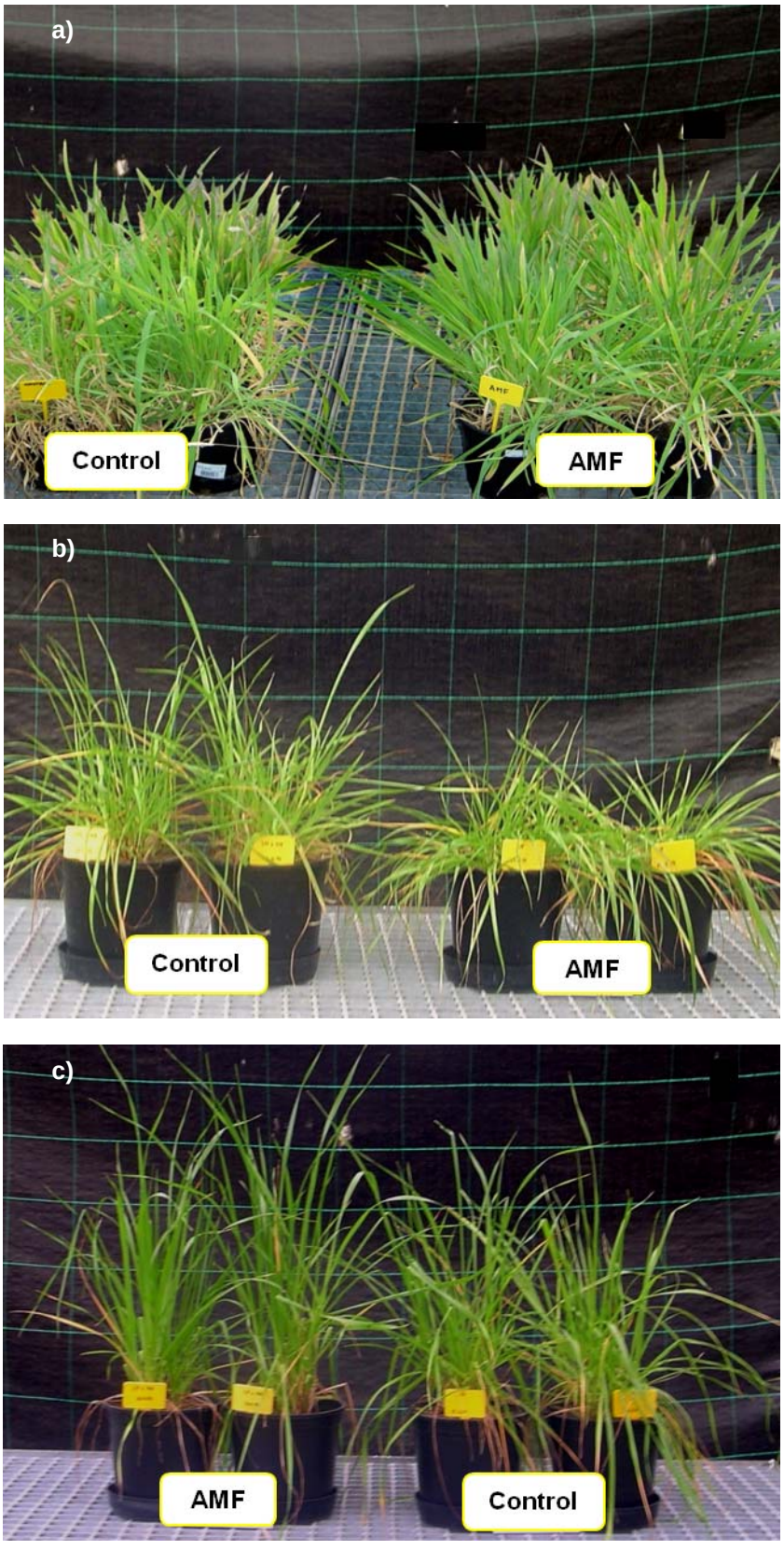

Figure 3. Experimental inoculation. *H. lanatus* mycorrhizal (AMF) and control plants (a); *L. multiflorum* mycorrhizal (AMF) and control plants (b); *L. perenne* mycorrhizal (AMF) and control plants (c).

The two-way Anova showed that the type of treatment as well as the plant species had a significant influence on plant parameters (Table 2).Thus there were highly significant effects of treatments (two-way Anova: F= 21.87; d.f.= 1, 78; p< 0.001), plant species (two-way Anova: F= 34.70; d.f.= 2, 78; p< 0.001) and the interactions between these 2 factors (two-way Anova: F= 10.46; d.f.= 2, 78; p< 0.001) on the fresh root weight (Table 2). The fresh root weight was negatively correlated with AMF root colonisation (*r= - 0.493,* p< 0.001). In fact, *L. perenne* control plants ($\bar{x}$ = 86.44 ± 2.74) had the highest fresh weigh root, and *H. lanatus* mycorrhizal plants ($\bar{x}$ = 52.63 ± 3.04) as well as, the *H. lanatus* control plants ($\bar{x}$ = 54.83 ± 3.71) the lowest fresh root weight (Tukey multiple comparison test p< 0.05) (fig. 4 a). However, fresh root weight of *L. perenne* control plants did not differ significantly from the *L. multiflorum* control ($\bar{x}$ = 81.99 ± 3.28) or mycorrhizal plants ($\bar{x}$ = 76.62 ± 2.95) (Tukey multiple comparison test p< 0.05) (fig. 4 a).

Table 2. *F* - and *p*- values from two-way ANOVA of dependent variables (fresh root weight, aerial fresh weight, total fresh weight and aerial fresh weight) to significance between treatment (control and AMF), plant spp. (*H. lanatus*, *L. multiflorum* and *L. perenne*) and interaction treatment x plant spp. * P < 0,05; ** P < 0,01; *** P < 0,001; n.s= not significant.

Main effects	Treatment		Plant spp.		Treatment x Plant spp.	
	F	*p*	*F*	*p*	*F*	*p*
Fresh root weight	21.87	***	34.70	***	10.46	***
Aerial fresh weight	45.81	***	36.85	***	12.15	***
Total fresh weight	4.33	*	5.63	**	2.58	n.s
Aerial dry weight	55.78	***	55.56	***	14.03	***

The same result was also observed in relation to aerial fresh weight, which also was significantly affected by the 2 main factors in question, as well as, by the interaction between them. This way, there were greater significant effects of treatment (two-way Anova: F= 45.81; d.f.= 1, 78; p< 0.001), plant species (two-way Anova: F= 36.85; d.f.= 2, 78; p< 0.001) and the interaction among these 2 factors (two-way Anova: F= 12.15; d.f.= 1, 78; p< 0.001) in aerial fresh weight among control plants and mycorrhizal plants (Table 2).

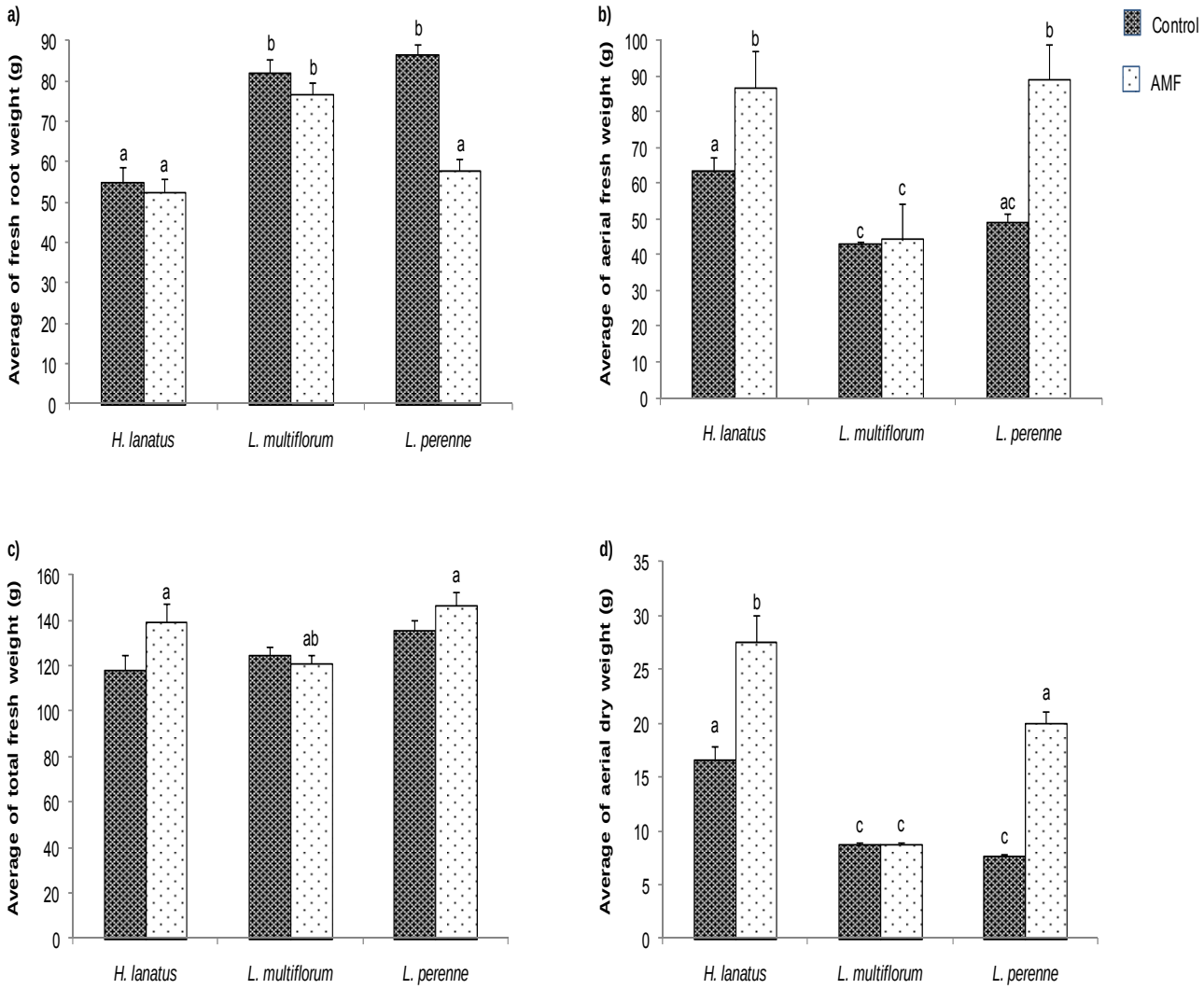

Figure 4. Experimental inoculation. Average of fresh root weight (a), aerial fresh weigh (b), total fresh weight (c) and aerial dry weight (d) from *H. lanatus, L. multiflorum and L. perenne* in non-mycorrhizal (control) and mycorrhizal (AMF) treatments. Standard errors of the mean (n= 14) are shown. For each parameter, different letters above the bars indicate significant differences between treatments after two-way ANOVA and Tukey's HSD test (P≤ 0.05).). ANOVA *P*-values are presented in Table 2.

The aerial fresh weight was significant and positively correlated with AMF root colonisation (*r= 0.375,* P< 0.05). Thus, the aerial fresh weight of *L. perenne* mycorrhizal plants ($\bar{x}$ = 89.13 ± 6.66) was significantly higher than *L. multiflorum* mycorrhizal plants ($\bar{x}$ = 44.33 ± 1.28) and control ones ($\bar{x}$ = 42.88 ± 0.94) (Tukey multiple comparison test p< 0.05), but was identical to the aerial fresh weight of *H. lanatus* mycorrhizal plants ($\bar{x}$ = 86.92 ± 5.05) (Tukey multiple comparison test p< 0.05) (Fig. 4 b).

The total fresh weight was affected by the presence/absence of AMF and by species of plants (Table 2). Then, there were significant differences between the 3 species of plants (two-way Anova: F= 5.63; d.f.= 2, 78; p< 0.01) (Table 2) on total fresh weight, which was significantly more high in *L. perenne* ($\bar{x}$ = 141.30 ± 3.78) than in *L. multiflorum* ($\bar{x}$ = 122.91 ± 2.64), but it was identical to *H. lanatus* ($\bar{x}$ = 128.90 ± 5.42) (Fig. 4 c). The total fresh weight also varied significantly between the 2 treatments performed, but much less markedly (two-way Anova: F= 4.33; d.f.= 1, 78; p< 0.05) (Table 2). In fact, the total fresh weight was higher in mycorrhizal plants ($\bar{x}$ = 135.79 ± 3.81) than in control plants ($\bar{x}$ = 126.28 ± 3.08) (Fig. 4 c). However, there were no significant differences of the interaction between the 2 main factors on total fresh weight (two-way Anova: F= 2.58; d.f.= 2, 78; p= 0.08) (Table 2).

The aerial dry weight was also significantly affected by the treatments (two-way Anova: F= 55.78; d.f.= 2, 78; p< 0.01), plant species (two-way Anova: F= 55.56; d.f.= 2, 78; p< 0.01) as well as, by the interactions between these 2 factors (two-way Anova: F= 14.03; d.f.= 2, 78; p< 0.001) (Table 2). Again, the effect of AMF was evident on this parameter, i.e. aerial dry weight was significant and positively correlated with the AMF root colonisation (*r= 0.534, p*< 0.001). In fact, the aerial dry weight of *H. lanatus* mycorrhizal plants was significantly higher than the others combinations ($\bar{x}$ = 27.50 ± 2.58) (Tukey multiple comparison test p< 0.05), and the opposite was observed in relation to aerial dry weight of *L. multiflorum* which was significantly lower in non-mycorrhizal plants ($\bar{x}$ = 8.69 ± 0.20) as well as in presence of AMF ($\bar{x}$ = 8.71 ± 0.25) (Tukey multiple comparison test p< 0.05) (Fig. 4 d).

4. Discussion

The establishment of the AMF in the root cortex is known to change many key aspects of plant physiology. These include the mineral nutrient composition of plant tissues, the hormonal balance, and the patterns of C allocation. Therefore, the AMF symbiotic status changes the chemical composition of root exudates, while the development of an AMF soil mycelium, which can act as a carbon source for microbial communities, introduces physical modifications into the environment surrounding the roots. AM-induced changes in plant physiology affect the microbial populations, both quantitatively and qualitatively, in either the rhizosphere and/or the rhizoplane. Therefore, the rhizosphere of a mycorrhizal plant can have features that differ from those of a non-mycorrhizal plant (Barea *et al*. 2005).

Root colonisation percentage

The three plant species used in this study showed multiple colonisation morphology, i.e. *Arum-type* and *Paris-type*. Thick wall of intercellular hyphae with projections and H connections and intercellular vesicles (*Arum-type*), as well as, intercellular coils and terminal arbuscules (*Paris-type*) were observed in the three host plant (Smith *et al*., 2003). On the other hand, the three plant species displayed different, but well described physiological alterations as a result of mycorrhizal colonisation.

In fact, the percentage of colonisation of almost all AMF structures was significantly higher in *H. lanatus* followed by *L. perenne* and *L. multiflorum*. Thus *H. lanatus* and *L. perenne* behaved as a typical mycorrhiza-responsive plant species, having relatively high levels of mycorrhizal dependency (40 % and 61 % respectively). Conversely*, L. multiflorum*, was overall less responsive to mycorrhizal colonisation, whereby it had lower mycorrhizal dependency (approximately 27 %). Smith and Read (1997) reported that the extent to which typical AMF colonise root systems varies with species of plants. Also, Bever (2002) showed in a microcosm experiment evident differences of host-specific in the AMF communities between 4 host plants (*P. lanceolata*, *A. odoratum* and *P. sphaerocarpon*). He reported that biovolume of AMF spores after the first generation was great with *Allium* and least with *Anthoxathum*. On the other, it was also noted that there are differences in the extent of infection between genotypes of the same species (Burleigh *et al*., 1998; 2002; Croll *et al*., 2008). Burleigh *et al*. (2002)

demonstrated that large differences may exist between plant species in how they respond at molecular level to AMF colonisation, since seven species on AMF varied widely in their influence on the root expression of the Mt4 gene from *Medicago truncatula*, whose expression is down-regulated by both phosphate starvation and mycorrhizal colonisation (Burleigh *et al*., 1998) as well as on the expression of LePT1 and TPSI1 of *Lycopersicum esculentum*. The same author noted that *M. truncatula* colonisation by *Glomus mosseae* resulted in the greatest reduction in MtPT2 and Mt4 gene expression and the highest level of P uptake and growth, but the opposite occurred in *M. Truncatula* root colonisation by *Gigaspora rosea*. However, little variation was detected in the expression of LePT1 and TPSI1 within roots of the seven mycorrhizas. Mathimaran *et al*. (2005) observed that host plant was an important determinant of AMF species composition, once *Glomus intraradices* and *Glomus caledonium* showed significant preferences to leek and sunflower respectively. Recently, Croll *et al*., (2008) found that among four different trap host species (*Allium porrum*, *Plantago lanceolata*, *Glycine max* and *Helianthus annuus*) the number of AMF genotypes was significantly higher in *A. porrum* and *H. annus* both with 9 isolates, than in *G. max* and *P. lanceolata* with 4 and 1 isolates respectively. They concluded that this fact could provide in field site a heterogeneous environment, since host plant may encourage certain genotypes.

Plant productivity

Mycorrhizal colonisation affects a wide range of morphological parameters in development root systems, a greater root branching being the most described effect (Regvar *et al*., 2003; Cho *et al*., 2009). Sohn *et al.* (2003) reported at transplanting stage of chrysanthemum plant (*Chrysanthemum morifolium* Ramat), that inoculation of AMF resulted in greater height, number of lateral roots, tap root length, and fresh and dry weight of shoots and roots compared to non-AMF treatment. Turkmen *et al*. (2008) noted that fresh and dry weights of shoots and roots of pepper seedlings were increased in presence of AMF species. However, our result did not agree with the previous work, we detected a decrease on fresh root weight of the 3 plants species in the mycorrhizal treatment compared to the control. A similar result was also found by Azcón and Ocampo (1981) who reported that mycorrhizal dependency among 13 wheat cultivars decreased as the root weight increased. Also Saif (1987) noted that

inoculation with mycorrhizal fungi favored the development of shoots more than roots, leading to a significant decrease in the root/shoot ratio of fourteen host plants. According to Azcón-Aguilar and Barea (1997) plants forming mycorrhizae tend to have a lower root:shoot (R:S) ratio, which means a greater biomass efficiency, since less energy is directed to root formation. Using micropropagated pineapple, Guillemin *et al*. (1992) were even able to demonstrate the existence of a negative correlation between the mycorrhizal effects on root and shoot development. According to the authors, the higher the root:shoot ratio, the less efficient the system for shoot production. This mycorrhizal effect on root development is partly due to architectural changes in the root system, as has been demonstrated in particular for micropropagated woody species such as grapevine (Schellenbaum *et al*., 1991) and *Prunus* (Berta *et al*., 1995). These studies show that mycorrhiza formation changes root topology from a herringbone pattern to a more dichotomous pattern, the latter being more efficient for scavenging of the soil nutrients. Pacovisky *et al*. (1986) reported that P-deficient plants lacking AMF symbiosis tend to have a high root:shoot ratio, which is usually associated with nutrient-stressed plants. More recently, van der Heijden *et al.* (2006) also noted in an experimental grassland microcosm with different AMF taxa and host plants, that root biomass was, on average, 26 % higher in control microcosm compared with microcosm inoculated with AMF.

It has been well documented that morphology of root is extremely important factor in determining mycorrhizal dependency. Plants with roots that are comparatively thick, with little branching and few or no root hairs tend to show greater positive growth responses to mycorrhizal colonisation than plants with fine, highly branched long roots with numerous root hairs (Hetrick *et al*., 1992; Wilson and Hartnett, 1998). Research has showed that the length and the density of roots hair are indicator of the mycorrhizal dependency (MD) of plants species or cultivar (Janos, 2007). Our result followed this trend, since fresh root weight was negatively correlated with AMF root colonisation, i.e. *L. multiflorum* had the largest root biomass and it was less mycorrhizal dependent than *H. lanatus* and *L. perenne*. Moreover, a significant correlation between root colonisation and mycorrhizal dependency was observed which may suggest that plant that do not rely on the symbiosis regulate mycorrhizal colonisation in some way and, thus, control carbon expenditure of the fungus (Wilson

and Hartnett (1998). In fact, the three grasses are described as cool-season (C_3), and particularly *L. multiflorum* was the less responsive and also the less colonised by the fungi.

The symbiosis with arbuscular mycorrhizal fungi (AMF) increases the performance of plants by improving nutrient acquisition, resulting in positive effects on growth and reproduction (Smith and Read 1997). In fact, the intensity of root mycorrhizal colonisation, which quantify the biomass of AMF in plants roots significantly differ between the 3 grasses. The high root colonisation observed in *H. lanatus* as well as in *L. perenne*, although less marked, promoted a significant increase in aerial fresh root, total fresh weight as aerial dry weight. This result could be associated with the increase of nutrient uptake by mycorrhizal roots. As the colonisation rate of roots increases, a mycorrhizal root system can explore a bigger soil extension. This result was consistent to previous works, among them Jaizme-Vega and Azcón-Aguilar (1995) who reported that growth of some tropical and subtropical cultures, like avocado, papaya, pineapple and banana, improved when they were inoculated with selected AMF during the first phase of growth. Likewise, Regvar *et al*. (2003) showed a significant increase on biomass parameters of pepper and parsley as a result of mycorrhizal inoculation.

Guissou (2009) also found a significant increase in shoot growth, total dry weight and nutrient uptake in seedlings of jujube (*Ziziphus mauritiana* Lam.) plants as result of the high rate of root colonisation observed in this plant.

Conversely, significantly lower total aerial fresh weight was recorded in *L. multiflorum* plants inoculated with AMF compared with non-mycorrhizal plants. Reduced growth as a result of mycorrhizal colonisation was also observed in some *Tagetes* cultivars (Linderman and Davis, 2004). No effect of root inoculation with *Glomus intraradices* on shoot biomass was earlier recorded in *Callistephus chinensis* grown in P-deficient soil under field conditions (Gaur and Adholeya, 2005). It is well known that mycorrhizal colonisation can depress plant growth primarily by sink competition for photosynthates (Douds *et al*. 1998). Some studies have indicated that plant growth depression due to mycorrhizal colonisation is attributed to greater carbon expenditure in a colonised root system (Peng *et al*., 1993).

In field cultivation mycorrhizal roots can explore more soil volume than non-mycorrhizal ones, due to their extramatricial hyphae (Sawaki and Saito, 2001); it is impossible in pot culture in very limited volume of growing media. Results obtained on *Typha latifolia* (Dunham *et al*., 2003) also suggest that under greenhouse conditions, AMF act to reduce plant growth despite increased mineral nutrition and photosynthetic activity. On the other hand, the time period of seedlings could have been too short to record a higher colonisation percentage, and consequently their effects on aerial biomass, since the root system infected normally increases with time sigmoidally (Smith and Read, 1997). Adding, according to van der Heijden *et al.* (2006) plant growth responses to AMF were temporally variable and some species obtained the highest biomass with different year, thus it may be beneficial for plant to be colonised by different AMF taxa in different seasons. However, though *L. multiflorum* is a weakly mycotrophic plant, there is evidence that it can maintain a viable AMF community in monoculture even when more strongly mycorrhizal plants are lacking (Teutsch *et al*., 2006).

To sum up, the inoculation with a mix of native AMF inoculum increased plant productivity. This agrees with other studies which show that native AMF are important contributors to plant biodiversity and ecosystem productivity (van der Heijden *et al*., 1998; Requena *et al*., 2001, Klironomos, 2003; Regvar *et al*., 2003). Furthermore, our results indicate that mycorrhizal inoculated at the first phase of development of the plant production system confer the greatest benefit to these plant species (Jaizme-Vega and Azcón, 1995; Regvar *et al*., 2003). However, the three grasses responded differently to AMF inoculation indicating that AMF is host-dependent (Eason *et al*., 1999; Hartnett and Wilson, 1999; Bever, 2002). This result underlines to the impact that cropping with a weak or non-mycorrhizal crop may have on AMF colonisation, nutrient uptake and yield of subsequent AMF reliant crop, especially if it is highly mycorrhizal dependent (Douds *et al*., 1997; Miller, 2000).

Our findings are of crucial importance to the agriculture systems of the Azores archipelago, a region where more than 80 % of the useful agricultural surface (SAU) is occupied by permanent pastures, where *L. perenne* and *H. lanatus* are the dominants plant species (Dias, 1996; Borges, 1997; Lopes, 1999). In these systems we have

assisted to a loose of biological diversity caused by the increasing input of fertilizers and pesticides to respond to the needs of high number of animals per

hectare (“livestock”). Therefore, in face to the relevant mycorrhizal responses of *L. perenne* and *H. lanatus*, the appropriate management of this symbiosis could enable the reduction of chemical fertilizer and pesticide inputs, a key aim of sustainable grassland plant production approaches (Azcón-Aguilar and Barea, 1997).

CHAPTER VI. GENERAL CONCLUSIONS

General conclusions

Arbuscular mycorrhiza is an ancient symbiosis between the majority of land plants and fungi from the phylum Glomeromycota. Arbuscular mycorrhizal fungi (AMF) colonise plant roots and contribute to the mineral nutrient uptake of the hosts in exchange for carbohydrates. AMF species diversity and identity was reported to have a decisive influence on the composition and productivity of natural plant communities. Only around 200 glomeromycotan species described so far were thought to colonise the majority of higher plant species and thus, their host specificity was thought to be very low. Determining how AMF biological communities are assembled and the factors that influence its distributions are fundamental challenges in ecology.

It is well documented that agricultures practices can affect the structure of arbuscular mycorrhizal communities. These changes reflect not only in species diversity but also in species composition, with the most of studies indicating a decrease with the level of land use intensification. However, our study showed that diversity of AMF spores as well as the diversity of AMF on *Holcus lanatus* L. roots did not change between the semi-natural and intensive land uses. The absence of different patterns of AMF species diversity, between the two land uses types could be related with the low level of land use intensification. In fact, it is known that the input of fertilisers and pesticides and several other management practices (e.g. grazing intensity) are important in defining the quality of the pasturelands for AMF. However, the input of fertilisers to Azorean pastureland is lower than average inputs to pastures on the European mainland, which enables the occurrence of endemic species even in most intensive land uses. On the other hand, striking differences in AMF spore composition were found between semi-natural and intensive land uses. Species in *Glomus* were dominant in intensive pastures, not only as reproductive spores but also in roots of *H. lanatus*, which conferred its status of “generalist” species due its high ecological plasticity occurring in a wide range of habitats inclusive in the most disturbed. In contrast, *Acaulospora* and *Scutellospora* were the two most representative genera in semi-natural pastures. Many of these species appeared to prefer or even to be restricted to a land use intensity or field site, and thus characterised as “specialist”. This was the case for most species of *Acaulospora* sp. and *Ambispora* sp. only found in semi-natural land use, while *Glomus etunicatum* and *Paraglomus* sp. just found in intensive ones. This suggests that these species have specific and rather narrow

niches. Distinct colonisation strategies adopted by AMF, even at family level and nutrient availability could explain these differences on species composition between land uses. Members of Glomaceae have a highly infective extra-radical mycelium that could allow colonising immediately plant roots. Hence, *G. mosseae* and *G. etunicatum* dominance may be related to competitive advantages for carbon uptake, which make them the most effective competitor due to its fast rate of colonisation and early peak in colonisation. Moreover, *Glomus* forms anastomose between mycelia therefore has the ability to re-establish an interconnected network after mechanical disturbance. Conversely members of Gigasporaceae, those are only capable of propagation via spore dispersal colonise plant roots more slowly than members of Glomaceae.

In order to better understand the role of AMF in natural ecosystems, as well as their basic biology, it is important to document seasonal changes of mycorrhizal colonisation. The fungal structure quantification in *H. lanatus* roots such as arbuscules, vesicles and hyphae was higher in semi-natural land use than in intensive ones, and showed a marked seasonality. This could be related with plant phenology and metabolic pathway, as well as with climatic conditions. Fungal structures were more abundant in the summer, the season with high temperature as well as solar radiation. Moreover, the higher abundance of fungal structure in *H. lanatus* root during this season could be related with plant phenology. This period matches with the start of the reproductive phase of *Holcus lanatus* at the sites. During flowering and/or fruiting the phosphorus demand is high and thus AMF colonisation levels were also high. This can also be demonstrated by higher percentage of AMF arbuscules colonisation during this season. Arbuscules are the place for exchange of nutrients between plant and fungus, and its high percentage of colonisation indicates an intense symbiotic activity.

Nutrient availability also affects the species composition and root colonisation of AMF communities. The density of spore of the most representative genera – *Glomus*, *Acaulospora* and *Scutellospora*, was correlated with soil nutrients. The percentage of AMF root colonisation showed the same pattern. Thus, the improvement of soil fertility, especially the availability of K, N, Ca and Mg, provided favourable environment for mycorrhizal formation and function in these systems. Among soil nutrients, phosphorus is widely known to inhibit the composition and diversity of AMF communities as well as spore and mycelium densities. However, a correlation between percentage of AMF colonisation and spore density with soil P content was not found. This may be due to low soil Pcontent in semi-natural and intensive pastures, which is

above the P concentrations that limit AMF colonisation and plant growth (P< 10 ppm), that could affect the absorption of other soil nutrients. Under P limited conditions, the investment of a host plant in AMF genus or species should be strengthened to maintain the uptake of limiting nutrients. So, as predicted by the functional equilibrium model, in low P soil, relative allocation to arbuscules, coils and extraradical hypae is generally increased by N enrichment and vice-versa. Then, changes in AMF community may be explained by changes in the soil N:P ratio.

In fact, the spore densities of *Acaulospora* and *Scutellospora* were positively correlated with N soil nutrient, but the opposite occurred with spore density of *Glomus* genus, which was reflected in a greater abundance of genera *Acaulospora* and *Scutellospor*a in semi-natural pastures, as well as, in higher abundance of *Glomus* genus in the intensively managed pastures.

The main AMF benefits to the plants is the growth improvement due to enhanced nutrient uptake, particularly immobile nutrients like phosphorus, because the hyphae can reach farther that the nutrient depleted zones that build up around plant roots. Thus, AMF may play an important role in ecosystems functioning, since their appropriate management can reduce the use of chemical and energy in agriculture and consequently lead to a more economical and sustainable production systems. However, host genotypes influence the response to mycorrhizal symbiosis both in terms of plant growth or protections against pathogens. According to their response to mycorrhizal colonisation, host plant can be classified in 3 categories: obligate, facultative or non-mycorrhizal depending if they show high, intermediate or non level of arbuscular mycorrhizal colonisation. Mycorrhizal response is usually measured in terms of mycorrhizal dependency. The tree plant studied – *H. lanatus* L., *Lolium multiflorum* Lam. and *Lolium perenne* L. are facultative mycorrhizal plants, but showed different degrees of response to native AMF inoculation. *L. perenne* was the most mycorrhizal dependent plant, followed by *H. lanatus*, which reflected in a higher root colonisation and thus higher plant biomass production. In contrast, *L. multiflorum* was the less responsive and also the less colonised by the fungi, which resulted in lower plant productivity when combined with mycorrhiza.

Mycorrhizal dependency among species has potentially important applications to agroecosystems functioning and management. In the Azores, upland semi-natural and intensive pastures are usually dominated by the grass species *Holcus lanatus*. In contrast, *L. multiflorum* and *L. perenne* are characteristic of lowland improved pastures

receiving regular imputs of fertiliser. Moreover, in lowland during the winter *L. multiflorum* is usually used in crop rotation with *Zea mays* L. an obligate mycorrhizal plant. Our findings suggest that in this soil management called “outonos” the AMF root colonisation of highly mycorrhizal dependant crop (*Z. may*), and subsequent production , can be committed due to lower AMF infectivity resulting from the previous weak mycorrhizal crop (*L. multiflorum*).In addition to increased inorganic nutrient acquisition, other potential benefits of AMF colonisation to host plant include enhanced resistance or tolerance to biotic stress, such as nematodes and root pathogens. In fact, the inoculation with native AMF increased the tolerance of *H. lanatus* to root-knot *Meloidogyne incognita*. Among the mechanism proposed to explain the protective effect of AMF, we suggest that plant growth promotion by AMF, and direct competition for host colonisation sites and photosynthates between AMF and nematode were involved.

AMF indigenous inoculum promoted the growth of the host plant, which was evident in a higher fresh and dry foliar biomass of mycorrhizal plants than in others treatments. Thus, there was an apparent increase in the vigor of the mycorrhizal plants that made them more resistant to nematode infection. However, root biomass was higher when plants were inoculated simultaneously with the two organisms, indicating that in presence of the nematode, AMF increase root biomass to compensate tissue damage and decay of root sections by the pathogen and consequently prevent significantly disease symptoms.

Both AMF and root-knot *M. incognita* share the same ecological niche, i.e. root of host plants. However, the nematode presence did not cause a decrease on mycorrhizal colonisation, indicating that probably a competitive interaction occurred between these two organisms on *H. lanatus* roots by space. On the other hand, as in mycorrhizal plants much carbon is used by the symbiotic AMF, less carbon could be available for nematode colonisation, preventing the infection by the nematode.

This was the first study about AMF community structure in Azores. It provides basic information about AMF species diversity and composition in semi-natural and intensive pastures of Azores, as well as the main factors that can contribute to differences between them. Moreover, the results also underline to the role of indigenous AMF as potential inoculums in pastures, either by its contribute to plant productivity or protection against nematodes. Thus, the correct use of AMF will help to decrease

fertiliser input yet maintain the same level of growth and production, which will be the crucial importance from the point of view of sustainable agriculture. However, much works will be needed to better clarify the patterns of AMF distributions in these systems. It will be interesting to further investigate whether these AMF differ in other functional traits that are of agronomic importance.

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