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Soil Fungi-Plant Interaction

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1. Introduction

One of the main European agricultural problems is the decline in soil fertility due to the reduction of the natural soil harmony. When natural soil composition is altered, few cultivated plants replace spontaneous populations of numerous species. Man becomes the only regulator of a new fragile equilibrium between the simplified biocenosis elements. Agronomic techniques (fertilisation, irrigation, soil tillage, etc.) become instruments to achieve such an improbable equilibrium. Frequently, these techniques are just the ones responsible for environmental pollution, disequilibrium among mineral elements and the general decrease in soil fertility. They are based on simplification of the relationships between the plant and other components of the natural habitat. This simplification should make agricultural systems easier to be controlled, but, indeed, it creates conditions of extreme weakness for plant life. It is clear that life on emerged lands has been possible thanks to complex relationships and, for plants in particular, to microorganism symbiosis. On the other hand, decreasing relationships between cultivated plants and other components of the natural habitat give rise to environmental degradation and pollution (due to the need of using high amounts of chemical inputs).

Soil fungi – plant roots have evolved forms of symbiosis, namely mutualistic associations. The partners in these relations are members of the *Zygomycota*, *Ascomycota* or *Basidiomycota* divisions and most vascular plants (Harley and Smith, 1983; Kendrick, 1985). In these symbioses, the host plant receives mineral nutrients, while the fungus obtains photosynthetically derived carbon compounds (Harley, 1989; Harley and Smith, 1983). At least seven different fungi – plants associations have been recognized, with distinct morphology patterns, involving different groups of organisms (Harley, 1989; Harley and Smith, 1983). The most common ones are: i) vesicular arbuscular mycorrhizas (VAM), in which *Zygomycetes* fungi produce arbuscules, hyphae and vesicles among root cortex cells, between cell wall and plasmatic membrane; ii) ectomycorrhizas (ECM), where *Basidiomycetes* and other fungi form a mantle around roots and a so-called Hartig net among root cells; iii) orchid mycorrhizas, where fungi produce coils of hyphae within roots (or stems) of orchidaceous plants; (iv) ericoid mycorrhizas, developing hyphal coils in outer cells of Ericales hair roots (Brundrett, 1991). Factors that can influence the establishment and persistence of mycorrhizal associations are various, besides symbiont compatibility: external factors - edaphic or microclimatic conditions, presence of further soil organisms, nutrient competition; and internal factors - organism phenology. Infective propagules must be present when root growth activity occurs, since roots have a limited period of susceptibility

(Brundrett and Kendrick, 1990) and rapid colonisation of the root system is required for an effective association (Bowen, 1987).

This chapter focuses on some effects of that symbiosis on plant physiology. The general positive influence of symbiosis is remarkable, especially in terms of nutritional function. Moreover, the microorganisms of the rhyzosphere play an important role in root hydraulics and plant water status: directly - through uptake and transport, exceeding or regulating part of the cellular barriers; or/and indirectly - the hyphae increase the water potentially available by exploring a bigger soil volume and interstices between little agglomerates. Possible mechanisms by which symbiosis improves plant performance are discussed. Experimentations have been carried out to estimate the progressive colonisation and persistence of the symbiotic microorganisms on roots in agricultural conditions. Studying artificial symbiosis, induced during the fruit tree production process, could concur to enhance the plant tolerance to abiotic and biotic stresses. In this regard, *Glomus intraradices* and *Trichoderma harzianum* fungi were found to be effective as native inoculants in many Mediterranean areas. Changes in morphological and physiological plant characteristics could increase the general quality and viability of planting material in sustainable management.

1.1 The genus *Trichoderma*

The genus *Trichoderma* characterises filamentous fungi that can be isolated from many soil types. As part of a healthy soil environment, *Trichoderma* species have been found worldwide. They are able to colonise plant roots and debris, including decomposition of organic substrates (saprobes) (Kendrick, 1985). The fungi of this genus are genetically quite diverse, with a number of different capabilities (Harman et al., 2004). The biocontrol mechanisms exercised by *Trichoderma* could be attributed to competition for nutrients, release of extracellular hydrolytic enzymes, and secondary metabolites toxic to plant pathogens at very low concentrations (Mathivanan et al., 2008). *Trichoderma* also induces defence responses in host plants (e.g., 'systemic acquired resistance' - SAR) (Mathivanan et al., 2008). In particular, *T. harzianum* produces a variety of antibiotic antifungal peptides that interact with cell membranes of plant fungal pathogens, so inhibiting their growth (Harman et al., 2004). Furthermore, *T. harzianum* has been shown to inhibit wood rots and other fungal plant pathogens by up to 60% through production of volatile antibiotics (Morrell, 1990).

Selected strains of *T. harzianum* showed to suppress plant diseases, but also not to be adaptable to various plant species and pathogens. To overcome these limitations, researchers at Cornell University produced a hybrid strain with enhanced vigour and larger adaptability, called strain T-22 (T22). It is the active ingredient of commercial biocontrol products. T22 acts as a deterrent, protecting the root system from the attack of pathogen fungi like *Fusarium*, *Pythium*, *Rhizoctonia* and *Thielaviopsis*.

The main applications of T22 are as preventive and defence of the root against pathogen agents. The biofungicide protects the plant by establishing itself in the rhizosphere and occupying that soil ecological niche. Since it is a living organism, T22, as saprophyte, can grow along the entire length of the root system, establishing a physical barrier against pathogen attack. As long as root systems remain actively growing, *T. harzianum* T22 continues to act by feeding plant wastes released. In this way, it also takes nutrients away from the pathogens. Plant tissues, which already are under attack before the application of T22 spores, will not experience the full benefits of using the biocontrol agent. T22 can

damage root-rotting pathogen fungi through the releases of digestive enzyme chitinases, which dissolve the chitin in cell walls. Once damaged, the pathogen itself becomes prey of other soil organisms. *Trichoderma* spp. can also attack and parasitize other fungi directly. Plant root system is even more efficient, by T22 enhanced root growth and development. A larger root system is likely able to explore soil, and to use water and nutrients efficiently. In fact, in addition to the disease control, T22 increases the overall health of a plant. Its use allows reducing the nursery bench time, mineral fertilisation and fungicide applications, and getting larger leaves and stems as well as faster rooting in outdoor conditions (Harman et al., 2004; Mathivanan et al., 2008; Morrell, 1990; Sofo et al., 2010, 2011).

1.2 The genus *Glomus*

Glomus is the largest genus of arbuscular mycorrhizal fungi (AMF). All species establish symbiotic relationships with plant roots. The establishment of a functional symbiosis involves a sequence of recognition events, leading to the morphological and physiological integration of the two organisms (Giovannetti & Sbrana, 1998). The life cycle of an AMF begins with spore germination, and follows with a pre-symbiotic mycelia growth phase, hyphal branching, appresorium formation, root colonisation, and finally arbuscule development (Giovannetti et al., 1994). Arbuscules, the interface for nutrient exchange, are formed by repeated dichotomous fine branching. They grow inside individual cells of the root cortex, but remain outside their cytoplasm, due to plasma membrane invaginations. Hyphae with arbuscules contain numerous nuclei and structures, polyvesicular bodies and electron dense granules inside small vacuoles (Fig. 1, 2), the site of intense alkaline phosphatase and ATPase activities (Gianinazzi et al., 1979). Mycorrhizal fungi form, also in soil, a hyphal network that can obtain and transport nutrients, propagate the association and interconnect plants (Newman, 1988). The production of plant-external hyphae varies with species and isolates of fungi, can be influenced by soil properties and is an important determinant of mutualistic effectiveness (Gueye et al., 1987).

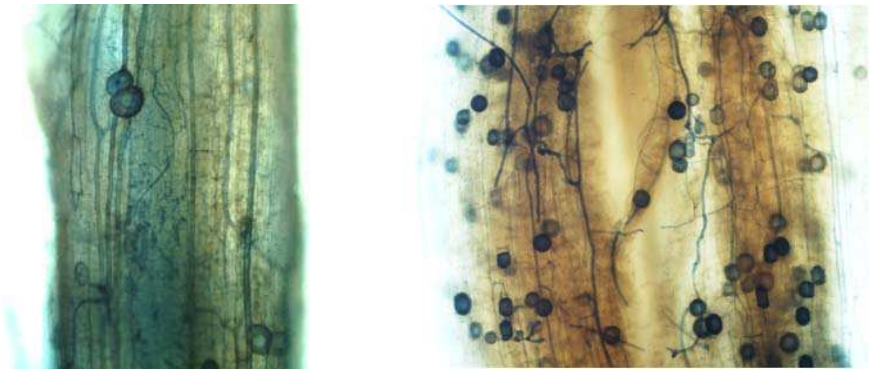

Fig. 1. Roots of *Prunus* rootstocks with hyphae and spores of *Glomus intraradices* (right; 100x magnification). In particular, (left) the multiple walls of spores (200x magnification).

Researchers have highlighted a particular infective capacity of the species *Glomus intraradices* (Estaùn et al., 2003). *G. intraradices* is autochthon to the Mediterranean basin; hence it is well adapted to the temperate climate.

Beneficial effects, in particular of some *Glomeromicota* spp. fungi, have been reported on growth, tissue hydration and leaf physiology (Allen, 2007).

The activity of VAM fungi in soils is usually quantified by measuring structures formation within roots with a microscope using a clearing step and staining procedures (Phillips & Hayman, 1970; Trouvelot et al., 1986).

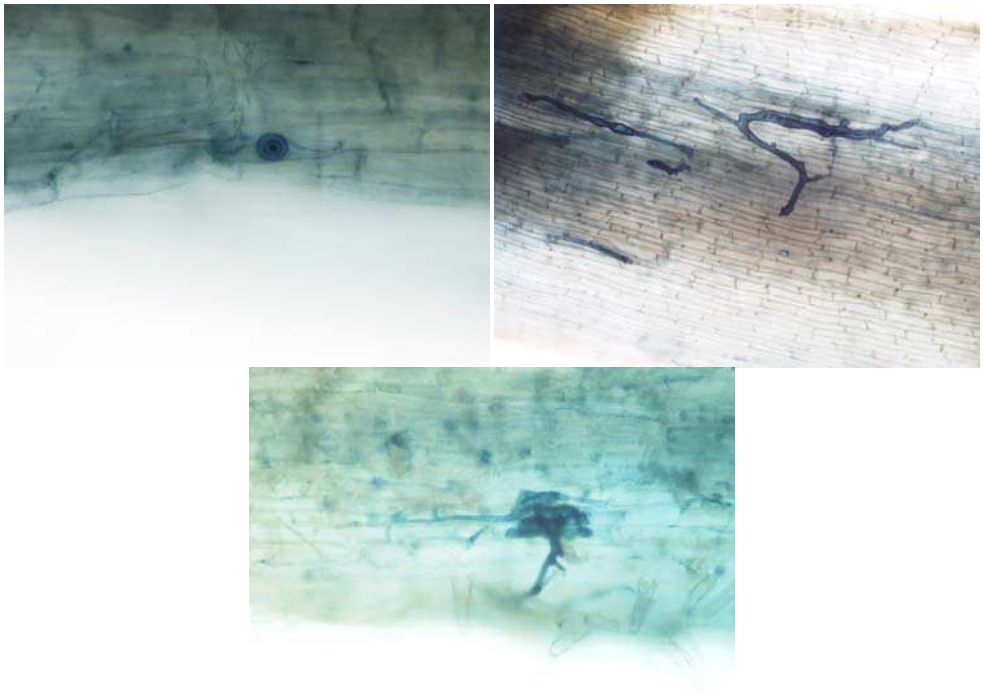

Fig. 2. Roots of *Prunus* rootstocks with *Glomus intraradices* spore (left), appresorium (right) and arbuscule (below; 400x, 200x and 400x magnification, respectively).

2. The influence of mychorrizas on plants

2.1 Use of *Trichoderma harzianum* strain T-22 in *Prunus* spp. micropropagation processes

T. harzianum is utilized as inoculant for crop production purposes, and for the improvement of plant nursery processes (Mathivanan et al., 2008). In fact, it is necessary to increase the quality of nursery plant material, in terms of higher and faster root and shoot development. To this end, the growth substrate (peat) can be inoculated with *Trichoderma* few days before rootstocks transplanting at the concentration of about 1 kg m^{-3} of substrate (see Section 3.1).

The inoculation of *T. harzianum*, during the rooting phase when plants are cultured in vitro under sterile conditions, could be another way to minimize losses in the acclimatisation phase. This method could avoid the competition of *T. harzianum* with other microorganisms

usually found in soil, hence allowing a better interaction with plant roots and a better induction of plant growth.

The first steps in understanding the interactions between a plant and *T. harzianum* are to define and optimize the most appropriate method and time of inoculation, in order to verify the effects of this fungal symbiont on the micropropagated plant material, in a way that fits existing production processes. Inoculation of T22 was applied on *in vitro*-cultured shoots of GiSeLa6® (*Prunus cerasus x Prunus canescens*) and GF677 (*Prunus amygdalus x Prunus persica*), important commercial rootstocks utilized for stone fruits (Sofo et al., 2010). The results showed that early inoculation with T22 (at the time of setting up the in vitro cultures) damaged the plants severely (Sofo et al., 2010). Early inoculation was not successful, as the fungus did not establish a symbiotic relationship with the plants but damaged them by acting first as a competitor for nutrients in the agar medium, then as a saprophyte. On the contrary, 7 days after shoot transfer in the root-inducing medium, plants survived and increased shoot growth and root development (about 160% of non-inoculated controls). A co-metabolic system with mutual benefits for plant and fungus was likely established (Sofo et al., 2010). In a different, aseptic hydroponic system, *Trichoderma* treated cucumber plants showed similar effects (Yedidia et al., 1999). Specific *Trichoderma* spp. strains have shown to be capable of plant growth enhancement, and of the bio-control of a range of wood-rot fungi when grown on a low-nutrient medium. *T. virens* or *T. atroviride* increased biomass production and stimulated production of lateral roots in *Arabidopsis* seedlings (Contreras-Cornejo et al., 2009). In our case, a simple MS medium was used, which is quite representative of fresh softwood (Harman et al., 2004). The colonisation frequency (number of colonised root fragments/total root fragments*100) of the *in vitro* system GiSeLa6®-T22 was 20%, applying the fungus 7 days after shoot transfer in the root-inducing medium. Although the measured fungal colonisation frequency was not high, the morphological effects of fungus colonisation on GiSeLa6® plants were evident, and inoculation mass and hyphae of T22 along roots of GiSeLa6® rootstock were clearly observed (Sofo et al., 2010). *T. harzianum* is capable of invading roots, but is typically restricted to the outer layers of the cortex (Yedidia et al., 1999).

T. harzianum cultured *in vitro* under controlled and stable conditions, without any nutrient limitations or other competing microorganisms, can display maximum growth and interact optimally with plants. Even if a further step in the micropropagation process, with a later treatment, might not be profitable.

2.2 Biochemical and morphological changes in *Prunus* rootstock inoculated with *Trichoderma harzianum* strain T-22

Trichoderma harzianum has been successfully used for the biological control of many plant pathogens through chemiotropic mycoparasitic interactions with some target fungal organisms (Thangavelu et al., 2004; Sahebani and Hadavi, 2008). Enzymes, which are active during mycoparasitism, degrade the cell wall (Belén Suárez et al., 2005; Yang et al., 2009). *T. harzianum* strain T-22 (T22) has the ability to directly enhance root growth and plant development also in the absence of pathogens (Harman, 2000; Sofo et al., 2010). It has also been suggested that this could be due to the production of some unidentified growth-regulating factors by the fungus or to the induction of the production of these factors in plants (Windham et al., 1986). All these findings indicate the versatility by which *T.*

harzianum can directly manifest biological control activity. In spite of their theoretical and practical importance, the mechanisms responsible for the growth response due to the direct action of *T. harzianum* on agronomic plants have not been investigated extensively.

Both endo- and ectomycorrhiza can enhance adventitious root formation. Ectomycorrhizas are able to produce auxins, gibberellins and other phytohormones (Hartmann et al., 2002).

It is well known that high values of the IAA/CKs ratio in plants promote root formation, whereas low contents induce shoot-bud formation. Thus, the growth-promoting action of T22 could be due to changes in phytohormone levels and balance in the plant. Another hypothesized positive direct effect of *T. harzianum* on plants is the solubilisation of some insoluble or sparingly soluble minerals by acidification of the medium, which could provide a better nutrient availability and uptake for the plants (Altomare et al., 1999; Kücük et al., 2008; Singh et al., 2010).

This research investigated the biochemical basis of the direct plant-growth-promoting activity of T22 on the genotype GiSeLa6® (*P. cerasus x P. canescens*), one of the most important commercial rootstocks used for sweet and sour cherry varieties.

The application of *T. harzianum* strain T-22 (T22) during the rooting phase of GiSeLa6® rootstocks resulted in greater mean root and shoot length if compared to un-inoculated plants (Sofo et al., 2010).

It is probable that the up-regulation of key genes for hormone biosynthesis or the down-regulation of the genes involved in hormone catabolism was induced by the T22 secretion of elicitors diffused into the medium or directly transferred from the fungal hyphae to the root cells, as suggested by Harman et al. (2004). There is evidence that the change in phytohormone levels is one of the direct mechanisms by which *T. harzianum* promoted root and shoot growth.

Both auxins and cytokinins are involved in shoot and root growth, and morphology. Indole-3-acetic acid (IAA) is the most widely naturally-occurring auxin in vascular plants, and it is involved in lateral and adventitious roots initiation and emergence, as well as in shoot development by changes in cell division, expansion and differentiation (Hedden and Thomas, 2006). The results showed that after T22-inoculation, IAA in leaves and roots significantly increased by 150 and 120%, respectively, whereas DHZR decreased by 80%. Increases in t-ZR were found only in leaves (90%). The auxin/cytokinins ratios changed from 29 to 47 in leaves, and from 15 to 21 in roots of un-inoculated and T22-inoculated plants, respectively. Root activity determined a decline of medium acidity, and this effect was more marked in T22-inoculated plants (up to pH 4) (Sofo et al., 2011). Among cytokinins, trans-zeatin (t-ZR) and dihydrozeatin (DHZR) - two of the most active in plants - control cell division and are involved in reducing apical dominance, inhibiting xylem formation and root growth, promoting leaf expansion and chloroplast development, and delaying senescence (Srivastava, 2002). As root induction and growth are stimulated by auxins and inhibited by cytokinins, the observed increase in IAA and decreases in t-ZR and DHZR could explain the higher root growth observed in T22-treated plants.

These results could also explain the higher shoot elongation observed (previous section) and the results of Sofo et al. (2010) who found increases in the number of leaves and in stem

diameter of T22-treated GiSeLa6® rootstocks. Generally, abscisic acid (ABA) acts as a general inhibitor of growth and metabolism, and negatively affects the synthesis of proteins and nucleic acids, even though these effects vary with tissue and developmental stage (Kobashi et al., 2001; Srivastava, 2002). Notwithstanding the significant differences in ABA levels between leaves and roots, T22 did not induce a higher ABA accumulation in both the tissues and thus did not determine growth inhibition (Sofo et al., 2010, 2011).

Altomare et al. (1999) emphasised that the plant-growth-promoting capacity of *T. harzianum* was associated with in vitro solubilisation of certain insoluble minerals accomplished via production of chelating metabolites and fungus redox activity. In our experiment, we clearly demonstrated that a strong acidification in the medium inoculated with T22 occurred. This acidification could determine the solubilisation of some salts and their higher availability for plants (Kücük et al., 2008; Singh et al., 2010). It is noteworthy that pH values markedly decreased also in the presence of T22 alone, but it appears that the synergistic action by plant and T22 caused a greater acidification of the medium (Sofo et al., 2011). This might depend on the fact that T22 enhances the acidifying capacity of the root due to the proton extrusion of the root cells through the plasmalemma (Kücük et al., 2008). The pH decrease could allow the solubilisation of MnO2, Fe2O3, metallic zinc, and calcium phosphate, and the reduction of Fe(III) and Cu(II), with evident benefits for plant nutrient uptake (Kücük et al., 2008; Singh et al., 2010). The minimum pH values found by Altomare et al. (1999) in sucrose-yeast extract liquid cultures plus *T. harzianum* but without plants, were approximately 5.0, then similar to our values. In our experiment, we observed a further pH decrease of approximately 1 unit of pH during T22-plants interaction (Sofo et al., 2011). We hypothesize that in natural soils, which usually have a pronounced buffering capacity, the acidification due to T22 could be less pronounced, as recently suggested by Singh et al. (2010).

Microscopic analyses were carried out to compare root systems of T22-inoculated and un-inoculated plants. Plant overall morphology of T22-inoculated and un-inoculated plants grown in cherry medium differed significantly (Sofo et al., 2011). These analyses revealed changes in root cell wall suberification of the exoderm and endoderm, with an increase in suberized cellular layers from 1 to 2-3, and an enhancement of cell wall epifluorescence (Sofo et al., 2011). Root tissues contain abundant alkaloids: berberine, chelerythrine, sanguinarine and chelidonine (along with other isoquinoline alkaloids), and some of them act as fluorochromes for suberin and lignin, providing numerous potential natural dye sources for fluorescence microscopic techniques (O'Brien and McCully, 1981; Brundrett et al., 1988). In our case, the observed cell wall epifluorescence (Fig. 3) indicated that T22 seems to induce the ex novo synthesis of phenolic compounds in the plants, likely by the secretion of elicitors and the following induction of defence responses, as suggested by Mathivanan et al. (2008). The enzymes of the phenylpropanoid pathway, involved in lignin biosynthesis (Gianinazzi-Pearson et al., 1994), are some of the compounds induced and involved in plant defence. The increased lignification of root endodermal cells induced by mycorrhiza was already found by Dehne (1982), but it was never demonstrated for T22. We suggest that the accumulation of protective molecules, such as lignin and suberin, in plants inoculated with T22 could accelerate their hardening. The accumulation of structural substances may be of key importance in the resistance process, by increasing the mechanical strength of the host cell walls (Dalisay and Kuc, 1995).

During the acclimatisation phase of nursery processes, all these observed biochemical and morphological changes induced by T22 could be an advantage. Plants could acclimatise better to new and hostile environments, so increasing plant survival in the absence of pesticide applications.

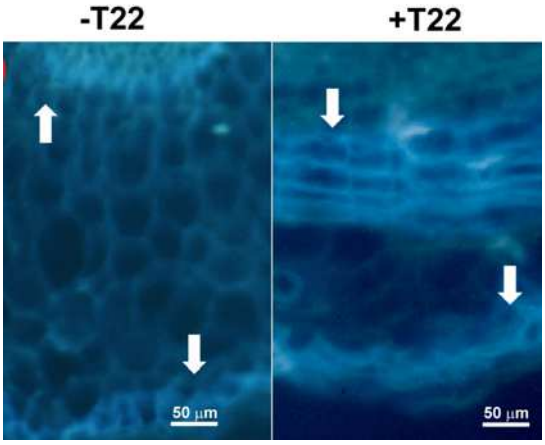

Fig. 3. Root cross-sections (diameter ≤ 1 mm; 6 mm from the root tip) of micropropagated plants inoculated with T22 (right) and un-inoculated plants (left) observed at 100x magnification with a mercury lamp. The arrows indicate the endodermic (above) and exodermic (below) layers (Sofo et al., 2011).

2.3 The influence of mychorrizas on vegetative growth

The artificial mychorrization, in the nursery phase, becomes useful particularly when the populations of native fungi are not present or have been reduced by intensive agricultural practices. Beneficial effects, in terms of positive plant growth responses and enhanced nutrient uptake, have been obtained with many agricultural crops.

The presence of arbuscular endo-mycorrhizal fungi was already evident in roots of olive tree plants, in many regions of the globe (Roldàn-Fajardo and Barea, 1986). The dependence of this species on these symbiotic microorganisms, relatively to mycotrophic function, is remarkable (Hayman et al., 1976; Roland-Fajardo and Barea, 1986; Briccoli Bati et. al., 1992). The positive influence of mycorrhizas was also demonstrated both on rooted olive cuttings (Di Marco et al., 2002) and during their growth in nursery (Briccoli Bati and Godino, 2002; Briccoli Bati et al., 2003). The study of the mycorrhizal symbiosis influence induced in olive tree, could concur to optimize the development of the plant and to improve its tolerance to some a-biotic and biotic stresses.

Trials were carried out on the effects of mychorrizic symbiosis induced in five cultivars of *Olea europaea*: Carolea, Coratina, Maiatica of Ferrandina, Leccino and Tondina. During the experimentation, self-rooted cuttings were inoculated, at two transplants in pot, with: *Glomus intraradices*, *Glomus* spp., *Glomus* spp. plus *Trichoderma* sp. plus bacteria (*Pseudomonas* spp., *Bacillus* spp.). Using the ordinary management techniques, the plants grew under optimal water status and limited fertilization (no phosphorus).

Morph-anatomically, by observations and microscopic analyses, the roots of the mycorrhized olive plants exhibited just few peculiar characteristics: the distribution in the substrate was always homogenous, the tip diameters were bigger, the adhesion of soil particles in the rhyzosphere was determined by a widespread presence of mycelium. Other authors do not give special particulars on that, but only report some differences related to root length and depth in other tree species (Andersen et al., 1988). Observations were made to compare root systems of different inoculated and un-inoculated plants just before sampling. Morphology and behaviour were significantly different. Control plants: the roots were branched, of 0.5-1.0 mm in mean diameter; their main colour was pale yellow-brown; they came out of the pot bottom; soil particles, especially of peat, tightly adhered to them. *Glomus intraradices* treatment: the roots, homogeneously of 0.2-0.5 mm in mean diameter, also in this case were branched, presented tip thickenings; root growths, even if coming out of the pot bottom, were more reduced in number than the control; their colour was the same, pale yellow-brown; mycelium seemed to wrap up and hold soil ryzosphere particles, which consequently didn't adhere directly to the epidermal layer. *Glomus* spp. treatment: the roots, homogeneously of 0.2-0.5 mm in mean diameter, well distributed in the pot volume and branched, presented tip thickenings; root growths, even if coming out of the pot bottom, were more reduced in number than the control; their colour was the same, pale yellow-brown; the soil ryzosphere particles didn't adhere directly to the epidermal layer. *Glomus* spp. + *Trichoderma* sp. + bacteria treatment: the roots were branched and well distributed in the whole soil volume; they came out of the pot bottom; the root number with a diameter smaller than 0.5 mm was greater; their colour did not change; the soil ryzosphere particles didn't adhere directly to the epidermal layer.

The percentage of mychorrizal colonisation reached values up to 100%, while the intensity of mychorrization was variable, from 20 to 50%. Destructive and not-destructive biometric measurements estimated the variations, if any, in terms of plant growing rate and stored dry matter. Altogether, in the first eighteen months, all the inoculated treatments showed greater increments, up to 20%, than the controls. *Glomus intraradices* gave the best result, 20% more (Fig. 4). However, a strong inoculum-cultivar interaction emerges; Maiatica cultivar, in fact, reached higher stem diametrical increments, 20% more, with the *Glomus* spp.; Carolea cultivar, 10% more, and Coratina, 30% more, with the *Glomus* spp. plus *Trichoderma* sp. plus bacteria; Leccino and Tondina confirmed the efficiency of *Glomus intraradices*, 30% and 25% more, respectively (Tataranni et al., 2010; Fig. 5). AM fungi prove to be better competitors than others when several isolates are inoculated together in pot culture experiments (Lopez-Aguillon & Mosse, 1987; Wilson, 1984). In these studies, the success is generally due to the ability to colonise roots most rapidly (Wilson, 1984). The outcome of competition should be expected to depend on the placement and amount of the inoculum, hyphal growth rates in soil and root interactions (Abbott & Robson, 1991; Hepper et al., 1988).

The quality of the plant material used to establish an orchard is of utmost importance for its success. The use of symbiotic and biocontrol microorganisms can help to improve rootstock production and quality (Whipps and Gerhardson, 2007; Kapoor et al., 2008).

Myrobolan 29C plants grown in pots, treated with *Glomus intraradices* and *Trichoderma harzianum*, showed better physiologic and development performance as compared to the control (Dichio et al., 2011). Mycorrhization with *Glomus* and soil colonisation with *Trichoderma* enhanced plant growth (total biomass) by about 24% and 38%, respectively. The

inoculation of micropropagated plantlets with selected fungi has been tested to improve the quality of many cash crops (Augé, 2001). Calvente et al. (2004) supported above all the effectiveness of native *G. intraradices* fungi as inoculants in many Mediterranean areas. Sofo et al. (2010) also demonstrated that the application of *T. harzianum* T22 increased the numbers of leaves, roots, and stem diameters.

Fig. 4. *Olea europaea* cv. Coratina control plants (left) and inoculated with *Glomus intraradices* (right) after about eighteen months (Tataranni et al., 2010).

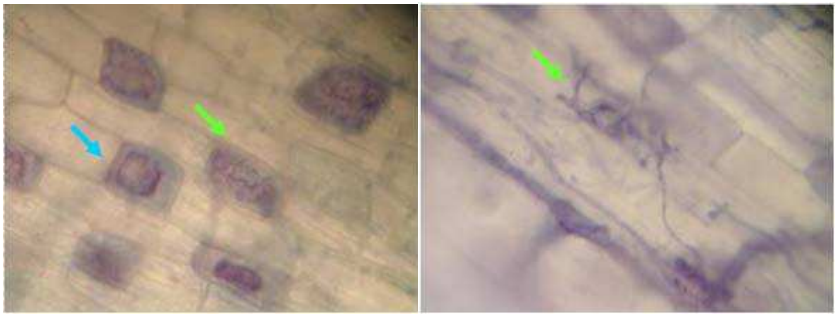

Fig. 5. *Glomus intraradices* colonisation. Hyphae and fungus structures in roots of *Olea europaea* cv. Coratina stained with Trypan blue; green arrows: arbuscules; blue arrow: spore in formation (400x and 600x magnification; Tataranni et al., 2010).

2.4 Effects of fungicides application on mycorrhization rate and persistence parameters of *Glomus intraradices*

Agricultural practices, such as pesticides, fungicide use and fertilizer applications, can eliminate or severely reduce the incidence of mycorrhizal activity. Mycorrhizal fungi can be quite sensitive to some fungicides, but not to at all of them. Some fungicides can actually stimulate mycorrhizal fungi. Two mechanisms were proposed to account for beneficial effects of fungicides on mycorrhizas: i) root colonization may be stimulated if the fungicide alters the host plant physiology by increasing root exudates (Jabaji-Hare & Kendrick, 1985; Schwab et al., 1982); ii) the fungicide reduces populations of organisms antagonistic to AMF (Hetrick & Wilson, 1991). However, the indiscriminate use of pesticides and fungicides usually leads to a reduction in spore numbers (Manjunath & Bagyaraj, 1984) and diversity (Schreiner & Bethlenfalvay, 1996). In addition, high levels of these substances also reduce

AMF colonisation of roots, resulting in reduced AMF activity (Newsham et al., 1995; Udaiyan et al., 1995). Phosphorus influxes into mycorrhizal roots are quickly reduced after the application of systemic fungicides (Hale & Sanders, 1982), such as benomyl [methyl 1-(btylcarbamoyl)-2-benzimidazole carbamate]. According to the literature, alterations induced by fungicides on the health and physiology of plants have significant effects on symbionts (Garcia-Romera & Ocampo, 1988; Schwab et al., 1982). The response of mycorrhizal fungi to chemical treatments can be influenced by the different species of host plants, the variety of compounds used, their modes of action, different application methods and rates, the growth phases of mycorrhizal fungi, environmental conditions (Giovannetti et al., 2006; Jalali & Domsch, 1975). Fungicides, applied as soil drench, reduced the proportion of active external hyphae, expressed as alkaline phosphatase (ALP) active fractions (Kjøller & Rosendahl, 2000). Internal fungal structures seem to be less vulnerable to toxicants, when these are applied to the soil. The loss of activity in external structures might inhibit the transfer of nutrients and water towards the plant, but the survival of fungal structures in the root cortex is important for the re-establishment of symbiosis.

Effects of fungicide applications applied in combination during acclimatisation - a crucial phase of micropropagated rootstock production - were evaluated on mycorrhization rate and persistence parameters (Phillips & Hayman, 1970; Trouvelot et al., 1986) of *G. intraradices*, in potted GF677 rootstocks (Fig. 6). Plants in the acclimatisation phase, despite fungicide applications, are particularly sensitive to diseases favoured by high relative humidity conditions. *G. intraradices* was able to better colonise the roots of non-fungicide plants, during the first months from inoculation (April – July). In July, the frequency of mycorrhization system revealed to be by 10% higher in non-treated plants. The intensity of root colonisation ranged from 80 to 85% without fungicide applications; it remained at 70% when applying fungicides. Fungicides also affected arbuscular formation; in fact, arbuscular abundance parameters revealed a constant 10% reduction as compared to non-treated plants. External hyphae of AM fungi should be more sensitive to fungicides, above all during presymbiotic growth and the establishment of symbiosis (Kjøller & Rosendahl, 2000). Compounds with a systemic mode of action were also reported to inhibit AM activity (Hale & Sanders, 1982; Newsham & Watkinson, 1995). Boscalid is a systemic fungicide belonging to the anilide class, effective against botrytis rot and crown rot by inhibiting the electron transfer of the succinate dehydrogenase complex in the mitochondrial inner membrane. Successively, in December, differences on mycorrhization parameters between fungicide- and non-treated plants became not significant. At that point, the colonisation intensity of plants with fungicides was found to be even higher by 30%. Arbuscular abundance parameters were equivalent. In non-host condition, the impact of Iprodione, a contact fungicide that inhibits DNA and RNA synthesis, was reduced on AM fungi (Giovannetti et al., 2006). According to some authors, Metalaxyl even increased radical colonisation and the length of external hyphae (Giovannetti et al., 2006; Groth & Martinson, 1983). So, the better root colonisation of treated rootstocks observed in December could be determined by indirect effects due to the control that fungicides have on pathogens and antagonists of AM fungi (Giovannetti et al., 2006; Hetrick & Wilson, 1991).

The combination of the used fungicides caused a decrease in hyphal colonisation by *Glomus intraradices* during the first months after the treatments. AMF show different sensitivity to fungicides at the various phases of the biological cycle. In particular, the presymbiotic growth and the establishment of symbiosis seem to be very delicate phases, since fungi

essentially develop outside the host coming into direct contact with residual products. The beneficial effects of fungicides on mycorrhizas, as verified later on, can probably be explained by the reduction in competition of antagonistic organisms and pathogens. For nursery plant production, although inoculations with AMF and fungicide treatments may protect against pathogens at the same time, in certain phases, it is necessary to consider all involved interactions carefully and prefer more environmental friendly methods.

Fig. 6. Fungicide phytotoxic effects in the acclimatisation phase of rootstock production process, after 20 days from application (left). Rootstock treated with biological agents (right).

2.5 Effects of mycorrhizas on plant water relation

Fungi are able to alter the water relations of the plant (Safir & Nelsen, 1985), but not all works compared symbiotic and control plants of similar size and/or nutritional status. So, mycorrhizal effects indirectly related to nutrition could not always be distinguished (Augé, 2001). However, non-nutritional symbiotic effects have been reported. As for the mechanism of influence, studies suggest hormonal involvement (Allen et al., 1982; Section 2.2); stimulation of gas exchange through increased sink strength (Kaschuk et al., 2009), reduction of resistances to water flow in plant (Allen, 2007; Uehlein et al., 2007). Substantial evidence has been obtained in *Bouteloua gracilis*, *Helianthus annuus*, *Citrus jambhiri* and *Prunus*, species in which mycorrhization increased the transpiration rate and, in some cases, the leaf or root conductance (Levy & Krikun, 1980; Allen, 1981, 1982; Koide, 1985; Tataranni et al., 2011).

2.5.1 Potentials, stomatal conductance and photosynthesis

Plant water status is typically quantified by measuring potentials (Ψ). Leaf Ψ of non-stressed plants is usually not affected by symbiosis (Levy & Krikun, 1980; Allen, 1982; Davies et al., 1993; Augé, 2001), but results are controversial in non water-limiting conditions. When host total leaf Ψ is similar to non-mycorrhized controls, because of frequently different photosynthetic rates observed, leaves of symbiotic plants might develop dissimilar osmotic potentials (Augé, 2001).

Symbiosis, instead, postponed declines in leaf Ψ during drought stress (Davies et al., 1993; Subramanian et al., 1997). Leaf Ψ was also reported to recover control levels more quickly in mycorrhized than control plants after drought (Subramanian et al., 1997).

Drought can cause an active accumulation of solutes in plant cells (osmotic adjustment; Dichio et al., 2006). This plant response to stress conditions was shown to be more marked during mycorrhization (Allen & Boosalis, 1983; Davies et al., 1993), resulting in higher leaf turgor (Davies et al., 1993).

When root systems is limited to small soil volumes (pots), leaf or shoot Ψ did not differ or declined more quickly in mycorrhized plants (Safir & Nelsen, 1985; Goicoechea et al., 1997).

Stomatal conductance and leaf potentials (Ψ) are related. Leaf hydration should be, in fact, naturally associated with altered stomatal behaviour. Despite information on potentials, symbiotic compared to control plants often display different transpiration rates and stomatal conductance to water vapour (Augé, 2001). Published data show different fungus–host species sensitivity, but mycorrhizas have, in general, induced increases in transpiration and stomatal conductance (Augé, 2001). Soil water was consequently observed to be depleted more in those plants. Symbiotic plants of similar leaf areas, transpiring at higher rates, led to more rapid soil water losses (Augé, 2001).

Stomatal parameters were altered by symbiosis also without changes in leaf potentials (Allen and Boosalis, 1983; Allen, 2006). In this case, mycorrhization probably influenced intrinsic hydraulic or biochemical properties of plant by signals directly (Augé, 2001). During drought, Duan et al. (1996) reported concentrations of ABA in xylem sap of mycorrhized plants lower than controls; Goicoechea et al. (1997) detected the same trend in leaves and roots.

In several experiments, differences in stomatal conductance between symbiotic and control plants were observed only under drought (Henderson & Davies, 1990; Davies et al., 1993), as it is the case for potentials. Stomata showed different thresholds at which they began to close or closed fully (Allen and Boosalis, 1983). Moreover, stomatal conductance in mycorrhized plants remained unaffected by declines in available soil water longer than control plants (Duan et al., 1996).

Photosynthesis is stimulated by symbiosis. Symbiotic plants often show higher photosynthetic rates, which is consistent with effects on stomatal conductance. Sink stimulation has been identified from various researches as one possible explanation for the differences in photosynthesis observed between nutrient-fertilised plants and symbiotic plants (Kaschuk et al., 2009). The inoculation of legumes with rhizobia and/or AM fungi resulted in sink mediated stimulation of photosynthesis, improving the photosynthetic nutrient use efficiency and the proportion of seed yield in relation to the total plant biomass. The carbon invested in the symbiosis was insufficiently compensated by enhanced nutrient acquisition, evidencing a nutrient-independent effect, in which the C costs are compensated directly by increased photosynthetic rates. Symbiosis could act by removing the limitation of rubisco activity and electron transport rates (increase in leaf N and P mass fraction and/or removal of the triose-P) (Kaschuk et al., 2009). Enzymatic activities and total protein concentrations were found to be typically higher in symbiotic compared to control plants during drought (Goicoechea et al., 1997; Subramanian & Charest, 1997).

Both non-hydraulic and hydraulic symbiosis - induced effects probably influence leaf water relations and gas exchange in host physiology at the same time, especially during drought (Davies et al., 1993; Saliendra et al., 1995). Stomata responses (variation in leaf Ψ, turgor or

water content) seem to involve ABA pathway (Saliendra et al., 1995), xylem - apoplast pH (Green et al., 1998; Hartung et al., 1998) and/or auxin/cytokinin balance (Sofo et al., 2010, 2011). These are among the factors that can further regulate hydraulic conductivity and, in particular, water channels activities (Tournaire-Roux et al., 2003).

2.5.2 Hydraulic conductivity

Sap flow depends on several plant and environmental factors (e.g. soil water availability, evaporative demand, etc.), and their interactions. Among plant factors, hydraulic capacity of the conductive tissues is involved in plant water relations since it contributes to leaf water supply. At least one-half of the resistance to hydraulic flow through plant vascular systems occurs in the root system (radial and axial pathways). These resistances are regulated by the physics of conduits (e.g. size and density of vessels), by some physiological traits, such as aquaporin-induced changes of membrane permeability, and depend on the time scale considered (Vandeleur et al., 2005). Directly, through absorption and transport, exceeding part of the cellular barriers, or indirectly, exploring a bigger soil volume and interstices between little agglomerates, the hyphae increase the water flow potentially available to plants. The experimental evidences are not always in agreement but, as Safir & Nelsen (1985) suggest, the positive effects due to the fungus could become important in limiting conditions of water stress. The best uptake of an essential element - phosphorus - seems to be the base mechanism proposed to explain these phenomena. Phosphorus could increase the permeability of root cells to water; at the same time, the development of hyphae, besides the already mentioned advantages, could reduce the inner root resistances that the water flow normally meets, above all crossing the walls (Safir & Nelsen, 1985). In optimal nutritional conditions (high phosphorus), no significant differences in root conductivity between mycorrhized plants and controls have been found; in *Fraxinus pennsylvanica,* differences of root conductivity were just found in relation to the accumulated phosphorus concentrations measured in the various plant organs, and in relation to dry matter partitioning (Andersen et al., 1988).

We tested the hypothesis that mycorrhizas can increase the hydraulic conductance (i.e. reduce resistance) of inoculated *Prunus* plants in pots without phosphorus applications. Myrobolan 29C micropropagated plants were treated with commercial *Glomus intraradices*, during the acclimatisation stage in the nursery, and grafted (Portici, apricot cv.) by chip budding technique. *G. intraradices* effectively and persistently infected the root in our system (Tataranni et al., 2011). Hydraulic conductance was measured in rootstock and rootstock + grafting point by a Hydraulic Conductance Flow Meter. A Hydraulic Conductance Flow Meter (HCFM; Dynamax, Inc. Houston, TX - U.S.A.) measured water flow (F) forcing distilled and degassed water into the excised "rootstock + grafting point" or root system (opposite direction to the transpiration stream) and changing the applied pressure (P) simultaneously. The slope of the linear regression (over the range of 0.1 - 0.5 MPa pressures applied) between water flux (F) and pressure (P) represented the hydraulic conductance (K). Values of K for each individual (rootstock + grafting point) were determined after cutting the scion 3-4 cm above the grafting point and carefully mounting the compression coupling head on the cut surface. After K was recorded, also rootstocks were cut, 1 cm below the grafting point, and rootstock conductance (K_R) was determined. Both K and K_R were standardised per unit of dry weight and referred to as conductivity (kg s^{-1} MPa^{-1} g^{-1}), thus

avoiding the effects directly due to the size (Augé, 2001). The rootstock conductivity of the inoculated plants was double compared to the control, reaching the value of 1.4×10^{-7} kg s^{-1} MPa^{-1} g^{-1}. The conductivity of the rootstock + grafting point combination of the inoculated plants was 5-times greater than the controls (Fig. 7, 8, 9; Tataranni et al., 2011). In order to understand possible mechanisms by which mycorrhizas enhance plant hydraulics, it is important to consider the resistances that water might meet in a symbiotic system. It is well known that hyphal tips penetrate the wall, reducing apoplastic resistances to water flow, but not the plasmalemma of cortical root cells. External hyphae extend into the soil, explore it, and constitute the largest biomass fraction of the fungus. Fungal hyphae, moreover, can wrap roots (Allen, 2006), rapidly transporting water and nutrients (Duddridge et al., 1980). Water can be taken up by a hyphal tip in the soil and transferred either through the cytoplasm or through the fungus inner wall layers to a cortical cell, without encountering further resistances. Membrane structures, indeed, are the site of extensive exchanges of mineral nutrients, carbohydrates and water. Marked changes in symbiotic membrane specialisation were already detected and related to aquaporins (Allen, 2007; Uehlein et al., 2007). Immunolocalisation showed the accumulation of aquaporin proteins in mycorrhized root fragments, in the vascular part and especially in the root cortex. A subsequent clear increase in water permeability was recorded, too (Uehlein et al., 2007). Symbiosis could so modify the cell-to-cell contribution to water transport and therefore influence root hydraulic conductivity.

Positive interactions between the root system and the *Glomus* fungi increased hydraulic conductivity, hence improved leaf specific conductivity and tree water use efficiency may be expected.

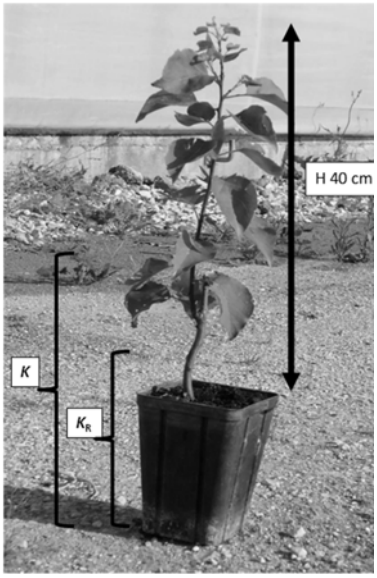

Fig. 7. Micrografted apricot plant on biotizated rootstock (Portici/Myrobolan 29C). The compression head of the HCFM was installed on the scion (K determination) and on the rootstock (K_R determination; Tataranni et al., 2011).

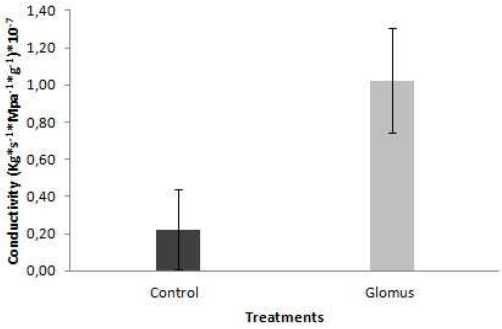

Fig. 8. Conductivity of rootstock + grafting point in control and *Glomus intraradices* inoculated plants (Portici/Myrobolan 29C). Mean ± SE, n=10 (Tataranni et al., 2011).

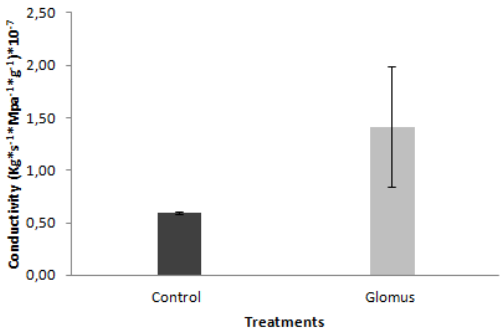

Fig. 9. Conductivity of Myrobolan 29C rootstock in control and *Glomus intraradices* inoculated plants. Mean ± SE, n=10 (Tataranni et al., 2011).

The effects of beneficial fungi, *Glomus intraradices* and *Trichoderma harzianum* T22, on plant water uptake and transport were also compared (Tataranni et al., 2011b).

Persistent *G. intraradices* - Myrobolan 29C root interaction and *T. harzianum* soil colonisation were established after six months from inoculation in acclimatisation. All the parameters assessed to evaluate colonisation were higher in the treated plants than in the controls (Phillips & Hayman, 1970; Trouvelot et al., 1986). For *Glomus* treated plants, the frequency of root system mycorrhization (F%) was 100% in the treated plants, while it was about 40% for the control. The colonisation intensity (M%) achieved 40% in the treated rootstocks, near 0% for the control. The arbuscular abundance in root segments (a%) was about double in the treated plants, reaching the value of 13%. For *Trichoderma* treated plants, CFUs measured from soil samples were 2.6 x 10^4 g^{-1}, significantly different compared to the controls, 0.4 x 10^4 g^{-1}. Control contamination, which was however uninfluential to our trials, can be due to the presence of natural microorganisms in the soil mix used (Briccoli et al., 2009). The root conductivity of the inoculated plants was double compared to the controls (Fig. 10). There were not significant differences between *Glomus* and *Trichoderma* treatments (Tataranni et al., 2011b).

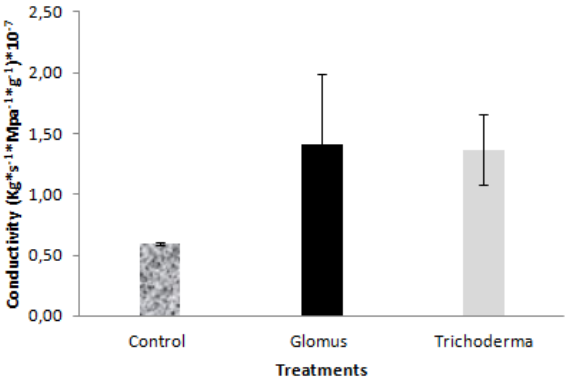

Fig. 10. Hydraulic conductivity of micropropagated Myrobolan 29C in control and inoculated plants. Comparison between *Glomus* and *Trichoderma treatments.* Mean ± SE, n=10 (Tataranni et al., 2011b).

3. Fungus application and persistence

3.1 Inoculation materials and methods

Criteria for optimizing biotisation methods in plant production processes must combine qualitative, environmental and economic benefits. The source of bioagents (commercial or fresh culture) and the possible combination with other microorganisms represent a key topic for setting up successful production procedures.

The main methods of inoculation, both for *Trichoderma* and *Glomus*, have been compared in experimentations during the acclimatisation phase of micropropagated rootstocks. The microorganism source can be dissolved into a water solution and plants dipped in it just before planting (dipping method). Alternatively, the source can be mixed with the peat substrate four days before planting (mixing method). Other studies evaluated *Trichoderma* application in micropropagation during the rooting phase (see Section 2.1).

The inoculation by dipping, on the basis of the experiments carried out, was more efficient and effective against pathogens (see Section 3.3; Bardas et al., 2011b).

Concerning the possibility to propagate and use fresh *Trichoderma,* T22 fresh culture revealed the most promising results in root colonisation, compared with commercial treatments at the same dose, either mixed in turf (fresh T22 colonisation reached 100%, 15 days after transplantation, commercial product was 50%) or inoculated by dipping (fresh T22 colonisation reached 100%, 15 days after transplantation, commercial 40%). Fresh T22, when mixed in turf, revealed the most promising results in substrate colonisation: colonisation reached 100%, 60 days after transplanting, 65% by dipping method. The specific enhanced colonisation rates of fresh T22 cultures also influenced several plant growth characteristics (see Section 3.3; Bardas et al., 2011b). That was probably due to an early activation of the "fresh" microorganisms and subsequent plant protection mechanisms.

3.2 Persistence of biocontrol agents

The presence of *Glomus intraradices* in roots and *Trichoderma harzianum* in pot soil was confirmed to be significant after six months from the inoculum carried out during the acclimatisation phase of some commercially used rootstock (GF677, Myrobolan 29C, GiSeLa®6, OHF 19-89; Fig. 11 and 12).

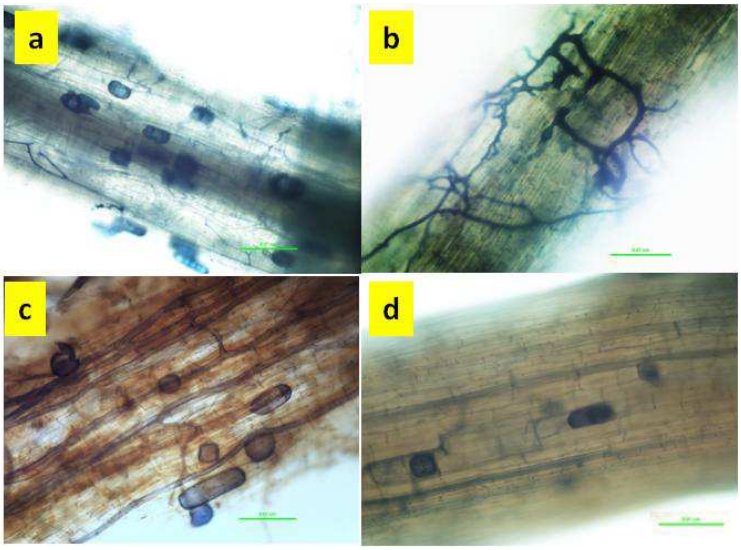

Fig. 11. *Glomus intraradices* colonisation (6 months from inoculation). Roots of GF677 (a), Myrobolan29C (b), GiSeLa®6 (c) and OHF19-89 (d), with variable fungus structures, observed under transmitted light microscope (200x magnification; scale bars = 0.01 cm).

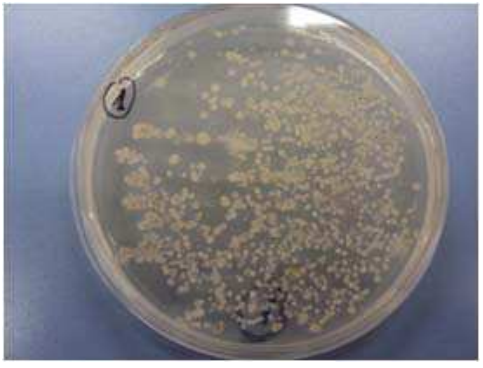

Fig. 12. CFUs of *Trichoderma harzianum* on semi-selective culture media.

The following parameters are calculated to evaluate *G. intraradices* colonisation (Trouvelot et al., 1986): F% is the frequency of root system mycorrhization; M% is the intensity of root colonisation; a% is the arbuscular abundance in root fragments analysed; A% is the root arbuscular abundance.

All treated rootstocks showed 100% mycorrhization frequency (F%), while controls about 50%. Controls were probably infected by natural agents already present in the soil or in the irrigation water. Colonisation intensity (M%), the most important parameter to evaluate the colonisation, ranged between 40 and 85%, reaching the highest values in GF677, compared to controls with only 20%; the differences observed were statistically significant. The arbuscular presence (a% and A%) was about 50% in *Glomus* treated GF677, 10% in Myrobolan 29C and 20% in OHF19-89. Arbuscular structures were limited in number for GiSeLa®6 and control plants.

T. harzianum CFU mean values ranged between 1.45 in GF677 rootstocks and 3.0 x 10^4 g^{-1} for GiSeLa®6, while controls contamination rate was low, between 0.2 and 0.55 x 10^4 g^{-1}.

The results of two-year experimentation on the persistence of microorganisms in potted plants showed a general colonisation trend of *Glomus intraradices* and *Trichoderma harzianum* with a peak in June-July months, followed by a decrease in December. These microorganisms, however, survive during the winter period and reactivate in spring (Fig. 13 and 14). Seasonal variations in the periodicity of root growth and mycorrhizal activity occur in ecosystems (Brundrett and Kendrick, 1988). Literature reports that there are no significant seasonal variations in the degree of mycorrhizal colonisation of many herbaceous plants, because only a fraction of their roots are replaced each year (Brundrett and Kendrick, 1988, 1990). Other species of deciduous plants have annual roots with well-defined periods of growth and senescence, resulting in abrupt transitions in mycorrhizal colonisation levels. Moderate seasonal variations in the extent of mycorrhizas for roots of European deciduous forest species (Mayr and Godoy, 1989) and salt marsh plants (Van Duin et al., 1989) were also associated with new root production during the growing season.

Moreover, mycorrhizal strategies of plants may be correlated with the environmental conditions prevailing when plants produce new roots, as was observed in a temperate deciduous community (Brundrett, 1991). In this community, most species with root growing in summer were mycorrhizal, while those with active roots in spring or fall had little or no mycorrhizas.

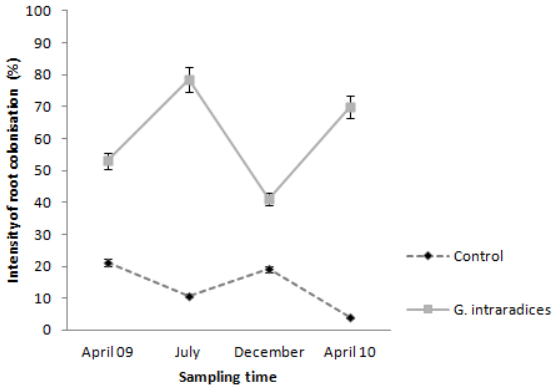

Fig. 13. *Glomus intraradices* trend of persistence (intensity of root colonisation, M%) during a year period in treated and control GF677 rootstocks.

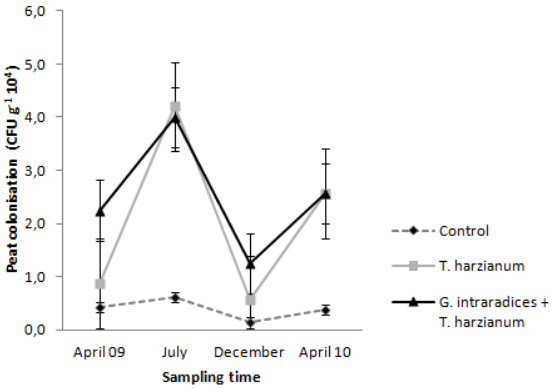

Fig. 14. *Trichoderma harzianum* trend of persistence (colony forming units, CFU) during a year period in treated and control Myrobolan 29C rootstocks.

3.3 Interaction of biocontrol agents with soil pathogens in nursery

Biological control has emerged as an important method in the management of plant pathogens. *Pythium ultimum, Fusarium oxysporum* and *Rhizoctonia solani* diseases are limiting factors during the acclimatisation phase of rootstock production, when further problems can be due to the absence of beneficial microorganisms on plants obtained by micropropagation and in greenhouse conditions. Mycorrhiza and *Trichoderma* species have long been recognized for their ability to reduce pathogen growth, survival or infections by different mechanisms (Harman et al., 2004). Chitinolytic enzymes play an important role in fungal – fungal interactions and especially in mycoparasitism (Duo-Chuan, 2006), hydrolyzing chitin, which is an essential component of fungal cell wall (Bowman et al., 2006). So, specific treatments can be a potential means in integrated rootstock production systems.

Micropropagated plants of Myrobolan 29C treated at transplantation with *Trichoderma harzianum* and *Glomus intraradices* alone or in combination, were inoculated with *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum* (Dichio et al., 2011). Disease incidence was measured 15 and 30 days after transplantation. However, the biocontrol agents induced a greater plant growth (see Section 2.3) compared to controls, with *T. harzianum* T-22 treatment showing the best promoting effect. *T. harzianum* T-22 decreased values of *P. ultimum* and *R. solani* diseased plants. All biocontrol treatments showed lower *F. oxysporum* disease incidence values compared with the untreated plants (Dichio et al., 2011).

Various *Trichoderma* species (*T. harzianum* T22 and *T. asperellum* B1) were also tested with GF677 rootstocks, *in vitro* and *in planta*, against single and combined inoculations of the pronounced plant pathogens (Bardas et al., 2011a). *In vitro* experiments revealed differentiations regarding the mode of action of the tested biocontrol agents. *In planta* experiments showed that both *Trichoderma* spp. are excellent root and peat colonisers, resulting in promotion of specific plant growth characteristics and suppression of *P. ultimum* and *R. solani* mediated disease development. *T. harzianum* T22 and *T. asperellum* B1 combined treatment was statistically different compared to single biocontrol treatments, showing higher colonisation rates, greater plant growth and enhanced pathogen control (Bardas et al., 2011a).

By employing especially *Trichoderma* as a biocontrol agent, it is possible to successfully control *P. ultimum* and *R. solani* without use of fungicides. This leads not only to lower production costs and better control of damping off diseases, but also to environmental benefits, such as minimal direct or indirect soil and ground water contamination, and improved personnel safety practically eliminating the restriction time for personnel re-entry after pesticide application. In addition, we observed that avoiding the use of fungicides, the quality and uniformity of the plants were improved and the duration of the acclimatisation phase became 4-5 days shorter. The integration of novel sustainable technologies in the production scheme of commercial rootstock production leads to significant economic, operational and environmental benefits, which could be extended to other crops.

4. Conclusion

The most appropriate inoculation time and method were defined to confirm the effect of mycorrhization on micropropagated rootstocks. At the time of planting from the jars to the soil, micropropagated rootstocks were inoculated with *T. harzianum* and *G. intraradices* by plant dipping, in inoculum – water solution, or by mixing the inoculum with the peat substrate before planting (four days before transplanting). There were low levels of *T. harzianum* detected shortly after application with dipping, compared to application by mixing with peat. T22 fresh culture revealed the most promising results in root colonisation, compared with commercial treatments at the same dose.

The use of biological agents (*T. harzianum* T22 and *G. intraradices*) in nursery production process by micropropagation, significantly decreased the percentage of dead plants during the acclimatisation phase, also compared to fungicide treated plants. It was evident that the growth rate of the inoculated plants, without fungicide applications, was not different from those treated with fungicides. Moreover, the treatment with fungicides reduced root colonisation and arbuscular formation. Therefore, the plant production process can be innovated by eliminating the use of fungicides during the acclimatisation phase.

The highlighted result was the control effect obtained for the biological agents against fungal pathogens. Both *Glomus* and *T. harzianum* (in combination and alone), but mostly *T. harzianum*, promoted plant growth and conferred protection from soil-borne pathogens (*Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum*).

Based on the results obtained, the higher shoot and root growth induced for plants inoculated with T22 could be due to a different phyto-hormonal balance or to acidification and redox fungal activity of the growth medium. The levels of IAA increased significantly after inoculation with T22 in both leaves and roots. The auxin/cytokinin ratios increased strongly in leaves and roots of plants treated with T22. Production of hormone-like metabolites and/or induction of the production of these compounds in the plants have been proposed as one of the direct mechanisms by which symbiosis promotes plant growth. Experiments demonstrated that strong acidification occurred in the medium inoculated with T22.

In pots, the presence of *G. intraradices* in roots and *T. harzianum* in soil was confirmed to be significant in June, after about one year from inoculation. The results of two-year experimentation on the persistence of microorganisms showed a general colonisation trend of *G. intraradices* and *T. harzianum* with a peak in June-July followed by a decrease in December. These microrganisms, however, survive during the winter period and reactivate in spring. In nursery fields, *G. intraradices* colonisation was very high in all treatments.

Mycorrhization of rootstocks showed effects on cultivar quality and general vigour after shoot development. The results of the plant growth characteristics showed positive effects of the inoculum on all parameters measured (plant height, number of leaves, shoot dry weight, root dry weight and the root-shoot ratio). *Glomus* and *Trichoderma* biotised plants reached a total mean dry weight greater than the controls, by about 20% and 30%, respectively, at the end of the production cycle in pots. The field management using biocontrol agents was shown to induce the best growth rates compared to the normal growers' management practices. In the nursery, in general, at the early stages of growth, mycorrhized plants showed high level of fresh and dry matter accumulation. There were no significant differences between the mineral element concentrations in the dry matter of the various treatments compared to the controls; but, considering the mean total amount per plant, quantities of macro-elements were greater in treatments with *Glomus* or *Trichoderma* than in the controls. This was due to the greater growth rate of the inoculated plants.

Beneficial fungi can enhance efficiency of plant roots to absorb water, macro- and microelements from the soil or container media. Plants treated with *G. intraradices* and *T. harzianum* showed an increase in water conductance. External inputs can thus be reduced. Plant tolerance to pathogens increases. The plant could be more efficient in surviving drought conditions. Other benefits include enhanced seedling growth, increased adventitious root formation of cuttings and enhanced transplant establishment. Mycorrhiza or biocontrol microrganisms enhance, in general, plant health and vigor. Advantages in utilizing mycorrhizal fungi or other beneficial microorganisms, during propagation and production, include marketing higher value plants.

In a vision of sustainable environment and new market demands, it becomes possible to improve plant quality and anthropic input efficiency. Symbiosis represents an excellent example of ecological adaptation. Plants and microorganisms interact in the same environment in order to obtain evolutionary advantages.

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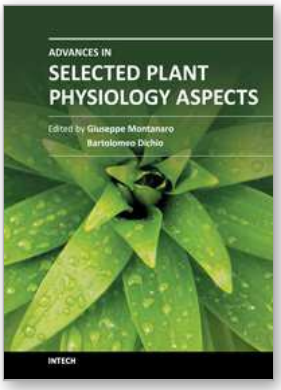

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The book provides general principles and new insights of some plant physiology aspects covering abiotic stress, plant water relations, mineral nutrition and reproduction. Plant response to reduced water availability and other abiotic stress (e.g. metals) have been analysed through changes in water absorption and transport mechanisms, as well as by molecular and genetic approach. A relatively new aspects of fruit nutrition are presented in order to provide the basis for the improvement of some fruit quality traits. The involvement of hormones, nutritional and proteomic plant profiles together with some structure/function of sexual components have also been addressed. Written by leading scientists from around the world it may serve as source of methods, theories, ideas and tools for students, researchers and experts in that areas of plant physiology.

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