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Role of Arbuscular Mycorrhizal Fungi in Nutrient Uptake and Growth of Durum Wheat

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Abstract of the thesis

Soil microbiome is involved at different levels in the food web, in bio-geochemical nutrient cycles and in several interactions with plants. Based on its key role in the in the agro-ecosystem processes, the soil microbiome has been identified as one of the principal factors in an agriculture addressed to the ecological intensification. Among the several relationships established between plants and soil microorganisms, arbuscular mycorrhizal (AM) symbiosis is the most widespread. Two out of three of all plant taxa (among others the main crops) are involved in the AM symbiosis which takes place between the plant root system and arbuscular mycorrhizal fungi (AMF), a monophyletic group of fungi belonging to the subphylum of Glomeromycotina. Although AM symbiosis can provide several positive services in the agro-ecosystem, the main benefit has always been highlighted in the increment of plant nutrition. However, the outcome of the AM symbiosis is context dependent. The greatest benefits ascribed to AMF has been observed on plant P acquisition under conditions of soil P-deficiency, whereas their contribution on plant N nutrition is still debate, since positive, neutral and negative effect have been observed. The reason of such contradictory results seems to rely on the soil N availability, since AMF have a notable N demand for their own metabolism and can even compete with the host plant for the soil N under soil N-deficiency. Additionally, although AMF can transfer N from organic source, no information is available on whether or not this ability change varying the organic source composition. Given the role of AMF in nutrient cycling, uptake and transfer to the host plant, increasing our knowledge about their role on plant nutrition is crucial in an agricultural addressed to the environment and economic sustainability. With the view to the agriculture sustainability, the reduction or the absence of tillage can provide several environmental and agronomical benefits. At the same time, different tillage system determines various pedo-climatic micro-environments which can profoundly influence the soil microbial community composition. Different pedo-climatic micro-environments are also observed along the soil profile with the consequence of drastic modifications in the community composition of bacteria and fungi (including AMF) along the soil depth. Modification in the community composition can differ in symbiotic efficiency and therefore drive the outcome of the interaction between crop and soil microorganisms. However, the potential effect of the different communities deriving from the above reported micro-environments on plant growth has not yet been investigated. In order to contribute to fill the above reported gap of knowledge a set of 4 experiments (described in the chapter 2) were carried out. Two experiments (paragraph 2.1 and 2.2) aimed to evaluate the effect AM

symbiosis outcome varying soil N and P availabilities, and the effect of AMF on plant growth, N uptake and N recovery from the applied fertilizer when N in soil was applied as mineral or organic source. The third experiment (paragraph 2.3) aimed to characterize the AMF community along the soil profile and to evaluate if the observed differences were able to affect the plant growth and nutrient uptake under adequate water availability or under drought stress. Finally, the fourth experiment (paragraph 2.4) focused on the effect of the soil microbial community deriving from different long-term tillage management and depth on plant growth an N uptake. In all the experiments durum wheat was used as focal plant.

Results have shown that under soil N-deficiency AMF compete with the host plant determining a decrement of plant growth and N uptake. A negative effect of AMF on plant growth was also observed under very high soil N availability, in absence of other limiting factors. Whereas, a positive AMF effect was observed at intermediate soil N availability, when the host plant is still under N-limiting conditions and the fungal component has satisfied its own demand. In the latter case, AMF have shown the ability to transfer a substantial amount of N derived from mineral fertilizer and organic matter. However, the organic matter composition has strongly affected the effect of AMF on plant performance. In fact, while AMF increased the plant N recovery from the organic patch with a low C:N ratio, a detrimental effect of plant growth and N recovery was observed in presence of an organic source with a high C:N ratio. Results have also shown that the AM symbiosis outcome in presence of different soil N availability conditions may change in relation to the availability of other elements. In fact, while under conditions of high P availability, the mycorrhizal outcome shifted along the entire spectrum of the ecological relationships (mutualism, commensalism or parasitism) depending on the availability of N, under soil P-deficiency, AMF have always provided a benefit to the host plant, regardless the soil N availability.

Results of the third experiment have highlighted a significant shift of AM fungal communities with depth and the existence of subsoil specific AM fungal phylotypes. The inoculation with living soil deriving from different depths resulted in variations in root colonization consistent with those detected by molecular analysis, but have had little or no effect on plant performance both with adequate water availability and in presence of drought. On the contrary, significant differences on root colonization, aboveground biomass production and N uptake were observed when pants were inoculated with living soil deriving from different tillage systems and soil depth (paragraph 2.4).

1. Introduction

The past century has witnessed a huge increment in agroecosystem productivity per unit area in order to try to satisfy the ever increasing worldwide food demand. These results were obtained thanks to the so called "green revolution" which consisted in agricultural research mostly focused on breeding and the release of new crop varieties, agronomic solutions and chemical-pharmaceutical products addressed to increasing the yield of the main world cereal crops. Recently, Grassini et al. (2013) have reported that crop yields under conventional agriculture (i.e. reliance on synthetic chemical fertilizers and pesticides, continuous tillage, monocropping, etc.) have reached their limits for further increases and this mostly due to the unsustainable management practices that determined land degradation and soil erosion (Pimentel & Burgess, 2013). In fact, the conventional agriculture applied in the past decades, alongside the yield increments, has determined several environmental problems, such as global warming, water eutrophication, biodiversity loss etc. (Foley et al., 2005; Tsiafouli et al., 2015). Certainly, agriculture is not the sole sector contributing to the cited environmental problems, but, as reported by the IPCC (2014) it is one of the dominant fields that have contributed and still contribute to them.

In the past decades, the scientific community has started to recognize the weaknesses of conventional agriculture and several authors have started to stress the need for a new and even revolutionary approach to agriculture or "ecological intensification" to meet the challenges of food security and, at the same time, guarantee environmental sustainability (Cassman, 1999; Foley et al., 2011; Tilman et al., 2011; Bommarco et al., 2013; Tittonell, 2014). Hitherto, ample work has been done to mitigate the environmental impact of agriculture and to find ecological agroecosystem management solutions (Pittelkow et al., 2015; Giller et al., 2015), but further investigations are needed to meet these current challenges of agronomic research. Clearly, such agroecosystem management needs a holistic approach, involving every single facet of agroecosystem management, in order to keep and, where possible, to increase food production and reduce environmental impact, inasmuch as soil is a "non-renewable" resource. According to Bender et al. (2016), among the different aspects, the belowground biome seems to be one of the most important components of the agro-ecosystem and one of the main factors able to contribute to an agriculture addressed towards ecological intensification. The assumption of Bender et al. (2016) is based on the fact that soil is one of the richest biodiversity ecosystems on earth with millions of different organisms, and all of them are involved in diverse ecological services that could result useful in the agroecosystem (Table 1; Bardgett & van der Putten, 2014). In fact, soil organisms are involved at different levels in the food web and in several bio-

geochemical nutrient cycles, and based on their key role in ecosystem services are often identified as "ecosystem engineers" as well as one of the principal factors in an agriculture addressing ecological intensification (Bender et al., 2016).

Table 1. Estimated abundance and diversity of soil taxa. Source: Bardgett and van der Putten, 2014.

Taxon	Diversity per amount soil or area (taxonomic units indicated below)	Abundance (approximate)
Prokaryotes	100-9,000 cm^{-3}	4-20 $\times 10^9$ cm^{-3}
Fungi	200-235 g^{-1}	100 mg^{-1}
AMF (species)	10-20 m^{-2}	81-111 m cm^{-3}
Protosts	150-1,200 (0.25 g)$^{-1}$	10^4-10^7 m^{-2}
Nematodes (genera)	10-100 m^{-2}	2-90 $\times 10^5$ m^{-2}
Enchytraeids	1-15 ha^{-1}	12,000-311,000 m^{-2}
Tardigrades	?	?
Collembola	20 m^{-2}	1-5 $\times 10^4$ m^{-2}
Mites (Oribatida)	100-150 m^{-2}	1-10 $\times 10^4$ m^{-2}
Isopoda	10-100 m^{-2}	10 m^{-2}
Diplopoda	10-2,500 m^{-2}	110 m^{-2}
Earthworms (Oligochaeta)	10-15 ha^{-1}	300 m^{-2}

1.1. Soil biome and its function in the agroecosystem

Soil fauna plays a key role in the cycle of the soil organic matter (SOM) and its fragmentation and degradation, making organic particles suitable for the successive food web steps (Wood & Bradford, 2018). Protist and nematodes regulate the population dynamics of bacteria and other different organisms, such as fungi, nematodes and other protists (Geisen, et al., 2015). However, even though the cited organisms perform a fundamental role in the agroecosystem processes, as reported by Bender et al. (2016), the key steps in the biogeochemical cycle of the most important nutrients in plant growth (e.g. N and P) are performed by soil microorganisms (i.e. bacteria and fungi). In fact, such organisms secrete different types of enzymes and are directly involved in different parts of the N (i.e. N-fixation, mineralization, nitrification, and denitrification) and P cycles, as solubilization and mineralization (Hayatsu et al., 2008; Richardson & Simpson, 2011). These processes are of key importance in the agroecosystem functioning since, in natural conditions, the nutrients in soil are usually present in organic complex, thus not available for plant acquisition and must be subjected to several processes (i.e. decomposition and mineralization) to become phytoavailable. Plants use the mineralized nutrients acquired directly and/or through mutualistic organisms (as it will be discussed below) to build up the organic matter which, when not part of the harvest, is again decomposed. In addition, the soil microbiome comprises a large part of the soil living biomass and is actively

involved in the soil carbon cycling (Fierer et al., 2012; He et al., 2014), hence, in the carbon dioxide (CO_2) emission from the agroecosystem, which due to its concentration in the atmosphere is considered the most important among the greenhouse gases. In particular, the microbial community can influence the soil carbon sequestration both directly and indirectly with the efficiency of this process depending on several parameters such as the microbial community balance (fungi:bacteria ratio; with fungal dominated communities retaining more carbon), temperature, salinity, substrate quality and quantity and nutrient availability (Six et al., 2006). The direct microbial contribution to soil carbon storage depends on the efficiency with which the organic carbon taken up by the microbial biomass is incorporated into the bacterial and fungal biomass, while the indirect contribution is essentially related to the improvement of the soil aggregation status (where the microbial community is actively involved as it will be discussed below) which physically protect the soil organic matter. At the same time, the improvement of the aggregation status played by the soil microbial community, especially by the soil fungal community, can affect the emission of the nitrous oxide (N_2O) a particularly dangerous greenhouse gas with a warming potential circa 300 times higher than CO_2 (IPCC, 2001). In particular, the improvement of the soil aggregation affects the abiotic soil environment influencing the bacterial community (Veresoglou et al., 2012) which can shift away from N_2O-producing nitrifiers towards organisms able of complete nitrification or N_2O reduction (Jones et al., 2014; Domeignoz-Horta et al., 2017).

Such aspects highlight the pivotal role of the soil microbiome in the agroecosystem and clearly underline that a thorough understanding of the soil microbial community and its role in the agroecosystem is essential to an agriculture aiming at sustainability and in order to maximize agroecosystem functioning.

Moreover, a deep understanding of the soil microbiome could lead to a solution for mitigation of damage from pathogens (Pieterse et al., 2014), one of the main problems in yield reduction for cornerstone crops such as wheat, soy, rice, corn, etc. (Oerke, 2006). In particular, an abundant presence of beneficial soil microorganisms in the rhizosphere can compete with plant pathogens for plant-derived nutrients, or plant-associated microorganisms can induce systemic resistance to a wide spectrum of pathogens and insects (Berendsen et al., 2012). At the same time, the presence of some groups of soil microorganism can improve the health plant status by improving the plant nutrients uptake. A wealth of literature is available about the symbioses established between plants and soil microorganisms to compensate the plant nutrient deficiencies that often occur due to soil nutrients depletion or due to the presence of nutrients forms unavailable for direct plant uptake (Gyaneshwar et al., 2002; Wang et al., 2012; Smith &

Smith, 2011; Miransari, 2013). One of the main examples of mutualistic symbiosis is the relationship between Fabaceae and rhizobium bacteria. In this symbiosis, there is a formation of particular structures in the root (nodules) that allow to fix N from the atmosphere and later such N is used by the plant for its growth. In exchange the plant transfers part of the fixed carbon to the symbiont microorganisms. However, such symbiosis is exclusive of plants belonging to the Fabaceae family. Other symbioses that can influence plant growth are more widespread among plant families (including Fabaceae). Among the others, the mycorrhiza symbiosis is the most widespread among plants (Smith & Read, 2008). The main benefit ascribed to the mycorrhizal symbiosis has always been highlighted as an increment in plant nutrition. However, several studies conducted in the last century have emphasized a multifunctionality effect of mycorrhizal symbiosis in the natural ecosystem and in the agroecosystem. Particularly, its role has been underlined in the SOM cycle, in the interactions with the rest of the soil microbial community and in soil aggregation (Fig. 1.1; Finlay, 2008).

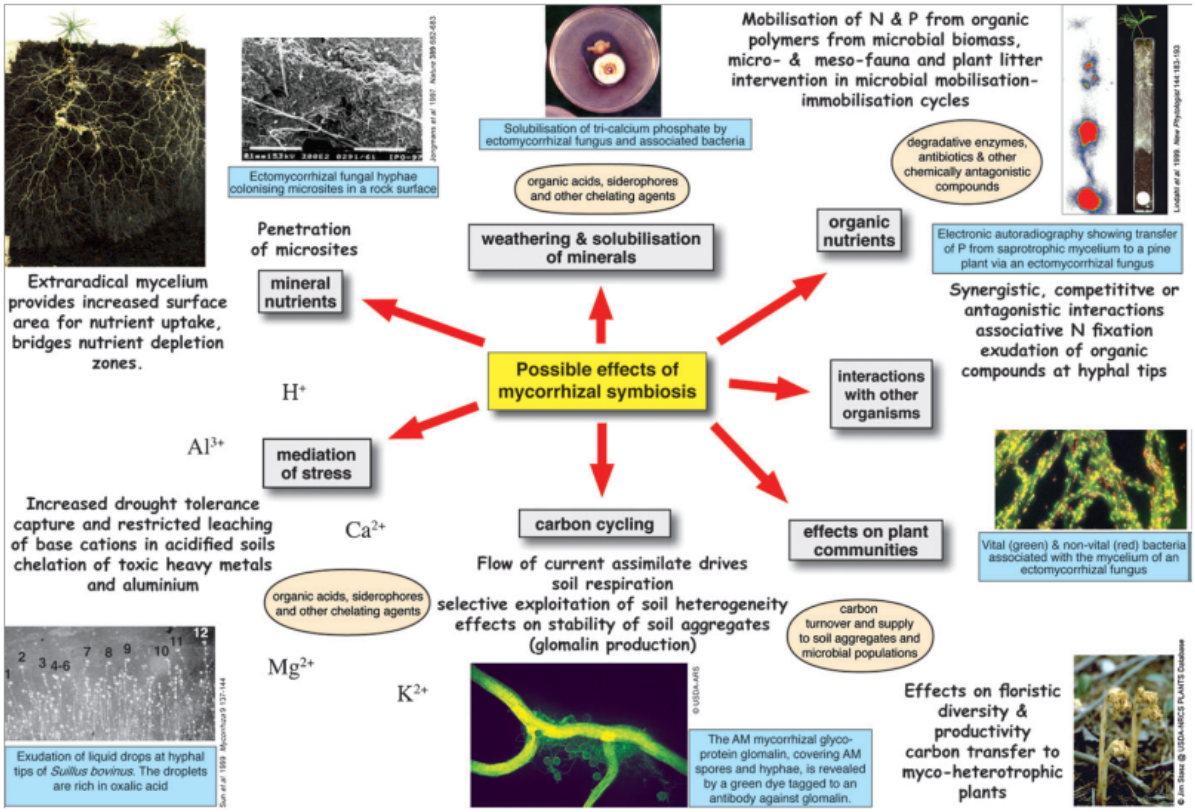


Fig. 1.1. Possible interaction involving the Extraradical mycelium of mycorrhizal symbiosis. Source: Finlay, 2008.

1.2. Mycorrhizal Symbiosis

The term Mycorrhizal was coined at the end of the 19th century by Frank (1885) to describe peculiar structures found in the plant root system which were associated with fungal mycelium. The term mycorrhiza is composed of two greek words ("mykes" = "fungus" and "rhiza" =

"root") that identify the association between the fungus mycelia and the root system. Later, several studies have been conducted on this symbiosis and its widespread diffusion was shown in almost the entire terrestrial ecosystem, either natural or human-managed (Brundrett, 2009). Mycorrhizas symbiosis occurs in more than 90% of vascular plants, and the small portion of vascular plants which is non-mycorrhizal consists of habitat specialists or nutritional specialists, such as carnivores, parasites and cluster-rooted species (Fig. 1.2; Brundrett & Tedersoo, 2018). Based on morpho-physiologic criteria, seven or more types or mycorrhiza have been identified. However, some of them are very similar, hence Brundrett & Tedersoo (2018) have proposed the following approach to classify the mycorrhiza types into four main categories: i) ectomycorrhizas, ii) ericoid mycorrhizas, iii) orchid mycorrhizas, iv) arbuscular mycorrhizas (AM) (Fig. 1.2).

Ectomycorrhiza is formed mostly between forest plant species (e.g. Pinus and Larix) and fungi belonging to the phylum of Ascomycota and Basidiomycota. The term ectomycorrhiza is referred to the development of the fungus mycelia that is mostly external to the root. In the ectomycorrhizas there is a formation of a particular structure that completely wraps the root (mantle) and an intraradical mycelia development (Hartig net) which has the transfer function of nutrient and other molecules between the two symbionts (Dell et al., 1994). Within the ectomycorrhizas, based on the mantle development and on the magnitude of the Hartig net colonization several subtypes are identified (Brundrett & Tedersoo, 2018).

Ericoid mycorrhizas take place between plants belonging to the order of Ericales and fungi from the phylum of Ascomycota. In such symbiosis, the fungus actively penetrates the cell wall, but never penetrates the host plasmalemma, and forms hyphal coils that allow the communication between plant and fungus.

Orchid mycorrhizas are confined to Orchidaceae plants and saprotrophic fungi from the Tulasnellaceae and Ceratobasidiaceae families. Orchidaceae are myco-heterotrophic for part of their life cycle which make them completely dependent on the fungus for carbon and other nutrients. In fact, in some cases, the fungal mycelia can link the orchid with other autotroph plants to transfer carbon to host orchid (Leake, 2004).

However, the mycorrhizas types reported above are confined to a small percentage (c. 15%) of vascular plants, while more than 70% establish symbiosis with Arbuscular Mycorrhizal Fungi (AMF) (Fig. 1.2); to which this thesis mostly refers. Details of AM symbiosis are discussed in the following sections.

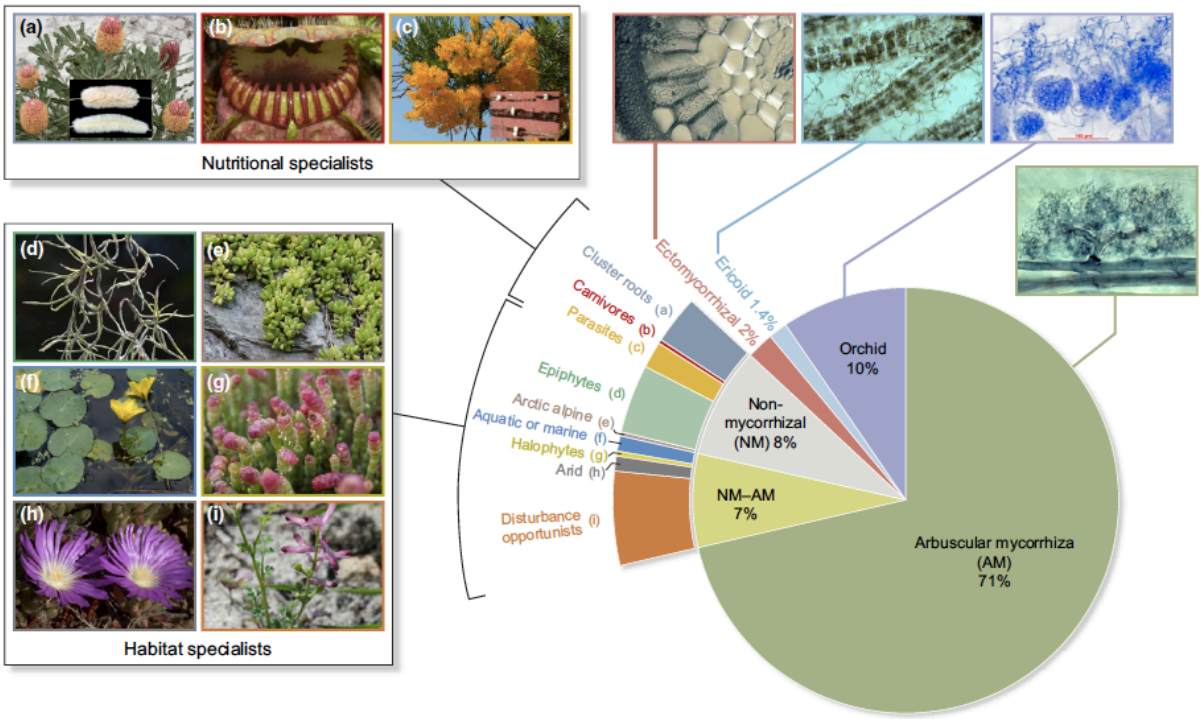


Fig. 1.2. Different mycorrhizas types and nonmycorrhizal plants habitat and nutritional specialist. Source: Brundrett & Tedersoo, 2018.

1.3. Arbuscular Mycorrhiza Symbiosis

Two thirds of all plant taxa (among others the main crops) are involved in the Arbuscular mycorrhiza symbiosis (Smith and Read, 2008). Such symbiosis takes place between the plant root system and a monophyletic group of fungi belonging to the subphylum of Glomeromycotina (Spatafora et al., 2016). Structures of AMF have been found in fossils dated c. 400 Myr (Taylor & Osborn, 1996). However, it is unclear if AM symbiosis evolved with the first plant terrestrial colonization or it evolved later in the Silurian with the plant complexity increment (Brundrett & Tedersoo, 2018).

AMF are obligate symbionts and therefore need a living host for their development. In fact, although AMF spores are able to germinate without a host, in this case, their growth is very contained and the organism collapses within a few days (Parniske, 2008). Vice versa, in the presence of a living host there is the production of a well extended mycelium which in contact with the host plant forms the appressorium (also called "hyphopodium") and penetrates the root epidermis (Buee et al., 2000; Bucher, 2007; Fig. 1.3). The stimulation of spore germination and fungal hyphae development has been ascribed to particular compounds (Strigolactones) found among other root exudates (Parniske, 2008). In addition, although the fungus actively penetrates the root epidermis, the plant actively prepares the intracellular environment (Pre-Penetration Apparatus) for the fungal infection (Genre et al., 2005; Fig. 1.3). Later, the hyphae develop inside the root until they reach the cortex cells, where, by subsequent division form

characteristic tree-shaped structures called Arbuscules (Fig. 1.3). Such structures represent a keystone in AM symbiosis since here the two symbionts transfer nutrients to each other as well as other signaling molecules (Harrison, 2005). Arbuscules are formed continuously and have a lifetime of a few days; after the plant cells digest the arbuscules and can be colonized again by a new hypha (Smith and Read, 1997).

Although there is transfer between the host plant and the fungus, the two symbionts are never in direct contact since between them there is a plant-derived membrane (Peri-Arbuscular Membrane). Such a membrane is formed by polysaccharides and other transport molecules that allow the transfer between plant and fungus (Parniske, 2008).

The AMF also have an extraradical development, which can reach more than 100 m cm^{-3} of soil (Miller et al., 1995). The extra-radical mycelium (ERM) is considered as an extension of the root system and has been shown that it can extend for more than 25 cm from the root with a remarkable increase of the accessible soil volume for nutrient uptake (Jansa et al., 2003a). In addition, the ERM's small diameter (1 to 10 µm; Bago et al., 1998) can allow exploration to a certain nutrient pool otherwise not accessible. The nutrients intercepted by the ERM are translocated in the intraradical mycelia and after, in part are used for its own metabolism and in part are transferred to the host plant. In exchange, the plant furnishes organic carbon, which can be up to 20% of the total carbon fixed by the plant (Bago et al., 2000).

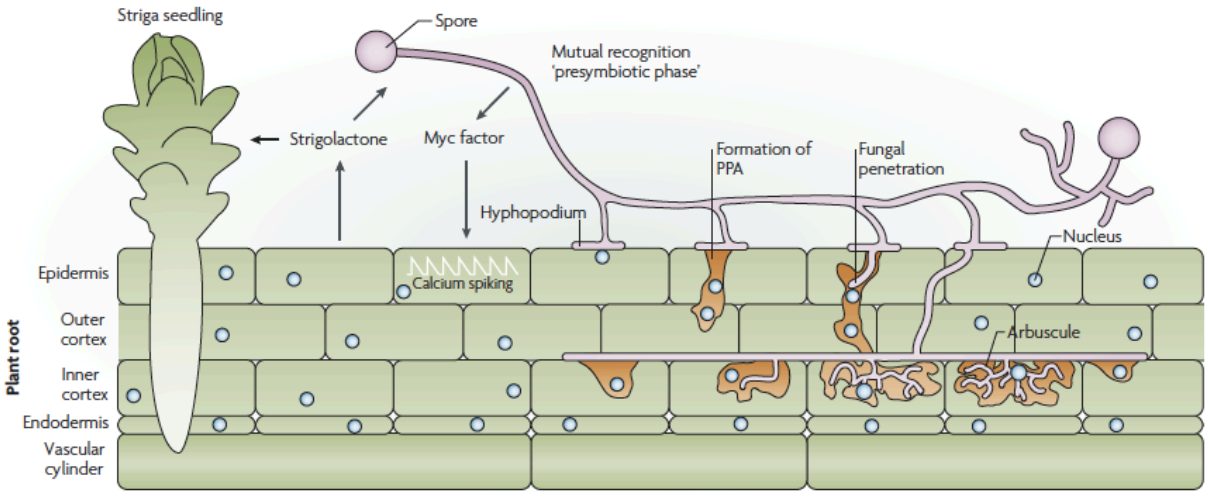


Fig. 1.3. Arbuscular mycorrhiza development. PPA (Pre-Penetration Apparatus). Source: Parniske, 2008.

1.4. AMF functions

Nutrient Uptake

The main role ascribed to AMF has been highlighted in plant nutrition, particularly in the uptake of nutrients with low soil mobility such as P, Zn, S etc. In particular, the vast majority of studies

have been conducted to elucidate the role of AMF in P plant nutrition, which in some cases has been quantified as contributing up to 90% of the total P acquired by the plant (van der Heijden et al., 2015). Often, P in soil is adsorbed to charged soil constituents (e.g. metal oxides, oxyhydroxides, and hydroxides) or bounded to metal cations (Ca in alkaline soils and Al/Fe in acidic soils) that extremely reduce its concentration in the soil solution and confine the plant P pool to the rhizosphere (a few mm from the root surface). As it is well known, P is one of the main essential macronutrients in plant nutrition and a notable amount is required during plant growth. Therefore, the rhizosphere is quickly P depleted and a deficiency during the crop cycle could compromise the productivity of the agroecosystem. In arbuscular mycorrhizal plants, the small diameter of the ERM can explore soil portions not accessible to the plant root hairs, increasing the volume of soil that can be explored and the absorption surface. In fact, the small diameter of the AM fungi ERM forms a dense network in soils, increasing absorption surface area per unit biomass up to two orders of magnitude greater than the plant root system alone (Raven and Edwards 2001), with the consequence of an increment in the P pool available for the plant (Smith & Smith, 2011). Such mechanisms can further affect the kinetics of inorganic P and the amount of P that can be released in the soil solution. In fact, P moves into the soil solution through diffusive flux following a concentration gradient (Frossard et al., 2000) and a major lowering of the soil solution P concentration by the ERM can increase the amount of P desorption and therefore increase the amount of P that can be intercepted and absorbed. Moreover, the ERM exudates determine the solubilization of P from insoluble sources that can be easily acquired by the ERM or the root hair (Tawaraya et al., 2006). The increment of P due to the presence of AMF has been also ascribed to an increment of N fixation in symbiosis with legumes (Larimer et al., 2014; Saia et al., 2014b; Püschel et al., 2017). In fact, several authors hypothesized that the increment in P plant nutrition can increase the plant N requirements and stimulate nodulation. Furthermore, part of the acquired P might be transferred to the rhizobium and stimulate N fixation (Saia et al., 2014b; Püschel et al., 2017). At the same time, the presence of AMF can influence the fate of the N fixed by the legume in intercropping (which is usually ascribed as one of the most productive agricultural systems and that can play a key role towards agricultural sustainable intensification; Brooker et al., 2015). In fact, AMF have a low host specificity, consequentially a single plant can be infected by several AMF species which at the same time can infect several plant individuals/species. In this case, the fungus mycelia create a hyphal network (so-called Common Mycorrhizal Networks) that can connect to neighboring plants and serve as a preferential way for nutrient transfer between these plants (He et al., 2003; Yao et al., 2003; van der Heijden & Horton, 2009; Fellbaum et al., 2014). In cases of legume

non-legume intercropping the Common Mycorrhizal Networks can serve as a preferential way to transfer N from the fixing N component (legume) to the non-fixing N component (He et al., 2003; Meng et al., 2015; Ingraffia et al., 2019). However, the impact of AMF on this process is unclear since N transfer from the legume to the non-legume, no effect, and, in some cases, even transfer from the non-legume to the legume have been all reported (reviewed by He et al., 2003). However, other than N-transfer, the presence of the Common Mycorrhizal Networks can alter the competition or/and the facilitation between the intercropped species transferring nutrients from the weaker component to the stronger or vice versa (van der Heijden & Horton, 2009; Fellbaum et al., 2014).

Concerning N plant nutrition, the contribution of AMF in such process is still unclear. In fact, often the contribution of AMF to N plant nutrition is quite limited or in some cases, the presence of the symbiont fungi even reduces plant growth (Reynolds et al., 2005; Corrêa et al., 2014; Wang et al., 2018). Such results have led to the hypothesis that even when the presence of AMF increases the plant's N uptake, this could be only a consequence of the increment in plant P uptake that increases the plant N demand (Reynolds et al., 2005). However, molecular approaches have highlighted the influence of AMF in the expression of ammonium and nitrate transporters in plants, corroborating the hypothesis of a direct involvement of AMF in N plant nutrition (Koegel et al., 2013; Saia et al., 2015a); in particular concerning ammonium, for which it has been shown that AMF extraradical mycelia have a five-fold affinity compared to the plant root system (Pérez-Tienda et al., 2012). Moreover, several pot experiments have shown a direct role of AMF in plant N uptake from inorganic and organic sources (Johansen et al. 1994; Hawkins et al. 2000; Mäder et al. 2000a; Leigh et al. 2009; Saia et al., 2014a; Thirkell et al., 2016). Aside from the direct involvement of AMF in nutrient uptake, the fungi can affect the SOM cycle. In fact, although during their evolution AMF lost their saprotrophic ability (Tisserant et al., 2013) and hence cannot directly decompose the SOM, the ERM can be a habitat for other microbes which can generate a synergistic association between these organisms (Gahan & Schmalenberger, 2015; Kaiser et al., 2015). In particular, concerning P, a cooperation between AMF and phosphate-solubilizing bacteria can occur and allow to access another P source. Indeed, phosphate-solubilizing bacteria can release several phosphatases hydrolyzing P from the organic matter (Jorquera et al., 2008), which can later be acquired by AMF and transferred to the host plant (Zhang et al., 2016). Moreover, the ERM exudation or the hyphal C itself, due to its fast turnover (a few days for fine hyphae; Staddon et al. 2003), can be a substantial carbon input enhancing the mineralization rate (Hodge & Storer, 2015). At the same time, the presence of AMF can influence soil aeration conditions by influencing soil

aggregation and therefore driving the microbial community composition with consequent repercussions on mineralization, nitrification and denitrification processes (Veresoglou et al., 2012).

Abiotic and biotic stresses

Alongside plant nutrition, AMF have shown to be able to improve plant performances in the presence of abiotic and biotic stress. As far as the abiotic stress is concerned, their role has been highlighted several times in the mitigation of the effects of salinity and drought (Porcel et al., 2012; Saia et al., 2014b; Fileccia et al., 2017; Quiroga et al., 2017); such stresses are often linked to each other, since salinity determines alterations of the hydraulic conductivity and soil water potential. In fact, excess of Na^+ and Cl^- ions in soil disturbs ionic balance in soil solution and hampers its original potential, therefore the uptake, the transport, and the utilization of essential nutrients. Also, soil salinity affects plant growth through toxic effects of Na^+ and Cl^- ions, which leads to denature enzyme structure, damage cell organelles, decrease respiration and photosynthesis, and disturb osmotic imbalance leading to physiological drought and nutrient imbalance in the plant. Salinity is also responsible for oxidative damage to the plant through the generation of reactive oxygen species (ROS) (Borde et al., 2017). Several mechanisms, direct and indirect, have been proposed in the mitigation of the cited stresses by AMF. The indirect effects are ascribed to the better nutritional state that is often observed in AM-plants compared to non-AM-plants (non-AM plants defined as the same plant species grown in absence of AMF), and hence more able to contrast the stress. Among the direct mechanisms, the ability of the ERM to explore a greater volume of soil than the sole root system is surely one of the main factors that can mitigate the stress of drought. Ruth et al. (2011) have estimated that this amount could reach up to the 20% of the total water acquired by the plant. Moreover, it has been shown that AMF can increase the uptake of several micronutrients (Clark, & Zeto, 2000). In particular, this process could be essential under salt stress to decrease the toxic effects of Na^+ and Cl^-. In fact, Na^+ and K^+ can be replaced, and under sodium salinity conditions, the elevated concentration of sodium in the soil solution can inhibit nutrient uptake by interfering with various K^+-selective ion channel transporters in the plasma membrane (Wild, 1988). In addition, Na^+ can interfere with various metabolic pathways, such as protein synthesis, and enzymatic activity and in the stability of the cell membrane (Giri et al., 2007; Estrada et al., 2013). Under high salinity, the presence of AMF can reduce the Na^+/K^+ ratio further reducing the deleterious effects of sodium (Mohammad et al. 2003). In addition, AM symbiosis can affect plant gene expression, and therefore plant physiology and biochemistry,

such as: increase in turgor potential, root hydraulic conductivity, stimulation of the synthesis of osmolytes (proline and betaine) and aquaporins, stimulation of the antioxidant enzyme activity etc. which increase the plant's ability of stress mitigation (Porcel et al., 2012; López-Ráez, 2016).

As regards biotic stress, the presence of AMF can remarkably reduce root infection by antagonistic endophytes and other telluric microbial pathogens (Larimer et al., 2010; Jung et al., 2012). Indeed, the complete carbon dependence of AMF on the host plant determines that these organisms occupy the same ecological niche, and therefore are direct competitors for carbon sources (Jung et al., 2012). Additionally, the presence of AMF affects the root exudate composition by stimulating the production of secondary metabolites (phenolic and allelopathic compounds; Jung et al., 2012). Moreover, Pozo & Azcón-Aguilar (2007) have highlighted the role of AMF in containing the aboveground antagonistic community as well. The latter seems to be due to morphological and metabolic alterations of the host plant (Jung et al., 2012), aside from the better nutritional status of the AM-plant compared to non-AM-plant. In fact, the presence of AMF influences the cell wall lignification and stimulates the synthesis of several plant defense phytohormones (salicylic acid, jasmonic acid, phytoalexins etc.; Jung et al., 2012).

Nutrients loss

The increment in plant nutrient uptake due to AM symbiosis and the amount of nutrients immobilized in the fungal biomass drastically reduce the amount of nutrients that can be lost through different pathways in the agroecosystem (Asghari & Cavagnaro, 2012; Cavagnaro et al., 2015; Köhl & van der Heijden, 2016). Furthermore, as previously mentioned, AMF can influence the surrounding microbial community and soil aggregation and, therefore, processes such mineralization, nitrification, and denitrification (Veresoglou et al., 2012; Cavagnaro et al., 2015). In addition, the preferential uptake and immobilization of ammonium reduce the amount of N that can be nitrified and lost via leaching, as nitrate is highly mobile in soil and easily leached. At the same time, the reduction of the N pool for the nitrification contains the production of N_2O. In this regard, several microcosm experiments have shown a substantial reduction in N_2O production in systems with the presence of AM-plants compared to when plants were grown in absence of AMF (Bender et al., 2014; Zhang et al., 2015a; Storer et al., 2017). In addition, the AMF extra-radical mycelia represent a relevant quantity of soil organic carbon. In fact, their intimate nutrient exchange, regards organic carbon with the host plant, make AMF one of the major organisms with a direct effect on soil carbon cycle (Johnson et al.,

2013). Moreover, recently a greenhouse experiment has shown that AMF can stabilize carbon within soil during litter decomposition (Verbruggen et al., 2016), showing the impact that AMF can have on CO_2 production, the main greenhouse gas involved in global warming (ICPP, 2001).

Soil aggregation

As previously mentioned, AMF can affect the formation and the stabilization of soil aggregates; fundamental aspects of soil quality and fertility and an essential element to consider for agricultural sustainability. In fact, the containment of soil erosion has become a global priority in the new agricultural approach and the aggregative status of soil particles plays a key role in such processes. Several studies have reported a direct linked between soil aggregation and extra-radical AMF mycelia growth in soil (Rillig et al., 2002; Wilson et al., 2009; Rillig et al., 2010; Leifheit et al., 2014). AMF seems to be able to affect soil aggregation through physical and chemical mechanisms, and through interaction with other soil biota (Lehmann et al., 2017). In fact, extra-radical hyphae growing into the soil exert pressure between the soil particles which will get closer and therefore compress each other. As regards the chemical mechanisms, it has been shown that the fungal mycelia can excrete several polysaccharides during their lifecycle (Hooker et al., 2007) and influence the presence of a particular class of proteins (glomalin-related soil proteins) (Rillig, 2004), which can serve as a cement-like stabilizing aggregate (Rillig et al., 2007; Lehmann et al., 2017). Moreover, hydrophobins (hyphae exudate compounds) can increase the aggregate stability by water repellency and prevent wetting of aggregates and regulate aggregates' aqueous/aerial phases. The latter, by influencing the physical environment of the aggregate influence the living microbial community, which together with the ERM's living bacteria can affect soil particle alignment (Caesar-Tonthat, 2002).

1.5. Effect of agricultural practices on AM Symbiosis

Although AM symbiosis is usually described as a mutualistic relationship, the outcome of the symbiosis is driven by the environmental conditions under which the symbiosis is established. AM symbiosis can cover the whole ecologic relationship spectrum (parasitism, commensalism, and mutualism; Johnson et al., 1997; Johnson et al., 2015), defining mutualism an increment, commensalism no difference and parasitism a decrement of plant biomass production due to the presence of AMF. In particular, parasitism can occur when the plant is grown in high nutrient availability and can satisfy its own nutrient requirements regardless of the presence of the symbiotic fungi. In such a scenario, the carbon transferred to the fungus could be allocated

in the plant biomass increasing its own performance (Smith et al., 2009). In turn, the presence of AMF can result detrimental for the plant growth when the symbiosis is established under very low nutrient availability, in particular of N. In fact, under such conditions both organisms (host plant and AMF) can compete for nutrient acquisition and the relationship become parasitic (Püschel et al., 2016).

Clearly, in agroecosystems the agricultural practices adopted can markedly influence the environmental conditions and therefore the outcome of the AM symbiosis.

Fertilization

Given the key role played by AMF in the acquisition of nutrients, surely fertilization is one of the main agricultural practices that can affect the AM symbiosis outcome. In particular, a strong depressive effect of fertilization has been found on ERM development and on the percentage of root infection by AMF (Mäder et al., 2000b). After all, when the plant is grown in high nutrient conditions the benefit of the AM symbiosis is markedly reduced and therefore the plant could tend to reduce colonization. In fact, the plant plays a key role in the symbiosis establishment and can reduce the amount of transferred carbon when no rewards occur (Kiers et al., 2011).

Fertilization is a keystone in the agroecosystem productivity and often inorganic fertilizers are required to replace nutrients removed by crops and obtain high productivity, as the yield may be subject to a severe decrement in absence of fertilization or when it occurs only with organic fertilizer (Giller et al., 2015; Connor, 2013). However, inorganic fertilizers usually have a low use efficiency; for instance more than 50% of N can be lost via leaching or volatilization (Tilman, 1998; Sharpley et al., 2003; Ruisi et al., 2016). Clearly, in light of ecological intensification of agroecosystems, this practice needs to be revised and particular attention needs to be put on nutrient loss containment and use efficiency increment. AMF could serve in this pathway since as previously reported it can markedly increment the uptake of several nutrients. However, it is clear that AMF cannot completely substitute fertilization, but a potential reduction of the amount of applied fertilizers is likely if the soil microbial community is considered in the context of fertilization management (Srivastava et al., 2017). In addition, due to the role played by AMF in nutrients cycling per se and in interaction with other soil microbial organisms (particularly with the bacterial community), it would be possible to apply fertilizers in a more stable form, for instance as organic manure, compost or compost extract, etc., which can increase soil quality other than serving as a nutrient source. Although, it should

be remembered that these latter forms of fertilization are not devoid of environmental drawbacks (Adesemoye & Kloepper, 2009).

However, the effect of fertilization on AMF community and on the AM-mediated plant growth is still debated, especially concerning N fertilization. In fact, although several studies have reported a strong reduction of the AMF root colonization, AMF abundance and diversity following high P supply (Olsson et al., 1997; Camenzind et al., 2014; Chen et al., 2014; Lin et al., 2012), controversial effects, shifting from negative to no impact, have been reported following N fertilization (Albizua et al., 2015; Verbruggen et al., 2013; Tian et al., 2013; Williams et al., 2013). Such aspects can affect the functionality of the AM symbiosis since evidence arising from a pot experiment points to AM fungi diversity as having a key role as drivers of plant productivity (van der Heijden et al., 1998). This is not surprising, in fact as reported by Parniske (2008), the different AMF species have different mycelia growth patterns and therefore can have complementarity in the benefits they may offer. Thus, the effect of the agricultural management, and in particular of the fertilization, on the AMF soil diversity can markedly affect the AM symbiosis output and therefore the crop production. Additionally, the soil nutrient availability may affect the functionality of the AM symbiosis. In particular, concerning the two most studied macronutrients (N and P), seems that a key role is played by the soil N and P availability ratios. Johnson (2010) proposed the "trade balance model" to explain how N and P soil availability can affect the AM symbiosis shifting the relationship between plant and fungi from mutualism to parasitism and vice versa. Essentially, the model suggests that N fertilization can only be beneficial if the plant is in a P limited condition and will therefore benefit from providing C to the roots and symbiotic fungi. Pot experiments have been conducted to verify the "trade balance model" manipulating P and N soil availability (Johnson et al., 2015; Püschel et al., 2016), however, experiments have been conducted on *Andropogon gerardii* a highly mycotrophic C4 grass, while no information is available on moderate mycotrophic C3 plants. As N utilization by AMF is concerned, it is established that AMF take up inorganic N either NH_4^+ and NO_3^- (Bücking & Kafle, 2015), but if and how AMF utilize organic N is still poor invetsigated. Moreover, organic matters deriving from different residues can differ in their behavior in the mineralization process (Cabrera et al., 2005) differing both in time and in the amount of nutrient that can be released from the process. Given the ecological role of AMF in nutrient cycling, uptake and transfer to the host plant, addressing this lack of information is now pressing in the light of an agricultural focused on the environment and economic sustainability.

Pest protection and weed management

Along with fertilization, plant protection from pests and post-sowing weed control are two agricultural practices that affect the productivity of the agroecosystem. Among the various strategies to combat pest and weeds, chemical-derived compounds are the least expensive, easiest and most effective way (Fartyal et al., 2018), and therefore, up to now, the widespread in conventional farms growing field crop. However, the application of such chemical-derived compounds affects the soil microbial community and thus also the AMF community (Li et al., 2013; Rivera-Becerril et al., 2017).

As far as pesticides are concerned, in vitro experiments have shown that such compounds can determine profound alteration in spore germination and infective processes, in the extraradical mycelium architecture and spore formation (Zocco et al., 2008). Furthermore, the presence of fungicides alters several enzymatic activities related to the extraradical mycelium (Zocco et al., 2011). Moreover, results from a field experiment have shown that fungicide applications can significantly alter the composition of the AMF community (Rivera-Becerril et al., 2017).

As for weed management, the use of herbicides can affect the AMF community both directly, by interfering with several metabolic pathways (Li et al., 2013), and indirectly, altering host quality and quantity (Lekberg et al., 2017). In fact, despite the low specificity fungal-host species in the AM symbiosis, if compared to other symbioses such as Fabaceae-Rhizobacteria, each host plant has a particular preferential association with some AMF species partner and vice versa (Sanders, 2003).

The alteration of the AMF community due to the applied chemical input into the agroecosystem could be a paradox. In fact, although the application of such chemical compounds protects the crop against pests, on the other hand, they can reduce the benefits provided by the AMF community, making the "system" even more dependent to the chemical input. Vice versa, pest control through natural enemies could, on the one hand, reduce the damages from pest and on the other hand not alter the eventual benefit deriving from the AM symbiosis. Several organisms belonging to the phyla Pseudomonas, Bacillus, and Trichoderma could markedly reduce the pest crop damage (Turlings et al., 2012; Pieterse et al., 2014) and at the same time maintain the benefits provided by AMF. In fact, since such organisms may do not have the undesirable effect exerted by the chemical-derived compounds, possibly, they would not exert any antagonistic influence in the plant-AMF interaction. Nevertheless, inoculation of microorganisms (either AMF or other ones), cannot be considered an agricultural practice to be applied indiscriminately since can have several undesirable consequences (Machado et al., 2017; Hart et al., 2018). In the light of the latter, the inoculation of microorganisms should be only applied in severely

degraded soils (i.e. restoration post mining) or in soils with very low inoculum potential (e.g. some situations in conventional agriculture or desertified landscapes), and avoid this practice in many other systems, where resident AMF communities are well-established, as in most agricultural soils (Oehl et al., 2010). Moreover, it would be very important to focus on the application and modification of agricultural practices aiming to maintain and increase the agroecosystem biodiversity. Indeed, an increment of biodiversity in sensu lato is always ascribed to an increment of the resilience of biological systems (Peterson et al., 1998; Mori, 2016), which, as previously reported, can have a key role on of plant productivity (van der Heijden et al., 1998).

Crop diversity and Tillage system

Based on the assumption that each plant species establishes symbiosis preferentially with some AMF species rather than others (Sanders, 2003), crop diversity in the agroecosystem plays a key role in increasing AMF diversity. With reference to this aspect, Johnson et al. (2004) in a pot experiment have shown a greater AMF diversity in Plantago lanceolata growing in soil previously hosting a mixture of 12 species than soil previously hosting a monoculture.

Within agroecosystems, intercropping and crop sequence are fundamental in increasing the plant community diversity, and hence key-players in increasing AMF community diversity. In addition, the different plant species can profoundly vary in the magnitude of root colonization (Hendrix et al., 1995). For instance, the extent of AM colonization varies between C4 and C3 plants species (Wilson & Hartnett, 1998) and among the C3 plants, in turn species belonging to different plant families (Barea et al., 1989; Ingraffia et al., 2019). Therefore, considering the AM compatibility in the choosing crops during the agroecosystem management could influence AMF diversity and the AMF inoculum potential left in the soil by the previous crop. Those parameters are also strongly affected by the soil tillage management (Jansa et al., 2002; and 2003b). Indeed, under conventional tillage (usually consisting as moldboard-plowing followed by one or two shallow harrowing; CT), extra-radical mycelia and root infected fractions are subjected to forceful physical pressure that can greatly reduce their integrity (Jansa et al., 2003b). Such structures, as reported by Klironomos & Hart (2002), in non-disturbed contexts are the main inoculation source and seem to be faster and more efficient than spores in the colonization of new plant root (Martins & Read, 1997). The impact of tillage on the extent of the extraradical mycelia can also affect the amount of glomalin-related soil proteins, which, as previously reported, can influence soil aggregation and hence affect soil erosion (Mardhiah et al., 2016), the latter already negatively affected by the CT management itself (Scopel et al.,

2005). Additionally, the impact of the tillage operations can reduce the AMF community diversity and the abundance of inoculum potential, compared to that observed under NT conditions, and favor the presence of species that invest more in sporulation than in symbiotic activity (Jansa et al., 2002; Säle et al., 2015). However, other studies have reported no effect of tillage on AMF abundance and diversity. For instance, Jansa et al. (2003b) by comparing the AMF communities deriving from three different tillage systems (CT, chisel plough, and NT) found great differences in the community structure, but did not detect differences in the diversity index. On the other hand, changes in AMF species that make up the AMF community structure may differ in their symbiotic efficiency (Jakobsen et al. 1992, Smith et al. 2000), and therefore can differently affect the host plant growth.

In the light of the foregoing, no-tillage seems to be the preferential soil management practice to increase the benefits obtainable from the AMF community. Moreover, NT can provide several environmental and agronomical benefits per se, reducing soil evaporation and improving soil aggregation and the water pool available (Kirkegaard, 1995; Madari et al., 2005; Lampurlanés & Cantero-Martínez, 2006). However, usually in such practice, sowing is preceded by the application of large spectrum herbicides (i.e. glyphosate-based herbicides) to reduce the competition of the spontaneous flora against the crop. Given its widespread use this class of herbicides has been object of several experiments and, notwithstanding its fast biodegradation and strong adsorption to soil particles (Vereecken, 2005), evidence reports that several classes of organisms, among others AMF, can be severely affected by glyphosate-based herbicides (Berger et al., 2013; Köhler et al., 2013; Zaller et al., 2014). Such aspects could be a deterrent for the application of the no-tillage system. However, a combination of the different soil management practices such no-till, reduce or strip tillage combined with cover crop, mulches, and crop rotation could reduce the need of recourse to herbicides (Pittelkow et al., 2015; Giller et al., 2015).

Although the conventional tillage practices are mostly concentrated in the first 30 cm (topsoil) of the soil profile, the effect could be extended to the subsoil (> 30 cm), reducing the porosity, the hydraulic conductivity etc. (Pagliai et al., 2004). Moreover, long-term conventional tillage can determine the formation of a plow pan which can reduce the subsoil root exploration (Ehlers et al., 1983), consequently reducing the possibility of establishing symbiosis with the subsoil microbial community. Subsoil has been shown to be a source of exclusive AMF species (Oehl et al., 2005; Sosa-Hernández et al., 2018a; Sosa-Hernández et al., 2018b) which could have evolved specific traits to explore the subsoil's hostile conditions and increase the nutrient availability for the crop, since subsoil has been also ascribed as a notable pool of mineral

nutrients (two thirds of the total plant requirements; Kautz et al., 2013) and water (Kirkegaard et al., 2007). Moreover, a recent review by Sosa-Hernández et al. (2019) have highlighted the potential contributions of subsoil AM fungi as a tool to reduce the fertilizer amount, increase the utilization efficiency of the supplied nutrients, and reduce the greenhouse gas emissions in agriculture.

However, although evidence from both spore identification techniques and molecular methods have shown different microbiome communities in NT compared to CT and in the topsoil compared to the subsoil, the role of the different communities deriving from these different environments on plant growth has not yet been investigated. Experiments assessing these potential differences are essential to fill this lack of knowledge since the effect of the different AMF communities on plant growth response is crucial for successful integration of the soil microbiome in the agroecosystem management.

1.6. Objectives of the thesis

In the last decades, several studies have shown that soil microbiomes could offer several services in agriculture, reducing the environmental impact and improving yields (Kloepper et al., 1989; Richardson & Simpson, 2011; Asghari & Cavagnaro, 2012; Bardgett & van der Putten, 2014; Bender et al., 2015, 2016; Cavagnaro et al., 2015). Among others, AMF can play a particular role in this scenario, as they are able to determine several benefits to the host plants and to the environment (Pellegrino et al., 2015; Cavagnaro et al., 2015; Köhl & van der Heijden, 2016; Ryan et al., 2018; Zhang et al., 2018a).

However, as previously reported, the extent of the benefits is context-dependent and, in some cases, the presence of AMF has led to negative results (Johnson et al., 1997, 2015). Furthermore, most of the available data derive from experiments conducted on highly mycotrophic C4 plant, while less information is available on moderate mycotrophic C3 plants. This thesis was aimed to contribute to the understanding of the relationship between a moderate mycotrophic C3 plants (durum wheat, a main crop for the Mediterranean environment) and soil microbial community (with particular regard to AMF) with the ultimate goal of providing information useful to develop agronomic solutions able to increase the potential benefits that soil microbiome can offer.

Actually, to date there are still several little-known aspects about the relationships between microbiome and crops; the present thesis, by means of different specific experiments, aimed to address the following gaps of knowledge:

- the direct and indirect involvement of AMF in plant N nutrition is still debated as experimental results are often contrasting mainly due to the different conditions of total soil N content and its availability for plants. To this end an experiment was carried out to test the hypothesis if AMF enhance durum wheat nitrogen uptake and nitrogen recovery from mineral fertilizer or organic matter added and if the effects differ at varying N availability and the characteristics of the organic matter added (different plant residues and C:N ratio);
- soil N and P availability ratios can affect the AM symbiosis shifting the AM symbiosis through the entire ecological relationship spectrum (ranging from mutualism to parasitism). In order to contribute to fill the knowledge gap on these aspects, two microcosm experiments were set up: the first was aimed to test if the AMF outcome in relation to the soil N availability presents a curvilinear response and to ascertain in which conditions of N availability the interaction plant-AMF shifts towards parasitic form; the second was aimed

at testing if the effects of the mycorrhizal response to the availability of N is affected by P availability.

- although modifications in the AMF community structure due to tillage system and soil depth have been observed, no information are available about the potential effect of the different communities on plant growth. Two different experiments were carried out to evaluate i) if, along a soil profile, AMF abundance, diversity and community structure is influenced by soil depth; ii) to test if the live inocula derived from different soil depths and from different soil tillage systems influence the wheat growth, its water use efficiency and N uptake.

2. Experiments

2.1. Effects of AM Symbiosis on Durum Wheat Growth and Nitrogen Uptake and Recovery when Organic or Mineral Nitrogen is applied

Abstract

Plants performance is strongly dependent on N nutrition, consequentially increment in N nutrition is essential in the agro-ecosystem productivity. AMF effects on plant N acquisition from both organic and inorganic sources is debated since contradictory results have been reported. The effect seems to rely on the soil N availability, since AMF have a notable N demand for their own metabolism and can even compete with the host plant under soil N-deficiency. Moreover, although AMF can transfer N from organic source, no information is available on whether or not this ability change varying the organic source composition. Using ^{15}N-labelled fertilizers as tracer, here was conducted an experiment aimed to evaluate the effect of AMF on N uptake and recovery from mineral or organic source in durum wheat. Moreover, two different organic sources were used to evaluate whether or not the organic matter composition can influence the AMF effect. A non-fertilized treatment was also included in the experiment.

Under sufficient N availability, AMF had no effect on plant biomass but have increased the N concentration, the plant N uptake and the N recovered from the fertilizer, whereas in soil N-deficient AMF determined a decrement of the aboveground biomass, highlighting a competition between plants and AMF for the element. Furthermore, AMF varied their effect varying the composition of the organic source composition. At low C:N ratio of the organic source applied AMF favored the plant N uptake and the N recovery. On the contrary, when the organic source had a high C:N ratio, a clear reduction of N recovery from the fertilizer was observed.

Introduction

Durum wheat is a keystone crop of the Mediterranean agroecosystem. As for other species, its growth is completely dependent on soil N and its performance is strongly subject to the soil N availability along all the phenological cycle. In fact, evidence from field experiments has reported a drastic decrease in crop yield by reducing the amount of N fertilizer (Giambalvo et al., 2004). However, it has been estimated that often 50% or less of the N fertilizer applied to soil is recovered by cereals and that this percentage decreases as the N fertilizer rate increases (Foulkes et al., 1998; Raun and Johnson, 1999; Blankenau et al., 2002; Ruisi et al., 2016). This

has also important agro-environmental implications as, due to the high N mobility in the soil–plant–atmosphere system, N not used by plants greatly contributes to agriculture-related pollution through leaching, volatilization, and denitrification (Drinkwater et al., 1998; Limaux et al., 1999).

Several studies have highlighted that AMF can play an essential role in plant N acquisition from both organic and inorganic sources (Johansen et al. 1994; Hawkins et al. 2000; Saia et al., 2014a; Thirkell et al., 2016); however, their role is often controversial (Corrêa et al., 2015; Wang et al., 2018). In fact, AMF have a notable N demand for their own metabolism (Hodge & Fitter, 2010) and can even compete with the host plant for soil N when the soil is N-deficient (Püschel et al., 2016). Moreover, although AMF can enhance the mineralization rate by releasing C to the mineralization site (Hodge & Storer, 2015; Bunn et al., 2019) and/or by influencing the soil microbial community (Veresoglou et al., 2012), little is known if this ability differs in presence of organic matter with different composition (i.e. different C:N ratio). In fact, contrary to what occurs when the organic matter has a relatively low C:N ratio, in the first step of the mineralization there is a temporary immobilization of N when the organic matter has a relatively high C:N, which reduce the soil N availability and therefore, possibly, also the AM symbiosis functionality.

The present experiment aimed to test if AMF enhance durum wheat N uptake and N recovery from mineral fertilizer or organic matter added and if these effects differ at varying N availability and characteristics of the organic matter added.

Materials and Methods

Pot and Plant management

The experiment was carried out outdoors in 6 liter sterilized pots (d=16 cm; h=30 cm) under sterilized artificial substrate growing durum wheat (T. durum Desf. cv. Anco Marzio). The growing substrate was composed as follow: 70% in river sand and 30% in agricultural soil. Both the substrate portions have been sieved through a 2 mm mesh, characterized and sterilized following the cycle: humidification, 24 hours at room temperature and 24 hours 130 °C, for a total of three cycles. Sand total N (Kjeldahl) and available P (Olsen P) were 0.11 g kg^{-1} and 7. 44 mg kg^{-1} respectively. Agricultural soil properties were as follows: 267 g kg^{-1} clay, 247 g kg^{-1} silt, and 486 g kg^{-1} sand; pH 8.0; 10.8 g kg^{-1} total C (Walkley— Black); 0.86 g kg^{-1} total N (Kjeldahl); 40.1 mg kg^{-1} available P (Olsen P), 598 mg kg^{-1} total P, 26 cmol kg^{-1} cation exchange capacity; 1.70 dS m^{-1} saturated electrical conductivity (EC) (25°C); 27.9% water content at field capacity, and 18.9% at the permanent wilting point.

Each pot has been filled with 7.5 kg of substrate. The crop was sown on February the 3rd 2016, distributing 15 surface sterilized seeds per pot. Ten days after the emergence the thinning was done to reach the final density of 7 plants per pot. All the pots and seeds were sterilized using sodium hypochlorite 3% for 3 to 5 minutes. After the sowing, all the pots were irrigated to reach the holding capacity. Afterward, the soil moisture was monitored twice a week through the gravimetric method and additional water was added when the soil moisture reached 70% of the holding capacity. The hydrologic parameters of the substrate were determined using the gravimetric method (Dobriyal et al., 2012). Briefly, 10 perforated crucibles were filled with 100 grams of soil and placed in a basin with water up to half of the crucible's height. The crucibles were let to absorb water by capillarity until each pot was saturated. The excess water was let drained away and the weight difference at field capacity and the following drying at 105 °C to a constant weight was monitored. The available water for the crop was obtained by subtracting the weight after the oven dry period from the field capacity weight. The studied factors were: i) fertilization: control not fertilized; two levels of mineral N supply; two different organic matter amendments; ii) inoculation: uninoculated control; AMF inoculation. A total of 50 pots were set up [2 (with or without AMF inoculum) *5 soil-N levels*5 replicas] in a completely randomized design. The plants were grown for 84 days after the sowing (DAS).

Fertilization treatments

Durum wheat in presence (+myc) or in absence (-myc) of AMF inoculum has been grown under four N fertilizer treatments (N-org1; N-org2; N-min1; N-min2) and in a non-fertilizer treatment (N0). 10% ^{15}N enriched ammonium sulphate ($(NH_4)_2SO_4$) was applied in the two treatments N-min1 and N-min2. The treatment N-min1 was obtained applying a total of 0.75 g of fertilizer per pot (equivalent to 78.37 kg of N ha^{-1}). In the treatment N-min2, the amount of N applied was doubled, hence, a total of 1.5 g of fertilizer per pot (equivalent to 156.75 kg of N ha^{-1}) was added. In both the mineral treatments, the fertilizer addition was split in two fertilization events: two third of the total fertilizer amount, 0.5 and 1 g per pot (equivalent to 52.25 and 104.5 kg of N ha^{-1}) for N-min1 and N-min2 respectively, was applied eleven days after the emergence; the rest (one third of the total amount), 0.25 and 0.5 g per pot respectively for N-min1 and N-min2, was applied 38 DAS (concomitantly with the durum wheat elongation phase beginning).
All of the organic N treatments have been done applying 13 grams of organic matter (OM) per pot (equivalent amount of 6.5 Mg ha^{-1}). This amount is approximately equivalent at the usual amount of biomass left by the previous crop in the field in the semi-arid agroecosystems.

The OM was chopped (c. 2 mm) and homogenously distributed at 5 to 10 cm depth one day before the sowing. Residues of two crops with different C:N ratio, Lolium multiflorum (Ryegrass; N-org1) and Vicia faba (Faba bean; N-org2), were used as organic N source. Both the OM were obtained from a pilot experiment where the two species were grown on ^{15}N-enriched soil. The pilot experiment ended when both the species reached the maturation phase. Later, the biomass was harvested, dried and characterized for the total C, N and ^{15}N concentration (Table 2.1.1). OM characteristics are reported in the Table 2.1.1. Therefore 0.118 and 0.363 g of organic N per pot were applied in N-org1 and N-org2 treatments, respectively.

Table 2.1.1. Properties of the Organic N sources used in the experiment.

Crop residual	Total C (g kg^{-1} dry weight)	Total N (g kg^{-1} dry weight)	C to N ratio	^{15}N content (g kg^{-1} total N)
Faba bean	447	27.9	16.2	4.3
Ryegrass	453	9.1	49.7	8.9

Inoculum

At sowing time, the natural soil microbial community, discarding AMF, was reintroduced to each pot. To this end, a soil filtrate solution was obtained through filtration of a soil suspension. Briefly, the soil was suspended in distilled water at the ratio of 1:3 and shacked for 20 minutes at 140 rpm. Later, after the decantation, the suspension was filtered through an 11 μm mesh to discard the natural AMF community. A total of 200 ml of soil filtrate solution was added per pot. Additionally, half of the pots (+myc) were also inoculated with arbuscular mycorrhizal fungi, adding 1 g per pot of a mix of two AMF species (*Rhizophagus irregularis and Funneliformis mosseae*), equally present at a density of 700 spores g^{-1}. The AMF inoculation has been done at the same time of the sowing time distributing the inoculum just below the sowing bed.

Biomass harvest and analysis

At the end of the experiment (84 DAS), the aboveground biomass (shoot) was harvested and the fresh and dry weight recorded. Later, the aboveground biomass was ground to fine powder and the total N and the ^{15}N concentration were determined. The total N concentration was determined using the Dumas method (flash combustion with automatic N analyzer; DuMaster D-480, Büchi Labortechnik AG, Flawil, Switzerland), while the ^{15}N concentration was determined through the method MIP 134 Rev. 00 2017 (Delta V Isodat Acquisition Flash EA).

The belowground biomass (root) was carefully extracted through sieving and consecutive washing, and thereafter oven dried at 40 °C until constant weight.
Two root biomass subsamples were extracted. One was used to quantify the root length using the modified Newman formula (Tennant, 1975):

$$\text{Root length} = (11/14) \times N \times G$$

where N is the total number of intercepts of root with vertical and horizontal grid lines, G is the grid square dimensions (cm).
The other root subsample was cleared with KOH 10%, stained with trypan blue 0.05% (Phillips and Hayman, 1970), and used to quantify the percentage of AMF infection using the method proposed by McGonigle et al. (1990). The AMF infection was assayed by scoring a minimum of 150 lines intersection for the presence of intra-radical AMF structures.

Soil sampling and analysis

During the root extraction, a representative soil sample was collected. The soil sample was sieved at 2 mm and immediately stored at -20 °C to minimize changes in nutrients. Later, soil mineral N (nitrate, nitrite and ammonium) content at the sampling stage was assayed through colorimetric method using the Bran+Luebbe AutoAnalyzer 3 (Norderstedt, Germany). Briefly, 10 grams of soil were extracted in 100 ml of a 2M KCl-extractable solution and shaking for 1 hour at 140 rpm. The solution was later filtered through filter paper (Whatmann 42) and used to assay the $N\text{-}NH_4^+$, and $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ concentration.

Calculation and statistical analysis

The total root length was calculated based on the known weight of both the subsample and the total root biomass. The specific root length (SRL) was calculated by dividing the total root length with the total root weight, while soil root density (SRD) was obtained by dividing the total root length with the pot soil amount (grams of soil per pot).
N uptake was obtained by multiplying the N concentration for the aboveground biomass production in each pot. The ^{15}N concentration was used to determine the amount of N recovered from the applied fertilizer ($^{15}N_{rec}$) and its percentage ($\%N_{rec}$) applying respectively the following equations 1 (eq.1) and 2 (eq.2) according to Hauck and Bremner (1976):

$$^{15}N_{rec} = N_{upt}\, X \frac{^{15}N_{fp} - {}^{15}N_{nfp}}{^{15}N_{fert} - {}^{15}N_{nfp}} \qquad (1)$$

$$\%N_{rec} = \frac{^{15}N_{rec}}{f} \; X \; 100 \qquad (2)$$

Where $^{15}N_{fp}$ is the ^{15}N atom% in the fertilized plant, $^{15}N_{nfp}$ is the ^{15}N atom% in the nonfertilized plant (N0) from the same inoculation treatment, $^{15}N_{fert}$ is the ^{15}N atom% in the fertilizer, and f is the fertilizer rate (g pot^{-1}).

A two-way factorial analysis of variance (ANOVA) was used to determine whether data collected were affected by N treatment, AMF inoculation or their interaction. Due to the negligible colonization of the non-inoculated treatments, statistical analysis of the response variable "AMF colonization" was performed on the subset of the inoculated treatment (+myc). The response variable was subjected to the one-way ANOVA using "fertilization" as explanatory variable. The analyses were performed using R software version 3.4.1 (R Core Team, 2017). Shapiro test and Bartlett test were used to assaying respectively the normality and the homoscedasticity of the model residuals. When response variables did not fulfill the ANOVA assumptions data were transformed accordingly. Following the ANOVA test, post hoc mean comparison Tukey's HSD test ($P \leq 0.05$) was performed. When interaction terms were significant, single degree freedom contrasts were conducted to investigate the effect of mycorrhization within each fertilization treatment.

Correlation between the percentage of root colonization by AMF and N applied (fertilizer N concentration x amount of fertilizer per pot) was performed using the subset of the inoculated treatments.

Untransformed data have been reported in tables and in graphical representations. Package "tidyverse" (Wickham, 2017) was used for data graphical representation.

Results

Mycorrhizal colonization

Although AMF colonization was observed in the non-inoculated treatments (-myc), the extent of the colonization was always less than 4% and very different from the values observed in the inoculated treatments (+myc) (Table 2.1.2). The colonization percentage in the inoculated treatment ranged from an average of 19.60 % observed in the treatment N-min2 to an average of 26.83% observed in the treatment N0. The percentage of AMF colonization was significantly higher in N0 treatment compared to all the other treatments; no significant differences were observed among all the other treatments. In addition, overall the parameter has shown a negative correlation with the N applied ($r = -0.66$; $P \leq 0.001$).

Table 2.1.2. AMF root colonization, total root length, soil root density (SRD), specific root length (SRL), root shoot ratio (R:S), aboveground N concentration, and total mineral N residual in soil observed in the different fertilization treatments in absence(-myc) or presence (+myc) of AMF inoculum.

		AMF colonization (%)	Root length (m)	SRD. (cm g^{-1})	SRL (m g^{-1})	R:S	Nitrogen concentration (%)	Soil mineral N (mg kg^{-1})
N0	**-myc**	-	730^{b}	9.74	138.3	$0.26^{\#}$	$0.63^{\#}$	$2.61^{\#}$
	+myc	26.83	844^{a}	11.26	142.50	0.32	0.55	2.02
N-org1	**-myc**	-	570	7.61	138.20	$0.25^{\#}$	$0.68^{\#}$	2.00
	+myc	22.25	568	7.58	143.00	0.29	0.79	2.02
N-org2	**-myc**	-	939	12.52	154.70	0.23	$0.74^{\#}$	$2.73^{\#}$
	+myc	21.56	992	13.22	157.30	0.24	0.89	2.27
N-min1	**-myc**	-	957	12.76	142.10	0.24	$0.76^{\#}$	$2.15^{\#}$
	+myc	21.64	1042	13.90	151.40	0.23	0.95	1.99
N-min2	**-myc**	-	1157	15.42	144.90	0.24	1.13	2.41
	+myc	19.60	1243	16.58	160.20	0.23	1.26	2.32
Source	**df**							
Fert	**4**	*** (4.29)	*** (76.03)	*** (1.01)	*** (12.02)	*** (0.027)	*** (0.09)	*** (0.14)
myc	**1**	-	* (48.09)	* (0.64)	ns	* (0.017)	** (0.06)	*** (0.09)
Fert x myc	**4**	-	ns	ns	ns	*	*	***

Data are means, (n = 5). *, ** and *** indicate significant differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences. Values in brackets indicate significant differences at P≤0.05 (Tukey's HSD test). # indicate significant difference between -myc and +myc within the same fertilization treatment (*P≤0.05*).

Plant biomass production

The fertilization treatment has clearly affected the plant biomass production in both the fractions above and below ground (Fig. 2.1.1a, b). The mineral N fertilization has always increased the biomass production compared to the non-fertilized treatment N0. In particular, an increment of 48% and 67% (overall means of +myc and -myc) was observed in the aboveground biomass production for the treatments N-min1 and N-min2 respectively compared to the non-fertilized treatment N0 (Fig. 2.1.1a). The same trend was observed for the two treatments in the belowground biomass production showing an increment of 21% and 40% compare to the treatment N0 in N-min1 and N-min2 respectively (Fig. 2.1.1b). By contrast, different responses were observed for the two organic sources addition (N-org1 and N-org2). In particular, in the treatment N-org2 was observed an increment of 34% and 10% in above and belowground biomass respectively compared to the treatment N0, while a decrement of 23% and 28% for the two biomass portions, above and below-ground respectively, was observed in the treatment N-org1 compare to the treatment N0 (Fig. 2.1.1a, b).

The AMF inoculation has shown its influence only in the aboveground biomass production for the treatments N0 and N-org1 (post-hoc comparison, $P \leq 0.05$), while any significant influence ascribed to the AMF inoculation was observed in the treatments N-min1, N-min2 and N-org2

compared to the respective non-inoculated treatment (interaction fertilization × AMF inoculation significant at $P \leq 0.05$; Table 2.1.3; Fig. 2.1.1a). In particular, the presence of AMF led to a decrement of 9.5% and 16% in aboveground biomass production for the two treatments N0 and N-org1 respectively compared to the same fertilization treatments non-inoculated. The AMF inoculation did not show any significant effect on the belowground biomass production (Fig. 2.1.1b). However, the presence of AMF inoculum determined an increment of the total root length and of the soil root density in the treatment N0 (Table 2.1.2). Also, the root:shoot ratio was significantly increased by AMF inoculation in the treatments N0 and N-org1 shifting the values respectively from 0.26 ± 0.013 and 0.25 ± 0.007 in absence of inoculum to 0.32 ± 0.015 and 0.29 ± 0.025 in presence of AMF (Table 2.1.2).

Table 2.1.3. ANOVA output of aboveground biomass dry weight, belowground biomass dry weight, N uptake and N recovery.

Source	df	Aboveground biomass	Belowground biomass	Nitrogen uptake	N recovery
Fert	**4**	*** (1.94)	*** (0.82)	*** (29.94)	*** (1.72)
myc	**1**	ns	ns	ns	*** (1.21)
Fert x myc	**4**	***	ns	**	***

*, ** and *** indicate significant difference at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences. Values in brackets indicate significant differences at P≤0.05 (Tukey's HSD test).

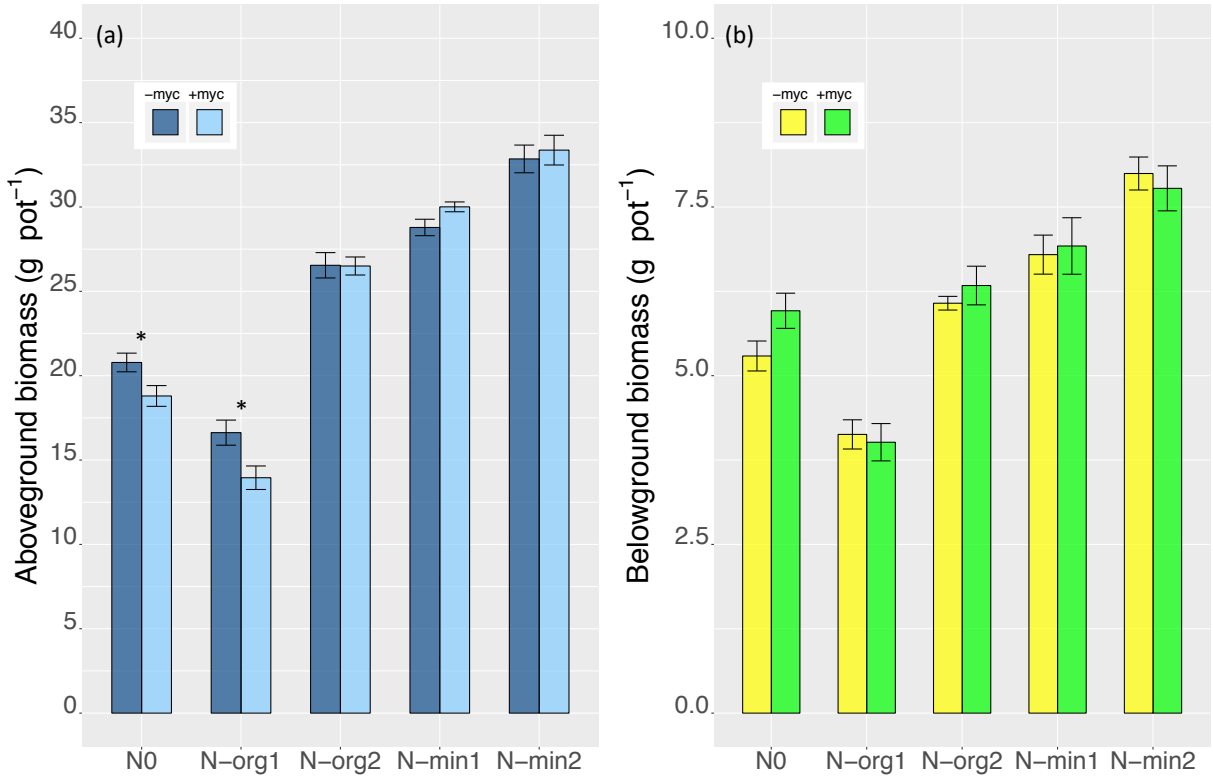


Fig. 2.1.1. Durum wheat response to the different fertilization treatments in absence (-myc) or presence (+myc) of AMF inoculum. Aboveground biomass dry weight (a); belowground biomass dry weight (b).
Bars represent means ± SE (n = 5). * above bars indicate significant difference between -myc and +myc within the same fertilization treatment ($P \leq 0.05$).

Plant biomass N Concentration, Uptake and Recovery, and N in soil

The fertilization treatments significantly affected the N concentration in the aboveground biomass, showing the highest values in the treatment N-min2 (overall means of +myc and -myc= 1.19 %) and the smallest in the treatment N0 (overall means of +myc and -myc= 0.59 %). The presence of AMF has determined a decrement of the parameter in the treatment N0, while a significant increment was observed in the other fertilization treatments except for the treatment N-min2, where, although an average increment was noted, the post-hoc comparison did not show a significant effect (Table 2.1.2). The same trend was noted for the N uptake except for the treatment N-org1, where no difference due to the inoculation treatment was observed (+myc and -myc; Fig. 2.1.2a). In particular, an increment of N uptake due to the AMF presence was observed in the fertilization treatments N-min1 and N-org2 (average of +20% and +12% respectively), while a decrement of 16.5% was observed in the treatment N0 and any difference in the treatments N-min2 and N-org1 (Fig. 2.1.2a). The values observed in the treatment N-org1 either in presence and absence of AMF did not differ from those observed in the N0 treatment in presence of AMF (112.8 ± 6.4 and 110.2 ± 7.3 and 103.4 ± 6.9 mg of N acquired per pot in N-org1 -myc and +myc, and N0 -myc respectively). The same trend was

observed in the residual soil mineral N ($N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$) assayed at the end of the experiment (Table 2.1.2). In particular, the total mineral N in soil was 2.00 ± 0.03 and 2.02 ± 0.03 mg per kg of soil in N-org1 -myc and +myc respectively, and 2.02 ± 0.08 in N0 in presence of AMF. Surprising, for this parameter, the N0 treatment has shown a lower value of mineral N in soil in presence of AMF, although the plant N uptake was higher in absence of inoculum. A significant reduction of the soil mineral N residual in soil ascribed to the AMF inoculum was also observed in the fertilization treatment N-org2, while no significant differences were found in the treatments N-min1 and N-min2, although lower average values were observed in both treatments.

The percentage of the biomass plant N derived from the applied fertilizer ($\%N_{rec}$) has shown an interaction effect between the fertilization treatments and the AMF inoculum ($P \leq 0.001$; Table 2.1.3; Fig. 2.1.2b). In particular, a remarkable increment of an average of 24%, 45.9% and 15.9% ascribed to the presence of AMF was observed on the N recovery of the treatments N-min1, N-min2 and N-org2 respectively. By contrast, in the N-org1 treatment the AMF inoculum has determined a severe decrement of this parameter shifting the percentage of N derived from the organic source from an average of 8.45% to an average of 2.99%.

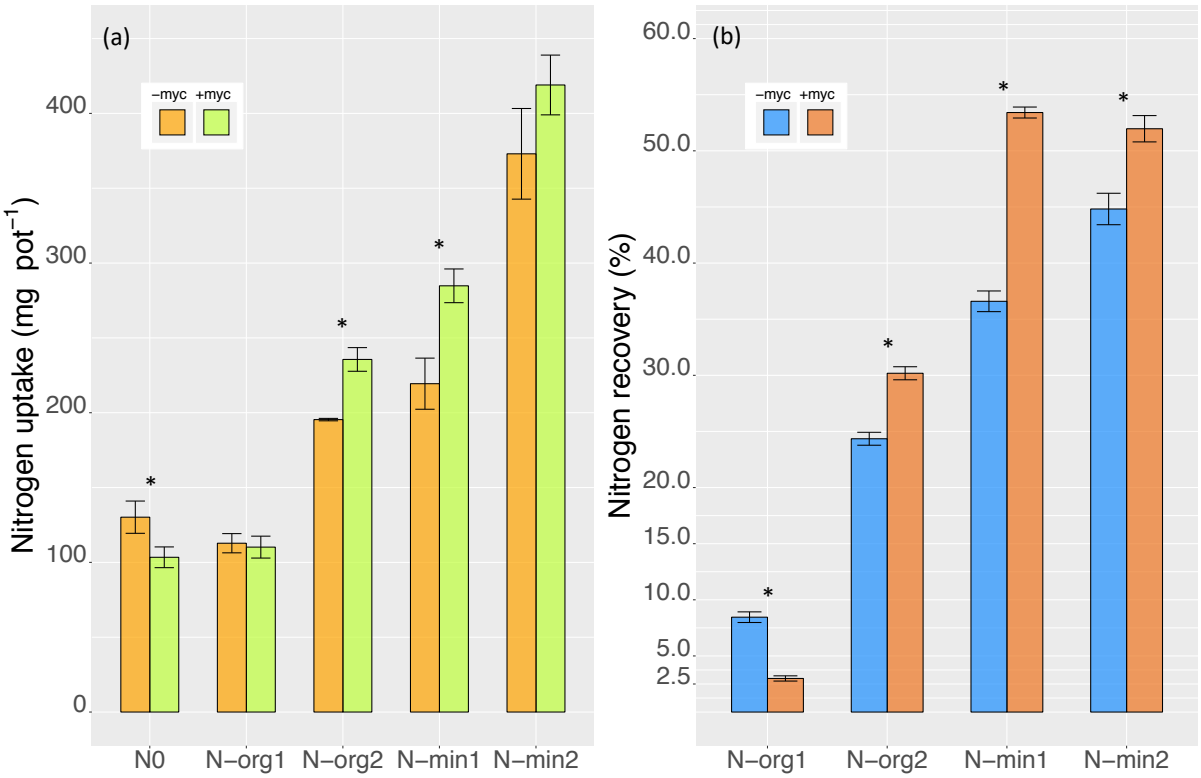


Fig. 2.1.2. Durum wheat response to the different fertilization treatments in absence (-myc) or presence (+myc) of AMF inoculum. N uptake (a); Percentage of N recovery (b).
Bars represent means ± SE (n = 5). * above bars indicate significant difference between -myc and +myc within the same fertilization treatment (*P≤0.05*).

Discussion

In the present experiment, mycorrhizal colonization, although lower than that observed in durum wheat in other studies (Saia et al., 2014a; Ercoli et al., 2017), determined significant effects on both quantitatively and qualitatively plant traits.

Among the fertilization treatments, the percentage of root colonization decreased with N enrichment, confirming the observation made by Ercoli et al. (2017) in durum wheat under field conditions. Probably in N-rich soils the plant can autonomously satisfy its nutritional needs without the cost of transferring photosynthates to the mycorrhizae. The AMF inoculation differently influenced the root:shoot ratio among the fertilization treatments by showing higher values in presence of low N availability. The increment in the root:shoot ratio ascribed to the presence of AMF was due to the detrimental effect observed in the aboveground biomass rather than a positive influence on the belowground biomass growth. Probably in N0 and N-org1, AMF altered the carbon allocation within the plant tissues, increasing the amount of carbon transferred in the root system and potentially to themselves. Actually, results from an experiment obtained using ^{14}C have shown that AMF can exert a strong C-sink effect modulating the carbon allocation among the plant tissues in a symbiosis involving barley and Glomus mosseae (nowadays F. mosseae) (Lerat et al., 2003). Probably, in the other treatments (N-min1, N-min2 and Norg-2) the photosynthate availability was sufficient to satisfy the fungal requirements and those for plant growth, thereby reducing the carbon partitioning effect.

The influence of AMF on biomass production was confined to a detrimental effect on the treatments N0 and N-org1. These results could be ascribed to the competition between the two symbionts for N when the symbiosis is established under low N availability in the growth substrate. The addiction of organic residues with a high C:N ratio (C:N = 49.7; N-org1 treatment) which could have caused a temporary sequestration of nutrients, as well as a reduction of the total amount available for the crop as also observed by Geisseler and Horwath (2009). AMF have a high N demand for their own metabolism (Hodge & Fitter, 2010) and under conditions of soil N deficiency can compete with the host plant for the available N (Püschel et al. 2016). However, although the detrimental effect on the aboveground biomass was observed in both treatments N0 and N-org1, the AMF inoculation differently affected the N concentration and uptake in the two treatments. In fact, AMF inoculation determined a decrement of both the N related parameters in N0, while an increment of N concentration was observed in N-org1 so that in this treatment no difference was observed in N uptake. Possibly, these results could be ascribed to the different N pools available in the soil in the two treatments. In fact, in treatment N0 the only N source was the original substrate concentration and, when

present, the AMF could have strongly competed for nutrient acquisition reducing plant uptake. By contrast, in the N-org1 treatment, the AMF could more efficiently use the N deriving from the organic matter as a N source, leaving the original substrate N supply for the plant. This hypothesis may be supported by the fact that the AMF can influence the mineralization process and use the nutrient derived from the process due to their favorable position (Hodge & Storer, 2015). Arbuscular mycorrhizal fungi are unable to mobilize organically bound nutrients (Bunn et al., 2019); however, they, through the release of labile C compounds in their hyphosphere can stimulate the activity of microbial decomposer, so increasing the decomposition rates of organic residues. The N recovery of the different treatments observed in the present experiment supports this hypothesis. In fact, the parameter was positively affected by the presence of AMF in all the fertilization treatments except for treatment N-org1. This highlights that although the plants acquired the same amount of N in presence or absence of AMF, the N source in the two inoculation treatments was different.

AMF determined an increment of all of the plant N related parameters (N concentration, N uptake and N recovery) in treatment N-org2, however without affecting plant biomass production. These findings agree with those observed in other pot experiments where AMF were shown to be able to transfer a substantial amount of N derived from the organic patch to the host plant without affecting plant biomass (Hodge et al. 2001; Herman, 2012). The difference observed between the two organic treatments in the present experiment could have been ascribed to the lower C:N ratio of the organic patch in treatment N-org2 compared to the one in N-org1 (16.2 vs 49.7 C:N ratio in N-org2 and N-org1, respectively). In fact, the mineralization of an organic patch with a relatively low C:N ratio (as in treatment N-org2) can reduce the competition between the plant and soil microorganisms, releasing a substantial amount of N to sustain the growth of both plant and fungi (Hodge et al., 2000). However, it should also be noted that the absolute amount of N was higher in treatment N-org2 compared to treatment N-org1 (118.3 vs 362.7 mg of N per pot in N-org1 and N-org2, respectively). This could have also affected the total amount of N available in soil and therefore the competition between plant and soil organisms, including AMF. Indeed, an increment of the N related parameter ascribed to the presence of AMF was also observed in the two mineral treatments, also in this case without affecting both above- and belowground plant biomass production. These results are in line with the findings by Reynolds at al. (2005) who hypothesized that the AMF carbon drain imposed on the plant could prevent the increase of the plant growth that is usually observed when more N is available. Indeed, as observed by Bago et al. (2000) the amount of carbohydrates transferred to the symbiont fungi can be up to 20% of total plant

carbon. However, the higher values of N concentration in the biomass, although not influencing the biomass production, could influence the yield quality of the grain at maturity. In fact, a portion of the biomass N is transferred to the grain (N remobilization) and this portion is directly influenced by the biomass N concentration. To date, however, there is a lack of information on these aspects.

In conclusion, the effects of mycorrhization varied depending on the applied fertilization treatments. Under conditions of not-limiting N availability, mycorrhization had no effect on the amount of plant biomass but resulted in a qualitative improvement, increasing the N concentration of plant tissues and, consequently, the overall plant N uptake. On the contrary, in limiting N availability (N0 treatment), the mycorrhization, although significantly higher than that found on the fertilized treatments (N-org1, N-org2, N-min1 and N-min2), determined a contraction of the aboveground biomass accumulation (-9.6% compared to –myc treatment) and particularly of plant N uptake (-20.6%), highlighting how in these conditions a competition is established between plants and AMF for the element. Supplying organic matter with high C:N ratio (N-org1 treatment) penalized plant growth compared to N0 treatment, probably due to the temporary decrease of N availability in the substrate; under these conditions, the presence of AMF further penalized the aboveground plant growth (-16.1% compared to –myc treatment), but determined an increment of N concentration in its tissues (+16.3%); these apparently conflicting results could be explained considering the variations in N availability in the substrate over time. In fact, it is known that the decay of organic matter with a high C:N ratio, is characterized by two phases: a phase of N net immobilization followed by a phase of N net mineralization. Therefore, it is possible to highlight that initial condition of N deficiency have increased the competition between plant and AMF, strongly penalizing the growth of plants, while the subsequent increase in N availability (AMF may have favoured the mineralization processes of the supplied organic matter, as previously described) was used by plants increasing the concentration of the element in the tissues without increasing the plant growth; probably an increment of the plant biomass could have been observed if the trial had lasted longer.

Furthermore, the mycorrhization has (indirectly) influenced the mineralization of organic matter even if the magnitude of this effect varied according to the type of organic matter supplied. In fact, the presence of AMF when the OM supplied had a low C:N ratio favoured the mineralization processes and, consequently, the N uptake by plants; on the other hand, when the supplied OM had a high C:N ratio, although increases in the mineralization was determined, a clear reduction of N recovery from OM was observed, suggesting that under N limiting

conditions the presence of AMF can have pronounced effects on the competition for different N sources among plants, microorganisms and AMF themselves.

Finally, the results revealed an active role of mycorrhizae in favouring N recovery from the substrate; this certainly has positive agro-environmental implications as it would reduce the risks that N can be released into the environment.

2.2. Balance in Soil N and P Availability Affect AM Symbiosis Outcome in Durum Wheat

Abstract

Although AM symbiosis is usually considered mutualistic, several factors affect the symbiotic outcome and under certain condition AMF have been shown to depress plant growth. One of the main factors affecting the symbiosis outcome seems to be the soil P availability, with positive outcome under soil P-deficient and negative outcome under P-rich soil. However, AMF have been shown to positively affect the plant N nutrition regardless the soil P availability. On the other hand, AMF have a high N request, consequently, under soil N-deficient competition between AMF and plant can occur, determining a parasitic outcome. Parasitism can also occur under N-rich soil since plant can autonomously satisfy its N request and the AMF C cost can exceed the benefit that the symbionts can provide. To evaluate the effect of soil N availability and the interaction between soil N and P availability on AM symbiosis in durum wheat two microcosm experiments have been carried out. In the Experiment 1 plants has been grown in presence (+myc) and in absence (-myc) of AMF inoculum along soil N gradient, ensuring high availability of all the other nutrients; in the Experiment 2 durum wheat in +myc and -myc was grown modulation the soil N and P availability.

Results have shown that soil N availability, under high availability of all the other essential nutrients, can drive the mycorrhizal outcome through a continuum from mutualism to parasitism and vice versa. On the contrary, AM symbiosis positively affect plant under P-deficient soil regardless the soil N availability. The same trend was observed on plant quantitative and qualitative traits, and on nutrients' efficiency parameters. Finally, results have shown that under certain conditions AMF may affect plant organography and the N allocation in the different plant organs.

Introduction

The main benefit ascribed to AM symbiosis is an increment of the acquisition of nutrients by plants. Nevertheless, the availability of the different nutrients in soil could differently affect the symbiosis. Indeed, low availability of P in soil often has shown to have a strong positive effect on the symbiosis outcome, while contrasting results have been reported concerning N (Smith & Smith 2011; Reynolds et al., 2005; Johnson et al., 2015). The different effects seem to be related to the different chemical properties of the two elements and their availability in the soil solution. In fact, P in soil is often absorbed to soil constituents or bounded to metal cations, so

that the soil P phytoavailable is not enough to satisfy the plant requirement. In such scenario, the small diameter of the mycorrhizal ERM can explore soil portions not accessible to the plant root hairs, increasing, at the same time, the absorption surface area per unit biomass up to two orders of magnitude greater than the plant root system alone (Raven and Edwards 2001). At the same time, such mechanisms can further affect the kinetics of inorganic P and its diffusive flux (Frossard et al., 2000), and therefore the amount of P in the soil solution. By contrast, N, in nitrate form, is in the soil solution also at low soil N concentration and therefore easily acquired by the plant itself. Additionally, AMF have a notable N demand for their own metabolism, which N concentration can be as high as 4 to 7 times the concentration of the aboveground plant biomass and up to 10 times the N concentration of the belowground biomass (Hodge & Fitter, 2010). This allows the consideration that under certain N availabilities competition between plant and symbiotic AM fungi can occur, shifting the interaction from mutualistic behavior to parasitism (Johnson et al., 1997, 2015; Püschel et al., 2016; Paragraph 2.1 of the present thesis). This notwithstanding, thanks to the above mentioned characteristics, the ERM could take up a portion of N in ammonium form retained by the exchange complex. This mechanism could increase the available soil N pool, which would limit the competition between the two symbionts due to a niche differentiation. By contrast, under high N availability, the plant could satisfy its N requirements by root N acquisition and the AMF net carbon cost could result higher than the AMF net benefit (Johnson et al., 1997; Corrêa et al., 2015). In turn, several authors have proposed a curvilinear hypothesis between outcome of the AM symbiosis and soil N availability (Gange and Ayres 1999; Janos 2007; Corrêa et al., 2015). Several pot experiments have been carried out aiming to test this hypothesis, however contradictory results have been reported (Bååth and Spokes 1989; Corrêa et al., 2014), showing a gap of knowledge in our understanding the AM symbiosis functionality. Possibly, the contradictory data obtained could be ascribed to that mycorrhizal responses is not based on the soil availability of only one nutrient, but rather on the availability of several nutrients (e.g. N, P or other) and their ratios into the soil (Chen et al., 2010; Johnson et al., 2015; Püschel et al., 2016). Indeed, Johnson et al. (2010) have proposed the "trade balance model" suggesting that the symbiosis functionality could be driven by the interaction between soil N and P availability with the availability of C to foraging the symbiont fungi. Recently, pot experiment conducted by manipulating soil N and P have reported evidence of the functionality of "trade balance model" (Johnson et al., 2015; Püschel et al., 2016). Both the experiments have been conducted on *Andropogon gerardii* a highly mycotrophic C4 grass, while no data are available on moderate mycotrophic C3 plants.

Since several C3 plants (varying in their mycotrophic attitude) are of a key importance for many countries, understanding their response in the above mentioned conditions seems to be crucial. To contribute to increase our knowledge on how soil N availability and soil N:P ratios can affect AM symbiosis, here two microcosm experiments were set up. The specific goals were: i) verify the existence of a curvilinear response of the AM symbiosis along a N gradient in a moderate mycotrophic C3 plant as durum wheat, pivotal crop of the Mediterranean agroecosystems, and proof if changes in soil N availability can shift the AM symbiosis outcome towards parasitic behavior; ii) test if such outcome changes varying the soil P availability.

Materials and Methods

Pot and Plant management

In both the microcosm experiments, durum wheat (*T. durum* Desf. cv. *Anco Marzio*) was grown in a rain protected wirehouse in 6 liter sterilized pots (d=16 cm; h=30 cm) filled with 7.5 kg of sterilized river sand. The sand used in the experiment have been sieved through a 2 mm mesh and sterilized following the cycle: humidification, 24 hours at room temperature and 24 hours 130 °C, for a total of three cycles. The sand was characterized for its nutrients content as follow: total N (Kjeldahl) was 0.11 g kg^{-1}; available P (Olsen) was 7.44 mg kg^{-1}; chloride content determined following the colorimetric AutoAnalyzer method proposed by Selmer-Olsen & Øien (1973) was 9 mg kg^{-1}.

In any case, the sowing has been done on February the 21st 2017, distributing 15 surface sterilized seeds per pot. All the pots and seeds were sterilized using sodium hypochlorite 3% for 3 to 5 minutes. After the sowing, all the pots were irrigated to reach the holding capacity. Ten days after the emergence the thinning was done to reach the final density of 7 plants per pot.

Soil moisture was daily monitored through gravimetric method. The hydrologic parameters of the substrate were determined using the gravimetric method (Dobriyal et al., 2012). Briefly, 100 grams of river sand were placed in forate crucibles and tap water was added to the water saturation. The excess water was let drained away and the weight difference at field capacity and the following drying at 105 °C to a constant weight was monitored. The available water for the crop was obtained by subtracting the weight after the oven dry period from the field capacity weight. From the sowing to the emergence distilled water was used to replenish the water losses. After, 150 ml of a modify Hoagland N and P free solution were added 3 times per week (every two days) and distilled water to bring the substrate back at the water holding capacity was applied in the other days. The modified nutritive solution used in the experiment was the follow:

5,12 mM K; 2,5 mM Ca; 1,64 mM Mg; 4,21 mM S; 0,042 mM B; 0,07 mM Fe; 0,016 mM Mn; 0,007 mM Zn; 0,004 mM Cu; 0,00097 mM Mo; pH 6<x<6,5 and E.C. 2<x<2,2 dS m^{-1}. Calcium was applied as calcium oxide; potassium, magnesium and copper were applied in the solution as sulphate salts; iron, zinc and manganese were applied as DTPA chelates.
In both the microcosm experiments, each treatment was set up in 5 replicates and arranged in a completely randomized design. The plants were grown for 72 days after the sowing (DAS).

Experiment 1

Four soil N levels ("N0"; "N1"; "N2"; "N3") were compared in the present experiment. In all the treatments the N concentration was determined by adding different amounts of ammonium nitrate (NH_4NO_3) to the modified nutritive solution. The nutritive solution N concentration was 0.75 mM (10.7 mg l^{-1}), 2.5 mM (35 mg l^{-1}), 7.5 mM (105 mg l^{-1}) and 15 mM (210 mg l^{-1}) for N0, N1, N2 and N3 respectively. All of the described treatments were set up in no-limiting P concentration, which was 1 mM (31 mg l^{-1}) applied as P_2O_5.
A total of 4.14 liters of nutritive solution were applied in the entire experiment for a total of 44.35 mg pot^{-1}, 143.41 mg pot^{-1}, 556.01 mg pot^{-1}, 1174.80 mg pot^{-1} of N potentially available for the crop (N applied) for N0, N1, N2 and N3 respectively.

Experiment 2

Two P levels combined with two N levels were compared in this microcosm experiment. The two N levels N1 and N3 described in the previous section (Experiment 1) were combined with two P levels (Low P and High P) so, the treatments in this microcosms experiment were: N1-Low P; N1-High P; N3-Low P; and N3-High P. In the High P treatments, the level of P in the nutritive solution was 1 mM (31 mg l^{-1}). The P deficiency in the Low P treatments was determined by reducing the amount of P in the nutritive solution from 1mM to 0.1mM (3.1 mg l^{-1}) in the first 44 DAS; after, due to the severe P stress, the P solution concentration was increased to 0.2 mM (6.2 mg l^{-1}) until the harvest (28 days). In any case, P was applied as P_2O_5.
A total of 4.14 liter of nutritive solution were applied during the entire experiment.
The total P potentially available for the crop (P-applied) in the entire experiment was 128.49 mg pot^{-1} in the High P treatments (N1-High P and N3-High P) and 18.74 mg pot^{-1} in the two Low P treatments (N1-Low P and N3-Low P).
The total N potentially available for the crop (N-applied) in the entire experiment was 143.41 mg pot^{-1} and 1174.80 mg pot^{-1} for N1 and N3 respectively.
The N and P applied were obtained by summing respectively the amount of N and P added in each nutritive solution application in the respective treatment along the entire experiment.

Inoculum

In both the microcosm experiments, half of the pots were inoculated with the two AMF species Rhizophagus irregularis and Funneliformis mosseae (+myc). The inoculation has been done in two steps applying a total of 3 grams of an inoculum where each of the two species was present at the rate of 700 spores g^{-1}. The first step was done contemporary to the sowing, distributing two grams of inoculum per pot just below the sowing bed. The second AMF inoculation was done immediately after the thinning distributing 1 gram of inoculum per pot in solution with 75 ml of distilled water. In the second inoculation step the treatment without AMF inoculation (-myc) received 75 ml of deionized water. In addition, 225 ml of a soil filtrate solution per pot was added in all the pots to establish the soil microbial community excluded AMF. The soil filtrate solution was obtained through filtration of agricultural soil suspension. Briefly, soil was suspended in distilled water at the ratio of 1:3 and shacked for 20 minutes at 140 rpm. Later, after the decantation, the suspension was filtered through an 11 μm mesh to discard the natural AMF community. The soil filtrate addition has been done in two steps contemporary to the AMF inoculation, adding 150 ml and 75 ml of soil filtrate in the first and second step respectively.

Biomass harvest and analysis

All of the analysis described below were carried out for both the microcosm experiments if not otherwise specified.

At the end of the experiment (72 DAS), the aboveground biomass (shoot) was harvested and the fresh and the dry weight recorded. The aboveground biomass weight was recorded separately for culms, green leaf limbs (green leaves), senescent leaf limbs (senescent leaves) and spikes in order to measure the incidence of each of those botanical fractions on the total aboveground biomass. In the experiment 1, N concentration was determined separately for each botanical fraction by ground each fraction to a fine powder and analysed using the Dumas method (flash combustion with automatic N analyzer; DuMaster D-480, Büchi Labortechnik AG, Flawil, Switzerland). In the experiment 2, due to the extremely low biomass produced in some treatments, all the botanical fractions were merged to a single sample, ground to fine powder and used to determine the total N concentration as described above and the total P concentration using the Kalra and Maynard method (Kalra and Maynard, 1991).

The belowground biomass (root) was carefully extracted through sieving and consecutive washing, and thereafter oven dry at 40 °C until constant weight. Successively, a representative root biomass subsample was extracted and used to quantify the percentage of AMF infection

using the McGonigle method (McGonigle et al. 1990), after being cleared with KOH 10% and stained with trypan blue 0.05% (Phillips and Hayman, 1970). The AMF infection was assayed scoring a minimum of 150 lines intersection for the presence of intra-radical AMF structures.

Calculation and statistical analysis

The N uptake was obtained by multiplying the N concentration for the aboveground biomass. N efficiency parameters were calculated adapting the method proposed by Moll et al. (1982) and Huggins and Pan (1993). N uptake efficiency (NUpE) was calculated as the ratio between aboveground N uptake (g pot^{-1}) and N supply (g pot^{-1}), where N supply was estimated as the amount of N applied in each fertilization treatment plus the substrate N content. N use efficiency (NUE) was obtained as the ratio of aboveground biomass production (g pot^{-1}) to N supply (g pot^{-1}). Here, given that the biomass was harvest before the grain maturity, NUE was determined using the aboveground biomass production instead of the grain yield.

Additionally, for the treatments of Experiment 2 the aboveground biomass P uptake was determined by multiplying the P concentration for the aboveground biomass production. Following the calculation applied for the N efficiency parameters, the P efficiency parameters were also obtained. P uptake efficiency (PUpE) was calculated as the ratio between aboveground P uptake (g pot^{-1}) and P supply (g pot^{-1}), where P supply was estimated as the amount of P applied in each fertilization treatment plus the substrate P content. P use efficiency (PUE) was obtained as the ratio of aboveground biomass production (g pot^{-1}) to P supply (g pot^{-1}).

The aboveground biomass mycorrhizal growth response (MGR) was calculated according to Gange and Ayres (1999) as follow:

$$\mathrm{MGR} = \frac{\mathrm{AM\text{-}NAM_{mean}}}{\mathrm{NAM_{mean}}} \mathrm{X}\ 100$$

Where: AM is the plant's aboveground biomass dry weight grown in presence of AMF inoculum; NAM_{mean} is the mean of the plant's aboveground biomass dry weight grown in absence of AMF inoculum from the same N treatment.

Data collected from the Experiment 1 were analyzed with a two-way ANOVA to test whether the response variables were affected by the "N level", "AMF inoculation" or their interaction. N supply was also used to assay the regression coefficient between N level and mycorrhizal growth response in the Experiment 1.

Correlation between the percentage of root colonization by AMF and N supply was performed using the subset of the inoculated treatment of all N treatments.

Data from the Experiment 2 were subjected to the three-way ANOVA testing the response variables against the explanatory variables "N level", "P level", "AMF inoculation" or their interactions.

Correlation coefficients between the percentage of root colonization by AMF and N supply and P supply, and between MGR and the percentage of root colonization by AMF were determined using the subset of the inoculated treatment of all nutrient combination treatments.

In any case, correlation analyses were carried out using a linear regression model.

In both the experiments, due to the negligible colonization of the non-inoculated treatments, statistical analysis of the "AMF colonization" response variable was performed on the subset of the inoculated treatment (+myc). In the experiment 1 the response variable was subjected to the one-way ANOVA using "N level" as explanatory variable. In the experiment 2 a two-way ANOVA was used to determine whether the response variable was affected by "N level", "P level" or their interaction.

All the analyses were performed using R software version 3.4.1 (R Core Team, 2017). Shapiro test and Bartlett test were used to assaying respectively the normality and the homoscedasticity of the model residuals. When response variables did not fulfill the ANOVA assumptions data were transformed accordingly. Following the ANOVA test, the post hoc mean comparison Tukey's HSD test ($P \leq 0.05$) was performed. When interaction terms were significant, single degree freedom contrasts were conducted to investigate the effect of mycorrhization within each fertilization treatment.

Untransformed data have been reported in tables and in graphical representations. Package "tidyverse" (Wickham, 2017) was used for data graphical representation.

Results Experiment 1

Mycorrhizal colonization

Mycorrhizal colonization in the non-inoculated treatment was always below 1%. In the inoculated treatment the values ranged from 25.62 (N0) to 14.63 (N3) (Fig. 2.2.1). Intermediate values, respectively 23.42% and 15.41%, were observed for the treatment N1 and N2. The differences among N treatments were highly significant ($P \leq 0.001$; Table 2.2.1). However, at the post hoc test no difference was observed between the treatments N0 and N1 as well as between the treatments N2 and N3. Negative correlation between AMF colonization and the amount of N applied ($r = - 0.84$; $P \leq 0.001$) was observed. Moreover, the percentage of root colonization by AMF resulted also negative correlated with the MGR ($r= -0.4787$; Fig. 2.2.2).

Indeed, the two parameters (AMF colonization and MGR) have shown different trends when analysed in relation to the different N level treatments.

Table 2.2.1. ANOVA output of AMF root colonization, aboveground biomass dry weight, belowground biomass dry weight, root shoot ratio (R:S), aboveground biomass N concentration, aboveground biomass N uptake, N uptake efficiency (NUpE) and N use efficiency (NUE)

Source	df	AMF colonization	Aboveground biomass	Belowground biomass	R:S	Nitrogen concentration	Nitrogen uptake	NUpE	NUE
N level	**3**	*** (4.34)	*** (0.35)	*** (0.50)	*** (0.035)	*** (0.08)	*** (15.15)	*** (0.01)	*** (0.25)
myc	**1**	-	* (0.17)	ns	ns	ns	ns	ns	ns
N level x myc	**3**	-	***	ns	*	ns	*	**	***

*, ** and *** indicate significance differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significance differences. Values in brackets indicate significant differences at P≤0.05 (Tukey's HSD test).

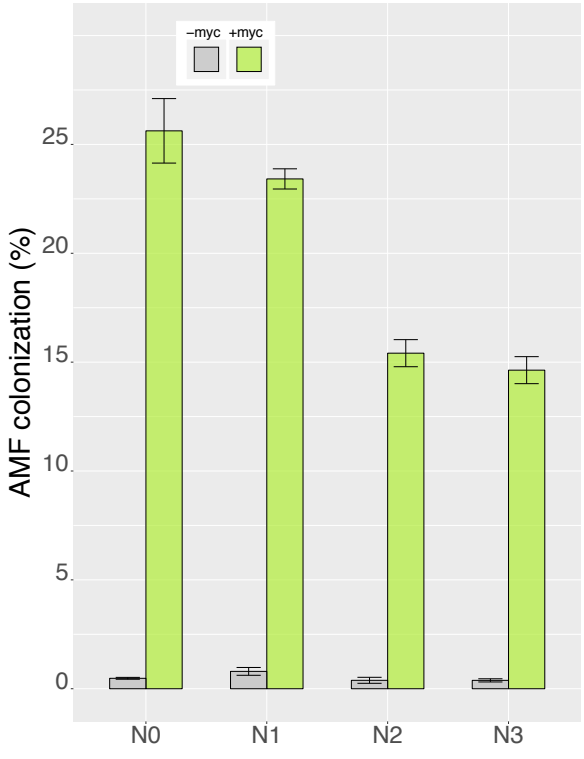


Fig. 2.2.1. AMF root colonization in durum wheat grown in the different Nitrogen levels in absence (-myc) or presence (+myc) of AMF inoculum. Bars represent means ± SE (n = 5).

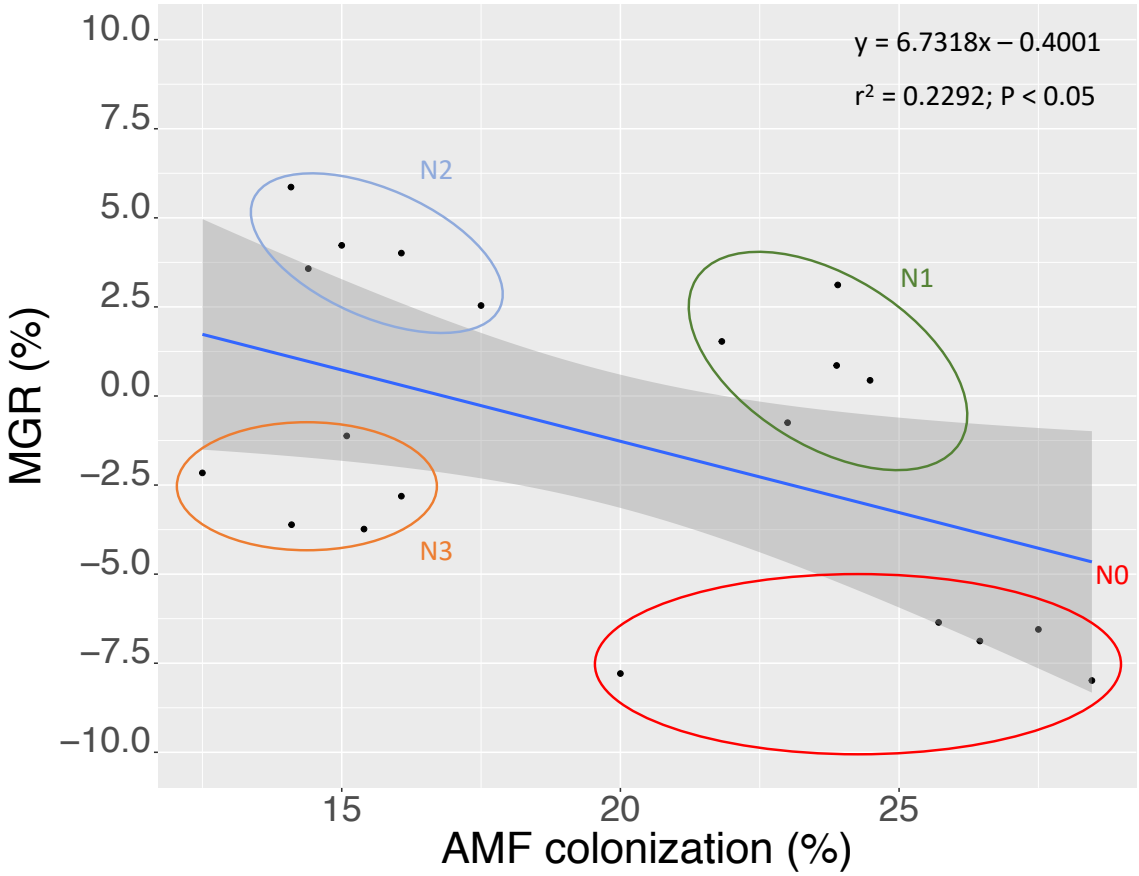


Fig. 2.2.2. Relationship between mycorrhizal growth response (MGR) of durum wheat and AMF root colonization in the different N treatments.

Plant response

Both the biomass compartments have shown the lowest values in the treatment N0 (3.93 and 10.35 g per pot for below and aboveground biomass respectively; average of -myc and +myc) and the highest in the treatment N2 (6.08 and 18.73 g per pot for below and aboveground biomass respectively; average of -myc and +myc; Fig. 2.2.3a, b). No significant AMF effect was observed on the belowground biomass production (Table 2.2.1; Fig. 2.2.3b). By contrast, AMF inoculation has differently affected the aboveground biomass production (interaction N level x myc significant at $P \leq 0.001$; Table 2.2.1), showing a curvilinear relationship between aboveground biomass and N supplied ($r^2 = 0.71$, $P < 0.001$; fig 2.2.4). In fact, MGR shifted from negative values, observed in the two extreme N levels N0 and N3, to positive values, in the two intermediate N levels (treatment N1 and N2). However, although a slight positive MGR was observed in the treatment N1, the difference between +myc and -myc in terms of aboveground biomass production was not significant. The aboveground biomass in the treatments N0 ranged from 10.73 and 9.97 gram per pot in -myc and +myc respectively. In the treatment N3 the average of shoot biomass was 18.02 and 17.53 grams per pot in -myc and +myc respectively.

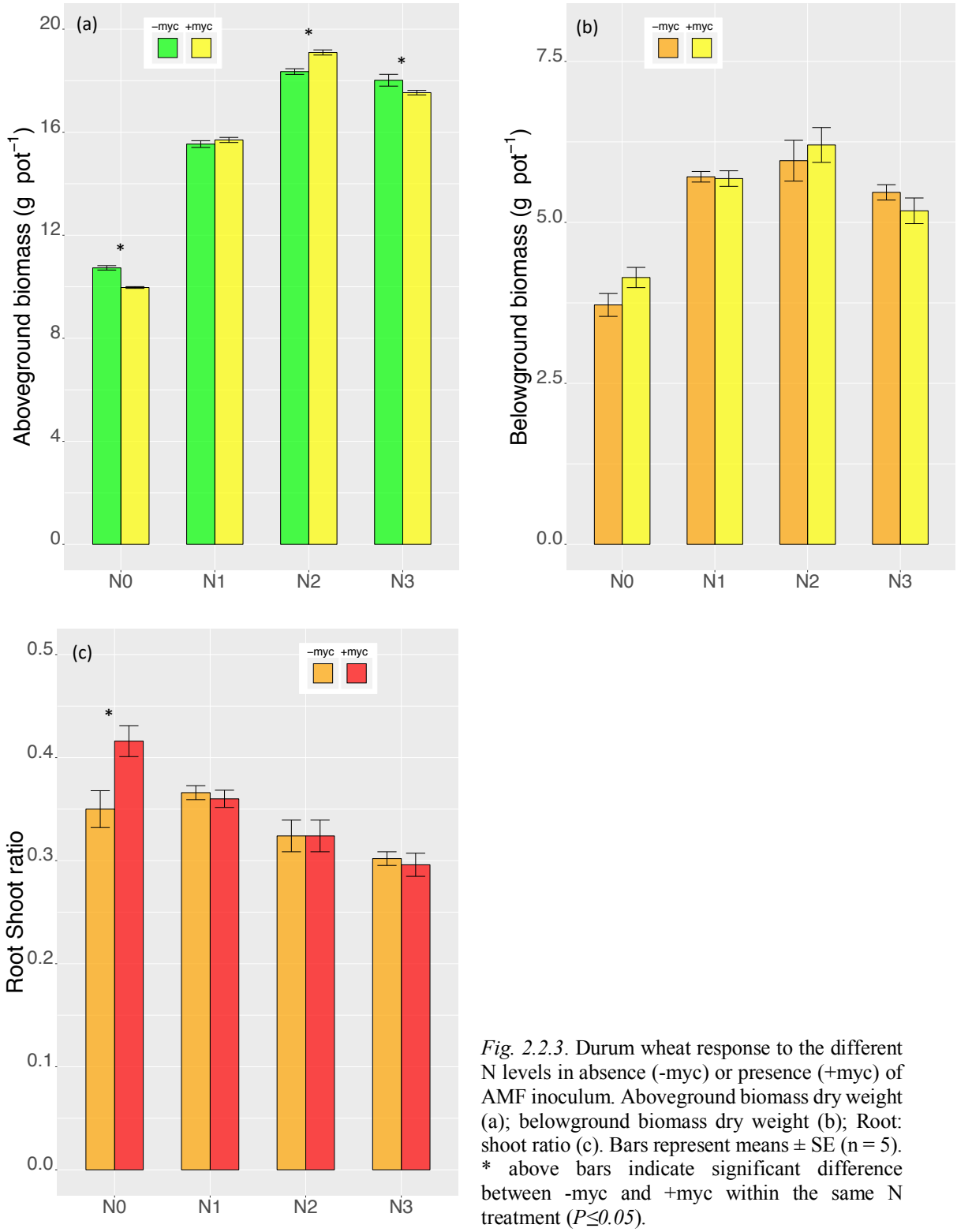


Fig. 2.2.3. Durum wheat response to the different N levels in absence (-myc) or presence (+myc) of AMF inoculum. Aboveground biomass dry weight (a); belowground biomass dry weight (b); Root: shoot ratio (c). Bars represent means ± SE (n = 5). * above bars indicate significant difference between -myc and +myc within the same N treatment ($P \leq 0.05$).

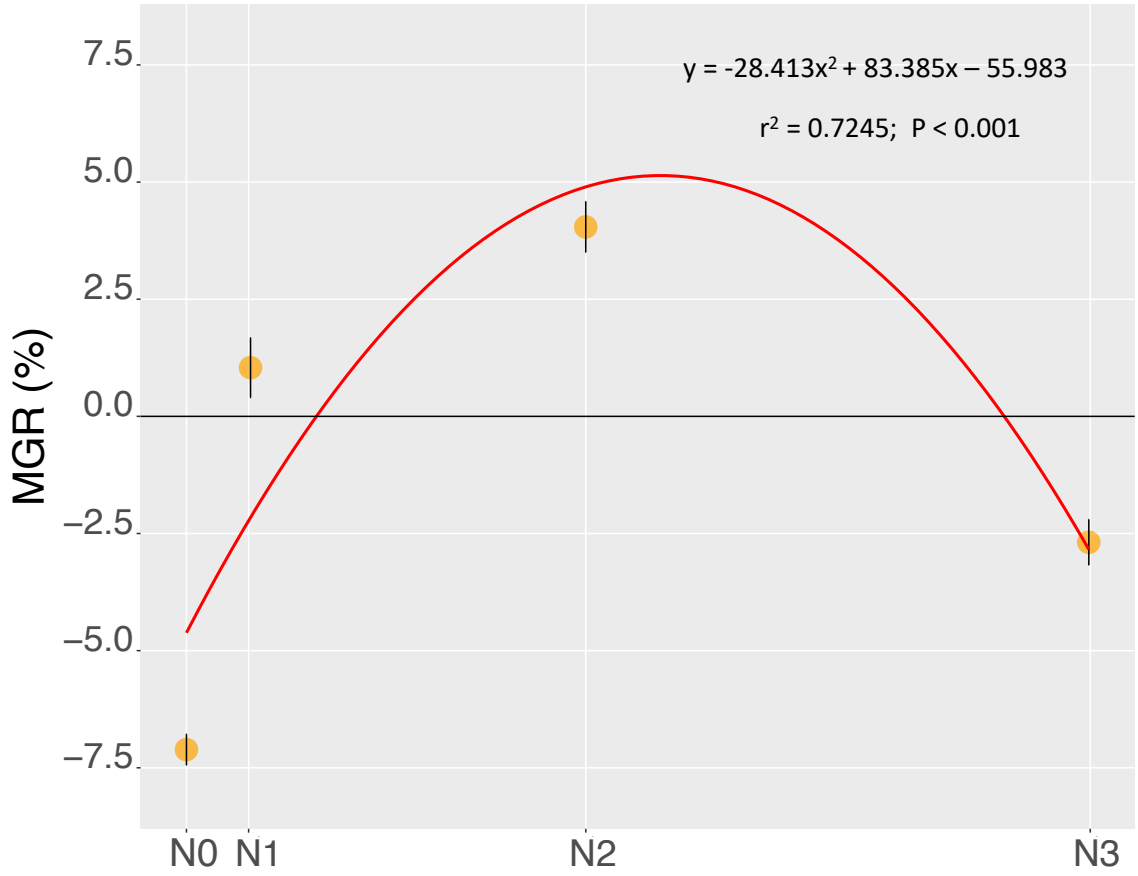


Fig. 2.2.4. Regression between mycorrhizal growth response (MGR) of durum wheat and N supply in the different N treatments. Points represent means ± SE (n = 5).

increment of 4.04% in shoot biomass compared to -myc treatment (Fig. 2.2.3a).

Although the contained effect on the total aboveground biomass, AMF inoculation have significantly influenced its composition determining an increment in the proportion of green leaves on the total aboveground biomass (-myc and +myc 11.47% and 12.28% respectively; average of the different N levels), whereas no effect was observed in the others plant botanical fractions (Table 2.2.2). A significant interaction between N levels and AMF inoculation have been observed on the root:shoot ratio (Table 2.2.1; Fig. 2.2.3c). In particular, the presence of AMF increased the value by the 19.8% in the treatment N0, while no AMF effect for this parameter was observed in the other treatments (Fig. 2.2.3c).

As far as concern the N related parameters, total aboveground biomass N concentration was not affected by AMF inoculation, whereas the parameter strongly varied among the N treatments. The highest value was observed in the treatment N3 (2.43%; average of -myc and +myc), while the lowest value was detected in the treatment N1 (1.17%; average of -myc and +myc; Fig. 2.2.5a). On the contrary, AMF inoculation have affected the N concentration in green leaves and in senescent leaves, while no effect was observed for the parameter in the others fractions of the aboveground biomass (culms and spikes; Table 2.2.2).

Table 2.2.2. Plant botanical fractions (green leaves, senescent leaves, culms and spikes) incidence on the total aboveground biomass and plant botanical fractions N concentration observed in the different N levels in absence (-myc) or presence (+myc) of AMF inoculum.

		Green leaves (%)	Senescent leaves (%)	Culms (%)	Spikes (%)	Green leaves N concentration (%)	Senescent leaves N concentration (%)	Culms N concentration (%)	Spikes N concentration (%)
N0	**-myc**	10.48	7.93	57.45	24.14	0.89	1.58	0.75	2.29
	+myc	11.19	7.77	58.29	22.66	0.94	1.57	0.76	2.40
N1	**-myc**	9.57	10.31	57.67	22.45	0.90	1.62	0.78	2.20
	+myc	10.14	9.53	57.84	22.49	0.90	1.62	0.76	2.07
N2	**-myc**	12.53	15.33	55.39	16.75	1.32	2.30	1.21	3.35
	+myc	13.65	15.10	55.02	16.24	1.48	2.25	1.27	3.28
N3	**-myc**	13.30	22.56	51.51	12.63	2.27	2.73	2.01	3.75
	+myc	14.17	21.54	51.14	13.15	2.42	2.63	2.04	3.67
Source	**df**								
N level	**3**	*** (0.87)	*** (9.09)	*** (2.21)	*** (1.80)	*** (0.20)	*** (0.08)	*** (0.08)	*** (0.33)
myc	**1**	* (0.80)	ns	ns	ns	* (0.87)	* (0.03)	ns	ns
N level x myc	**3**	ns	ns	ns	ns	ns	ns	ns	ns

Data are means, (n = 5). *, ** and *** indicate significant differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences. Values in brackets indicate significant differences at P≤0.05 (Tukey's HSD test). # indicate significant difference between -myc and +myc within the same fertilization treatment (*P≤0.05*)

In particular, the presence of AMF has significantly increased the N concentration in the green leaves (1.34% and 1.43% respectively for -myc and +myc; average of the different N levels), while a decrement was detected in the senescent leaves (2.00% and 2.06% for -myc and +myc respectively; average of the different N levels).

AMF inoculation affected the aboveground N uptake differently in relation to N treatments (interaction N level x myc significant at P ≤ 0.001; Table 2.2.1; Fig. 2.2.5b). In fact, N uptake in the treatments N1 and N3 was unresponsive to the presence of AMF. On the contrary, in N0 a decrement of 6.8% in N uptake was observed in +myc compared to –myc, whereas, in the treatment N2, the presence of AMF increased by the 8.4% the amount of N acquired (Fig. 2.2.5b). A strong interaction between the two main factors was also observed concerning the two N efficiency parameters (interaction N level x myc significant at P ≤ 0.01 and P≤ 0.001 for NUpE and NUE respectively; Table 2.2.1). The presence of AMF affected the NUpE only in the treatment N2 shifting the value from 0.23 observed in absence of AMF inoculum to 0.25 g of N acquired per g of N supplied when plants were grown in presence of AMF (Fig. 2.2.5c). NUE was negatively affected by the presence of AMF in the treatment N0 (12.34 vs 11.46 g of aboveground biomass per g of N supplied respectively in -myc and +myc; Fig. 2.2.5d), which determined a decrement of 7.80%. On the contrary, the inoculation treatment has positively affected the parameter in the treatment N2 (13.3 vs 13.8 g of aboveground biomass per g of N supplied respectively in -myc and +myc; Fig. 2.2.5d).

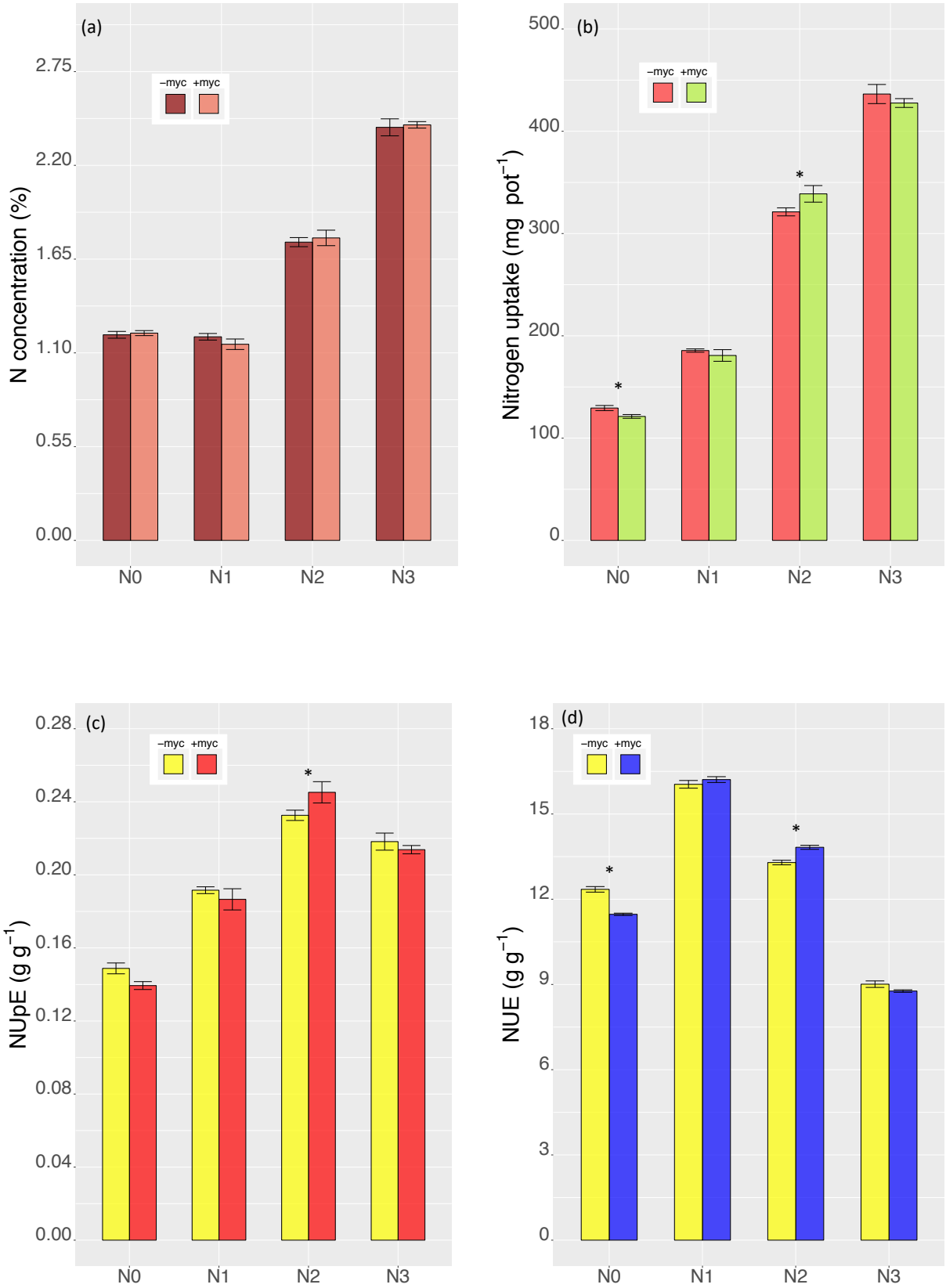


Fig. 2.2.5. Durum wheat response to the different N levels in absence (-myc) or presence (+myc) of AMF inoculum. Aboveground biomass dry weight N concentration (a); Aboveground biomass N uptake (b); N uptake efficiency (NUpE; c); N use efficiency (NUE; d). Bars represent means ± SE (n = 5). * above bars indicate significant difference between -myc and +myc within the same N treatment ($P \leq 0.05$).

Results Experiment 2

Mycorrhizal colonization

Mycorrhizal colonization in all the non-inoculated treatments was negligible showing values always below 3%. In the inoculated treatments the highest mycorrhizal colonization was observed in the treatment N1-Low P and the lowest in the treatment N3-High P (Fig. 2.2.6). Therefore, the root colonization was negatively affected by the increment of both the nutrients in soil, although the extent of the negative effect ascribed to the N addition was higher than that observed for P addition. AMF root colonization did not show any correlation with the MGR (r= -0.0001; Fig. 2.2.7).

Table 2.2.3. ANOVA output of AMF root colonization, aboveground biomass dry weight, belowground biomass dry weight, mycorrhizal growth response (MGR), root shoot ratio (R:S), aboveground biomass N concentration and aboveground biomass P concentration

Source	df	AMF colonization	Aboveground biomass	Belowground biomass	MGR	R:S	Nitrogen concentration	Phosphorus concentration
N level (N)	**1**	*** (2.07)	*** (0.40)	*** (0.15)	* (16.71)	ns	*** (0.05)	*** (0.05)
P level (P)	**1**	*** (2.07)	*** (0.40)	*** (0.15)	*** (16.71)	* (0.03)	*** (0.05)	*** (0.05)
myc	**1**	-	** (0.40)	ns	-	ns	* (0.05)	ns
N x P	**1**	ns	***	***	*	***	***	***
N x myc	**1**	-	ns	ns	-	ns	ns	ns
P x myc	**1**	-	***	**	-	ns	*	***
N x P x myc	**1**	-	*	ns	-	ns	ns	ns

*, ** and *** indicate significance differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significance differences. Values in brackets indicate significant differences at P≤0.05 (Tukey's HSD test).

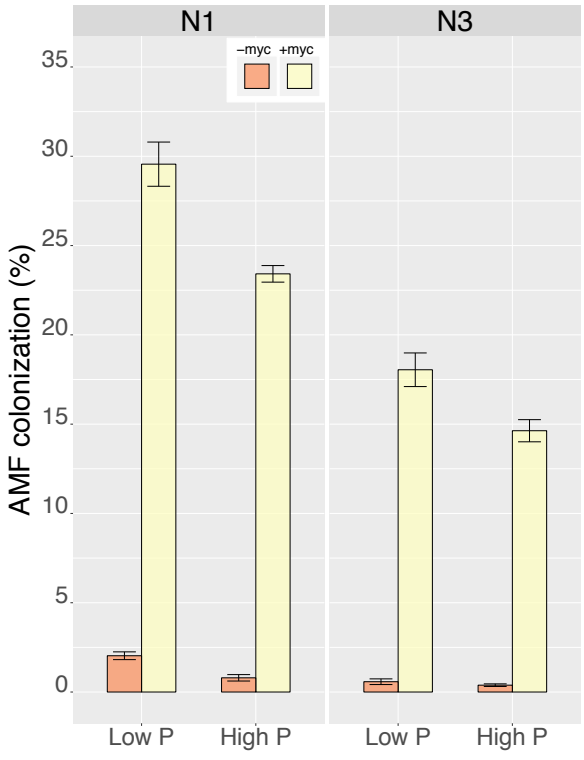


Fig. 2.2.6. AMF root colonization of durum wheat in the different N and P applied combinations in absence (-myc) or presence (+myc) of AMF inoculum. Bars represent means ± SE (n = 5).

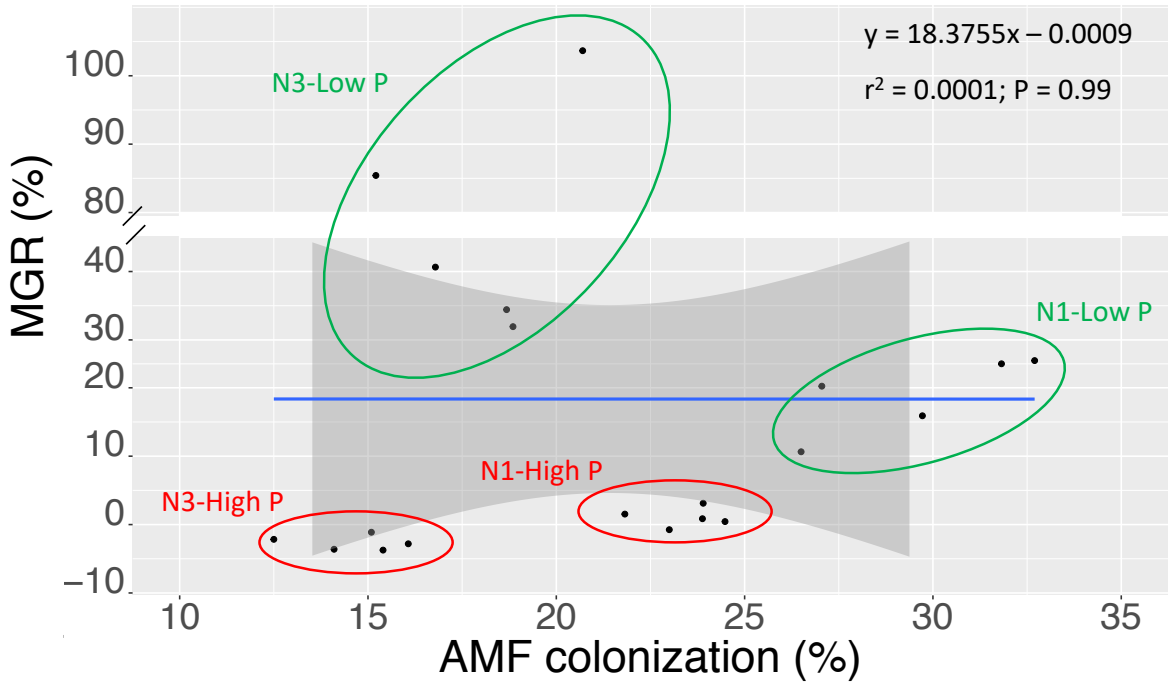


Fig. 2.2.7. Relationship between mycorrhizal growth response (MGR) of durum wheat and AMF root colonization in the different N and P applied combinations.

Plant response

Plants growth was significantly compromised under Low P conditions in both N levels. In Low P treatments, compared to High P, above and belowground biomass have shown a decrement of 40% and 47% respectively in the N1 treatment (average of -myc and +myc). The decrement was even more pronounced in the treatment N3, where the production of above and belowground biomass was reduced by 82% and 76 % respectively (average of -myc and +myc; Fig. 2.2.8a, b). AMF inoculation has shown interaction between inoculation and P treatment for the belowground (P x myc significant at P≤ 0.05; Table 2.2.3). A significant increment of belowground biomass ascribed to the inoculation treatment was observed when plants were grown under Low P treatments. In particular, the presence of AMF increased the biomass production by the 14% (1.93 vs 2.32 in -myc and +myc respectively; average of both the N treatments Low P), whereas no effect of AMF was observed when plants were grown under High P condition (Fig. 2.2.8b). Aboveground biomass has resulted strongly affected by the interaction between inoculation and both N and P treatments (N x P x myc significant at P≤ 0.05; Table 2.2.3). In particular, in the Low P treatments an increment of 19% and 56% ascribed to AMF was observed respectively in the treatments N1 and N3. AMF inoculum did not show any effect on plant growth under High P condition in N1, whereas determined a detrimental effect in the treatment N3-Low P (Fig. 2.2.8a). In fact, the MGR was significantly affected by the soil N and P balance (interaction N x P significant at P≤ 0.05; Table 2.2.3). In particular, in

the two High P treatments the MGR was very contained varying from -2.7% in N3 to 1.03% in N1. By contrast, a very large MGR was observed in the two Low P treatments, where the values varied from 18.85% in N1 up to 56.21% in N3 (Fig. 2.2.9).

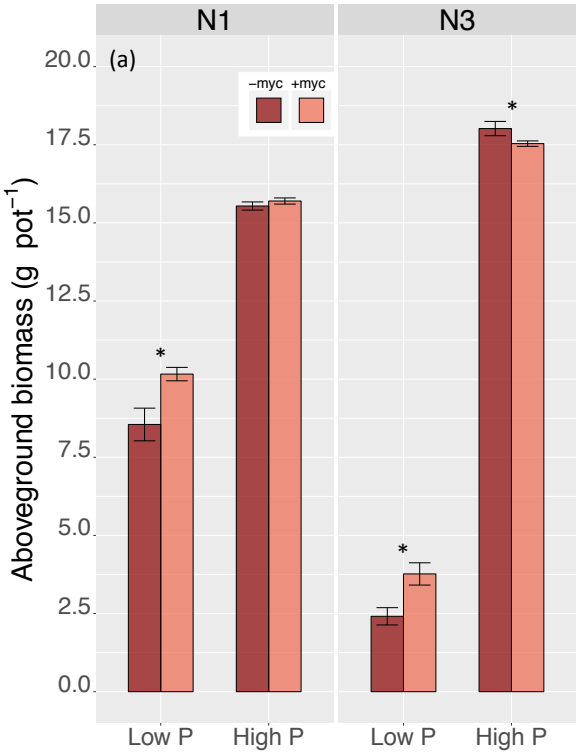
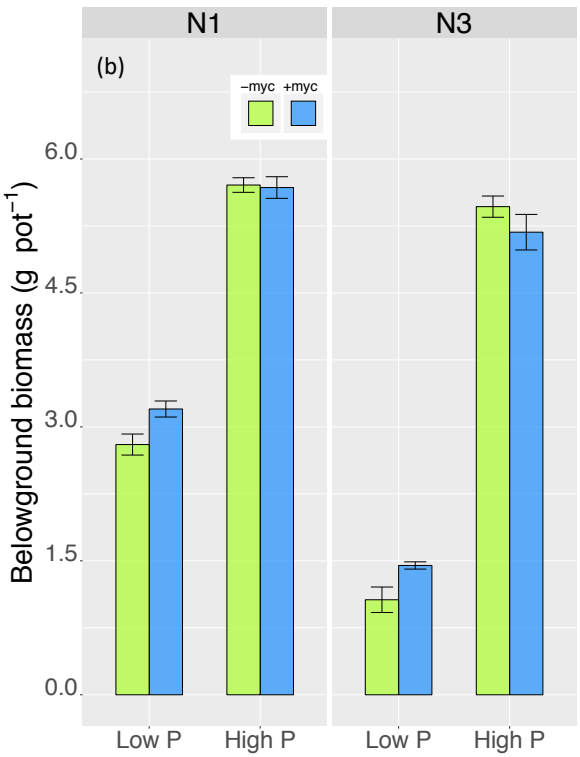
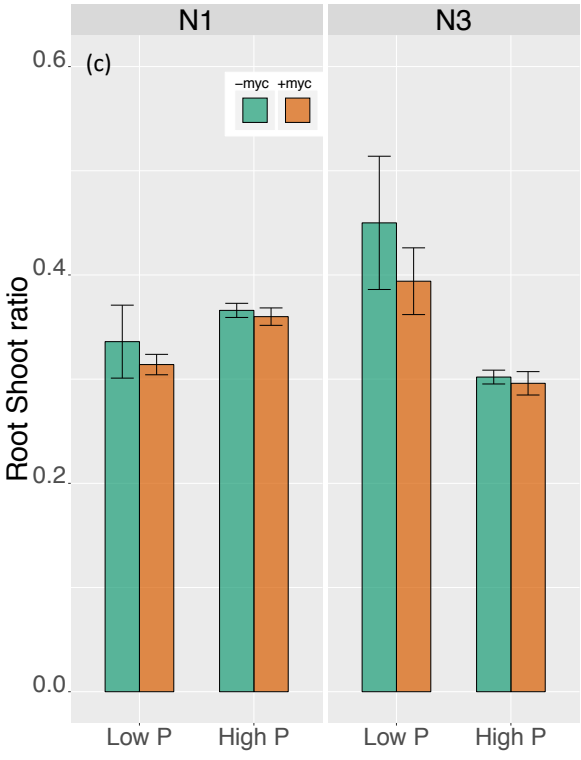


Fig. 2.2.8. Durum wheat response to the different N and P applied combinations in absence (-myc) or presence (+myc) of AMF inoculum. Aboveground biomass dry weight (a); belowground biomass dry weight (b); root shoot ratio (c). Bars represent means ± SE (n = 5). * above bars indicate significant difference between -myc and +myc within the same N treatment (*P≤0.05*).





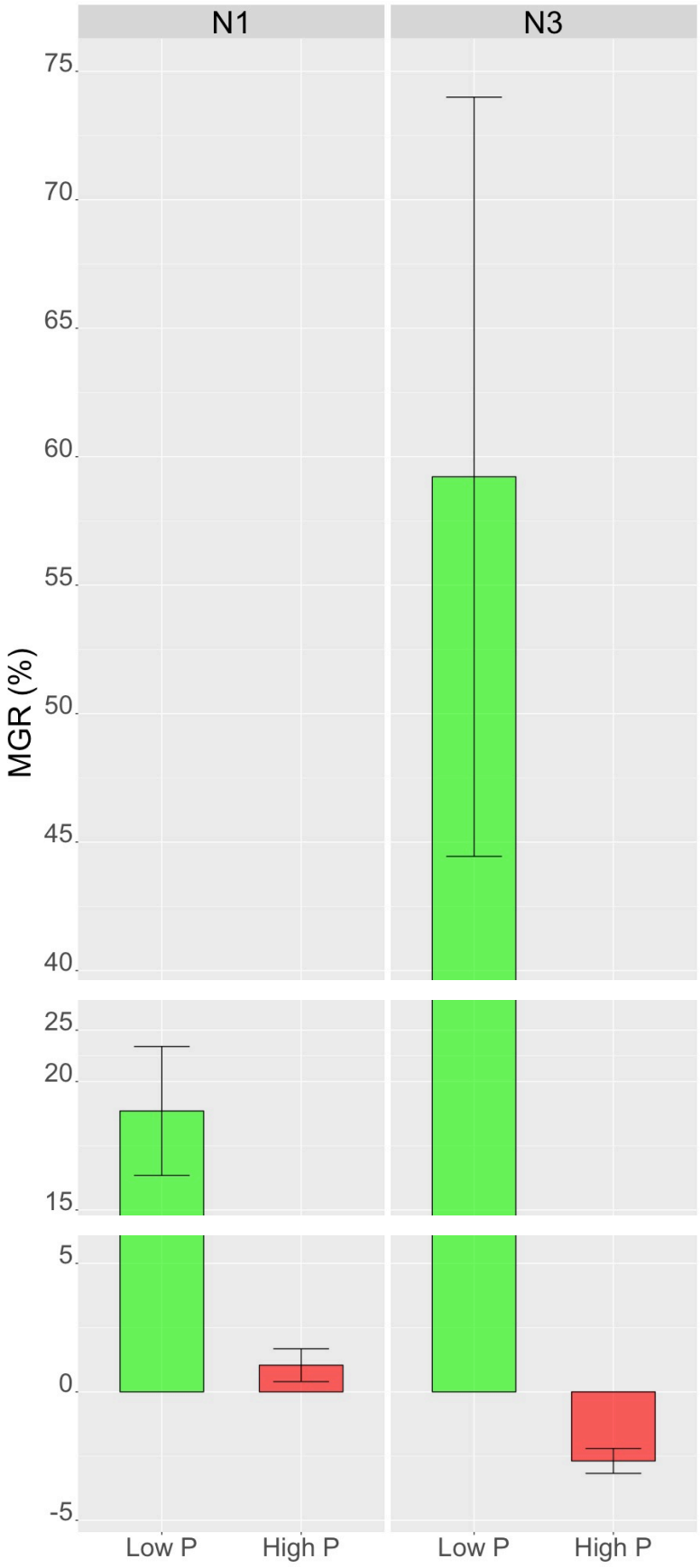


Fig. 2.2.9. Mycorrhizal growth response (MGR) of durum wheat to the different N and P applied combinations.

Table 2.2.4. Plant botanical fractions (green leaves, senescent leaves, culms and spikes) incidence on the total aboveground biomass observed in the different N and P applied combinations in absence (-myc) or presence (+myc) of AMF inoculum.

		Green leaves (%)	Senescent leaves (%)	Culms (%)	Spikes (%)
N1 High P	**-myc**	9.57	10.31	57.67	22.45
	+myc	10.14	9.53	57.84	22.49
N1 Low P	**-myc**	14.95	8.07	54.61	22.37
	+myc	14.47	7.08	56.59	21.86
N3 High P	**-myc**	13.30	22.56	51.51	12.63
	+myc	14.17	21.54	51.14	13.15
N3 Low P	**-myc**	21.71	24.89#	49.70	6.17#
	+myc	19.32	17.24	50.89	12.55
Sourse	**df**				
N level (N)	**1**	*** (0.96)	*** (1.17)	*** (0.93)	*** (1.27)
P level (P)	**1**	*** (0.96)	*** (1.17)	** (0.93)	** (1.27)
myc	**1**	ns	*** (1.17)	ns	* (1.27)
N x P	**1**	ns	*	ns	**
N x myc	**1**	ns	*	ns	**
P x myc	**1**	*	**	ns	*
N x P x myc	**1**	ns	*	ns	*

Data are means, (n = 5). *, ** and *** indicate significant differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences. Values in brackets indicate significant differences at P≤0.05 (Tukey's HSD test). # indicate significant difference between -myc and +myc within the same N treatment (*P≤0.05*).

The presence of the AMF inoculum has also affected the composition of the aboveground biomass (Table 2.2.4). Interaction between N level and presence of AMF was observed concerning the green leaves proportion on the total aboveground biomass (interaction P x myc significant at P≤ 0.05; Table 2.2.4). The latter was positively influenced in High P treatment (11.43% vs 12.15% for -myc and +myc respectively), whereas a detrimental effect was observed under Low P conditions (18.33% vs 16.89% for -myc and +myc). In the treatment N3-Low P, the presence of AMF was also determining a decrement in the senescent leaves proportion (24.89% vs 17.24% for -myc and +myc respectively), while no effect was detected when plants were grown in the others treatments (interaction N x P x myc significant at P≤ 0.05; Table 2.2.4). Interaction between the three main factors was also observed for the spikes incidence on the total aboveground biomass (interaction N x P x myc significant at P≤ 0.05; Table 2.2.4). The inoculation in the latter parameter determined a strong effect only in the

treatment N3-Low P, where, compared to the not inoculated treatment, an increment of 103.73% was observed (Table 2.2.4).

Both N concentration and N uptake of the aboveground biomass were significantly affected by the interaction between AMF inoculation and soil P availability (interaction P x myc significant at P≤ 0.05 and 0.001 respectively for N concentration and uptake; Table 2.2.3, and 2.2.5). Aboveground N concentration was negatively affected by the presence of AMF in the Low P treatments showing values of 2.16 to 1.95 in –myc and +myc respectively. By contrast, AMF determined a positive effect on N uptake (96.5 vs 117.3 in -myc and +myc respectively). No differences for both the parameters have been observed in the High P treatments (Fig. 2.2.10a, b). Both P concentration and uptake have been strongly influenced by the interaction between inoculation and soil P availability (interaction P x myc significant at P≤ 0.0001; Table 2.2.3, and 2.2.5). Moreover, P uptake resulted also affected by the interaction between inoculation and N level (interaction N x myc significant at P≤ 0.05; Table 2.2.5).

In Low P levels, mycorrhizal plants compared to plants grown in absence of AMF inoculum have shown higher P concentration, whereas significantly lower values were observed in +myc plants when grown under no limiting soil P availability (High P treatments; Fig. 2.2.10c). The same trend was observed in the aboveground P uptake where the presence of AM fungi determined an increment from 3.22 to 5.67 in Low P, while a reduction from 27.72 to 23.29 was observed in the High P treatments (Fig. 2.2.10d).

Table 2.2.5. ANOVA output of aboveground biomass N uptake, aboveground biomass P uptake, N uptake efficiency (NUpE), N use efficiency (NUE), P uptake efficiency (PUpE), P use efficiency (PUE)

Source	df	Nitrogen uptake	Phosphorus uptake	NUpE	NUE	PUpE	PUE
N level (N)	**1**	*** (8.18)	*** (0.76)	*** (0.006)	*** (0.35)	*** (0.006)	*** (5.05)
P level (P)	**1**	*** (8.18)	*** (0.76)	*** (0.006)	*** (0.35)	*** (0.006)	** (5.05)
myc	**1**	ns	* (0.76)	* (0.006)	*** (0.35)	ns	*** (5.05)
N x P	**1**	***	ns	***	***	***	***
N x myc	**1**	ns	*	ns	ns	*	ns
P x myc	**1**	**	***	**	***	***	***
N x P x myc	**1**	ns	ns	ns	ns	ns	ns

*, ** and *** indicate significance differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significance differences. Values in brackets indicate significant differences at P≤0.05 (Tukey's HSD test).

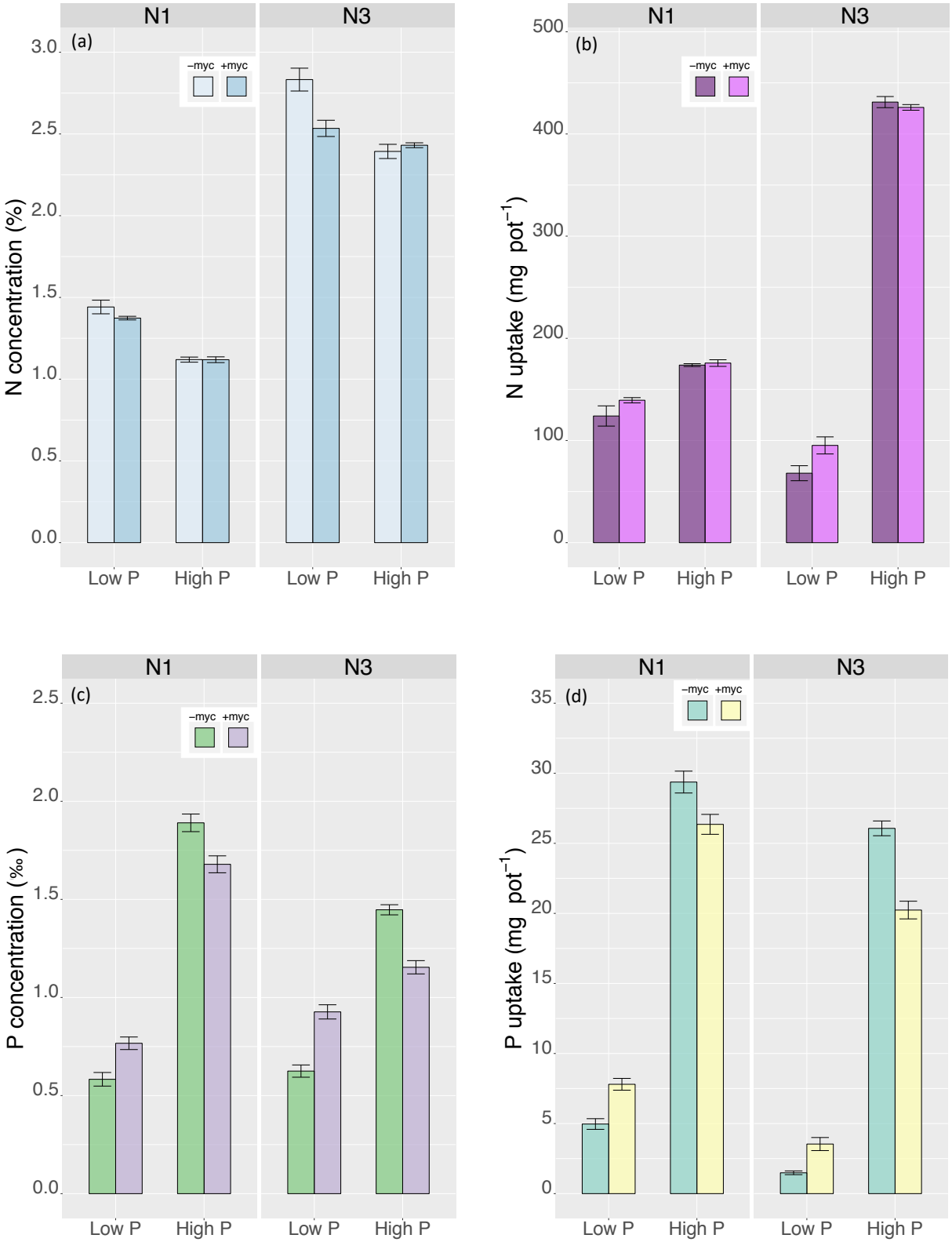


Fig. 2.2.10. Durum wheat response to the different N and P applied combinations in absence (-myc) or presence (+myc) of AMF inoculum. Aboveground biomass N concentration (a); aboveground biomass N uptake (b); aboveground biomass P concentration (c); aboveground biomass P uptake (d).
Bars represent means ± SE (n = 5).

The indices of uptake and utilization efficiency for both the nutrients investigated have always shown a strong interaction between soil P availability and AMF inoculation (interaction P x myc significant at P≤0.01 for NUpE and P≤0.001 for NUE, PUpE and PUE; Table 2.2.5). Only PUpE was also affected by the interaction between N treatment and AMF inoculation (interaction N x myc significant at P≤0.05; Table 2.2.5).

N efficiency parameters have shown the same trend, showing that the AMF inoculation has significantly increased the two parameters in both the N levels under Low P, whereas no effect was observed in both the N treatments under High P (Fig. 2.2.11a, b). In the Low P treatments, NUpE ranged from 0.081 to 0.096 in absence and presence of AMF inoculation respectively (average values of both the N treatments under Low P; Fig. 2.2.11a), whereas AMF increased the NUE from 5.01 to 6.19 in -myc and +myc respectively (average values of both the N treatments under Low P), determining an increment of 23.5% (Fig. 2.2.11b).

PUpE was positively affected by the AMF inoculation in the two Low P treatment regardless the N treatment, showing values of 0.04 and 0.08 in -myc and +myc respectively (average values of both the N treatments under Low P; Fig. 2.2.11c). Whereas, a negative effect of AMF was observed in the two High P treatments (0.15 vs 0.13 in -myc and +myc respectively, average values of both the N treatments under High P; Fig. 2.2.11c). In the two Low-P treatments, similarly to what observed for the PUpE, a positive effect of AMF was observed for the PUE, whereas no effect was observed in the two High-P treatments (Fig. 2.2.11d). In particular, compared to the non-inoculated treatments, the presence of AMF determined an increment in PUE by the 27% when plants were grown under P-deficiency (treatment Low P; Fig. 2.2.11d).

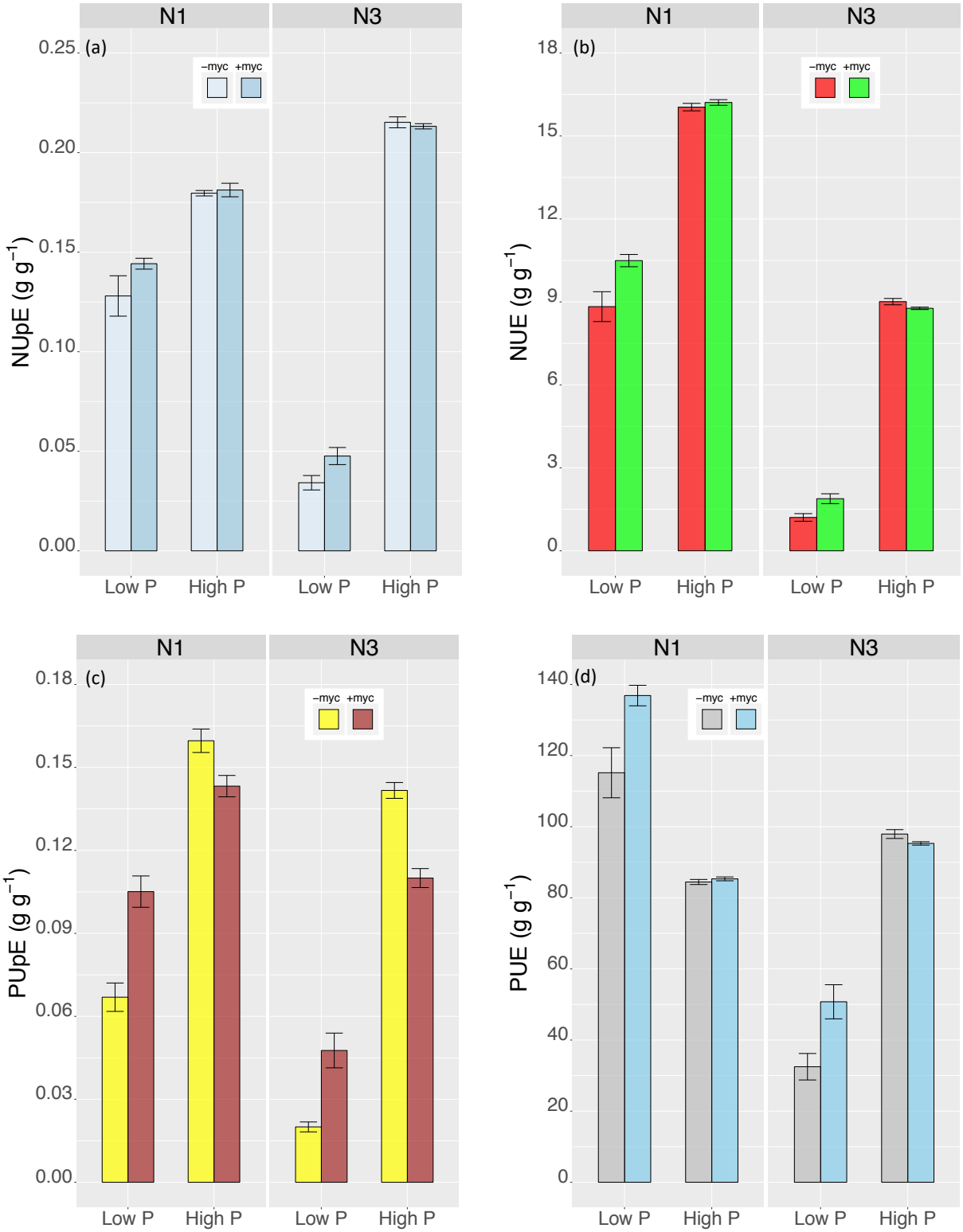


Fig. 2.2.11. Durum wheat response to the different N and P applied combinations in absence (-myc) or presence (+myc) of AMF inoculum. N uptake efficiency (NUpE; a); N use efficiency (NUE; b); P uptake efficiency (NUpE; c); P use efficiency (PUE; d). Bars represent means ± SE (n = 5).

Discussion

The "trade balance model" based on the soil N:P ratios have been proposed to explain the shifting of AM symbiosis outcome from mutualism to parasitism and vice versa (Johnson, 2010). Authors providing experimental evidences of this model (Johnson et al.; 2015; Püschel et al., 2016) have explained their results pointing at a high N:P ratio as the main driver for having a positive mycorrhizal growth benefit. Indeed, in N-rich soil, P can most likely be the limiting factor and the ability of AMF in P uptake result crucial for the plants growth. Data observed in the Experiment 2 seem to confirm this hypothesis, since the highest MGR was observed under the treatment with the highest soil N:P ratio among the compared experimental treatments (N3-Low P), while the lowest MGR was observed in the treatment N3-High P which had the lowest soil N:P ratio. However, the same trend of MGR was not observed in the Experiment 1, where P was not a limiting factor. In the latter case, MGR has shown the highest value in a treatment with an intermediate N:P ratio among the compared treatments (treatment N2). However, the effect size of the highest MGR was very different in the two experiments (modest in the Experiment 1 and substantial in the Experiment 2), highlighting once more time the crucial role of AMF on plants growth when soil is P-deficient. By contrast, less clear is the effect of AMF when plants are grown in N-deficient soil. Indeed, in the Experiment 1, where plants were grown along a gradient of soil N availability, MGR resulted negative when the amount of N supplied was very limited (treatment N0). This result was probably due to the high N demand of AM fungi (Hodge and Fitter 2010), confirming that the two symbionts can compete for the N in soil when N is a limiting growth factor (Püschel et al., 2016; Paragraph 2.1 of this thesis). However, in the Experiment 1, a negative MGR was also observed when plants were grown in presence of very high N availability, highlighting that when plants are grown in soil with an amply availability of mineral N the C cost for the plant exceeds the benefit that AMF can provide. Indeed, assuming a high availability of all the others essential soil-nutrients, a curvilinear relation of mycorrhizal growth response along the soil N availability gradient has been hypothesized (Gange and Ayres, 1999; Janos 2007; Corrêa et al., 2015). In the Experiment 1, although the overall effect size was quite modest, results of MGR were in accordance to this hypothesis. In fact, the MGR switched from negative to positive and vice versa based on soil N availability.

Johnson et al. (2015) and Püschel et al. (2016) in experiments carried out manipulating soil N and P availability have found a positive correlation between percentage of AMF root colonization and MGR. Here, a negative correlation was observed in the Experiment 1 and no correlation whatsoever was found in the Experiment 2. A possible explanation of this

discrepancy could be ascribed to the fact that both the cited studies have used a highly mycotrophic C4 grass species (*A. gerardii*) as focal plant, while in both the present experiments a moderate mycotrophic C3 species (*T. durum*) was used. Moreover, Püschel et al. (2016) pointed to a possible P limitation that could have affect the AMF root colonization along the N gradients, while here, in the Experiment 1, given the P concentration of the nutritive solution, a P limitation was highly unlikely. Moreover, in the experiments part of the N was applied as ammonium, which in high concentration has been shown to have deleterious effects on AMF colonization and on both AMF and plant growth (Valentine et al. 2002; de Graaf et al., 1998). This might also explain why in both the experiments a reduction of root AMF colonization was observed with increasing the amount of N applied. However, although in the two N3 treatments (Low P and High P) of the Experiment 2 the same amount and form of N was applied, a slight higher value of AMF root colonization and a large increment in MGR were observed in Low P compared to the treatment High P. Possibly, in N3-Low P, AMF by increasing the P uptake have at the same time mitigated the ammonium toxicity and the plant intensified the C flux to the roots and AM fungi. In fact, plants in the treatment N3-Low P, already deficient in P nutrition, were severally affected by the ammonium toxicity. Certainly, the increment in P nutrition due to AMF has improved the plant healthy status mitigating the toxicity effect and increased the plant growth. Thus, the plants could have possibly supplied more carbohydrates to fungal partners following the "reciprocal rewards model" proposed by Kiers et al. (2011). Vice versa, in N3-High P the presence of AMF resulted a net cost for the plant as shown by the negative MGR.

Although both the two Low P treatments have shown an increment in MGR and in AMF root colonization compared to the respective High P treatment (N1-Low vs N1-High P and N3-Low P vs N3-High P), as previously reported no correlation between the two response variables has been observed. Certainly, to this result has contributed the fact that the treatment N3-Low P, although showing the highest MGR, had largely lower values of AMF colonization than that observed in the treatments N1-low P and N1-High P. Moreover, differences in the degree of AMF colonization are not always unequivocally related to the AM symbiosis functionality and/or to the benefit that AMF could provide to the host plant (Smith & Smith, 2011). In fact, in studies carried out on various plants AM fungi combinations the AMF root colonization and the MGR have sometimes been found positive correlated (Graham & Abbott, 2000; van der Heijden et al., 2006; Blanke et al., 2011; Maiti et al., 2011), some others negative correlated (Jackson et al., 2002; Veiga et al., 2011; Walder et al., 2012; Corrêa et al., 2014), and some other times no correlated (Ryan & Angus 2003; Gao et al. 2006; Grace et al. 2009; Büscher et

al. 2012; Fellbaum et al. 2014). Also here, in both the experiments, although the degree of AMF colonization was lower than what observed in other studies using durum wheat as focal plant (Ryan et al., 2002; 2005; Saia et al., 2014a; 2015a; 2015b), the presence of AMF has affected plant biomass production. Moreover, in both the present experiments, the presence of AMF affected plants organs incidence on the aboveground biomass. In particular, the AM symbiosis has increased the incidence of green leaves, except for plants grown under the two Low P treatments in the Experiment 2, where a detrimental effect was observed. Interestingly, the negative effect of the AMF inoculation on the green leaves incidence was observed in the two treatments which have had the higher MGR (treatments N1-Low P and N3-Low P), whereas a positive effect was observed where the MGR was very contained or even negative. Under these latter conditions AMF seem may influence the allocation of the fixed C in the different plant organs within the aboveground biomass. Indeed, when AM symbiosis benefit is relatively high, plant is presumed to fix enough C to offset the fungus C cost and address the exceed C to its own growth. Vice versa, when the AM symbiosis C cost exceeds the benefit, the two symbionts may compete for the C allocation. In such scenario, AMF would benefit from increasing the leaves portion which has the highest photosynthesis capacity among the plant organs (Lupton, 1968). This hypothesis could also be supported by the increment due to the presence of AMF on green leaves N concentration. In fact, RUBISCO (ribulose-l,5-bisphosphate carboxylase-oxygenase), the primary CO_2-fixing enzyme in C3 plant accounts for as much as 75% of leaf N (Chapin et al., 1987); consequently, leaves N concentration controls the plant photosynthetic capacity, and therefore the amount of C that AMF can demand. Contrary to what observed in the leaves portion, no difference ascribed to AMF inoculation were observed in the total aboveground N concentration. The only difference in the aboveground biomass N concentration was observed in the treatments Low P where the presence of AMF determined a significant decrement of the parameter. However, the same effect was not observed in N uptake. In fact, in the same treatment the presence of AMF determined an increment in the amount of N acquired. Differences ascribed to the AMF inoculation in N uptake were also observed in the treatments of the Experiment 1. In particular, a detrimental effect was observed at N0, whereas no effect was observed at N3. This result confirms that under low soil N availability the two symbionts can compete for the N in soil, as already observed by Püschel et al. (2016) and in the previous experiment (Paragraph 2.1). In treatment N1 no significant differences were observed between the plants grown in the presence or absence of AMF, whereas in treatment N2 a slight, positive MGR was observed. Similar results have been observed by Püschel et al. (2016) in big bluestem grown under N supply similar to that of this experiment. Increments ascribed to AMF

inoculum in N uptake and in biomass production in durum wheat under high soil P availability have also been previously observed by Saia et al. (2015a).

The absence of the detrimental effect observed in the treatment N3 highlight that, in the present experiments, under high soil N availability, the plant was grown under no limiting N condition and the detrimental effect observed in the +myc treatment could be ascribed to the corresponding AMF C drain. On the other hand, possibly, AMF could have affected also the uptake of other nutrients determining nutrient(s) imbalance in plant tissue, and thus reducing plant growth (Corrêa et al., 2014). Indeed, looking at the data of P uptake of the Experiment 2, it emerges that the increase in the dose of N fertilizer has always led to a significant reduction in P uptake; therefore, high levels of N in soil seem to determine negative effects on plant P acquisition. This has led to the hypothesis that the reduction in P uptake has penalized growth when plants were grown under critical lack of P, while the same amount of N in soil did not have any effect when P was not a limiting factor. However, under this latter condition, AMF have determined a decrement in P concentration and uptake. A possible explanation of this strange result can be found on the fact that mycorrhizal seems to be able suppress the direct P uptake by the root system (Smith and Smith 2011) and the mycorrhizal contribution to the P uptake seems to decrease in line with the AMF colonization as P supply increase (Nagy et al., 2009). Also PUpE has shown the same trend, however, no differences ascribed to the AMF inoculation in PUE were observed in the two High P treatments of the Experiment 2. These results are very surprising since highlight that, although an increment in the uptake efficiency plants were unable to translate the better nutritional status in growth increase. By contrast, in the two Low P treatments the differences ascribed to the AM symbiosis observed in PupE resulted confirmed in PUE. Possibly, in this specific context, the difference in the PUE might be due to the no limiting P availability in the two High P treatments that determined luxury consumption, and at the same time, to the presence of a different limiting factor which suppress further plant growth. By contrast, in P-deficiency condition P was a limiting factor and therefore increment in the uptake efficiency due to the AM symbiosis was translated in increment in the nutrient use efficiency. Once more, the mechanisms that regulate the functionality of AM soil symbiosis related to the soil N seem to be different from that observed for the soil P. In fact, as for the P efficiency parameter, AM symbiosis has positively affected the N efficiency parameters in the two Low P treatments (N1-Low P and N3-Low P), whereas no effect was observed in the two High P treatments of the Experiment 2. However, in this case, the amount of N supplied in the two High P and Low P treatments was exactly the same, therefore these results were probably influenced by some other factor. Most likely, in P-limitation, plant tissues

were strongly unbalanced and, although N was available in soil, plants were not able neither to use nor even to acquire the soil N. Indeed, as reported by Güsewell (2004), the plant tissue N:P ratio is a driver of plant biomass production and alterations of its equilibrium can severally affect the plant growth.

In conclusion, soil N and P availability and their ratios have affected the mycorrhizal functionality in durum wheat shifting the mycorrhizal outcome though the entire spectrum of the ecological relationships. In fact, under low soil P availability the mycorrhizal outcome was positive either in low and high N availability. By contrast, limitation or excess in N availability in high soil P availability determined a depression of plant growth due to the presence of AMF, and only at intermediate level of N in soil an appreciable positive effect of AM symbiosis was observed, confirming the curvilinear hypothesis. Moreover, regardless the soil N availability the AMF effect was observed in both the P availability conditions (high and low), however, the size of the effect on the detected parameters was very contained at high soil P availability and large under soil P deficiency. Such trend was observed on both quantitative and qualitative parameters, and on both nutrients' efficiency parameters.

Finally, another noteworthy result was the effect of AMF on the aboveground biomass organography, as well as on the relative N allocation among the different aboveground biomass plant organs, which would provide useful information to the understanding of the mechanisms which rule the effects of AM symbiosis on plant growth.

2.3. Changes in the AMF Community deriving from different Soil Depths and Their Implications on Durum Wheat Growth

Abstract

Subsoil (namely soil below 30 cm) pedo-environmental conditions are very different and often more hostile to that observed in the topsoil. Several studies have shown drastic modifications in the microbial community along the soil profile and soil depth AMF taxa specialization. Hitherto, no data are available concerning whether and how these differences can affect plant growth. The present experiment aimed to evaluate the differences on AMF community composition along soil depth using a high-throughput approach and to verify if the observed differences were able to affect plant growth performance under well-watered (WW) and stress-watered (SW) conditions in a pot experiment.

Results have shown a variation in the AMF communities along soil depths and the existence of subsoil specific AMF phylotypes. In the pot experiment, root colonization decreased according to the sampling depth of the soil inoculum in both WW and SW. In SW a decrement of the root colonization degree in plants inoculated with soil from the topsoil was observed. By contrast, when the inoculum derived from the subsoil the degree of colonization increased, highlighting specific traits of the subsoil fungal community. Effects of the different soil inocula on plant growth were observed only in WW, resulting in a decrement in plant biomass and in WUE ascribed to the presence of the soil inoculum deriving from the fist 15 cm compared to when plants were grown in the presence of the other soil inocula.

Introduction

In its 2014 report IPCC envisioned possible future scenarios. Particularly, the report highlighted an increment of atmospheric CO_2 (from the current c. 400 to c. 1000 μmol l^{-1}) (IPCC, 2014; NOAA-ESRL, 2015) and an expected average temperature rise of more than 2 °C within the end of the century. In addition, an alteration of the frequency and the amount of rainfall is predicted (ICPP, 2014). These scenarios led to the hypothesis that crops would be subjected to several abiotic stresses in the near future (Mittler and Blumwald, 2010). Among others, the report pointed at drought and temperature rise as the two climatic parameters that can severely affect crop production. Kirkegaard et al. (2007) highlighted the role that the subsoil could have as a hydric reservoir and its essential role in crop development, especially in arid and semi-arid environments. However, the subsoil conditions are often hostile and its root exploration is limited (Bengough et al., 2011). Possibly, plants could benefit from the interaction with subsoil-

dwelling organisms that could have evolved particular traits to explore such hostile conditions and increase the water uptake under drought stress. In fact, several studies aimed at characterizing the microbial community along the soil profile have shown drastic modifications in the community composition of bacteria and fungi by depth (Oehl et al., 2005; Eilers et al., 2012; Sanaullah et al., 2016; van Leeuwen et al., 2017; Sosa-Hernández et al., 2018a). Additionally, Sosa-Hernández et al. (2018b) have highlighted evidence of soil depth AMF taxa specialization. By comparing data from mixing top and subsoil along a chronosequence and from undisturbed topsoil and subsoil, the authors highlighted that the abundance of AMF species living in the undisturbed subsoil were decreasing over time in the chronosequence, while the same trend was not observed for the topsoil AMF taxa. The environmental specialization of AMF taxa allows one to hypothesize changes in functionality traits as well. Therefore, the microbial community located in the distinct soil layers could affect crop growth differently. However, although several studies have focused on its characterization and have highlighted differences in the soil microbial community according to soil profile, to my knowledge, hitherto no data are available concerning whether and how these differences can affect plant growth. In order to address this gap in understanding, the present experiment aimed at: i) evaluating the differences on AMF community composition by soil depth using a high-throughput approach; ii) and verifying if the observed differences were able to affect plant growth performances under well-watered and stress-watered conditions in a pot experiment. Durum wheat was the focal plant species in the pot experiment since it is the main crop both in the long-term experiment, from which the inocula derive, and in the entire semi-arid Mediterranean area.

Materials and methods

Site description and inoculum sampling

The soil samples used as inoculum were taken in a long-term field experiment in Pietranera farm (approx. 30km north-west of Agrigento, Sicily, Italy; latitude: 27.54, longitude 13.51, elevation: 221 m). Briefly, the experimental design of the long-term experiment was a strip plot design with two replicates, where three tillage systems and three crop sequences are evaluated since 1991. In any case, the crops management match the respective combination of tillage and crop rotation regularly applied in the Mediterranean area. Details of the long-term experiment are reported in Giambalvo et al. (2012) and in Amato et al. (2013). A total of 30 plots with an area of 370 m^2 (18.5 m x 20 m) each were sampled at 4 different depths (0-15 cm, A; 15-30 cm, B; 30-60 cm, C; and 60-90 cm, D), giving a total of 120 samples. The sampling was

performed in the last ten days of May 2016. Per each plot, four holes to a depth of 100 cm were opened using a mini digger. Before the sampling, the first vertical soil layer was removed, in order to avoid contamination or dry soil. One single sample was taken from each of the four soil layers of each hole, placed into individual sample bags and immediately sieved through a 2 mm sieve. All depths from a given sample were sampled at the same point. The 4 technical samples from the same depth and the same plot were subsequently pooled to generate a single analytical sample per depth per plot. Each analytical sample was subsequently split in two samples consisting in 100 grams of soil each. One set of samples was immediately stored at -20 °C and used for the molecular analysis. The second was stored at 4 °C and used as inoculum in the pot experiment. The latter was also characterized for the total N concentration using an Elemental Analyser (EuroEA, HekaTech, Germany) with acetanilide (Merck, Darmstadt, Germany) as internal standard, and the average of each soil depth resulted as follow: 1.81 g kg^{-1}, 1.52 g kg^{-1}, 1.05 g kg^{-1} and 0.67 g kg^{-1} respectively for the soil layer A, B, C and D.

Molecular investigation

DNA was extracted from 250 mg of soil using PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's instructions. Nested-PCR approach described in Sosa-Hernandez et al. (2018a) using the primer sets SSUmAf-LSUmAr and SSUmCf-LSUmBr (Krüger *et al.*, 2009) was used to amplify the partial SSU, the entire ITS region and the partial LSU region in Glomeromycotina. Briefly, three nested PCRs were all performed with the Kapa HiFi PCR Kit (Kapa Biosystems, Woburn, MA, USA) following manufacturer's recommended procedures. The first PCR was performed using 1 µl of normalized DNA extract as DNA template and primer set SSUmAf-LSUmAr. The second PCR was performed using 1 µl of a 1:10 dilution of the previous PCR result as DNA template and primer set SSUmCf-LSUmBr. Finally, 1 µl of a 1:10 dilution of the second PCR was used as DNA template for the third PCR using the primers LR3 and LR2rev (Hofstetter *et al.*, 2002). The first and the second PCRs thermo-cycle conditions were as follow: 25 cycles 98ºC - 20 s., 60ºC - 30 s., 72ºC - 50 s.. The third PCR thermo-cycle conditions were as follow: 20 cycles 98ºC -20 s.,47ºC - 30 s., 72ºC - 50 s..

Amplicons were later gel-separated, band-excised and purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and used for a fourth PRC where the amplicons were tagged with indexing sequences and adaptors suited for Illumina MiSeq sequencing using the sequencing primers. The product was purified using magnetic beads (GC Biotech, Alphen aan den Rijn, The Netherlands), DNA concentration was determined and a

library was created pooling equimolar amounts of each sample. The amplicon pool was sequenced using 2 × 300 bp paired-end MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA) at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv, Berlin, Germany). Sequences were filtered using a single sequence variant (SSV) approach as implemented with the R package "dada2" (Callahan et al., 2016).

Pot experiment

Pot and plants

The pot experiment was carried out in a greenhouse under 20 °C +/- 2 °C and a photoperiod of 16 h per day. Additional light at a photosynthetic photon flux density of 60000 lumen was provided if necessary. In the experiment, 2.5 l sterilized pots were used. Each pot was filled with 2 l of artificial substrate composed V/V in 90% of 2 mm sieved soil and 10% of sand. The soil had a sandy silt texture (Albic Luvisol following FAO classification) with the following characteristics: 76 g kg^{-1} clay, 188 g kg^{-1} silt, 736 g kg^{-1} sand, pH 5.9, 18.7 g kg^{-1} total C, 1.2 g kg^{-1} total N, 69 mg kg^{-1} available P, 30 mg kg^{-1} K. Both the substrate portions have been steam pasteurized applying two cycles at 90 °C for 24 hours interrupted by 24 h at room temperature.

Before starting the experiment, a pilot experiment was carried out to assess the hydrologic parameters and the specific weight of the substrate using the gravimetric method (Dobriyal et al., 2012). Briefly, 10 perforated crucibles were filled with 100 grams of soil and placed in a basin with water up to half of the crucible's height. The crucibles were let to absorb water by capillarity until each pot was saturated. The excess water was let drained away and the weight difference at field capacity and the following drying at 105 °C to a constant weight was monitored. The available water for the crop was obtained by subtracting the weight after the oven dry period from the field capacity weight.

The sowing was done on August the 27th 2016, sowing 6 surface sterilized seeds of durum wheat (*Triticum durum* Desf., var. *Anco Marzio*) per pot. All the seeds and the pots were sterilized using a 2.5% sodium hypochlorite solution for 3 to 5 minutes. Later, five days after the emergence the thinning was done leaving 3 plants per pot.

A completely randomized design was adopted. Treatments were: i) inoculum (collected at 4 different depths as previously described); ii) two water regimes (well-watered, WW; stress watered, SW). To effectively address the influence of depth, all the plots (30) present in the long-term experiment were sampled and used as field replicates; so a total of 240 pots were set up; pots were re-randomized every two weeks.

Inoculum

30 grams per pot of each soil sample described above were used as inoculum. The inoculation was done in two steps, separating the inoculum into two equal parts (15 grams each). The first 15 grams were added during the pot filling, mixing the inoculum homogenously with the artificial substrate. The second part was added during the sowing phase, placing the inoculum just below the seeds. This inoculation method was used to assure the contact between the plant's root system and the inoculum at the early growth stage and along the entire experiment.

Water treatments

All the pots were maintained at the holding capacity during the first twenty days after the emergence of the wheat seedlings. Afterward, two water treatments have been applied: i) well-watered (WW), where the soil moisture was kept in the range between 80% to 100% of the holding capacity; ii) stress-watered (SW), where the soil moisture was kept in the range between 40% to 50% of the holding capacity. The soil moisture was adjusted three times per week using the gravimetric method, so that soil water content did not follow below 80% or 40% of the holding capacity. At each irrigation event, tap water was added in the adequate quantity to reach the upper threshold of each treatment.

Harvest and Measurements

62 days after the emergence, when the plants reached the flowering phase, a leaf core sample (about 2 mg weight) was taken in the middle part of a flag leaf of one of the principal culms and the aboveground biomass (shoot) harvested. The core sample and the aboveground biomass were oven dried at 85 °C until constant weight and the dry weight recorded. Then, the aboveground biomass was ground to fine powder for future analysis.

The flag leaf samples were used to assay the N and C concentration using the Elemental Analyser described above. A representative subsample of the total aboveground biomass was used to determine the total N concentration using the Dumas method (flash combustion with automatic N analyser; DuMaster D-480, Büchi Labortechnik AG, Flawil, Switzerland).

Belowground biomass (root) was carefully extracted through sieving and consecutive washing, oven dried at 40 °C until constant weight and the dry weight recorded. Successively, two root subsamples were extracted. One (about 2 mg) was ground to a fine powder and then used to assay the N and C concentration using the Elemental Analyser (EuroEA, HekaTech, Germany) with acetanilide (Merck, Darmstadt, Germany) as internal standard

The second was used to assay the root traits. The root subsample was extracted by taking very gently 4 to 5 first order roots ensuring to take all the others root orders to it attached. The subsample was rehydrated at 4 °C for 24 h and analyzed using the WinRhizo^{TM} scanner-based system (v.2007; Regent Instruments Inc., Quebec, Canada). This method, as reported by Bergmann et al. (2017), avoids root degradation and is strongly related to the data obtained on fresh root. In the analysis, root average diameter and root length were considered as root traits. Finally, a root sample of 80 to 150 root pieces of 1-2 cm was extracted to assay the root infection by AMF and other fungi endophyte. In this case, the subsamples were extracted from a subset of 136 pots as follow: 68 per each water treatment that is 17 replicas per each soil depth inoculum. All the four depth from each site were used to assay the root infection. The samples were stained with Trypan Blue according to a modified staining protocol (Phillips & Hayman, 1970). Briefly, the roots were cleaned with 10% KOH at 80 °C for 10 minutes, acidified in 1% HCl for 15 minutes at room temperature and stained for 40 minutes in 0.05% Trypan Blue at 80 °C. Root colonization was quantified according to the method proposed by McGonigle et al. (1990).

Calculation and statistical analysis

Eight pots were excluded from analysis for the following reason: two pots showed infection symptoms (classified as powdery mildew) and 6 pots presented extreme values in terms of soil dry weight. The latter pots were excluded since differences in soil dry weight could determine different nutrient availability and therefore being a confounding factor in the results.

Thus, the number of pots considered in the dataset was 232 as follow distributed: well-watered treatment 115 pots (29, 29, 29 and 28 respectively for the soil inocula A, B, C and D); stress-watered treatment 117 pots (30, 28, 30 and 29 respectively for the soil inocula A, B, C and D.

Data obtained from the imagine analysis were used to determine the total root length which was calculated based on the known weight of the root sample and the total root biomass; the soil root density (SRD) and specific root length (SRL) were obtained as the total root length divided respectively by the pot soil dry weight and the total belowground biomass. The N concentration determined on the total aboveground biomass and on the belowground biomass was used to obtain the N uptake of each respective fraction by multiplying the N concentration of each fraction for the respective amount of biomass produced. Finally, the total biomass (aboveground plus belowground biomass) and the amount of water consumed per each pot during the entire experiment were used to calculate the Water Use Efficiency (WUE) by applying the formula:

$$WUE = \frac{total\ biomass\ (g)}{water\ consumed\ (kg)}$$

Data were analyzed in R version 3.4.1 (R Core Team, 2017). ANOVA repeated-measures using the "aov" function was used to perform the analysis. This procedure was used since allow to distinguish between nested factors. The hierarchical structure was: water treatment nested in depth, with depth nested in sampling site, assuming site as a random factor. Each single field plot was used as a single unit in the analysis.

In addition, the pot soil dry weight was introduced in the model as a covariant in order to correct for small differences in soil dry weight due to the pot filling phase using wet soil (soil humidity due to the steam pasteurization).

Shapiro test and Bartlett test were used to check the normality and the homoscedasticity respectively. When the variables did not fulfill the ANOVA assumptions data were transformed accordingly. Following the ANOVA test, the post hoc mean comparison Tukey's HSD test ($P \leq 0.05$) was performed using the function "HSD.test" from the "agricolae" package (de Mendiburu, 2017). The "HSD.test" was performed based on the second or the third ANOVA error strata respectively in absence or in presence of the interaction between the two explanatory variables "Inoculation" and "Water- treatment". Bonferroni adjustment was used to prevent an inflation of P values. In details, two subsets based on the water treatment were created and the differences within the explanatory variable "Inoculation" assayed within each water treatment. Untransformed data have been reported in tables and in graphical representations. Package "tidyverse" (Wickham, 2017) was used for data graphical representation.

AMF community analysis was performed using the R software (R Core Team, 2016) and the package "vegan" (Oksanen et al., 2016). The identification of SSVs specific for each depth was performed on a non-normalized SSV table. Subsequent analyses were performed on a SSV table normalized by the minimum amount of reads per sample (1471). A Bray-Curtis (Bray & Curtis, 1957) dissimilarity matrix was generated with the function "vegdist", nonmetric multidimensional scaling (NMDS) was generated with the function "metaMDS", multivariate permutational analysis of variance (PERMANOVA) was carried out with the function "adonis". Graphical representations were generated using packages "ade4" (Dray & Dufour, 2007) and "vegan". Univariate analysis on the effect of depth was calculated using a generalized linear model (GLM) with a quasi-Poisson distribution.

Results

Molecular investigation

A total of 2162 AM fungal SSVs were identified. 282 fungal SSVs were exclusively found at the depth A, and 203, 110, and 87 at depths B, C and D respectively (Fig. 2.3.1). In total, 197 SSVs were exclusively found in the two soil layers C and D (subsoil, below 30 cm), whereas 485 SSVs were found in the topsoil (soil layers A and B). Richness and Shannon diversity values for each depth after normalization are reported in table 2.3.1. Both parameters have been reduced with increasing depth. In particular, richness value ranged from 135 (depth A) to 42 (depth D) whereas Shannon index varied from 3.83 to 1.90. Also, the analysis of community ordination showed a shift in community composition along depth (Fig. 3.3.2). The effect of depth on community composition was significant (PERMANOVA; $P<0.001$). Taxonomic assignment could only be carried at the phylum level, due to technical issues and therefore, no distribution of AM fungal families can be presented yet.

Table 2.3.1. Richness and Shannon indexes for each depth (means ± standard deviation). Richness is given in number of SSVs, sd stands for standard deviation

Depth	Richness		Shannon	
A (0-15 cm)	134.6 a	±38.9	3.83 a	±0.40
B (15-30 cm)	96.1 ab	±26.8	3.37 ab	±0.43
C (30-60 cm)	61.5 bc	±26.2	2.40 bc	±0.80
D (60-90 cm)	41.5 c	±24.4	1.90 c	±0.76

Different letters denote significant differences ($P \leq 0.05$)

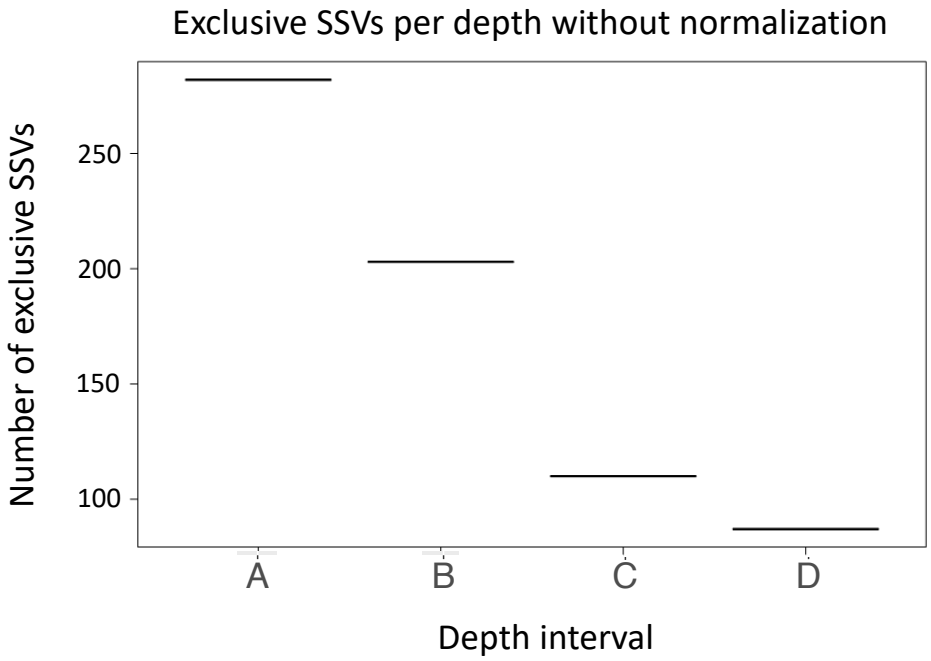


Figure 2.3.1. Number of SSVs found exclusively at each depth using a non-normalized table (i.e. without accounting for different sampling efforts).

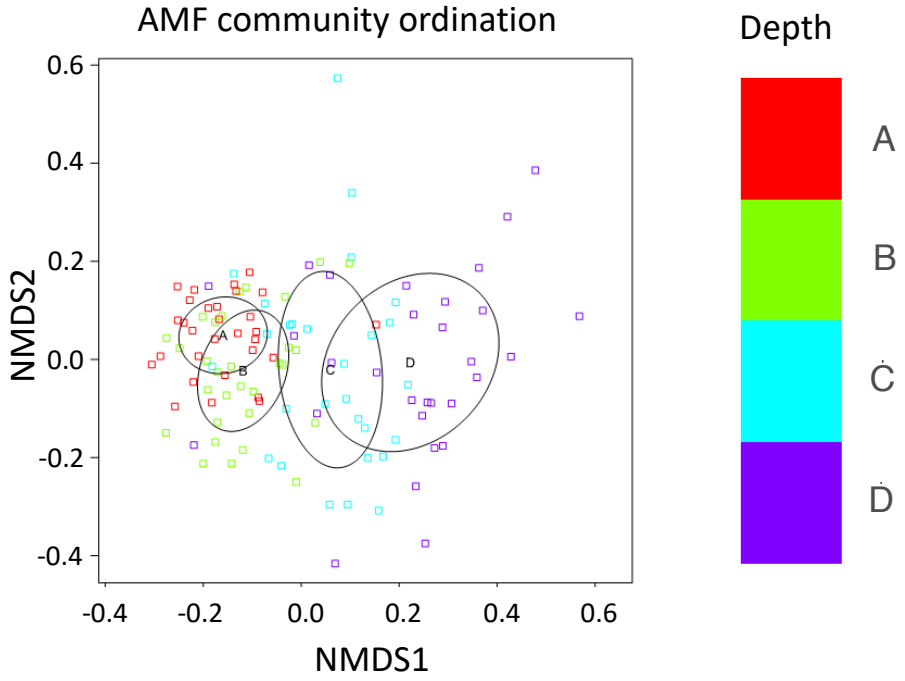


Figure 2.3.2. Non metric multidimensional scaling (NMDS) of the AM fungal communities at different depths, based on Bray-Curtis dissimilarities

Pot experiment

Plant root colonization

The different soil inocula applied significantly affected plant root colonization by AMF showing a continuous decrement along depth (A>B>C>D) either in WW and in SW. An interaction effect between the two explanatory variables (inoculation and water-treatment) was observed (Table 2.3.2). In fact, while the water stress application, compared to WW treatment, determined a reduction of the AMF root colonization when plants were grown in presence of the two topsoil inocula (layers A and B), the opposite was observed with the subsoil inocula (layers C and D; Fig. 2.3.3a). Furthermore, the values of root mycorrhization varied largely by depth in WW treatment, whereas the differences among depth inoculum were markedly smaller in SW treatment.

As well as the AMF colonization, the percentage of root colonized by others endophyte fungi significantly reduced by depth (P <0.001; Table 2.3.2), while, on the contrary, increased when water stress was applied (WS>WW; P <0.001; Fig. 2.3.3b).

Table 2.3.2. ANOVA output of AMF root colonization, other endophytes root colonization, aboveground biomass dry weight, belowground biomass dry weight and water use efficiency (WUE).

Source	df	AMF colonizzation	Endophytes colonization	Aboveground biomass	Belowground biomass	WUE
water treatment (1)	**1**	***	***	***	***	**
Inoculation (2)	**3**	***	***	***	***	***
(1) x (2)	**3**	***	ns	*	**	ns

*, ** and *** indicate significant differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences.

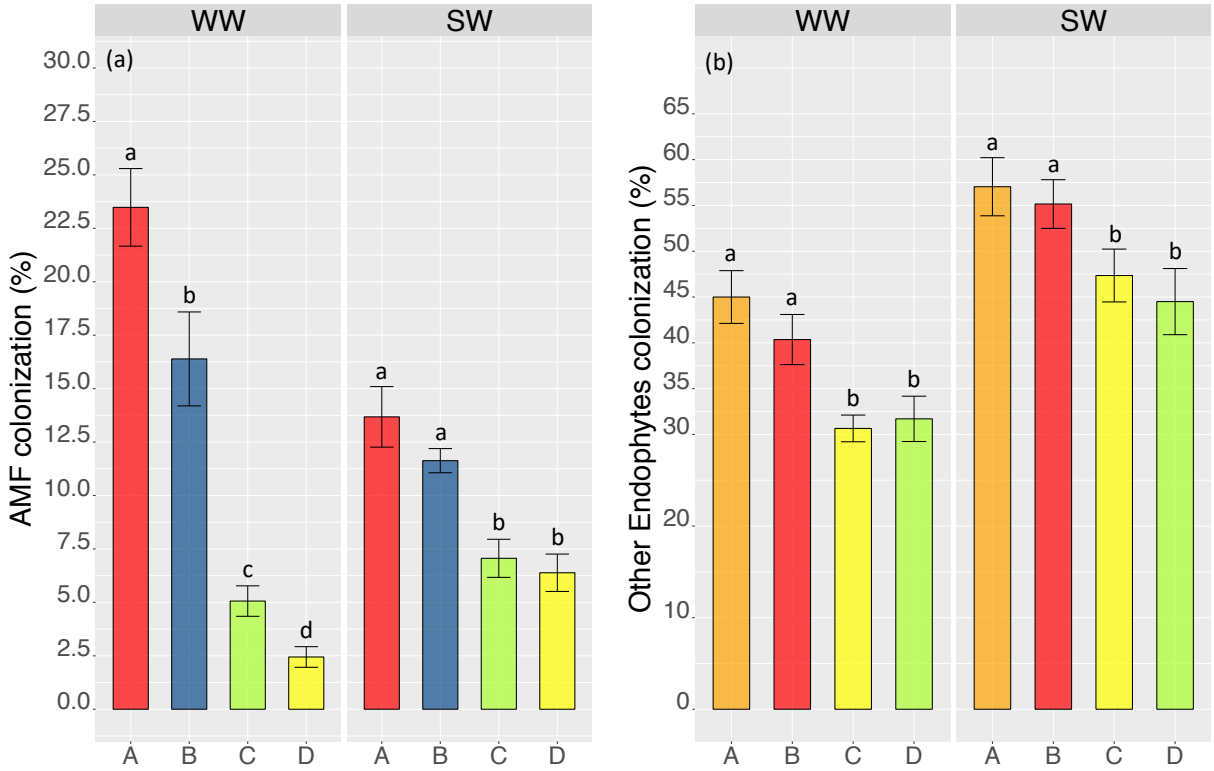


Fig. 2.3.3. Durum wheat response under well-watered (WW) and stress-watered (SW) conditions in presence of the different soil inocula. AMF root colonization (a); Other endophytes root colonization (b).
A, B, C and D represent soil inocula deriving from the soil layer 0-15 cm, 15-30 cm, 30-60 cm and 60-90 cm respectively. Bars represent means ± SE (n = 17). Bars with different letters within the same water treatment differ significantly from each other (*P≤0.05*).

Plant response

Interaction between the two predictors variables, inoculation and water treatment, was observed for both above and belowground biomass production (interaction P≤ 0.05 and P≤ 0.01 for above and belowground biomass respectively; Table 2.3.2). In both biomass portions, significant differences between the different soil inocula were found when plants were grown in well-watered conditions, while no differences between soil depth inocula were observed when plants were grown under drought stress (Fig. 2.3.4a, b). In particular, in the well-watered treatment both the biomass portions were lower in plants when grown in presence of the soil inoculum A compared to when grown in presence of the inocula B and D (post hoc comparison $P \leq 0.05$); intermediate values for both biomass portions were observed when plants were grown in presence of the soil inoculum C (Fig. 2.3.4a, b). In WW, aboveground biomass ranged from an average of 13.7 to an average of 15.04 gram per pot observed in plants grown in presence of the inocula A and B respectively (Fig. 2.3.4a). Belowground biomass ranged from an average value of 2.77 gram per pot in plants grown in presence of the inoculum A to an average of 3.40 gram per pot when inoculated with the soil inoculum D (Fig. 2.3.4b).

In the SW treatment, above and belowground biomasses were drastically reduced compared to the WW treatment, showing average values of 4.64 and 0.88 grams per pot respectively (average of plants grown in presence of all the four soil inocula), with no differences between the inoculation treatments, as previously mentioned (Fig. 2.3.4a, b). By contrast, the plant WUE was significantly affected by the presence of the different soil inocula in SW as well as in WW. In particular, in both water treatments the WUE was significantly lower when plants were grown in presence of the soil inoculum A compared to when grown in presence of the others three soil inocula, with no differences observed among the latter (B=C=D; Fig. 2.3.4c). The WUE was higher in the SW plants compared to the WW plants.

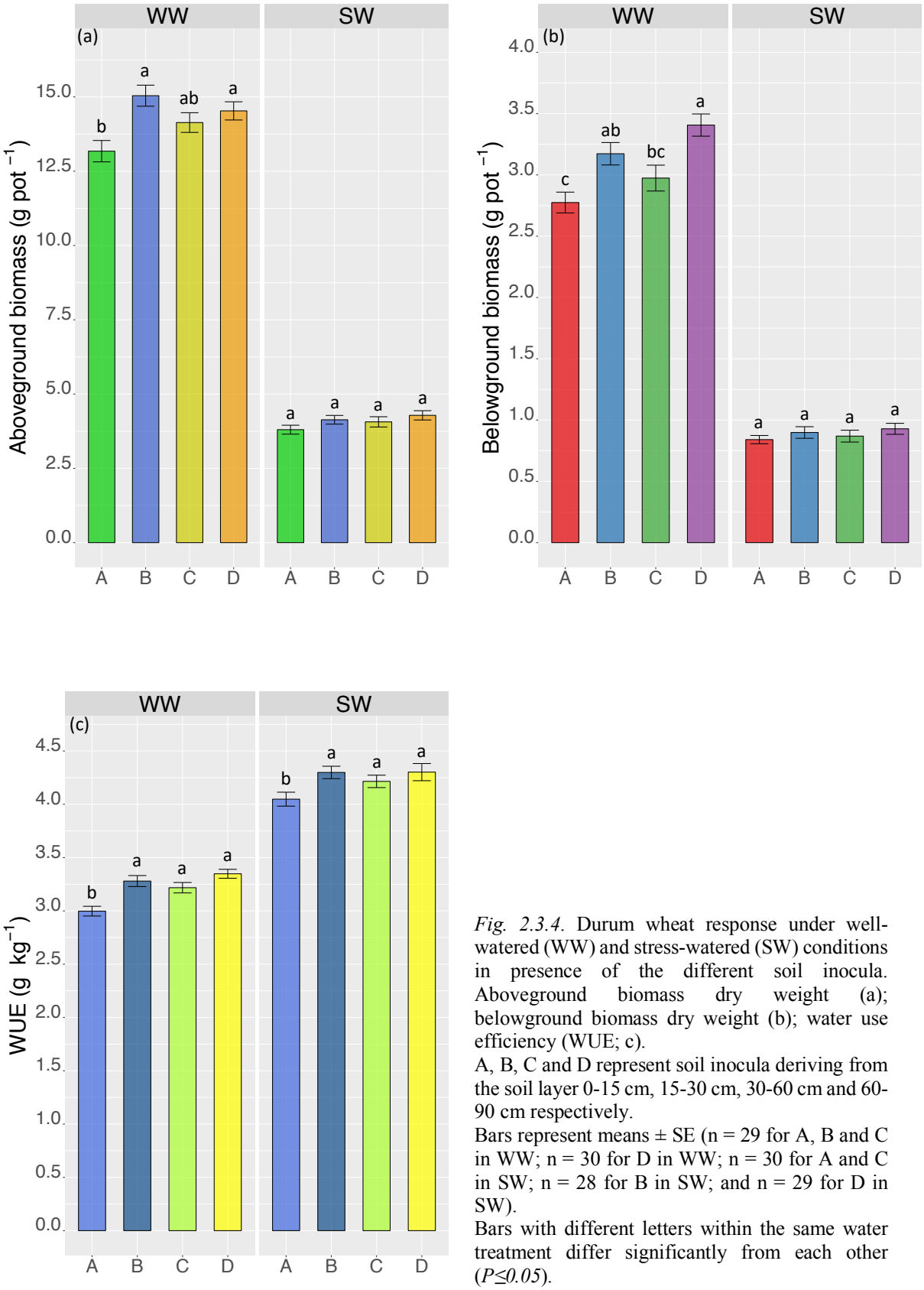


Fig. 2.3.4. Durum wheat response under well-watered (WW) and stress-watered (SW) conditions in presence of the different soil inocula. Aboveground biomass dry weight (a); belowground biomass dry weight (b); water use efficiency (WUE; c).
A, B, C and D represent soil inocula deriving from the soil layer 0-15 cm, 15-30 cm, 30-60 cm and 60-90 cm respectively.
Bars represent means ± SE (n = 29 for A, B and C in WW; n = 30 for D in WW; n = 30 for A and C in SW; n = 28 for B in SW; and n = 29 for D in SW).
Bars with different letters within the same water treatment differ significantly from each other ($P \leq 0.05$).

Table 2.3.3. Root shoot ratio (R:S), aboveground biomass N concentration, aboveground biomass N uptake, belowground biomass N concentration, belowground biomass N uptake, belowground biomass C concentration, belowground biomass C:N ratio, flag leaf N concentration, flag leaf C concentration and flag leaf C:N ratio of durum wheat grown in presence of the different soil inocula under well-water (WW) and stress-water (SW) conditions.

			Aboveground biomass		Belowground biomass				Flag leaf		
		R:S	N concentration %	N uptake mg pot^{-1}	N concentration %	N uptake mg pot^{-1}	C concentration %	C:N	N concentration %	C concentration %	C:N
WW	**A**	0.212	1.24	163.3	0.88	24.46	45.12	51.97	3.78	47.20	12.66
	B	0.211	1.15	173.3	0.86	27.49	45.33	53.07	3.75	47.32	12.82
	C	0.210	1.17	164.5	0.85	25.00	45.78	55.08	3.85	47.55	12.50
	D	0.235	1.12	162.5	0.85	28.97	45.43	53.62	3.81	47.77	12.72
SW	**A**	0.223	2.13	79.9	1.23	10.12	43.62	36.34	4.46	46.78	10.56
	B	0.316	2.11	86.8	1.18	10.34	43.26	37.38	4.67	47.01	10.11
	C	0.213	2.13	85.4	1.17	9.92	43.27	37.67	4.67	46.81	10.16
	D	0.217	2.09	88.9	1.20	11.02	42.82	36.11	4.79	46.85	9.81
Source	**df**										
water treatment (1)	**1**	ns	***	***	***	***	***	***	***	*	***
Inoculation (2)	**3**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1) x (2)	**3**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Values with different letters within the same water treatment differ significantly from each other (*P≤0.05*).
*, ** and *** indicate significant differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences.

N concentration, both in the above and belowground biomass, was significantly higher in the SW treatment compared to the WW (table 2.3.3), whereas the opposite was observed for N uptake (WW>SW). No significant effect of inoculation was observed on all the N related parameters in the above and belowground biomass both in WW and in SW (table 2.3.3).

Root diameter have shown an average of 0.133 mm in the treatment WW, significantly lower than the mean value observed in SW treatment (0.215 mm). In both water regimes, no appreciable difference ascribed to the presence of the different soil inocula was observed (table 2.3.4). Specific root length strongly varied between the two water treatments (on average, 239 and 129 m per gram of root in WW and SW, respectively) but not among soil inocula (table 2.3.4). On the contrary, significant differences among plants grown in presence of the four soil inocula were observed in the total root length and in the root soil density in WW but not in SW. In particular, the total root length ranged from 663 to 834 in WW and from 113 to 120 m per pot in SW in presence of the two soil inocula A and D respectively. The same trend was observed for the root soil density, which ranged from 29.48 to 37.20 in WW and 5.05 and 5.37 cm per gram of soil in SW, respectively in presence of the inoculum A and D (table 2.3.4).

Table 2.3.4. Root diameter, specific root length (SRL), total root length and root soil density (RSD) of durum wheat grown in presence of the different soil inocula under well-water (WW) and stress-water (SW) conditions.

		Root diameter mm	SRL m g^{-1}	Root length m pot^{-1}	RSD cm g^{-1}
WW	**A**	0.134	239	663^{b}	29.48^{b}
	B	0.136	235	740^{b}	32.70^{b}
	C	0.133	241	706^{b}	31.43^{b}
	D	0.131	244	834^{a}	37.20^{a}
SW	**A**	0.213	135	113^{a}	5.05^{a}
	B	0.214	129	116^{a}	5.13^{a}
	C	0.219	127	110^{a}	4.98^{a}
	D	0.221	128	120^{a}	5.37^{a}
Source	**df**				
water treatment (1)	**1**	***	***	***	***
Inoculation (2)	**3**	ns	ns	***	***
(1) x (2)	**3**	ns	ns	**	**

Values with different letters within the same water treatment differ significantly from each other (*P≤0.05*). *, ** and *** indicate significant differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences.

Discussion

Preliminary data of the molecular investigation on AMF across different soil depths have shown a significant shift of AM fungal communities by depth, and the existence of subsoil specific AM fungal phylotypes.

Increasing soil depth, a decline of AMF spore number, biomass and root colonization have been observed (Higo et al., 2013; Jakobsen and Nielsen, 1983; Muleta et al., 2008; Säle et al., 2015); however it has been estimated that over 50% of AMF total biomass is located below 30 cm (Higo et al., 2013). Data of the present experiment revealed the presence of unique SSVs in the different soil layers and a reduction of Richness and Shannon indexes diversity with of soil depth increase, confirming the observations of several authors (Muleta et al., 2008; Oehl et al., 2005; Sosa-Hernández et al., 2018a). According to Sosa-Hernández et al. (2018b; 2019), evidence exists for subsoil ecological specialization in some AM fungal taxa, so that topsoil and deeper community could respond differently to environmental changes and agronomic managements. The role of the community of the deeper soil layers has so far been little considered, but actually it could represent an important component of the agro-ecosystem contributing to the performance of the plants and to an improvement of the agroecosystem functioning. Considering the environment where they live it is possible to hypothesize that this fungal community is more tolerant to the anaerobic conditions, are able to colonize the smallest soil pores and are able to put in place a greater resources use efficiency, favouring at the same time a better of soil structure and the re-allocation of nutrients. All this could have positive agronomic effects, offering opportunities to improve the sustainability of the production processes, adding resilience to the agroecosystems. In the pot experiment, root colonization of AMF and other endophytes decreased according to the sampling depth of the soil inoculum, in concordance with the lower presence of symbiont microorganisms with increasing soil depth. However, although differences were observed in root colonization and preliminary results of the molecular investigation have shown the presence of diversity in the mycorrhizal community structure in the different soil layers, the effects of the different soil inocula on plant growth were rather modest.

Overall, the presence of the soil inoculum collected in the soil layer 0-15 cm determined a reduction in biomass production and in WUE compared to when plants were grown in the presence of the other three soil inocula. However, although the same trend was observed in both water treatments, the significant difference in the two compartments (above- and belowground) biomass production was observed only in the WW treatment, whereas WUE significantly differed in both water treatments (Fig. 2.3.4a, b and c). These results could seem surprising

since the plants exposed to soil inoculum A have shown the highest percentage of AMF root colonization. A possible explanation for this result could be found in the high fertility of the growth substrate used in the experiment (e.g. 18.7 g kg^{-1} total C, 69 mg kg^{-1} available P in the soil portion which was the 90% of the substrate), which could have substantially reduced the extent of the benefits that AMF can offer and, moreover, shift AM symbiosis towards parasitic behavior (Johnson et al., 1997; Johnson et al., 2015; paragraph 2.2 here). In fact, under conditions of high availability of resources, plants have the ability to autonomously satisfy their own needs and the carbon cost that the plant must support for mycorrhizal symbiosis would not be offset by the benefits that symbiosis could potentially offer.

AMF-root colonization can substantially decrease under water-stressed conditions (Ryan and Ash 1996; Al-Karaki and Al-Raddad 1997; Al-Karaki, 1998), and such effect was observed in the present experiment exposed to the two topsoil inocula (A and B, layers 0-15 and 15-30 cm respectively); on the contrary when the two sub soil inocula were used (B and C, layers 30-60 cm and 60-90 cm, respectively) the AMF colonization increased under water stress conditions compared to the well-watered treatments. This represents a further evidence that, compared to the topsoil AMF community, the subsoil community presents specific traits, showing an increased ability to activate symbiotic relationship even in a limiting condition environment. On the whole, under water stress condition the differences observed among the inocula treatments on AMF root colonization were limited and this could explain why in such conditions plants grown in presence of the different inocula did not show substantial differences. Moreover, in this experiment, the method of inoculation adopted (living soil inoculation) determined the addition of the entire soil microbial community (including saprotrophic endophytes) and the biological interaction between plant-AMF-other soil organisms could have reduced or overridden the AMF benefits, especially under drought conditions. In fact, saprotrophic endophytes can shift from commensalism to parasitism in the presence of weakened plants (Thomma, 2003; Hardoim et al., 2015).

2.4. Microbial Community from different Soil Tillage management and Depth Affect Durum Wheat Growth

Abstract

No-tillage (NT), compared to conventional tillage (CT), can offer several benefits to the agro-ecosystem sustainability. Among the others, increments in richness, diversity and activity of the soil microbial community have been found. However, no data are available concerning weather and how the microbial communities deriving from different tillage systems affect plant growth. Here, a pot experiment was carried out to verify whether soil microbial community deriving from NT and CT at two different depth (0-15 cm and 15-30 cm) differently affect durum wheat performance.

Results have shown better performance in durum wheat inoculated with soil from NT compared to when inoculated with that from CT when inoculum derived from the first 15 cm of soil, whereas no differences were observed when the inoculum was deriving from the soil layer 15-30 cm. Moreover, no differences were observed between the two soil inocula deriving from different depth in NT, while plant performance was positively affected by the inoculum deriving from the soil layer 15-30 cm compared to that deriving from the first 15 cm in CT. In any case, differences in plant performance were in accordance to that found on root colonization.

Introduction

The FAO (2011) defined three main principles in conservative agriculture: i) crop rotation; ii) permanent soil cover; iii) minimum soil disturbance by direct planting. The latter, usually defined as no-tillage (NT), has been ascribed as the central concept of conservative agriculture (Pittelkow et al., 2015). However, often no-tillage has shown less crop yield compared to conventional tillage (CT) and this aspect has limited the widespread adoption of this tillage system. On the other hand, NT can offer several benefits and several authors have often reported a reduction of the negative effects on crop yield when NT is combined with other conservative agriculture practices (Pittelkow et al., 2015; Giller et al., 2015; Giambalvo et al., 2018). Compared to CT, no-tillage protects soil from erosion, improves soil aggregation, reduces soil evaporation and increases the water pool available for the crop (Kirkegaard, 1995; Scopel et al., 2005; Madari et al., 2005; Lampurlanés and Cantero-Martínez, 2006). In addition, increments in richness, diversity and activity of the soil microbial community have been found with NT compared to CT (Jansa et al., 2002; Mbuthia et al., 2015; Sengupta & Dick, 2015).

Many experiments worldwide have demonstrated higher concentration of Phospholipid Fatty Acids (PLFAs; bioindicators used to investigate the structure of the microbial community) in untilled soils than in those conventionally tilled (Shi et al., 2012; Sun et al., 2016; Wang et al., 2017). Moreover, some have highlighted the increase of gram-positive (Zhang et al., 2014; Mbuthia et al., 2015; Zhang et al., 2018b) or gram-negative bacteria (Zhang et al., 2015b; García-Orenes et al., 2013), total bacteria and fungi (Feng et al., 2003; García-Orenes et al., 2013; Shi et al., 2012; Helgason et al., 2010; Sun et al., 2016) due to the different residue placements, water regimes as well as less disturbance. However, the effects on microbial community are controversial showing unclear trends or no differences across experiments, especially in the relative abundance of specific microbial groups and their ratios (Helgason et al., 2009; Helgason et al., 2010; Sun et al., 2016; Zhang et al., 2018b). Since both bacterial and fungal communities play a key role in the organic matter cycling and therefore in the nutrient availability for the crop, this could have implications on plant productivity. However, to my knowledge, hitherto no data are available concerning whether and how these differences can affect plant growth.

In order to contribute to the understanding of this knowledge gap, the present experiment aimed at verify whether the differences induced by tillage on the microbial community were able to affect plant growth performance in a pot experiment. The inocula were sampled in a long- term experiment (over 25 years) where different tillage techniques (CT and NT) were applied; the inocula were previously studied and differences in the composition and abundance of bacterial, fungal and mycorrhizal communities, due to the tillage had already been ascertained along with the soil depth (Badagliacca, 2016; Sosa-Hernández et al., unpublished data). Durum wheat was the chosen plant species since it is the main crop in the long-term experiment from where the inocula derive and the keystone crop of the rainfed Mediterranean agroecosystems.

Materials and methods

Inoculum sampling

The soil samples were taken in a long-term field experiment in Pietranera farm (approx. 30km north-west of Agrigento, Sicily, Italy; latitude: 27.54, longitude 13.51, elevation: 221 m). Briefly, the long-term experiment was a strip plot design with two replicates, where three tillage systems (conventional tillage, reduce tillage and no tillage) and three crop rotations (continuous wheat, wheat in rotation with faba bean and wheat in rotation with berseem clover) were evaluated since 1991. In any case, the crops were managed applying the regular agriculture practices used for each respective crop in the Mediterranean area. Details of the long-term

experiment are reported in Giambalvo et al. (2012) and in Amato et al. (2013). The sampling was done on January the 9th 2018 in all the plots where durum wheat was grown in the previous growing season under the two tillage systems CT and NT. A total of 12 plots with an area of 370 m^2 (18.5 m x 20 m) each were sampled at 2 different depths: 0-15cm and 15-30cm. Per each plot, 6 holes to a depth of 40 cm were opened using appropriate sterilized equipment and about 100 grams of soil from each hole and each soil layer were collected and placed into individual sample bags. Afterward, the soil was immediately sieved at 2 mm and all the samples deriving from the same soil depth and tillage system were merged to generate a single representative sample to be utilized as inoculum in the pot experiment. All the four final representative samples (NT 0-15; NT 15-30; CT 0-15; CT 15-30) were characterized for the total mineral N content (nitrate, nitrite and ammonium) using the Bran+Luebbe AutoAnalyzer 3 (Norderstedt, Germany); which result as follow: 19.9 mg kg^{-1}, 12.6 mg kg^{-1}, 12.7 mg kg^{-1} and 14.3 mg kg^{-1} respectively for NT 0-15 cm, NT 15-30 cm, CT 0-15 cm and CT 15-30 cm. Samples were stored at 4 °C until the beginning of the pot experiment.

Pot and plant management

Durum wheat (T. durum Desf. cv. Prospero) was grown in a rain protected wirehouse in 6 liter sterilized pots (d=16 cm; h=30 cm) filled with 7 kg of a sterilized artificial substrate. The growth substrate was composed for the 43% (3 kg) in river sand and for the 57% (4 kg) in agricultural soil. Both the substrate portions have been sieved through a 2 mm mesh, characterized and sterilized following the cycle: humidification, 24 hours at room temperature and 24 hours 130 °C, for a total of three cycles. Sand total N (Kjeldahl) and available P (Olsen) were 0.11 g kg^{-1} and 7.44 mg kg^{-1} respectively. Agricultural soil properties were as follows: 267 g kg^{-1} clay, 247 g kg^{-1} silt, and 486 g kg^{-1} sand; pH 8.0; 10.8 g kg^{-1} total C (Walkley— Black); 0.86 g kg^{-1} total N (Kjeldahl); 40.1 mg kg^{-1} available P (Olsen), 598 mg kg^{-1} total P, 26 cmol kg^{-1} cation exchange capacity; 1.70 dS m^{-1} saturated electrical conductivity (EC) (25°C); 27.9% water content at field capacity, and 18.9% at the permanent wilting point.

Durum wheat was grown in presence of the four soil inocula previously described and in a non-inoculated treatment (control). The sowing was done on January the 23rd 2018, distributing 15 surface sterilized seeds per pot. All the pots and seeds were sterilized using sodium hypochlorite 3% for 3 to 5 minutes. After the sowing, all the pots were irrigated to reach the holding capacity. Afterward, the soil moisture was monitored twice a week through the gravimetric method and additional water was added when the soil moisture reached the 70% of the holding capacity. Ten days after the emergence the thinning was done to reach the final density of 7 plants per

pot. A total of 25 pots were set up [5 (4 soil inocula+1 control) *5 replicas] in a completely randomized design. The plants were grown for 93 days after the sowing (DAS), coincidentally with the durum wheat flowering phase.

Inoculum

150 grams of each soil inoculum obtained as described in the "Inoculum sampling" section, were applied to each pot. The inoculation was done in two steps. 80% of the inoculum (120 grams) was added during the pot filling, mixing the inoculum homogenously with the artificial substrate. The other 20% (30 grams) was added during the sowing phase, placing the inoculum just below the seeds. This inoculation method was used to assure the contact between the plant's root system and the inoculum at the early growth stage and along the entire experiment. Following the same method, 150 grams of a mix of the four soil inocula (ratio of 1:1:1:1) was sterilized and added to the control treatment.

Biomass harvest and biomass analysis

At the end of the experiment (93 DAS), maximum height was measured and the aboveground biomass (shoot) was harvested and the fresh and dry weight recorded. Green leaf limbs were collected and used to determine the leaf area using an area meter (LI-3100C Area Meter, LI-COR GmbH, Germany). Later, all of the aboveground biomass fractions were merged to a single sample ground to a fine powder for determining the N concentration using the Dumas method (flash combustion with automatic N analyzer; DuMaster D-480, Büchi Labortechnik AG, Flawil, Switzerland).

The belowground biomass (root) was carefully extracted through sieving and consecutive washing, and thereafter oven dried at 40 °C until constant weight. Two representative subsamples were extracted from the belowground biomass. One was ground to a fine powder and used to determine the N concentration using the method previously described. The second was clarified with KOH 10%, stained with trypan blue 0.05% (Phillips and Hayman, 1970) and used to assay the percentage of root colonization by AMF and other fungi endophytes using the method proposed by McGonigle et al. (1990).

Calculation and statistical analysis

N uptake of both above and belowground biomass was obtained by multiplying each N concentration for the respective plant biomass portion (aboveground or belowground).

Data collected were analyzed using R software version 3.4.1 (R Core Team, 2017).

The data subset comprising response variables of the treatments (tillage: CT and NT; soil depth: 0-15and 15-30) were subjected to a two-way ANOVA. Following the ANOVA test the post hoc mean comparison Tukey's HSD test ($P \leq 0.05$) was performed. Student's t-test comparison was used to compare means of each soil inoculation treatment with the non-inoculated control. Shapiro test and Bartlett test were used to assay respectively the normality and the homoscedasticity of the model residuals. When response variables did not fulfill the ANOVA assumptions data were transformed accordingly.
Untransformed data have been reported in tables and in graphical representations. Data graphical representation was performed using Microsoft Excel.

Results

Plant root colonization

Unexpectedly, root colonization by both AMF and other fungi endophyte was observed in the control treatment; however, the percentage of root colonized was always far below to those observed in the presence of the four soil inocula (Fig. 2.4.1a, b).
In the inoculated treatments, on average, the root mycorrhization and the colonization of other endophytes was approximately 9% and 41%, respectively (fig 2.4.1a, b). For both parameters, a significant interaction tillage x depth was detected ($P \leq 0.001$ and $P \leq 0.05$ for AMF and others fungi endophytes, respectively; table 2.4.1). In particular, the inoculum deriving from the first soil layer (0-15 cm) of the NT treatment, determined a significant greater colonization of both AMF and other endophytes compared to that of CT from the same layer, whereas no differences between the two tillage techniques were observed in the deeper layer (15-30 cm). Moreover, it should be noted that, while AMF root colonization of NT significantly decreased with soil depth (from 11.6 to 8.8%), exactly the opposite occurred for CT (from 6.0 to 9.3%). Moreover, the inoculum of the deep layer determined an increase of other endophytes colonization in CT whereas no variation was observed between the two inocula 0-15 and 15-30 deriving from NT (Fig. 2.4.1a).

Table 2.4.1. ANOVA output of AMF root colonization, other endophytes root colonization, aboveground biomass dry weight, belowground biomass dry weight, aboveground biomass N uptake and belowground biomass N uptake.

	df	AMF colonization	Endophytes coloniazation	Aboveground biomass	Belowground biomass	Aboveground biomass N uptake	Belowground biomass N uptake
Tillage (T)	**1**	***	ns	ns	ns	ns	ns
Depth (D)	**1**	ns	*	ns	ns	**	ns
T × D	**1**	***	*	***	ns	**	ns

*, ** and *** indicate significant differences at level P≤0.05, P≤0.01 and P≤0.001 respectively; ns indicate no significant differences.

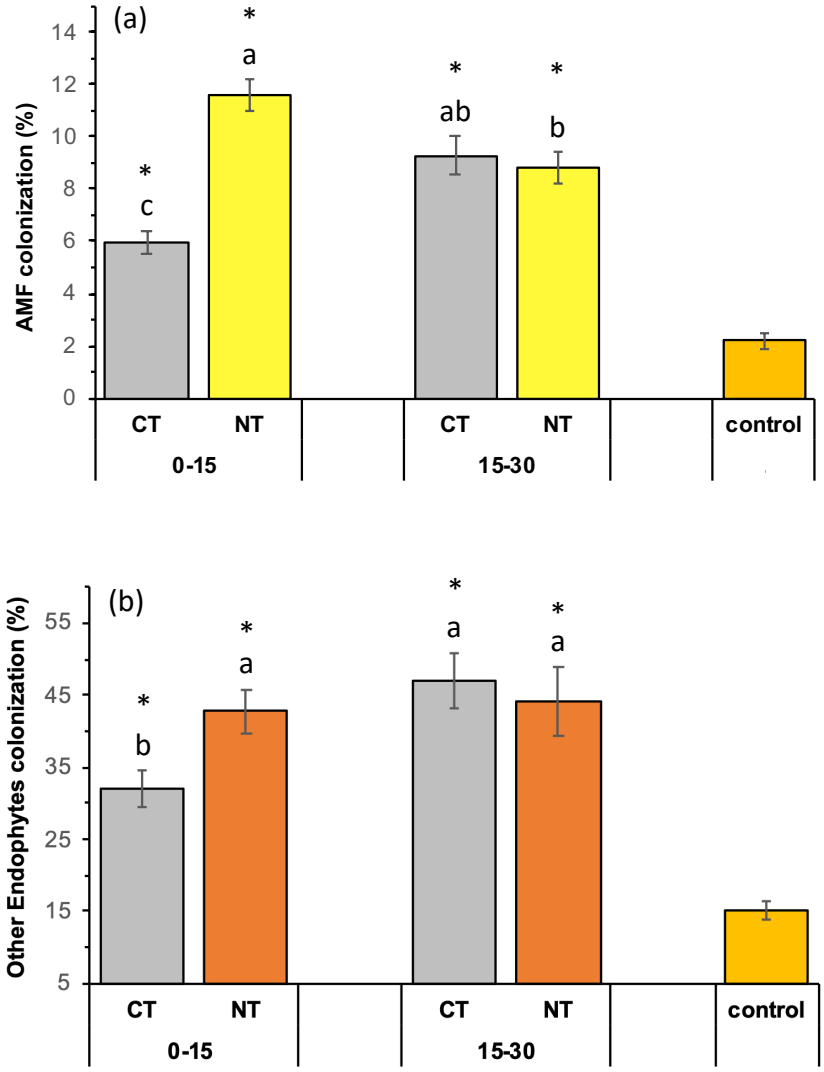


Fig. 2.4.1. Durum wheat response in presence of the different inoculation treatments. AMF root colonization (a); Other endophytes root colonization (b). Bars represent means ± SE (n = 5). Bars with different letters differ significantly from each other (*P≤0.05*). * above bars indicate significant differences between each treatment and the control according to the *t-test* (*P≤0.05*).

Plant response

The inoculum collected in the layer 0-15 cm in NT plots determined a higher aboveground biomass production compared to the inoculum collected at the same depth in CT plots (41.1 vs 36.6 g per pot, respectively), whereas no significant difference was observed when pots were inoculated with soil coming from the layer 15-30 cm (interaction Tillage × Depth significant at P≤ 0.001; table 2.4.1; Fig. 2.4.2). No significant difference was observed between plants grown after inoculation of soil coming from both layers of NT, whereas a significant increment of 12.5% was observed using the inoculum taken in CT plots from the layer 15-30 cm compared to the layer 0-15 cm (41.2 and 36.6 grams per pot, respectively). The two inocula NT 0-15 and CT 15-30 significantly increased the plants aboveground biomass production compared to the control, while no differences were noted between the control and the two treatment CT 0-15 and NT 15-30 (Fig. 2.4.2). The inoculation treatment did not influence other plant traits such as the maximum height, the root:shoot ratio (table 2.4.2), and the accumulation of belowground biomass (Fig. 2.4.3).

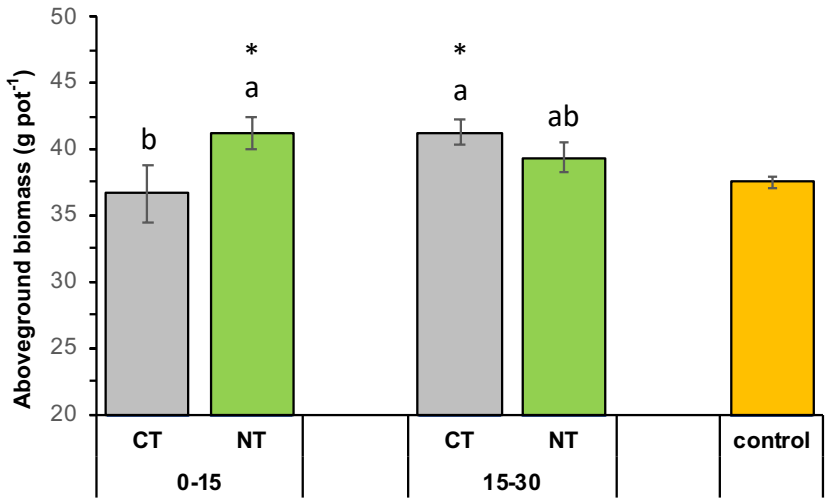


Fig. 2.4.2. aboveground biomass dry weight of durum wheat in presence of the different inoculation treatments. Bars represent means ± SE (n = 5). Bars with different letters differ significantly from each other (*P≤0.05*). * above bars indicate significant differences between each treatment and the control according to the *t-test* (*P≤0.05*).

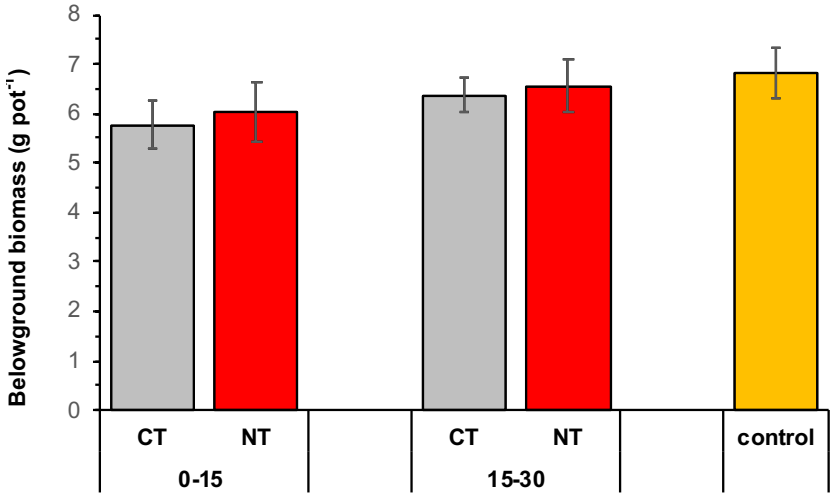


Fig. 2.4.3. belowground biomass dry weight of durum wheat in presence of the different inoculation treatments. Bars represent means ± SE (n = 5).

The N uptake in the aboveground biomass was significantly affected by the interaction between the two main factors (Tillage × Depth significant at P≤ 0.01; table 2.4.1) with a trend similar to that observed for the aboveground biomass. In particular, when the inocula deriving from the conventional tillage system was added, a marked decrement of 21% was observed if the inoculum was collected in the 0-15 cm layer compared to the inoculum 15-30 cm (Fig. 2.4.4a); whereas no difference was observed between plants grown with both soil inocula deriving from NT. No significant difference was also observed when plants were grown in presence of the inocula deriving from the soil layer 15-30 cm between NT compare to CT. By contrast, in presence of inocula deriving from the soil layer 0-15, NT compared to CT increased the aboveground N uptake by the 11% (443 and 400 mg of N per pot in NT and CT respectively). Again, as observed for the aboveground biomass the plants grown in presence of the two soil inocula NT 0-15 and CT 15-30 significantly differed from the non-inoculated control (Fig.2.4.4a). In particular, an increment of 11.8 and 22.5% compared to the control was observed in plants grown in presence of NT 0-15 and CT 15-30 respectively. The percentage of N in the aboveground biomass was not affected neither by tillage system nor soil depth; only the inoculum CT 15-30 has shown a significant effect compared to the control (table 2.4.2). By contrast, the presence of each of the soil inocula determined a significant increment in N concentration in the belowground biomass compared to the control (table 2.4.2). Plants grown in presence of the inocula deriving from CT always increased significantly the concentration of N in the belowground biomass compared to the inocula deriving from NT (table 2.4.2). However, the increment observed in the N concentration did not have any repercussion on the belowground N uptake. In fact, although slight increments were observed in plants grown in

presence of the two inocula CT deriving from both soil layer compared to the presence of the respective NT, no difference was observed in belowground N uptake neither among the soil inocula nor between each of the soil inocula and the control (table 2.4.1; Fig. 2.4.4.b).

Table 2.4.2. Plant height, root shoot ratio (R:S), leaf area, aboveground biomass N concentration and belowground N concentration of durum wheat in presence of the different inoculation treatments.

		Plant height (cm)	R:S	Leaf area (cm^2 pot^{-1})	Aboveground biomass N concentration (%)	Belowground biomass N concentration (%)
0-15 cm	**CT**	60.8	0.156	1551^{b}	1.09	1.11^{a} †
	NT	61.8	0.147 †	1687^{ab}	1.08	0.98^{b} †
15-30 cm	**CT**	61.2	0.154	1754^{a}	1.18 †	1.08^{a} †
	NT	58.0	0.167	1580^{ab}	1.12	0.97^{b} †
control		59.8	0.179	1629	1.04	0.72
Source	**df**					
Tillage (T)	**1**	ns	ns	ns	ns	*
Depth (D)	**1**	ns	ns	ns	ns	ns
T × D	**1**	ns	ns	**	ns	ns

* and ** indicate significant differences at level P≤0.05 and P≤0.01 respectively; ns indicate no significant differences. Value with different letters differ significantly from each other (*P≤0.05*). † indicate significant differences between each treatment and the control according to the *t-test* (*P≤0.05*).

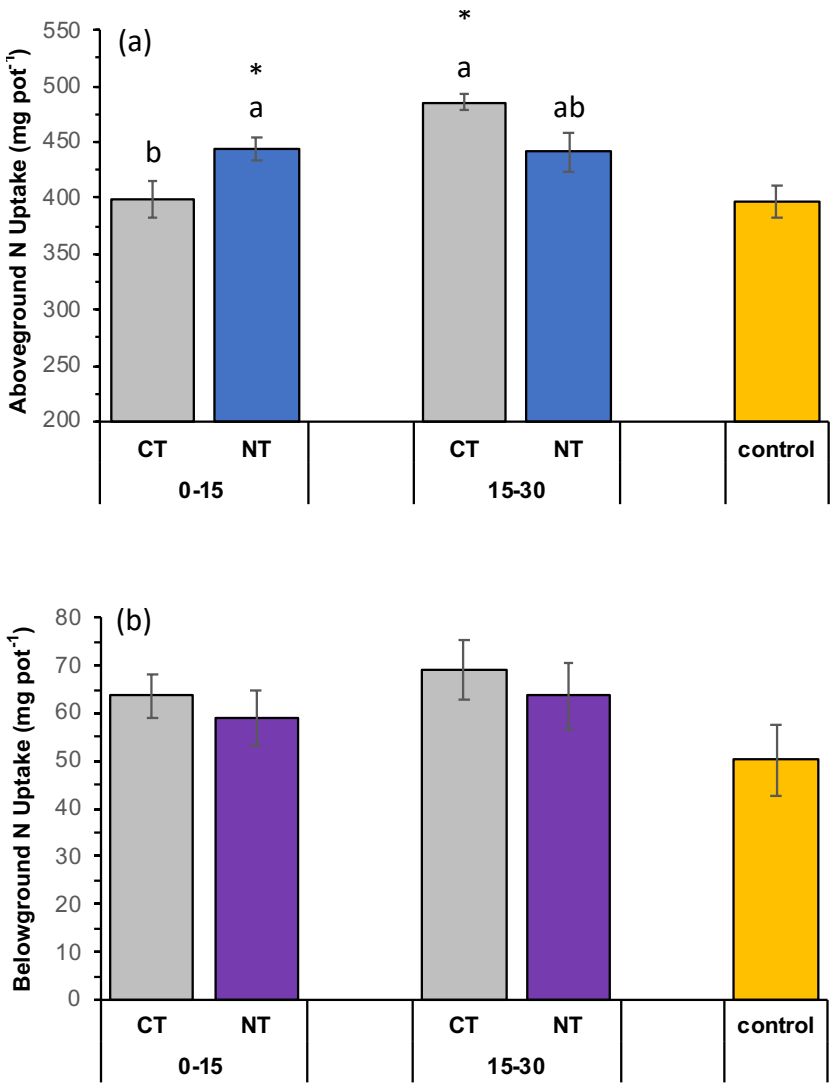


Fig. 2.4.4. Durum wheat response in presence of the different inoculation treatments. Aboveground N uptake (a); belowground N uptake (b). Bars represent means ± SE (n = 5). Bars with different letters differ significantly from each other ($P \leq 0.05$). * above bars indicate significant differences between each treatment and the control according to the t-test ($P \leq 0.05$).

Discussion

Overall, the extent of AMF root colonization was quite low in plants grown in the presence of each of the four inocula. Such low values could be ascribed to the sampling time, as the samples were collected in January before the sowing and after a period of about six months during which the soil remained bare (the previous crop was harvested in July). AMF are obligate symbionts and the absence of a host plant for a long time could have affected the inoculum potential (Harinikumar Bagyaraj, 1988). However, the percentage of AMF and other endophyte root colonization significantly differed among the applied inocula. In particular, higher values were observed in presence of NT soil compared to the CT soil when the inocula derived from the first soil layer (0-15 cm), whereas no difference was observed between CT and NT inocula deriving from the deeper soil layer (15-30 cm). This result agrees with the findings of Badagliacca (2016) who, on samples taken in the same season and in the same experimental units, found a greater fungal biomass in NT than CT in the upper soil layer and no or very small differences in the deeper layer. Moreover, Sosa-Hernández et al. (unpublished data), working on samples taken in the same field experiment, found a marked shift in AMF community composition due to the tillage management in the first 15 cm of soil, and a reduction in the community dissimilarity in the second soil layer (15-30 cm). Possibly, the differences in community composition observed by Sosa-Hernández et al. (unpublished data) could also be related to the infectivity potential of the different communities which could have affected the variations in the observed root colonization in the pot experiment. Moreover, other studies in different pedoclimatic conditions have highlighted a greater abundance of fungal structures (particularly concerning AMF) in untilled than in conventional tilled soil (Drijber et al., 2000; Spedding et al. 2004; Brito et al., 2012; Wetzel et al., 2014; Säle et al., 2015). The observed differences have been explained by the destruction of AMF hyphal networks and the dilution of AMF propagules due to tillage (Jansa et al., 2003b). Moreover, it should be considered that the continuous application of no tillage can cause variations in weed population; this can have caused modifications of the composition and dynamics of the mycorrhizal community as suggested by Jansa et al. (2003b; 2006) and Säle et al. (2015). Other possible explanations may lie in the variations induced by tillage on nutrient availability in the different soil layers and on the microbial community (Jansa et al., 2003b).

The differences observed in AMF root colonization are in accordance with the findings on aboveground biomass production and N uptake, namely higher values of both parameters in presence of NT 0-15 compared to CT 0-15 and no difference between the presence of the inocula deriving from the two tillage systems in the soil layer 15-30 cm. After all, several

studies showed that variations in the AMF community structure and abundance can lead to changes in crop productivity and nutrient uptake (Jakobsen et al., 1992; Smith et al., 2000). Moreover, the plant biomass production increased in presence of CT 15-30 compared to CT 0-15, while no difference was observed between the inocula deriving from the two soil layers in NT. This result could be ascribed to differences in the activity of the microbial community involved in the mineralization processes present in the different inocula, which could have determined different conditions of nutrient availability in the substrate. In fact, as reported by several authors in various pedoclimatic conditions (Doran, 1987; Kandeler and Böhm, 1996; Kandeler et al., 1999), the incorporation of the crop residues in CT can affect the microbial community, increasing its activity and the mineralization potential at 20-30 cm (moldboard plowing depth), compared to that observed in the first 10 cm, whereas the opposite was observed in NT.

3. Conclusive remarks

The aim of the present research was to study the role of arbuscular mycorrhizal symbiosis on the growth and N uptake in durum wheat, a pivotal crop of the rainfed agroecosystems in the Mediterranean area, as well as to evaluate how the changes imposed on the microbial community, with particular regard to AMF, by different agricultural practices (soil tillage) and the soil depth affect crop performances. To this end, a set of experiments with different specific goals was planned and performed providing the following main results.

The plant responses appeared to be extremely variable depending on the N availability in the substrate: under extreme deficiencies of the element in soil, the relationship between plant and AM fungi have assumed parasitic connotations (exp. 2.1 and 2.2); in fact, the strong competition for the element between the two symbionts (plant and fungi) drastically penalized the plant's response both in terms of growth and N uptake, favoring the fungal component, certainly more efficient in capturing and using the scarce amount of nutrient available in the substrate. On the other hand, also high soil N availability, in the absence of other limiting factors, can make the symbiotic relationship inefficient, penalizing the host plant that must sustain a carbon cost to feed the fungi without a gain in N uptake, available at a low energy cost in the substrate. The same research has shown that the benefits of the symbiosis take place only when the host plant is still under N limiting conditions and the fungal component has satisfied its own demand, as also highlighted in other experiments (Bååth and Spokes, 1989; Püschel et al., 2016). This result was confirmed by the values of ^{15}N fertilizer recovery which were significantly higher in mycorrhizal plants. From an agro-ecological point of view these findings can have positive implications: in fact, the above-reported results, have shown that favoring the establishment of an adequate mycorrhizal symbiosis with wheat crop can improve the nitrogen use efficiency and the utilization of the fertilizers supplied (N recovery) by the plants (allowing, moreover, a reduction of fertilizers supply) thus reducing the potential losses of the element from the agricultural system. Regarding this last aspect, it should be remembered that, on the one hand, nitrogen gas emissions into the atmosphere (such as ammonia, nitrogen oxide and nitrous oxides), contribute in a global climate change (nitrous oxide is a particularly potent greenhouse gas as it is over 300 times more effective at trapping heat in the atmosphere than carbon dioxide) and, on the other hand, that the nitrate losses from the soil through leaching represent an important source of pollution of the aquifers.

Overall, the results confirm the hypothesis regards the existence of a curvilinear response in mycorrhizal outcome along the soil N availability gradient (Gange and Ayres, 1999; Janos 2007; Corrêa et al., 2015).

Another specific goal was to evaluate the contribution of mycorrhizal fungi in the N uptake from organic sources. The data observed on this topic showed that the effects of mycorrhizal symbiosis vary widely in relation to the organic patch characteristics. In fact, AMF have shown the ability to transfer a substantial amount of N derived from organic matter, thus increasing all the N related parameters (N concentration, N uptake, N recovery), but only in the presence of an organic source with a relatively low C:N ratio. On the contrary, completely opposite results were observed when an organic source with a high C:N ratio was added. This appears to be related, also in this case, to variations in the substrate availability of N for the two organisms, confirming what was described above. In fact, applying an organic matrix with a low C:N ratio has certainly favored the mineralization processes, releasing adequate amounts of N to satisfy the fungal community requirements and, at the same time, guaranteeing a transfer of the element to the host plant as well. The addition of organic matter with a high C:N ratio has certainly led to a temporary reduction in the availability of mineral N in the substrate, consequently exacerbating the competition between plant and fungi for the element, and therefore causing the parasitic phenomena. Again, all this appeared confirmed by the analysis of the values of ^{15}N fertilizer recovery, which diminished with the addition of an organic patch with a high C:N ratio and increased when organic matter with a low C:N ratio was added. The results have also shown that the response of the mycorrhizal symbiosis in the presence of different soil N availability conditions may change in relation to the availability of other elements. In fact, while under conditions of high P availability, the mycorrhizal outcome shifted along the entire spectrum of the ecological relationships (mutualism, commensalism or parasitism) depending on the availability of N, with effects similar to those previously described, under scarce soil P availability, AM fungi have always provided a benefit to the host plant, regardless the N availability in the substrate. Furthermore, the research highlighted how the mycorrhizal symbiosis can exert significant effects on the aboveground plant biomass organography, as well as on the N allocation among the different organs, with an increased incidence of leaves and of the relative N concentration. This certainly represents a benefit for both symbionts, as the increase in the photosynthetates production can on one hand support plant growth and on the other fully satisfy the fungal carbon cost. This represents useful information in understanding the mechanisms that regulate the effects of AM symbiosis on plant growth.

One of the experiments aimed at evaluating the differences in AMF community composition with increasing soil depths (using a molecular approach) and verifying if the observed differences were able to affect plant growth performances in a pot experiment under stress-

watered and well-watered conditions. To this end, soil samples were taken at different depths and used as inoculum (exp. 2.3). The research highlighted a significant shift of AM fungal communities with depth and the existence of subsoil specific AM fungal phylotypes. Moreover, richness and Shannon diversity values for each depth (calculated on the basis of the number of the observed single sequence variants) decreased with increasing soil depth. This result is in line with the findings of Säle et al. (2015) and Oehl et al. (2005) who observed also a progressive reduction of the fungal spore abundance with increasing soil depths. The inoculation with soil samples taken at different depths resulted in variations in root colonization consistent with those detected by molecular analysis. The results have also showed how the AMF-root colonization decreased under water-stressed conditions, compared to the well-watered treatments, when topsoil inocula were used, whereas the contrary was observed with the sub soil inocula. This represents a further evidence that, compared to the topsoil AMF community, the subsoil community presents specific traits, showing an increased ability to activate symbiotic relationship even in a limiting condition environment; this suggests that the shift in AM community composition with depth may also reflect a change in their functional traits. In light of this, it emerges how it is necessary to take into account the subsoil when management practices are planned and also show how the deeper soil layers can represent a potential reservoir of biodiversity still little investigated.

However, although large differences were observed in AMF community composition and root colonization, very little or no effects of the different soil inocula on plant response in terms of growth, N uptake and water use efficiency, both with adequate water availability and in presence of a marked and prolonged water stress were detected.

On the contrary, the differences observed in the microbial and mycorrhizal community due to tillage (found in other research at the same experimental site and not the subject of this thesis), besides influencing root colonization, also determined variations in the aboveground biomass production and N uptake (exp. 2.4). In particular, the inoculum taken from the upper soil layer (0-15 cm) in plots continuously managed for over 25 years with no tillage showed a higher mycorrhizal infectivity compared to the inoculum sampled from the same soil layer but managed with conventional tillage for the same length of time, showing, as a consequence, benefits in terms of growth and N uptake for the wheat plants. No substantial difference was found in the effects of inocula deriving from the deeper layer (15-30 cm) whether tilled or not. This is a further confirmation of how the microbial and mycorrhizal community can influence the plant performance, having observed large and significant differences between inocula deriving from the two tillage systems in the surface layer (NT> CT), and modest in the deeper

layer. The greater diversity and abundance of the microbial and mycorrhizal community existing in the inocula taken from the upper soil layer managed under the no-tillage system (Badagliacca, 2016; Sosa-Hernández et al., unpublished data) can have positively affected, on one hand, the mineralization processes of the organic matter and the N cycle patterns, increasing their availability (effects predominantly at the expense of the bacterial community) and, on the other hand, improved the efficiency in N uptake (effect related to the mycorrhizal community). Therefore, from this research it emerges how the advantages often observed in the Mediterranean environment with the application of conservative soil management strategies as an alternative to the conventional ones depend to some extent also on the effects that the application of these techniques has on the structure and diversity of the microbial and mycorrhizal communities.

Furthermore, it should be highlighted that the two experiments (2.3 and 2.4), both evaluating soil inocula that varied widely in terms of community composition and abundance (taken at different depths along the soil profile and on different management systems: NT and CT), have provided contrasting results. One of the possible causes of this discrepancy lies in the different substrate used in the two pot experiments: fertile, nutrient-rich substrate in the first trial (soil depth inocula) and less fertile substrate in the second (tillage inocula). This also is in line with the results obtained in experiment 2.2.

Overall, the research showed that the effects of mycorrhizal symbiosis on the performance of durum wheat change according to several factors (soil N availability, type of fertilizer applied, presence of other limiting factors) and contributed to increasing the knowledge base about the mechanisms and the principles that underlie such changes. The research has also shown that the structure and abundance of soil microbial and mycorrhizal community is able to significantly influence plant performance; therefore, the choices of crop management techniques have to take into account their impact on the soil community.

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5. Other contributions

During the PhD I have also contributed to the following scientific communications:

Ingraffia, R., Amato, G., Frenda, A. S., & Giambalvo, D. (2019). Impacts of arbuscular mycorrhizal fungi on nutrient uptake, N2 fixation, N transfer, and growth in a wheat/faba bean intercropping system. PloS one, 14(3), e0213672.

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Ingraffia R., Giambalvo D., Ruisi P., Di Miceli G., Frenda A.S., Amato G. 2018. Nitrogen transfer is enhanced by AMF in a faba bean/wheat intercropping. Proc. XLVII Conference of the Italian Society of Agronomy - "L'Agronomia nelle nuove Agriculturae", Marsala (TP) 12-14 settembre 2018, 126-127

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