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AGRONOMIC ROLE OF ARBUSCULAR MYCORRHIZAL FUNGI IN AGRO-ECOSYSTEMS MANAGED WITH ORGANIC WASTES AND LOW QUALITY WATER

Coordinatore: Ch.mo Prof. Sergio Casella
Supervisore: Ch.mo Prof. Maurizio Borin
Co-Supervisore: Ch.mo Prof. Antonio C. Barbera

Dottoranda: Caterina Caruso

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Riassunto

Nei sistemi agricoli convenzionali o intesivi, il diffuso impiego di fertilizzanti minerali e agro-farmaci (fungicidi e insetticidi), a supporto della crescente domanda di prodotti agricoli, ha spesso compromesso la sostenibilità dell'agro-ecosistema, principalmente a causa di una minore fertilità e biodiversità del suolo agrario. In questo contesto, una valida alternativa può essere offerta dall'adozione di sistemi agricoli a basso-input e/o biologici, basati sull'impiego di fertilizzanti e/o ammendanti organici e sulla valorizzazione del ruolo svolto dai microrganismi indigeni del suolo e/o introdotti tramite biofertilizzanti, nel mantenimento della produttività delle colture.

I funghi micorrizici arbuscolari (AMF) sono un importante gruppo di microrganismi della rizosfera che instaurano una relazione simbiotica con circa 80-90% delle piante. Questi funghi svolgono un ruolo importante a supporto della sostenibilità ambientale, incrementando la disponibilità di nutrienti per la pianta ((in particolare fosforo (P)), migliorando la tolleranza e/o resistenza agli stress abiotici e biotici, mantenendo così la produttività delle colture agrarie. I benefici effetti correlati ai funghi micorrizici possono essere inibiti e/o ridotti dalle pratiche agricole convenzionali/intensive quali cospicue fertilizzazioni chimiche, impiego di agro-farmaci (es. fungicidi) e lavorazioni del terreno. Tuttavia questi effetti negativi possono essere mitigati dall'impiego come fertilizzanti/ammendanti di matrici organiche derivanti dai prodotti di scarto dell'agro-industria (acque di vegetazione) e produzione di biogas (digestato).

Il digestato ((frazione liquida (FLD) e solida (FSD)) è un sottoprodotto derivante dagli impianti di digestione anerobica di matrici organiche per la produzione di biogas, caratterizzato da una composizione chimico-fisica variabile in relazione alla "dieta" impiegata per alimentare i digestori. In generale, la componente liquida del digestato è caratterizzata da un elevato rapporto NH_4:TN, e un basso contenuto di sostanza organica, con un rapporto N:P a favore dell'azoto (N), mentre, caratteristiche opposte sono riscontrabili nel digestato solido. La composizione chimico-fisica del digestato determina il suo valore agronomico, potendo essere direttamente sversato (FLD) o distribuito (FSD) al suolo come fertilizzante/ammendante organico. Sebbene numerosi studi riportano un effetto positivo del digestato sulle comunità dei microrganismi del suolo, sulla base delle nostre conoscenze non sono stati effettuati studi sull'effetto della distribuzione di FLD e FSD su piante inoculate con AMF.

Nel bacino del Mediterraneo, dalla molitura delle olive residuano come sottoprodotti di scarto, elevati volumi di acque di vegetazione (AV), prodotte nel breve periodo della

stagione molitoria (2-4 mesi l'anno). Anche le AV, in relazione alla loro composizione chimico-fisica possono essere smaltite sversandole nei terreni agricoli con funzione di fertilizzante/ammendante del suolo, in particolare per apportare sostanza organica e potassio (K). Nonostante ciò, se non ben gestite agronomicamente, le AV hanno effetti fitotossici e antimicrobici legati al loro elevato contenuto in composti fenolici. Fin ad ora l'effetto delle AV sulla simbiosi micorrizica è stata poco studiato. Tuttavia, alcuni autori riportano un effetto negativo delle AV sulla simbiosi micorrizica, con una riduzione della colonizzazione delle radici.

Nelle regioni semi-aride e aride del Mediterraneo soggette a limitata disponibilità idrica, in particolare nella stagione primaverile-estiva, l'uso di acqua di scarsa qualità ai fini irrigui sta diventando una valida alternativa per il mantenimento delle rese e lo sviluppo agricolo. Sebbene l'elevato contenuto di sali disciolti in queste acque può influenzare negativamente le comunità microbiche del suolo e la resa delle colture, è stato ampiamente dimostrato che la simbiosi micorrizica può mitigare tali effetti negativi migliorando, in condizioni di salinità, l'assorbimento di nutrienti ed acqua delle colture, nonostante i meccanismi fisiologici e biochimici coinvolti, non sono ancora chiari.

In considerazione di quanto riportato, lo scopo di questo Dottorato di ricerca è stato quello di studiare e valutare in due differenti contesti bioclimatici (Veneto e Sicilia), l'effetto dell'inoculazione dei funghi micorrizici arbuscolari sulla produzione di colture erbacee da biomassa, fertilizzate con digestato (frazione liquida e/o solida), e da foraggio fertilizzate con acque di vegetazione, o irrigate con acqua di scarsa qualità.

Le attività di ricerca condotte in Veneto sono state finalizzate alla valutazione del ruolo agronomico degli AMF, considerando anche l'impatto ambientale in termini di emissioni di CO_2 e lisciviazioni di azoto dal suolo, in relazione a: i) produzione di colture erbacee da biomassa fertilizzate con FLD; ii) resa in biomassa di triticale conciato con fungicida, confrontando differenti tipi di fertilizzazione (minerale, FLD, FSD ed assenza di fertilizzazione). I risultati ottenuti mostrano quanto segue:

- su colture da biomassa: l'inoculazione con AMF non ha incrementato la resa, eccetto in topinambur gestito con doppio taglio; nel trattamento non inoculato è stata riscontrata una colonizzazione delle radici da parte di funghi micorrizici indigeni presenti nel suolo. L'inoculazione con AMF ha contribuito a ridurre le perdite per lisciviazione di NH_4-N, ma di contro ha incrementato la lisciviazione di NO_3-N e le emissioni di CO_2 dal suolo. Tra le specie studiate, l'arundo è stata quella più produttiva in termini di biomassa, seguita da miscanthus, sorgo, mais, topinambur e lolium, tuttavia con una minore

efficienza d'uso dei nutrienti e maggiori emissioni di CO_2-C cumulate. Il miscanthus e sorgo hanno mostrato altresì il migliore utilizzo di N e P, con moderate perdite per lisciviazione di NO_3-N e basse emissioni di CO_2-C cumulate.

- In triticale: l'effetto dell'inoculazione di AMF è stato inibito dal fungicida presente nei semi conciati. Tuttavia, la maggiore percentuale di colonizzazione delle radici è stata rilevata in presenza del trattamento con FDS, probabilmente a causa del contenuto di sostanza organica, che ha mitigato l'effetto del fungicida, e per la presenza di P in forma poco disponibile per la pianta. La fertilizzazione minerale ha determinato la maggiore produzione di biomassa delle piante di triticale rispetto alla fertilizzazione organica (FDL e FDS), anche se è necessario considerare gli aspetti ambientali (elevate emissioni di $CO_{2(eq)}$) ed economici (costo dei fertilizzanti) connessi a tale trattamento.

Le attività di ricerca condotte in Sicilia sono state focalizzate sull'effetto degli AMF su: i) successione triennale per la produzione di foraggio (consociazione *Triticum durum* Desf. - *Medicago scutellata* L.) e granella di favino (*Vicia faba* L. minor) e cece (*Cicer arietinum* L.), utilizzando differenti volumi di AV come unica fonte di fertilizzazione; ii) due genotipi di miglio per la produzione di foraggio, irrigati con acqua a due livelli di salinità e due regimi di restituzione idrica. I risultati ottenuti mostrano quanto segue:

- nella successione colturale: nel primo anno, l'inoculazione con AMF ha incrementato la resa di frumento, ma non quella di medicago scutellata. I volumi di AV non hanno influenzato la colonizzazione delle radici da parte dei funghi micorrizici inoculati, determinando solo in frumento, un maggiore assorbimento di N e P. Nel secondo anno, gli AMF non hanno influenzato la resa in granella del favino, determinando però un significativo maggiore assorbimento di P nella granella. Inoltre, gli AMF, in assenza di AV, hanno promosso l'azotofissazione attraverso un maggiore numero e peso di noduli radicali della leguminosa. Considerando i due anni sperimentali, le AV hanno incrementato la produzione di biomassa in scutellata e la resa in favino, ma non hanno influenzato la produzione di biomassa in frumento. Le AV hanno ridotto la presenza di infestanti al primo anno e il numero di noduli radicali in favino al secondo anno.
- In miglio: l'effetto dell'inoculazione di AMF sulla produzione di foraggio è stato negativamente influenzato dall'irrigazione con acqua salina, indipendentemente dai due genotipi studiati (Unikum e Kinelskoje); mentre in assenza di stress salino, gli AMF hanno incrementato la produzione di foraggio solo in Unikum. In condizioni di stress idrico (restituzione al 25% dell'ETm), gli AMF hanno promosso la produzione di foraggio, mentre nessun effetto è stato osservato in condizioni ottimali di irrigazione.

Unikum è stato il genotipo maggiormente produttivo nelle condizioni sperimentali siciliane, con una produzione di foraggio più che doppia rispetto al Kinelskoje.

Summary

In the conventional or intensive agriculture systems, to support the ever-increasing demand for agricultural products, the widespread use of mineral fertilizers and agro-chemicals (fungicides and insecticides), has led to the loss of soil fertility and soil biodiversity with a negative impact on the sustainability of the agro-ecosystem. In this context, a viable alternative to conventional or intensive agriculture systems, can be considered the low-input and/or organic ones, based on the use of organic fertilizer and/or amendment, and on the promotion of the role played by native soil microorganism or by bio-fertilizers introduced ones, to maintain the crop productivity.

Arbuscular mycorrhizal fungi (AMF) are an important group of soil microorganism that establish a symbiotic relationship with about 80-90% of plants. AMF play an essential role to support a sustainable environment, improving the nutrient availability (particularly P) to the plant, enhancing tolerance and/or resistance to abiotic and biotic stress, thus maintaining the crop productivity. Their beneficial effects can be inhibited or reduced by conventional or intensive agriculture practices. However, these negative effects can be mitigated by the use of organic wastes as fertilizer/amendment produced by agro-industrial activities (olive mill wastewater) and biogas process (digestate).

Digestate ((liquid (DLF) and solid fraction (DSF)) is a by-product of anaerobic digestion process for biogas production, characterized by a different physical-chemical composition mainly due to degraded feedstock. Generally, DLF is characterized by a high NH_4-H:TN ratio, lower organic matter contents and N:P ratio shifted to nitrogen (N), while opposite characteristics are found in DSF. The physical-chemical characteristics of digestate determine its agronomic value, which can be spreaded (DLF) or applied (DSF) directly in soil as organic fertilizer/amendment. Although several studies reported a digestate positive effect on soil microbial communities, to our knowledge the effect of DLF and DSF distribution on AMF inoculated plants has not yet been investigated.

In Mediterranean basin, from olive oil process, large amounts of olive mill wastewater (OMW) are produced in a short-period of time (2-4 months during year). Also the OMWs, in relation to their physical-chemical composition, can be used in agriculture as fertilizer/amendment in soil, especially providing organic matter and potassium (K).

Nevertheless, OMW has antimicrobial and phytotoxic effects due to the high contents of phenolic compounds. So far, the OMW spreading effect on AMF symbiosis is poorly investigated. However, some authors reported a negative OMW effect on AMF symbiosis with a decrease of root colonization.

In semi-arid and arid Mediterranean regions with limited freshwater resources, especially during spring-summer period, the use of poor quality water for irrigation is becoming a viable alternative to maintain crop yield and agriculture sustainability. Although this wastewaters contain dissolved salts which can negatively affect the soil microbial communities and crop production, it is widely demonstrated that AMF symbiosis improves nutrient and water uptake under saline conditions, even if the physiological and biochemical mechanisms involved are still unclear.

Considering the above mentioned, the aim of this Ph. D. research was to study and evaluation, in two different Italian bioclimatic contexts (Sicily and Veneto), AMF inoculation effects on biomass production on energy crops fertilized with digestate (liquid and solid fraction) and forage crops fertilized with olive mill wastewater or irrigated with poor quality water.

The research activities conducted in Veneto has been focused on the agronomic role of arbuscular mycorrhizal fungi inoculation, considering also the environmental impacts in terms of soil CO_2 emissions and nitrogen leaching, on: i) energy crop biomass production using DLF as fertilizer; ii) triticale biomass production under different fertilization rates (mineral fertilizer, DLF, DSF and not fertilizer) using seed-coated fungicides. The most relevant findings are the following:

- on energy crops: AMF inoculation did not increase the biomass production, except in Jerusalem artichoke managed with double biomass cutting. In the un-inoculated plots, a root colonization by indigenous mycorrhizal community was found. AMF inoculation contributed to NH_4-N leaching reduction but increased NO_3-N leaching and soil CO_2 emission. Among the studied species, giant reed was the most productive energy crop in terms of biomass production, followed by miscanthus, sorghum, maize, Jerusalem artichoke, and lolium; however it showed a lower nitrogen use efficiency and higher cumulative soil CO_2-C emissions. Miscanthus and sorghum showed the best N and P use efficiency, with a moderate NO_3-N leaching and lower cumulative soil CO_2-C emissions.
- In triticale: AMF root colonization was inhibited by the presence of seed-coated fungicide. Nevertheless, in DSF treatment, the highest AMF colonization observed was

probably due to the low available P form and the high organic matter content which probably contributed to mitigate fungicide negative effect. Mineral fertilization determined the highest triticale dry biomass production compared to organic fertilizer (DLF and DSF) even if the environmental (e.g. higher CO_2(eq) emission) and economical (e.g. fertilizer costs) aspects should be considered.

The researches conducted in Sicily have been focused on AMF inoculation effects on: i) a three years legume-based succession producing forage (*Triticum durum* Desf.-*Medicago scutellata* L. intercropping), broad bean (*Vicia faba* L. minor) and chickpea (*Cicer arietinum* L.) grain, using different OMW volumes as the only fertilization source; ii) a yearly evaluation of millet genotypes for forage production using irrigation water at two salinity levels and two water restitution regimes. The most relevant findings are the following:

- on crop succession: in the first year, AMF inoculation increased the durum wheat biomass yield, but not affected the medicago scutellata biomass production. AMF root colonization was not influenced by OMW volumes, with a significant higher N and P uptake observed in inoculated durum wheat; while, no statistical effect of AMF was found on N and P uptake in scutellata. In the second year, AMF inoculation did not affect broad bean grain yield and determined a higher P uptake in broad bean grain. Moreover, AMF inoculation promoted root nodule number and weight in broad bean in absence of OMW volumes. Considering the two years, OMW spreading promoted the fabaceae productions with a higher biomass in scutellata and grain yield in broad bean, whereas it did not show any effect on durum wheat biomass production. OMW spreading reduced the weed presence in the first year and broad bean nodulation in the second one.
- On millet: salt stress conditions negatively influenced AMF inoculum effects on forage production, without any difference between the two studied genotypes (Unikum and Kinelskoje). In absence of salinity treatment, AMF inoculation increased the forage production only in Unikum. Under water stress (25% ETm restitution) AMF inoculation promoted the forage production, whereas no significant AMF effect was observed in well-watered condition. Unikum proved to be the best millet genotype in experimental field conditions with more than double forage production compared to Kinelskoje.

Chapter I

General background and the aim of the Ph. D. thesis

Current conventional/intensive agriculture practices, mainly based on the widespread use of mineral fertilizers and agro-chemicals (such as fungicides and insecticides), play an important role to sustain and increase food and fiber production, in relation to the ever-increasing world population demands (Bhardwaj et al., 2014). However, in the long-time these anthropogenic activities if not well managed, lead to low soil fertility and to the loss of agro-ecosystem biodiversity (Vimal et al., 2017). A sustainable crop production has progressively led to a shift from conventional/intensive agricultural management systems to low-input ones, mainly based on the use of organic fertilizer/amendment and minimum tillage practices or no-tillage. In this context, the soil microorganisms' activities, particularly bacteria and fungi, play an important role in low-input cropping systems without increasing environmental burdens, improving nutrient availability, tolerance and/or resistance to abiotic (mainly salinity and drought) and biotic (soil-borne pathogens) stress, thus maintaining the crop yield with substantial economic and environmental benefits (Johansson et al., 2004; Vimal et al., 2017).

Arbuscular mycorrhizal fungi (AMF) are an important group of soil microorganisms that establish a symbiotic relationship with the major terrestrial vascular plant species (about 80-90%) (Brundrett, 2002; Smith and Read, 2010; Shah, 2014; Berruti et al., 2014; Berruti et al., 2016), including several agricultural crops (Berruti et al., 2016), except for some families such as Brassicaceae, Chenopodiaceae, Cyperaceae (Shah, 2014). The Greek term mycorrhiza (literally "myco" means fungus and "rhiza" means root) was introduced in the 1885 by Albert Bernard Frank (Frank, 1885). This symbiosis is considered very ancient (over 460 million years ago - Ordovician period) (Redecker et al., 2000; Bonfante and Genre, 2008); probably for this reason, AMF have lost their ability to live and complete their life cycle in absence of host plants (Requena et al., 2007). Arbuscolar mycorrhizal fungi belong to the phylum *Glomeromycota* (Schüßler et al., 2001), with less than 250 AMF species described up to date (Öpik et al., 2013).

The establishment of AMF symbiosis occurs through different phases and it can be briefly described as follows: 1) under favorable soil water and temperature conditions, AMF spores germinate spontaneously using their triacylglyceride and glycogen reserves to support the growth of hyphal germ tube (Bago et al., 2000). This behavior occurs independently by plant-derived signals such as root exudates (strigolactone) and volatile compounds (i.e. CO_2) (Harrison et al., 2005); 2) once in contact with the roots of host plant, the AMF forms an hyphopodium through which penetrates into root cells (Bonfante and Genre, 2008); 3) subsequently, the AMF hyphae colonized the root cortex and form

highly branched structures termed arbuscules, which are considered the exchange site of mineral nutrients and organic carbon between host plant and fungi (Garg and Chandel, 2010; Balestrini et al., 2015). In particular, arbuscules are short-living structures and begin to senesce after 4–10 days of activity (Strack et al., 2003). When it collapses, the remnants encapsulated inside the cell wall and is degraded (Sawers et al., 2008). After this process, the plant cell returns to the pre-arbuscular state and can be re-colonized at a later time (Sawers et al., 2008); 4) the life cycle of AMF is completed after formation of asexual chlamydospores on the external mycelium (Garg and Chandel, 2010).

The obligate biotrophs nature of AMF, contributes to improve crop production, plant nutrition (Bücking and Kafle, 2015; Tarraf et al., 2015; Langeroodi et al., 2017), especially as concerns phosphorus (Berruti et al., 2016; Williams et al., 2017), and nutrient use efficiency (Bender and Heijden, 2015), thus representing a key component of organic and/or sustainable soil-plant system (Gianinazzi et al., 2010; Bender et al., 2016). Nevertheless, the symbiosis provides several benefits to host plant, it has a cost in term of organic carbon. In fact, it is estimated that over 20% of the C fixed is delivered from host plant to fungi (Bago et al., 2000).

It was widely reported that intensive agricultural systems characterized by high fertilization input (particularly N and P) negatively affect AMF symbiosis, leading to a significant decrease in root colonization, mycorrhizal fungal richness and diversity (Mäder et al., 2000; Egerton-Warburton and Allen, 2000; Verbruggen et al., 2012; Sheng et al., 2013; Xiang et al., 2014; Albizua et al., 2015). Under enriched N and P soil conditions, while the carbohydrates costs remain the same for AMF symbiosis, the relative nutrient advantages for crops are reduced, and the colonized plants performance can be lower when compared to un-colonized ones (Janos, 2007).

Other advantages are provided by AMF symbiosis to host plant, such as improved tolerance to salinity and drought (Augé, 2001; Augé, 2004; Porcel et al., 2011; Augé et al., 2015). Although the physiological and biochemical mechanisms involved in the salt tolerance of AMF plants are still unclear, the improved nutrient uptake could be one of reasons (Al-Karaki, 2000). Furthermore, AMF are capable to improve crop water uptake through the hyphal network that not only increase the root absorption surface area but also leads to go beyond root's depletion zone through the mycelium extra-radical (Allen, 2007; Finaly, 2008), thus improving stomatal control and reducing transpiration rates (Allen, 2007; Aroca et al., 2007; Augé et al., 2015). It was also found that AMF symbiosis can increase the host plant root hydraulic capacity, stimulating the expression aquaporines which

facilitate the passive water flow (Bàrzana et al., 2015); the better crop water use efficiency in AMF plants promotes an enhance in drought tolerance (Augé, 2001; Maralunda et al., 2006; Aroca et al., 2008; Bàrzana et al., 2015).

AMF also play an important role on soil aggregate formation and stability (Rillig and Mummey, 2006; Leifheit et al., 2014; Leifheit et al., 2015; Rillig et al., 2015) through the glycoprotein production (glomalin) (Balota et al., 2016), contributing to protect soil C stocks (Rillig, 2004) and soil aggregates against erosion factors (García-Orenes et al., 2012).

Currently, little is known on the effect exercised by soil microorganisms on plant health. Understanding the microbial consortia and mechanisms involved in disease suppression may help to better manage plants while reducing fertilizer and pesticide inputs. There are two types of disease suppression in soils: i) a general, worldwide type effective in every soil, based on competitive activities of the overall micro- and macroflora; ii) specific suppression attributed to the enrichment of specific subsets of soil microorganisms (Raaijmakers and Mazzola, 2016). The figure 1 show plants exposed to the fungal pathogen in two different soil context: i) in a conducive soil with a low abundance of antagonistic microbial consortia, the fungal pathogen causes disease; ii) whereas, in a suppressive soil with a high abundance of antagonistic microbial consortia, most seedlings remain healthy (Raaijmakers and Mazzola, 2016).

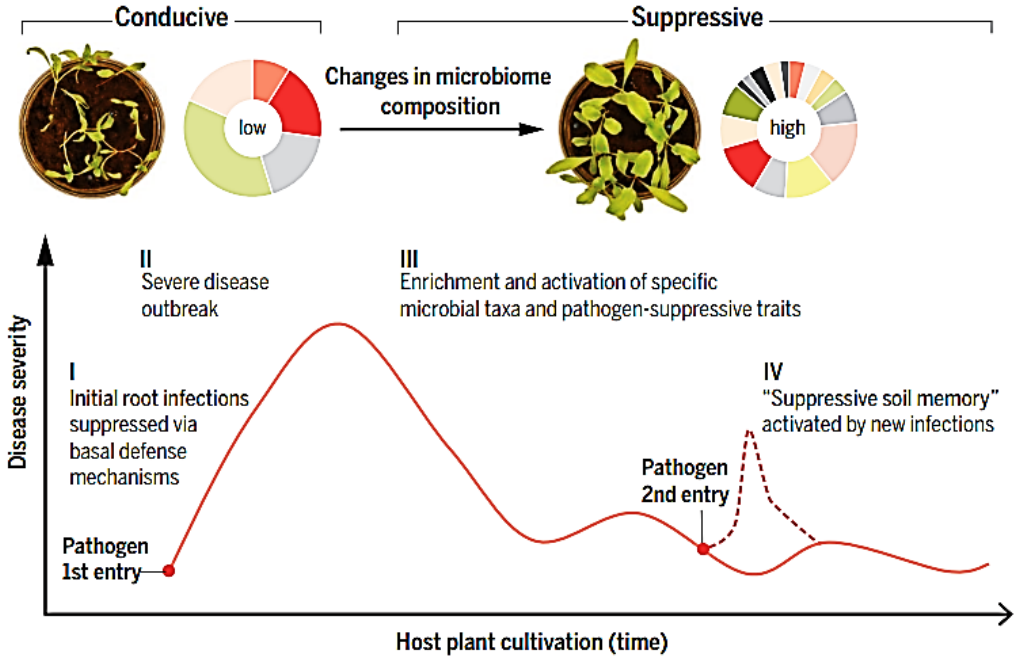


Figure 1 – Lines of difense: plants exposed to a fungal pathogen in disease-conducive and -suppressive soils (Raaijmakers and Mazzola, 2016).

Moreover, enanching the soil microbial consortia by AMF strains, through the beneficial plant-AMF symbiosis, it was reported an improves resistance to disease by soil-borne pathogens (i.e. nematode and fungal pathogens) (Linderman, 1992; Harrier and Watson, 2004; Aliasgarzad et al., 2006; St-Arnaud and Vujanovic, 2007; Pozo and Azcón-Aguilar, 2007, Ipsilantis et al., 2009), contributing to reduce the use of the fungicides and nematocide (Bender et al., 2016).

Moreover, it is also well-known that the interactions between host plant and soil microorganisms influence soil greenhouse gas (GHG) emissions (CO_2, N_2O and CH_4) (Jackson et al., 2008; Philippot et al., 2008; Frank and Groffman, 2009). Cavagnaro et al. (2012) reported an increase the soil CO_2 emissions in AMF colonized plants, probably due to direct fungal respiration or indirect influence on heterotrophic microorganisms (Johnson et al., 2002; Langley and Hungate, 2003; Zhu and Miller, 2003; Cavagnaro et al., 2008). Furthermore, some authors demonstrated that AMF symbiosis have a positive effect on N_2O emissions reduction (Bender et al., 2014) and N_2O emissions regulation at high soil moisture (Lazcano et al., 2014), thus suggesting that they may contribute to climate change mitigation (Berruti et al., 2016).

Crop management practices such as intensive soil tillage and mineral fertilization, the use of fungicides and insecticides, and low crop diversity due to the monoculture, negatively affect soil microorganisms' biodiversity, particularly AMF association (Verbruggen et al., 2010). Low-input agriculture system based on organic fertilizer/amendment such as digestate and olive mill wastewater, can be considerate a viable alternative to mitigate the negative effect of conventional or intensive crop management on AMF association.

The worldwide energy demand is growing rapidly (about 50% by 2040) mainly due to the increasing emerging nations energy consumption. Actually, over 85% of world energy demands is based on fossil-fuels, which has promoted to more than 90% of CO_2 emissions from their combustion related to greenhouse gas (GHG) emissions (International Energy Agency (IEA), 2016). In this context, the use of renewable energy sources can be considerate a viable alternative to fossil-based fuels; among them, the lignocellulosic biomass (e.g. agricultural residues and energy crops, etc.) is one of the most interesting source currently being used to produce energy through the anaerobic digestion processes (Sawatdeenarunat et al., 2016).

In the recent years, thanks to the incentive European environmental policies, the anaerobic digestion (AD) processes for biogas production is expanding in several countries (Perazzolo et al., 2016), reaching 17.376 AD plants in Europe, mainly localized in

Germany (10.846 AD plants) and in Italy (1.555 AD plants) (European Association Biogas, 2015).

Anaerobic digestion is a natural processes, where in anaerobic conditions about 20-95% of the feedstock organic matter is degraded by micro-organisms, to produce two by-products: biogas effluents (~ 70% CH_4 and~ 30% CO_2) and digestate (liquid and solid fraction) (Lukehurst et al., 2010; Möller and Müller, 2012; Insam et al., 2015; Romero-Güiza et al., 2016). The physico-chemical digestate characteristics varies strongly in relation to: i) feedstocks; ii) digestate design and operation conditions; and iii) digestate post-treatment (Holm-Nielsen et al., 2009).

Digestate is considerate an important source of residual organic-C and essential plant nutrients for the agriculture system, in fact it could be used as organic fertilizer or soil amendment in agro-ecosystems (Alburquerque et al., 2012b; Bachmann et al., 2014; Nicoletto et al.; 2014; Nkoa et al., 2014), reducing or replacing completely mineral fertilizer application with less environmental and economic costs (Walsh et al., 2012; Maucieri et al., 2017). The digestate agronomic value is manly related to phisycal-chemical composition (Kuusik et al., 2017), characterized by higher NH_4-N (about 60-80%):TN ratio and pH value and lower organic matter contents, total and organic carbon contents, biological oxygen demand C:N ratio and viscosities than undigested materials (Möller and Muller, 2012; Kuusik et al., 2017). Furthermore, it contains also a P source, as orthophosphates, rapidaly available for uptake by crops and a slow-release form (struvite crystals), due to precipitation of magnesium, ammonium and phosphate ions during AD, with a N-P-K-Mg rating of 5.7-28.9-0.0-9.9 (Tao et al., 2016).

Digestate solid fraction (DSF) is easily transportable (Al Seadi and Lukehurst, 2012), hygienically safe (Saveyn and Eder, 2014) and can be applied directly as organic fertilizer in soil (Al Seadi and Lukehurst, 2012), leading to enhanced plant–available P (Nest et al., 2015) with the support of the degradative activity of soil microorganisms (Richardson and Simpson, 2011). Although the digestate liquid fraction (DLF) generates higher interest than the solid one, because it contains more nitrogen and potassium (Romero-Güiza et al., 2016), we have to consider that the DLF soil distribution methods usually adopted (mainly splah-plate distribution), lead to a loss of N, due to its volatilization. In fact, the direct contact of DLF with high pH with the atmosphere determines the NH_4-N conversion into ammonium, which is released into air (Maurer and Müller, 2012; Nkoa, 2014).

Several potential benefits derive by digestate soil application in agro-ecosystem since it improves: 1) crop yield (Abudaker et al., 2012; Vaneeckhaute et al., 2013; Nicoletto et al., 2014); 2) crop nutrient uptake (Bachmann et al., 2014; Koszel and Lorencowicz, 2015); 3) soil fertility through higher nutrients availability (mainly N, P and K) (Arthurson, 2009; Möller and Müller, 2012); 4) soil aggregation due to the organic matter applied, whose content can vary between 30 and 80% in relation to the used digestate (Vaneeckhaute et al., 2017) – thus indirectely enhancing soil structure and improving water infiltration rates, necessary for a good crop production (Arthurson, 2009; Wager-Baumann, 2011; Nkoa et al., 2014; García-Sánchez et al., 2015); and 5) soil microbial activity and its microbial diversity (Petersen et al., 2003; Oldare et al., 2008; García-Sánchez et al., 2015).

It must be also considered the potential risk of inappropriate application of digestate in agriculture soil related to its salinity (i.e. Na^{+} and Al^{3+}) content and stability degree (Romero-Güiza et al., 2016). High volumes or continued spreading of digestate with high salt contents, can lead to an excessive salt and heavy metals accumulation in soil, thus inhibiting the plant growth (Alburquerque et al., 2012a; Restrepo et al., 2013; Mata-Alvarez et al., 2014); while, the use of the unstable digestate can exert a negative effect on organic matter mineralization and nutrient turn-over in plant-soil system (Alburquerque et al., 2012b; Abdullahi et al., 2008; Restrepo et al., 2013).

It is well-known that soil microorganisms represent a fundamental living and metabolically active component of soil quality (Arthurson, 2009; Watts et al., 2010), contributing not only to the organic matter decomposition and nutrients mineralization, but also to new organic matter production (Bachmann et al., 2014). Although several authors have investigated the digestate spreading e effect on soil microbial populations, reporting a positive or nil effect on soil microbial biomass, enzyme activities and changes in microbial community composition (Abubaker et al., 2012; Chen et al., 2012; Walsh et al., 2012; Johansen et al., 2013; Bachmann et al., 2014), to our knowledge, the effect of digestate liquid fraction (DLF) and solid fraction (DSF) application on mycorrhized plants has not yet been investigated.

Olive tree is the most important cultivated in Mediterranean basin, and the Spain is the main producing country (2.5 million ha), followed by Italy (1.13 million ha), Greece (0.85 million ha) and Portugal (0.35 million ha) (FAOSTAT, 2013).

The olive oil production in these countries represents almost 98% of the total world's production (Magdich et al., 2016). Consequently, about 30 106 m^3 of olive mill

wastewater (OMW) are annually produced during a short period of time, since the volume of OMW per 1000 Kg of olives ranges from 0.5 to 1.5 m^3 according to the oil extraction method (Barbera et al., 2013). The high amounts of OMW produced, must be properly managed to avoid the negative environmental impacts associated with their disposal, due to the amount of phenolic compounds (Barbera et al., 2013; Di Bene et al., 2013), that exert phytotoxic and antimicrobial effects (Obied et al., 2005; Saadi et al., 2007). These risks led olive oil-producing countries to enact some directives to regulate the direct OMW spreading. In particular, in Europe Union (UE) each member countries established different limits for OMW spreading (according to d.lgs 152/2006 in Italy the legal limit is 80 m3 ha^{-1}) (Barbera et al., 2013).

OMW chemical composition is highly variable due to the regional olive varieties characteristics, the harvesting period and oil extraction method (traditional or continuous systems) (Belaqziz et al., 2016; Magdich et al., 2016). The OMW are constituted mainly by: 1) water (about 83-94%); 2) organic matter compounds (about 14-16%); 3) mineral nutrients (about 0.4-2.5%) (Ammar et al., 2005), especially high phosphorus, potassium, calcium, magnesium and iron levels (Sierra et al., 2007), but low nitrogen content (Barbera et al., 2013); 4) sub-acid pH manly due to the presence of organic acid (Poiana and Mincione, 2002); 5) high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Galanakis, 2011; Rahmanian et al., 2014); high EC value (from 5.5 up to 12.0 dS m^{-1}) (Roig et al., 2006); and 6) inhibiting substances (i.e. lipidic and phenol compounds), particularly polyphenols contents at the levels ranging from 0.79 (El Hajjouji et al., 2008) to 13.5 g L^{-1} (Achak et al., 2009), that may have negative environmental impact when applied to soil (Barbera et al., 2013).

It is recently reported that the direct OMW application exerts a positive effect on soil structure proprieties, increasing the micro- and macro porosity and aggregate stability, mainly due to the organic matter applied with OMW spreading (Barbera et al., 2013) that represents approximately 65% of their dry weight (Paredes et al., 1999; Poiana et al., 2004), and improving the nutrient contents and soil fertility (Gargouri et al., 2014). For these reason and the OMW lower cost, it can be used as a soil amendment and/or organic fertilizer in agriculture systems (Barbera et al., 2013), particularly in semi-arid climatic conditions, such as Sicily region, affected by soil organic matter depletion and water deficiency (Garcìa et al., 1994; Alianello et al., 2001).

Although several authors showed a negative OMW influence during the seed germination phase (El Hadrami et al., 2004; Isidori et al., 2005; Quaratino et al., 2007; Pierantozzi et

al., 2011) and plant seedling emergence (Hanifi and El Hadrami, 2008) as result of the high OMW polyphenol content, while Barbera et al. (2013) reported beneficial effects on crop yield.

The OMW effect on soil microbial components is related to the volumes applied, that can on the one hand, influence temporary soil C enrichment, which is easily degradable and thus stimulates the microflora growth, and on the other hand inhibits certain microorganisms such as soil-borne fungal pathogens (Barbera et al., 2013). So far the OMW spreading effect on AMF symbiosis are poorly investigated (Mechri et al., 2008). However, some authors (Mechri et al., 2008; Piotrowski et al., 2008; Ipilantis et al., 2009; Di Bene et al., 2013) reported that OMWs exerted a negative effect on AMF symbiosis, consequently decreasing AMF root colonization, mainly due to their high phosphorus content, C:N ratio, pH, EC values and phytotoxic substances.

The sustainability of water resources is a critical problem for satisfying the increase water demands of different competitive sectors, mainly agriculture which globally uses over 70% of fresh water (Singh, 2015). Although in the recent past, the irrigation with poor quality water has been considered a limiting environmental factor for sustainable development (Cordoba et al., 2010; Gaudino et al., 2014), today the use of this water for irrigation is becoming a viable alternative for crop production and agriculture development, especially in semi-arid and arid Mediterranean regions subjected to limited fresh water resources (Dorta-Santos et al., 2016). However, we have to consider that poor quality water contains dissolved salts (mainly chloride and sulphate) (Belaid et al., 2009) which can negatively affect soil microbial community (Zalidis et al., 2002), organic matter mineralization (Bouksila et al., 2013), crop productivity and economic returns (Hu and Schmidhalter, 2004; Mahajan and Tuteja, 2005; Shrivastava and Kumar, 2015). Salinity is one of the major causes of soil degradation in the world (Hasegawa et al., 2000; Zhu, 2003 Giri et al., 2003; Al-Karaki, 2006; Evelin et al., 2009), that affects approximately 20% of irrigated land (Qadir et al., 2014). Several studies reported that poor quality water irrigation determined a physical and chemical soil degradation in the short and medium-term distribution (2–20 years) in arid regions (Lado and Ben-Hur, 2009). Salt negative effects on agricultural soil are also linked to the improper use of fertilizers or seawater intrusion into groundwater aquifers (Zalidis et al., 2002; Shrivastava and Kumar, 2015). Under low rainfall or poor drainage conditions, the salt accumulates over time on soil layer due to reduced leaching salts losses, thus resulting in soil salinity (Blaylock, 1994). It is well-known that all plant physiological stages are

negatively affected by salinity (Shrivastava and Kumar, 2015) due to three distinct physiological stresses: i) toxic effects of specific ions such as sodium and chloride, which disrupt the structure of enzymes and other macromolecules, damage cell organelles, reduce photosynthesis and respiration, inhibit protein synthesis, and induce ions deficiency (Ruiz-Lozano et al., 2012); ii) osmotic effect due to physiological drought because plants must maintain lower internal osmotic potentials to prevent water from moving from the roots into the soil (Aggarwal et al., 2012); iii) nutrient imbalance caused by depression in uptake and/or transport (Marschner, 1995; Adiku et al., 2001). Furthermore, under salinity conditions plant phosphorus uptake is significantly reduced because phosphate ions precipitate with Ca, Mg and Zn then being unavailable to plants (Wang et al., 2008; Park et al., 2009; Evelin et al., 2009; Bano and Fatima, 2009).

In this context, the use of salt-tolerant crops and soil microorganism such as AMF, could represent a sustainable solution to maintain crop productivity in semi-arid areas affects by salinity conditions.

It is demostrated that AMF symbiosis increase plant tolerance to abiotic stress such as drought (Ruiz-Lozano, 2003; Miransari, 2010) and salinity (Evelin et al. 2009; Miransari, 2010; Porcel et al., 2012). Possible AMF-mediated adaptation mechanisms inducing plant tolerance to saline conditions (Wu et al., 2010) are as the followings: 1) nutrient uptake improvement, especially phosphorus (P) (Al-Karaki 2000; Al-Karaki et al., 2001; Asghari et al., 2005); 2) accumulation of soluble sugars into the mycorrhizal roots (Feng et al. 2002); 3) K^+/Na^+ ratio adjustment (Giri et al., 2007; Asghari, 2012); 4) changes in antioxidant enzymatic activates (such as CAT, APX, POD and SOD) (He et al., 2007); 5) increase in the photosynthetic efficiency (Elhindi et al., 2016; Shamshiri and Fattahi, 2016).

Although AMF are widely found in saline soils (Yamoto et al., 2008; Wilde et al., 2009), some of their features such as spore germination, fungal hyphae growth (Porcel et al., 2012), arbuscules formation (Tian et al., 2004; Sheng et al., 2008), and root colonization levels (Juniper and Abbott, 2006) may be negatively affected by high salinity. Nevertheless, under saline conditions, several studies reported a positive effect on plant growth and yield production in presence of AMF in both glycophyte and halophyte plants (reviewed by Evelin et al., 2009 and Ruiz-Lozano et al., 2012). Furthermore, it is known that native AMF can perform better in the soils they were isolated from such as agricultural (Calvente et al., 2004) or semi-arid degraded soils (Caravaca et al., 2003). Estrada et al., (2013) reported that although maize shoot biomass production was

negatively affected by increasing salt concentrations in the soil solution, the presence of native AMF inoculums isolated from a dry and saline soil, improved maize production as compared to un-inoculated plots. A meta-analysis carried out on 60 papers to evaluate AMF response on C3 and C4 plant growth and nutrient uptake under saline conditions (Chandrasekaran et al., 2016), reported a general positive increase on shoot (67.1%), roots (57.8%) and total plant biomass (71.1%) and N (93.2%), P (86.8%) and K (42.7%) uptake in presence of AMF inoculation.

Despite these reported positive mycorrhizal behaviors, the positive influence exerted by AMF inoculum on crop production under saline conditions is not yet fully understood and demonstrated in the Mediterranean areas where native strains of michorrhizas are not yet isolated . In order to maximize the crop performance in semi-arid conditions, the isolation of AMF strains from Mediterranean saline soil and their study, could contribute to explain better the response and ecophysiology of AMF in response to use of poor quality water.

In view of the above mentioned, the aim of this Ph. D. research, in two different pedo-climatic Mediterranean region (Veneto and Sicily), is to assess AMF inoculation effect on biomass production in relation to organic fertilizer (digestate and olive mil wastewater) and irrigation with poor quality water, as follows:

Veneto region:

i) six perennial and annual energy crop for biomass production fertilizer with digestate liquid fraction; ii) triticale biomass production under different fertilization rates (mineral fertilizer, digestate liquid fraction, digestate solid fraction and not fertilizer), using seed-coated fungicides; iii) environmental impacts in terms of soil CO_2 emissions and nitrogen leaching.

Sicily region:

i) three years legume-based succession producing forage (durum wheat-scutellata intercropping), and broad bean and chickpea grain, fertilized with different OMW volumes; ii) a yearly evaluation of millet genotypes for forage production using poor quality water for irrigation at two salinity levels and two water restitution regimes.

Chapter II

Effects of mycorrhizal inoculation and digestate liquid fertilization on biomass production of six energy crops under rainfed condition

Abstract

The ecosystem services provided by arbuscular mycorrhizal fungi (AMF) and the use of dedicated energy crops and organic waste (as digestate), are a viable alternative to reduce the environmental negative impacts of intensive agriculture, opposing the climate change ongoing. The aim of this work was to evaluate AMF inoculation effects on both biomass production and soil CO_2 emissions of six energy crops fertilized with digestate liquid fraction (DLF). The trial was carried out in an experimental farm of the north-east Italy (Legnaro), and it lasted three years. The results showed, that in all studied crops, AMF inoculation did not affect biomass production. The AMF root colonization was variable during the experimental years as a result probably of the high N input and weather conditions. AMF treatment significantly reduced NH_4-N leaching (-32.8%), but conversely it increased NO_3-N leaching (+70.0%), soil CO_2 emission (+23.1%) and cumulative CO_2-C emissions (+17.0%) released into the atmosphere. AMF exerted a plant-specific effect on soil CO_2-C emissions, determining the highest emissions increment (+30.4%) in giant reed and Jerusalem artichoke, while the lowest one (+7.75%) in sorghum. Giant reed showed the highest biomass production (42.7 ± 3.73 Mg ha^{-1}), followed by miscanthus (29.1 ± 2.58 Mg ha^{-1}), sorghum (26.2 ± 1.36 Mg ha^{-1}), maize (22.6 ± 1.47 Mg ha^{-1}), Jerusalem artichoke (21.6 ± 1.46 Mg ha^{-1} in the average of the two cuts) and lolium (6.29 ± 0.39 Mg ha^{-1}). Sorghum showed the highest N (5.03% ± 0.11) and P (0.67% ± 0.03) concentrations in the biomass among the studied crops, and at the same time a lower N uptake compared to giant reed and a lower P uptake compared to maize. Sorghum (208.9 ± 14.9 g N_{ue} g^{-1} and 1592.6 ± 118.5 g P_{ue} g^{-1}) and miscanthus (152.7 ± 11.6 g N_{ue} g^{-1} and 1628.6 ± 130.5 g P_{ue} g^{-1}) showed the best nitrogen and phosphorus use efficiency, with a moderate NO_3-N leaching. One hour after digestate spreading, soil CO_2 emission levels significantly raised but just after 24 hours, they returned to pre-digestate spreading condition. Plant-specific effects on soil CO_2 emissions were observed, with the highest emissions in giant reed and Jerusalem artichoke, whereas the lowest in sorghum. The CH_4 yields derived by biomass gasification must be considered with caution because of a single year experiment for annual and perennial crops was considered. Concluding, the AMF inoculation exercised only a marginal effect, whereas the DLF effect on biomass production can be compared to mineral fertilization.

Introduction

The global energy demand is growing rapidly and it is presently satisfied by fossil fuels (Weiland, 2010). Due to climate change, limited fossil energy resources and the increase of the atmospheric CO_2, there is a global consensus that future consumption of energy should be directed to renewable energy sources (Johansen et al., 2013). In this context, the use of biomass to produce energy is a viable alternative to energy from fossil sources and it can be used at global scale in the world (McKendry, 2002). Anaerobic digestion (AD) of the biomass allows production of biogas as a renewable energy source from one hand and a high quantity of digestate as system waste from the other hand. This latter can exert negative environmental impact if it is not adequately treated or re-used in a proper way (Galvez et al., 2012). Digestate is characterized by higher NH_4-N:TN ratio and pH, lower organic matter contents, total and organic carbon contents, biological oxygen demand, C:N ratio and viscosities than undigested materials (Möller and Muller, 2012). Due to its high nutrients content, digestate (Tambone et al., 2010) is used as organic amendment or fertilizer in different agro-ecosystems (Bachmann et al., 2014; Nicoletto et al.; 2014; Nkoa, 2014). The application of organic amendments in agro-ecosystems has been widely recommended to improve the soil physical fertility and the soil carbon stock (Thangarajan et al., 2013; Ryals et al., 2014). However, at the same time, amendments influence soil greenhouse gas emission (Thangarajan et al., 2013) and so particular attention should be paid for the evaluation of environmental impact from all points of view. Several authors reported positive fertilizer effects of digestate on crops (Montemurro et al., 2010; Haraldsen et al., 2011; Alburquerque et al., 2012; Garfi et al., 2011), replacing inorganic fertilizer application with less environmental cost (Walsh et al., 2012; Maucieri et al., 2017).

Symbiotic mycorrhizal fungi, such as arbuscular mycorrhizal fungi (AMF), are a significant component of the soil microbial populations and they influence soil fertility and crops yield (Garg and Chandel, 2010; Cozzolino et al., 2013) and ecosystem sustainability (Gianinazzi et al., 2010). AMF play an important ecosystem service improving plants nutrition (Smith and Smith, 2011) and water uptake (Augé et al., 2015), nutrient mobilization from organic substrates (Finlay, 2008), soil C content (Rillig et al., 2001; Zhu and Miller, 2003; Orwin et al., 2011; Cheng et al., 2012; Averill et al., 2014), plants' resistance to abiotic stresses (Ruiz-Lozano et al., 2012; Aroca et al., 2013; Augé et al., 2014), soil aggregates stabilization (Rillig, 2004) and soil erosion reduction (Mardhiah et al., 2016). AMF natural functions have been marginalized in intensive agriculture due to

negative effect of tillage and high inputs of inorganic fertilizers and herbicides (Lumini et al., 2011; Zocco et al., 2011; Borriello et al., 2012; Berruti et al., 2014). AMF influence soil CO_2 emission directly through their respiration and indirectly influencing heterotrophic microorganisms. These last can be: 1) negatively influenced considering that AMF increases plant nitrogen (Miransari, 2011) and water uptake (Augé, 2004; Augé et al., 2015); or 2) positively influenced by AMF carbon release in the soil. Lazcano et al. (2014) reported that AMF colonization of tomato roots did not have a significant effect on total CO_2 emissions but the same amount of CO_2 emitted for root biomass unit was higher in AMF colonized plants. Several authors have studied the digestate effect on soil microbial populations (Abubaker et al., 2012; Chen et al., 2012; Walsh et al., 2012; Johansen et al., 2013; Bachmann et al., 2014), but to our knowledge the effect of digestate liquid fraction (DLF) application on plants mycorrhization has not yet been investigate.

The understanding of the interactions between AMF and digestate spreading on both plant production and soil CO_2 emissions are crucial for the development of sustainable agriculture.

With this in mind the aims of this work were to evaluate in open field conditions the AMF inoculation effects on both biomass production and soil CO_2 emissions of six perennial and annual energy crops fertilized with DLF.

Materials and Methods

Experimental description

The experiment has been carried out from January 2014 to March 2017 at the "L. Toniolo" experimental farm of the University of Padova at Legnaro (45° 21' N; 11° 58' E; 6 m a.s.l.), north-east Italy. The adopted experimental design was a split-plot with AMF inoculation as the main-plot (AMF-Y = inoculated and AMF-N = un-inoculated) and six herbaceous perennial or annual crops as the sub-plots replicated four times, for a total 48 concrete growth boxes (2x2 m side) and 12 treatments. The perennial species were *Arundo donax* L. (Giant reed), *Miscanthus x giganteus* Greef et Deu (Miscanthus), *Heliantus tuberosus* L. (Jerusalem artichokes), *Lolium perenne* L. (Lolium) and the annual species were *Zea mays* L. (Maize) and *Sorghum bicolor* (L.) Moench (Sorghum). The growth boxes were installed with the top surface at 1.3 m above ground level, to avoid water table influence, and the bottom open, to allow water percolation and were filled with fulvi-calcaric Cambisol soil, according to FAO-UNESCO classification. The first 50 cm soil profile in all boxes has been replaced using fulvi-calcaric Cambisol with

chemical-physical characteristics reported in Table 1 in January 2014, before the beginning of experiment.

Table 1 – Chemical-physical characteristics of the soil

Parameters	Deep soil (cm)
	0-50
Sand (%)	23.7 ± 2.83
Silt (%)	57.1 ± 2.51
Clay (%)	19.2 ± 1.13
pH	7.48 ± 0.06
ECe (µS cm^{-1})	271.3 ± 13.0
TNK (mg Kg^{-1})	1425 ± 47.8
NO_3-N (mg kg^{-1})	22.2 ± 3.71
NO_2-N (mg kg^{-1})	0.03 ± 0.002
NH_4-N (mg kg^{-1})	2.95 ± 0.29
PO_4-P (mg kg^{-1})	1.14 ± 0.18
P (g kg^{-1})	0.89 ± 0.01
Ca (g kg^{-1})	70.3 ± 1.46
K (g kg^{-1})	7.94 ± 0.56
Mg (g kg^{-1})	35.6 ± 0.61
Na (g kg^{-1})	0.36 ± 0.02

The DLF was distributed once a year (April 1st 2014, March 19th 2015 and April 1st 2016) at dose of 250 kg N ha^{-1} in all boxes. Main DLF chemical characteristics are reported in table 2. AMF inoculation (mix granular inoculum of *Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus etunicatum* and *G. clarum* obtained from MycAgro Lab., France) were carried out during sowing or plants transplanting at dose of 500 propagules m^{-2}, only in 2014 for perennial herbaceous crops, and in 2014 and 2015 for annual ones. No AMF inoculation was carried out at the beginning of 2016 crop season to evaluate, from previous two years' inoculation, the persistence and success of AMF inoculum in the experimental soil. After an adequate soil preparation, giant reed and Jerusalem artichoke (local ecotypes) were transplanted on February 26th 2014 at 7 rhizomes m^{-2}, miscanthus (local ecotype) was transplanted on February 26th 2014 at 7 plants m^{-2}, lolium (cv. Mathilde) was sowed in April 24th 2014 using 5 g seeds m^{-2}. Sorghum (hybrid Sugar Graze II) was sowed (4.5 g seeds m^{-2}) on April 24th 2014, April 13th 2015 and April 19th 2016, maize (DKC 5401) was sowed on April 24th 2014, April 27th 2015 and April 19th 2016 and it was thinned to obtain 7 plants m^{-2}. During experiment, the boxes with maize

and sorghum were annually interchanged. Growth boxes were kept free of weeds manually during spring seasons in both years, then weed control was not necessary.

Table 2 – Chemical-physical characteristics of the DLF spreaded during three experimental years

Parameters	Years		
	2014	2015	2016
EC (mS cm^{-1})	25.2	24.9	24.9
pH	7.4	7.6	7.8
COD (g L^{-1})	44.1	40.3	41.3
Dry matter (DM) (%)	4.8	5.0	6.0
Ash (%)	1.4	1.5	1.6
Total Carbon (% DM)	36.3	32.4	38.5
Sulfur (% DM)	1.0	0.7	1.0
TKN (mg kg^{-1})	4906.4	4874.6	5230.8
NH_4-N (mg kg^{-1})	3374.5	3199.6	2927.8
NO_3-N (mg kg^{-1})	17.9	4.5	-
NO_2-N (mg kg^{-1})	1.5	1.6	-
PO_4^{3-} (mg kg^{-1})	422.4	297.2	47.1
TP (mg kg^{-1})	537.3	589.1	640.5
K (mg kg^{-1})	3072.2	2619.4	3287.1
Ca (mg kg^{-1})	895.6	999.4	900.7
Na (mg kg^{-1})	222.4	293.1	264.5
Mg (mg kg^{-1})	590.0	464.1	500.7

Meteorological variables

Over the experimental period, the main meteorological variables (rainfall, maximum, minimum and average temperature, wind speed and solar radiation) and potential evapotranspiration (ET_0) were collected using a weather station located about 500 m far from the experimental site (ARPAV, Legnaro).

Bio-agronomic parameters

The main bio-agronomic traits (culm height and diameter, leaf number) were measured weekly on three plants for each growth box over the first two growing seasons. Plants were randomly selected and marked after plants emergence. At each harvest shoot density was detected.

Biomass sampling and analysis

During each experimental year plants harvest was scheduled considering plants species and meteorological conditions. Giant reed, miscanthus and maize were managed adopting single cut; sorghum was managed adopting multiple cuts (three); Jerusalem artichokes were managed adopting both single and double cuts. Lolium was managed with five cuts

during the three experimental years (Tab. 3). At the harvest time, fresh biomass production for each growth box was measured in the field. A subsample of fresh biomass was used to determine the biomass moisture content drying it in a thermo-ventilated oven at 65 °C until constant weight. Then dry biomass was milled at 2 mm (Cutting Mill SM 100 Comfort, Retsch, Germany) to determine total nitrogen (N) by Kjeldahl method and phosphorus (P) content (Balthrop et al., 2011).

Table 3 - Harvesting schedule during the trial

Specie	Year								
	2014			2015			2016		
A. donax	03/10	-	-	01/10	-	-	04/10	-	-
M. x giganteus	03/10	-	-	01/10	-	-	04/10	-	-
H. tuberosus (single cut)	03/10	-	-	01/10	-	-	04/10	-	-
H. tuberosus (double cut)	17/06	03/10	-	17/06	01/10	-	17/06	04/10	-
Z. mays	20/08	-	-	06/08	-	-	12/08	-	-
S. bicolor (multiple cut)	11/07	04/09	30/10	10/07	03/09	29/10	21/07	12/09	03/11
L. perenne	11/07	04/09	-	13/05	-	-	30/05	04/10	-

Nitrogen and phosphorus uptake and nutrient use efficiency

Nitrogen and phosphorus uptake were calculated as the product of nutrient concentration and dry biomass yield.

Nutrient use efficiency indicates the total biomass produced per unit of nutrient absorbed, and it is expressed as the ratio of dry matter production and nutrient content (g g^{-1}) (Beale and Long, 1997).

Soil moisture measurement

Volumetric soil water content was measured every 10 cm in the 0-90 cm soil profile with a Diviner 2000 device (Sentek, Stepney, Australia) which consists of a probe and hand-held data logging display unit, allowing measures onsite. Data were collected weekly in the two years between the 1st and the 3rd DLF distribution (from April 2014 to April 2016).

Water sampling and analysis

A porous ceramic plate (Ø 27 cm) was placed at 0.90 m depth in 18 boxes. The plates had air-entry suction of 50 kPa, saturated hydraulic conductivity of $1.25*10^{-5}$ cm s^{-1}. They were connected to a suction system by a network of Rilsan plastic thin (Ø 2 mm) pipes, protected by bigger (Ø 20 mm) and more rigid PVC pipes. This system consented the conduction of vacuum and the collection of percolation water samples. The central

components were placed in a small building close to the growth boxes (Fig.1) and consist of:

- 1 electric vacuum pump (power 0.37 KW) provided with a mechanical vacuum gauge. The pump was connected to a tank (50 L), provided with 2 pressure switches that allow the regulation of minimum and maximum thresholds;
- 1 pair of 5 L bottles to collect overflows;
- 18 pairs of 1 L bottles to collect samples; each pair was connected to one ceramic plate by a plastic pipe;
- 1 panel to control distribution of the vacuum, each ceramic plate was handled separately by means of a valve.

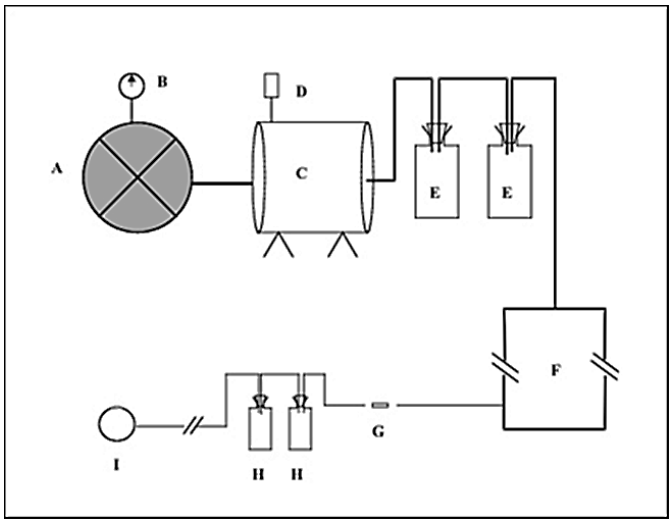


Figure 1 – Layout of the suction system to collect percolated water samples. A) electric vacuum pump; B) mechanical vacuum gauge; C) tank; D) pressure switches; E) bottles for overflows; F) panel to control distribution of the vacuum; G) valve; H) bottles to collect samples; I) porous ceramic plate

The system was started by manual activation of the pump, which, once it reached the set power, stabilized the suction intensity and began samples collection in the bottles.

The percolation water samples were taken, at 3 different times, in both the 60 days after DLF spreading in 2014 and the 45 days after DLF spreading in 2015; and 5 times after the first growing season from December 2014 to 2015 DLF spreading and 2 times after the second growing season.

A total of 223 percolation water samples were collected and analysed to detect total nitrogen (TN), ammonium nitrogen (NH_4-N), nitric nitrogen (NO_3-N), total phosphorus (TP) and orthophosphate (PO_4-P). All samples were frozen immediately after collection and stored until laboratory analysis. TN and TP were determined using Valderrama method (Valderrama, 1981), PO_4-P with Olsen method (Olsen et al., 1954), NO_3-N by modified Cataldo method (Cataldo at al., 1975) and NH_4-N by Fiastar 5000.

Root sampling and analysis

Root samples from three randomly selected plants per box were collected during each growing season in June with a hand-operated soil probe (5 cm diameter) in the first 20 cm depth for all crops, except for giant reed (in the first 30 cm depth). Subsequently, roots were washed clean of soil with some drops of Tween 20 and then rinsed several times in tap water in agreement with Vierheilig et al. (1998). Root samples were cleared and stained as reported in Annex I, for estimating the percentage of AMF colonization according to Trouvelot (1986) as follows: F%= mycorrhization frequency (the percentage of root fragments showing fungal colonization), M%= AMF colonization intensity (the percentage of fungi structures referred to the whole root system), m%= AMF colonization intensity (the percentage of fungi structures referred to colonized root fragments), a%= abundance of arbuscules (percentage of arbuscules presence referred to the root fragments showing fungal colonization); A%= abundance of arbuscules (percentage of arbuscules presence referred to the whole root system).

Soil CO_2 emission

Soil CO_2 emission was weekly monitored in each growth box from April 2014 (1st DLF distribution) to April 2016 (3rd DLF distribution). For CO_2 measure device maintenance soil CO_2 emission was not measured from mid-October to December 2014. Considering literature data on soil CO_2 emission peak in the first hours and days after DLF distribution (Grigatti et al., 2011) the frequency of soil CO_2 emission measures after DLF distribution was increased. The CO_2 flux was measured with the static non-stationary chamber technique (Maucieri et al., 2016a) using a chamber with a volume of 5 L and 10 cm square base. Soil CO_2 flux was determined by measuring the temporal change in CO_2 concentration inside the chamber using a portable IR instrument (Geotech G150), detecting CO_2 concentrations at levels of parts per million.

CO_2 flux was calculated using the following formula:

$$CO_2 = V/A * dc/dt$$

where CO_2 flux is expressed in mg CO_2 m^{-2} s^{-1}; V (m^3) is the volume and A (m^2) the footprint of the flux chamber; 'c' is the CO_2 concentration (mg CO_2 m^{-3}) and 't' the time step (s).

In each CO_2 measurement point, soil temperature and moisture (TDR 100 Soil Moisture Meter) in the first 7.5 cm were also detected.

Biochemical Methane Potential (BMP)

The BMP was estimated by the CREA laboratory (Modena – Italy) according to Owen et al. (1979), by monitoring cumulative methane production from a mixture (substrate + inoculum) incubated in anaerobiotic conditions at 35°C and pH 7. The mixture was constituted by: 1 g of volatile solids (VS) for sample, 50 mL of substrate (liquid pig manure) and 50 mL of standard inoculum.

Statistical analysis

The normality of data was checked using the Shapiro–Wilk test. Data that were normally distributed were analyzed using the analysis of variance (ANOVA) and significant differences were determined by Fisher's LSD test. Instead, for the data that didn't show normal distribution, Kruskal-Wallis and Mann-Whitney nonparametric tests were used to check the significant differences. Correlations between not normally distributed dataset were evaluated using Spearman Rank correlation.

Results

Meteorological variables

The experimental site is characterized by a sub-humid climate, with a mean annual rainfall of about 838 mm, quite evenly distributed throughout the year. The mean annual temperature is about 13.5 ºC. During the three experimental years, air temperature ranged from -5.6 to +36.8°C (Fig. 2a), the highest amount of rainfall was recorded in 2014 (1311 mm $year^{-1}$) whereas the lowest one in 2015 (-59.4% of rainfall). Intermediate rainfall was recorded during 2016 (-26.4% compared to 2014) (Fig. 2b). The maximum solar radiation and ET_0 were measured between June and July with fairly similar values in all three years (Fig. 2b and 2c).

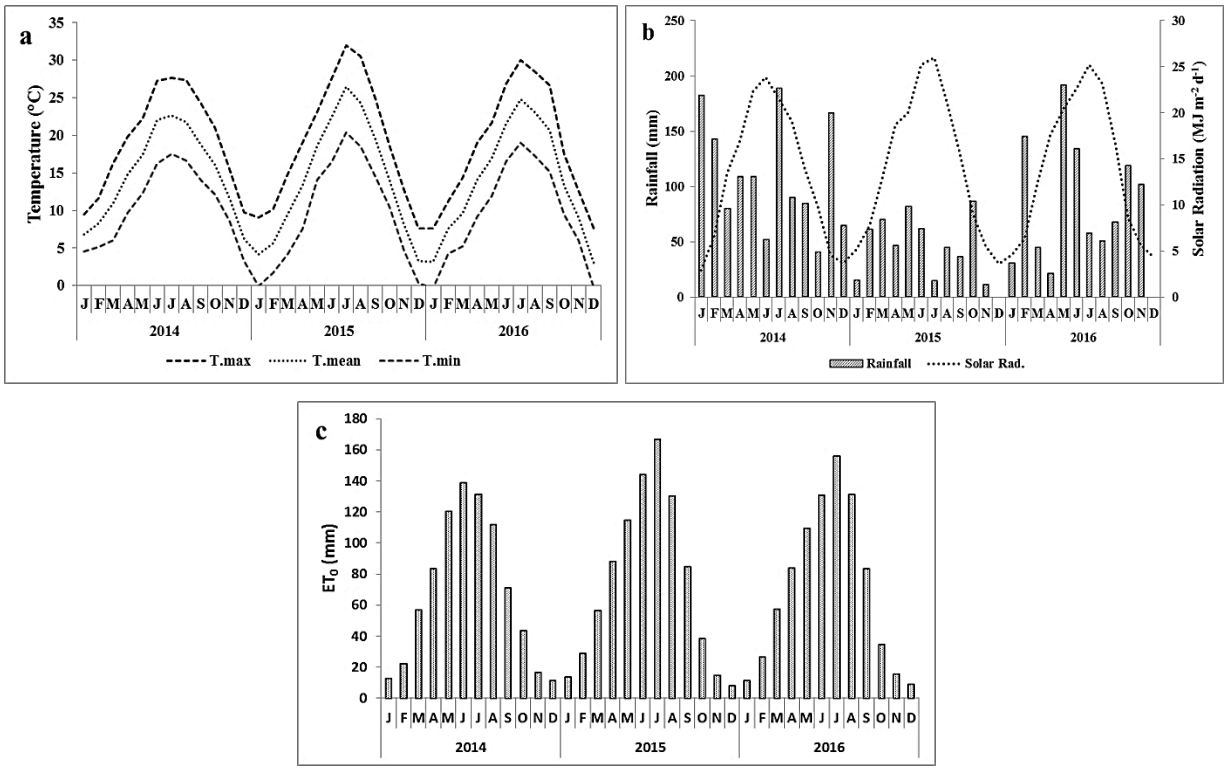


Figure 2 – Meteorological data recorded during the three experimental years: a) Maximum, minimum and average temperature; b) Rainfall and solar radiation; c) Potential evapotranspiration (ET_0).

Bio-agronomic traits: culm height, culm diameter and leaf number as affected by AMF inoculation, species, experimental year and cutting management

As reported in table 4, AMF inoculation did not affected significantly the bio-agronomic traits of all the crop species, with the only exception of miscanthus, where AMF inoculation determined a significant ($p<0.05$) culm height decrease (-4.90%) as compared to un-inoculated treatment.

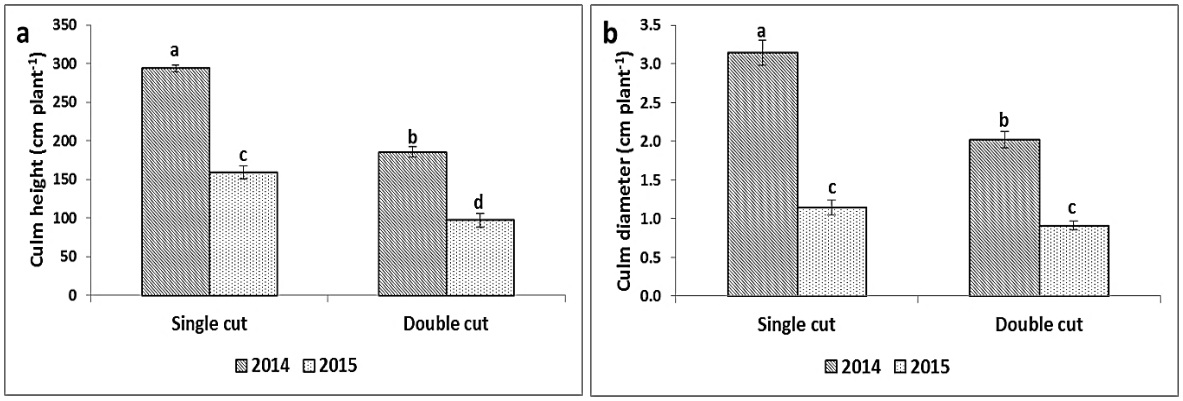
Considering the first two experimental years, in 2014, giant reed, miscanthus, Jerusalem artichoke managed with single and double biomass cutting and maize showed a significant increase in all the bio-agronomic traits as compared to 2015, except for giant reed culm height which was not different in both years (Tab. 4).

Table 4. Main effects of experimental variables on bio-agronomic traits. Different letters show statistical differences using LSD – Fisher Test

Species	Cut	Trait	AMF treatment				Years			
			AMF-N		AMF-Y		2014		2015	
A. donax	SC	Culm height (cm)	305.9 ± 10.9	ns	298.7 ± 10.7	ns	308.6 ± 7.96	ns	296.1 ± 13.0	ns
		Culm diameter (cm)	1.59 ± 0.10	ns	1.68 ± 0.08	ns	1.92 ± 0.07	a	1.36 ± 0.07	b
		Leaf number	38.0 ± 1.35	ns	37.0 ± 1.45	ns	40.0 ± 0.78	a	35.0 ± 1.71	b
M. x giganteus	SC	Culm height (cm)	293.5 ± 5.11	a	279.1 ± 8.22	b	311.7 ± 4.44	a	261.0 ± 519	b
		Culm diameter (cm)	1.38 ± 0.10	ns	1.36 ± 0.08	ns	1.78 ± 0.03	a	0.96 ± 0.03	b
		Leaf number	18.0 ± 0.38	ns	18.0 ± 0.47	ns	20.0 ± 0.16	a	16.0 ± 0.26	b
H. tuberosum (single cut)	SC	Culm height (cm)	223.4 ± 21.8	ns	229.5 ± 21.0	ns	293.8 ± 4.44	a	159.1 ± 8.56	b
		Culm diameter (cm)	2.09 ± 0.36	ns	2.20 ± 0.29	ns	3.14 ± 0.16	a	1.14 ± 0.10	b
		Leaf number	78.0 ± 13.1	ns	82.0 ± 12.1	ns	121.0 ± 2.16	a	39.0 ± 2.97	b
H. tuberosum (double cut)	1	Culm height (cm)	142.9 ± 6.05	ns	150.7 ± 6.79	ns	159.9 ± 4.46	a	133.7 ± 5.87	b
		Culm diameter (cm)	1.69 ± 0.19	ns	1.79 ± 0.25	ns	2.42 ± 0.12	a	1.06 ± 0.07	b
		Leaf number	38.0 ± 3.40	ns	39.0 ± 4.66	ns	51.0 ± 1.60	a	25.0 ± 1.28	b
	2	Culm height (cm)	133.4 ± 24.4	ns	138.0 ± 23.0	ns	211.1 ± 6.61	a	60.3 ± 6.94	b
		Culm diameter (cm)	1.16 ± 0.15	ns	1.22 ± 0.14	ns	1.62 ± 0.07	a	0.76 ± 0.07	b
		Leaf number	45.0 ± 4.40	ns	31.0 ± 4.42	ns	59.0 ± 2.57	a	35.0 ± 3.63	b
Z. mays	1	Culm height (cm)	252.7 ± 5.90	ns	258.5 ± 5.84	ns	268.8 ± 3.94	a	242.5 ± 6.26	b
		Culm diameter (cm)	3.43 ± 0.15	ns	3.39 ± 0.15	ns	3.82 ± 0.13	a	3.00 ± 0.11	b
		Leaf number	17.0 ± 0.18	ns	17.0 ± 0.16	ns	18.0 ± 0.12	a	17.0 ± 0.08	b
S. bicolor (multiple cut)	1	Culm height (cm)	209.7 ± 5.51	ns	206.4 ± 6.61	ns	223.9 ± 5.65	a	192.3 ± 4.54	b
		Culm diameter (cm)	1.57 ± 0.07	ns	1.53 ± 0.05	ns	1.41 ± 0.05	b	1.70 ± 0.05	a
		Leaf number	10.0 ± 0.22	ns	10.0 ± 0.23	ns	10.0 ± 0.20	ns	10.0 ± 0.24	ns
	2	Culm height (cm)	181.2 ± 13.8	ns	166.2 ± 15.5	ns	236.4 ± 7.93	a	110.9 ± 5.62	b
		Culm diameter (cm)	1.15 ± 0.05	ns	1.04 ± 0.04	ns	1.23 ± 0.03	a	0.97 ± 0.04	b
		Leaf number	9.0 ± 0.28	ns	9.0 ± 0.29	ns	9.0 ± 0.29	ns	9.0 ± 0.28	ns
	3	Culm height (cm)	90.1 ± 2.88	ns	91.8 ± 10.5	ns	98.3 ± 3.27	a	83.6 ± 2.14	b
		Culm diameter (cm)	0.78 ± 0.03	ns	0.80 ± 0.03	ns	0.86 ± 0.03	a	0.71 ± 0.02	b
		Leaf number	7.0 ± 0.31	ns	7.0 ± 0.22	ns	6.0 ± 0.16	b	7.0 ± 0.25	a

***SC = single cut management**

In Jerusalem artichoke, as concerns the effects of the interaction biomass cutting numbers x years, all the bio-agronomic traits were significantly ($p<0.001$) higher in the single biomass cutting (Fig. 3a, 3b and 3c) in both years, with the only exception for the culm diameter in the 2015 (Fig. 3b).



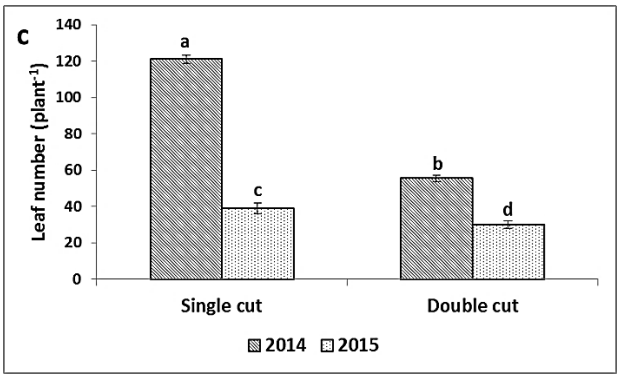


Figure 3 – Interaction between biomass cutting numbers x years in Jerusalem artichoke on: a) culm height; b) culm diameter and c) leaf number, in both experimental years. Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

When only the double biomass cutting was considered in Jerusalem artichoke on the first year (2014), a significantly (p<0.01) higher (+32.0%) culm height was registered at the second cut compared to the first one; an opposite trend was observed in 2015, with a significantly (p<0.01) higher (+121.7%) culm height at the first cut as compared to the second one (Fig. 4a). In both experimental years, Jerusalem artichoke showed at the first cut a significantly (p<0.01) higher (+49.2% for 2014 and +40.1% for 2015) culm diameter as compared to the second one (Fig. 4b). Only in the second growing season at the second cut, AMF inoculation induced a significant (p<0.01) leaf number increase (+19.7%) compared to un-inoculated plots (Fig. 5).

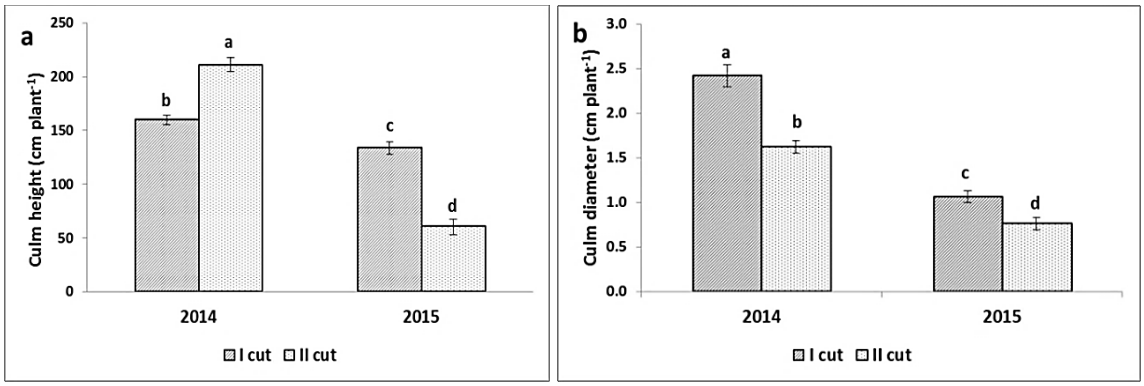


Figura 4 - Culm height (a) and culm diameter (b) in Jerusalem artichoke as affected by biomass cutting numbers and cropping season. Different letters show statistical differences at p<0.01 (LSD – Fisher Test).

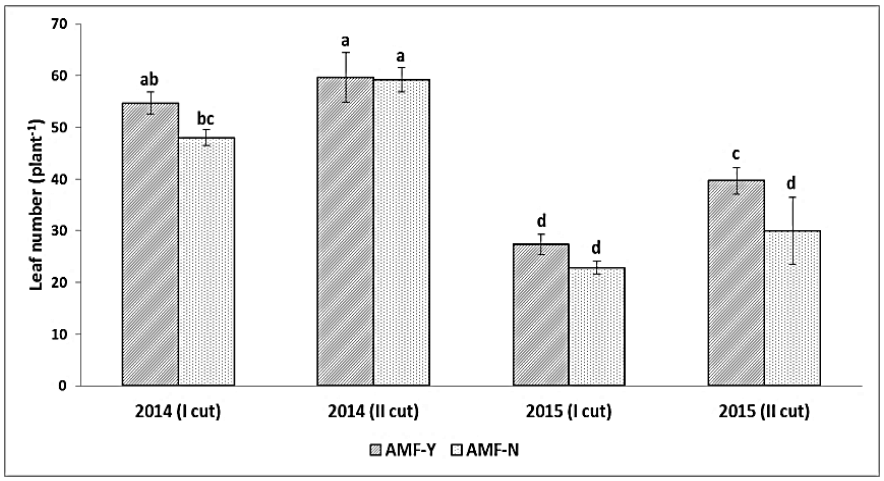


Figure 5 – Leaf number in Jerusalem artichoke as affected by mycorrhizal inoculation, biomass cutting numbers and cropping season. Different letters show statistical differences at p<0.01 (LSD – Fisher Test).

In sorghum, for all the three biomass cuttings, culm height was significantly (p<0.001) higher in 2014 (+16.5%, +113.1% and +17.6% for first, second and third cut, respectively) compared to 2015 (Fig. 6a). Culm diameter was significantly (p<0.001) higher (+20.5%) in 2015 than 2014, only at the first cut (Fig. 6b); while, in the second and third cut, it was significantly (p<0.001) higher in 2014 (+26.7% and +20.9% for second and third cut, respectively) than 2015 (Fig. 6b). The same leaf number was determined in the first and the second cutting in 2014 and 2015 (Fig 6c); in the third cutting leaf number resulted significantly (p<0.001) higher (+27.0%) in 2015 than 2014 (Fig. 6c).

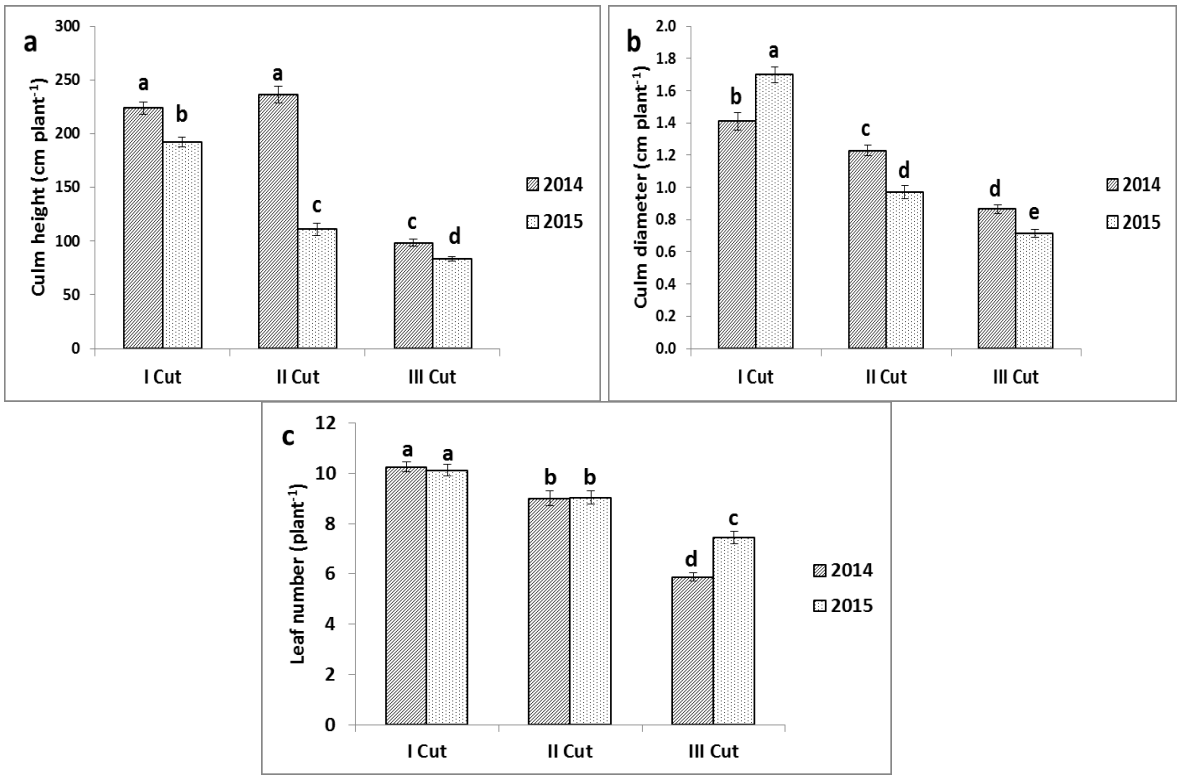


Figure 6– Sorghum bio-agronomic traits in both experimental years as affected by biomass cutting number and cropping seasons on culm height (a); culm diameter (b) and leaf number (c). Different letters show statistical differences at $p<0.001$ (LSD – Fisher Test).

In lolium, the first two biomass cuttings were made in 2014 and the third one in 2015. The AMF inoculation did not determine any difference on plant height (grand mean = 67.2 ± 2.0 cm plant^{-1}). At the third cut (the only cut made in 2015), lolium plant height showed a significant ($p<0.001$) increase (+30.4% and +78.5%) compared to first and second cut, respectively (Fig. 7).

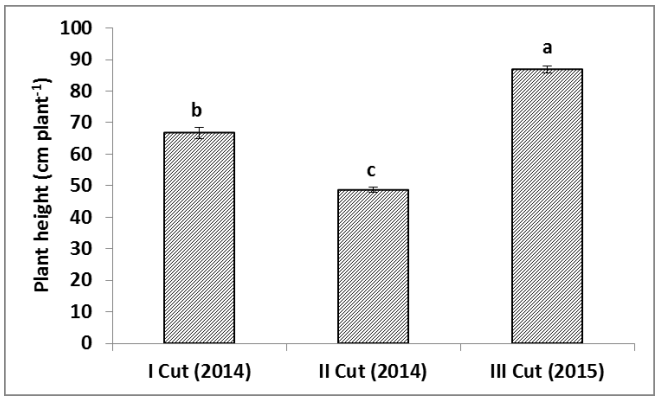


Figure 7 – Plant height in lolium as affected by biomass cutting number Different letters show statistical differences at $p<0.001$ (LSD – Fisher Test).

Shoot density

AMF inoculation affected shoot density only in miscanthus and sorghum (Tab. 5).

In particular, in miscanthus, AMF inoculation determined a significantly ($p<0.05$) lower (-27.1%) shoot density than un-inoculated treatment only in the first year; but not in the following two years (Fig. 8). In sorghum, a positive AMF inoculation effect was observed on shoot density, with a significant ($p<0.001$) increase (+15.4%) as compared to un-inoculated treatment (Tab. 5).

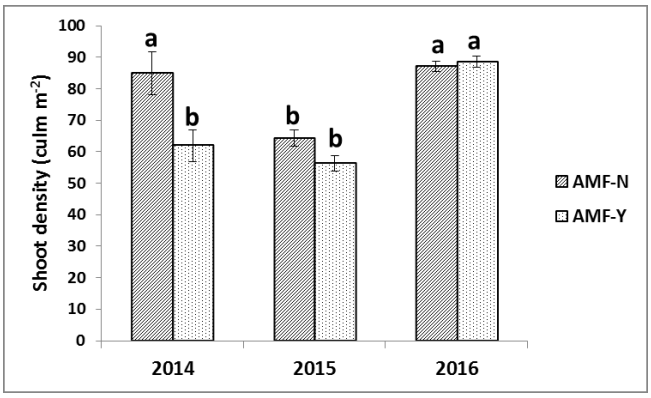


Figure 8 – AMF inoculation effect on shoot density in miscanthus during experimental years. Different letters show statistical differences at $p<0.05$ (LSD – Fisher Test).

Considering all the experimental years, the same trend in shoot density was found in giant reed and Jerusalem artichoke when managed with the double biomass cutting, with a significant ($p<0.01$) increase from the first to the third year (Tab. 5); while, the shoot density in Jerusalem artichoke managed with a single biomass cutting, significantly increased only between the first and the second year, whereas it did not show any difference between the second and third year (Tab. 5). Miscanthus showed a significant ($p<0.001$) decrease (-17.9%) in shoot density from the first to the second year, with the highest density reached at the third year (Tab. 5). An opposite trend was found in sorghum with a significantly ($p<0.001$) higher shoot density on the first year compared the other ones (Tab. 5).

Table 5. Main effects of experimental variables on shoot density (culm m^{-2})

Species	AMF treatment		Years			Biomass Cuttings		
	AMF-N	AMF-Y	2014	2015	2016	1st	2nd	3rd
A. donax	109.0 ± 14.9 ns	113.0 ± 3.7 ns	12.0 ± 1.16 c	34.0 ± 0.93 b	38.0 ± 1.26 a			
M. x giganteus	79.0 ± 3.83 a	69.0 ± 4.60 b	74.0 ± 5.85 b	60.0 ± 2.24 c	88.0 ± 1.16 a			
H. tuberosum (single cut)	73.0 ± 18.4 ns	80.0 ± 16.9 ns	23.0 ± 5.74 b	97.0 ± 3.01 a	109.0 ± 8.56 a			
H. tuberosum (double cut)	117.0 ± 15.4 ns	109.0 ± 15.0 ns	53.0 ± 10.8 c	128.0 ± 3.55 b	160.0 ± 10.1 a	109.0 ± 19.1 ns	117.0 ± 9.85 ns	
S. bicolor (multiple cut)	111.0 ± 10.2 b	128.0 ± 11.7 a	154.0 ± 13.0 a	102.0 ± 11.9 b	103.0 ± 13.3 b	62.0 ± 3.64 c	104.0 ± 11.2 b	193.0 ± 5.90 a

Jerusalem artichoke managed with double biomass cutting showed a significant interaction cuts x experimental years, with an opposite shoot density trend at the first and the third year; no difference was found between the first and the second cut in 2015 (Fig. 9).

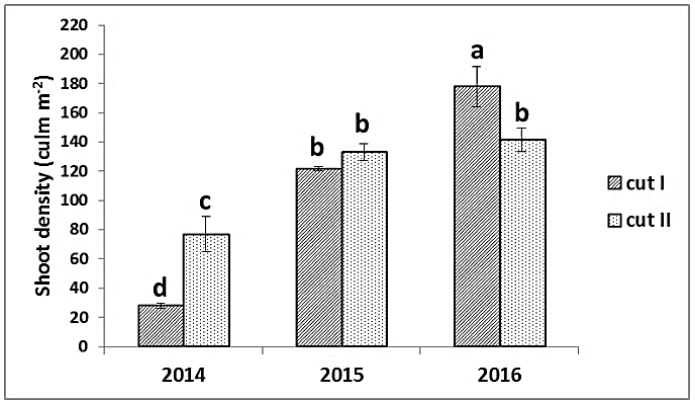


Figure 9 – Effect of the two cuts on shoot density in Jerusalem artichoke during experimental years. Different letters show statistical differences at $p<0.05$ (LSD – Fisher Test).

Comparing single and double biomass cutting, Jerusalem artichoke showed a significant ($p<0.001$) shoot density increase (+48.0%) when managed with the double cutting, as shown in Fig. 10.

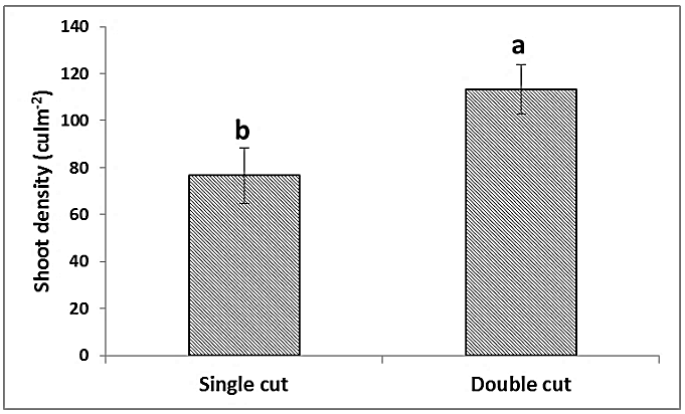


Figure 10 – Comparison of the effect between single and double biomass cutting on shoot density in Jerusalem artichoke. Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

Sorghum showed a significant (p<0.001) higher shoot density at the third cut in all the three experimental years (Fig. 11). In the first year, shoot density significantly increased from the first to the third cut, whereas, in the second and third year, no differences was observed between the first and second cut, highlighting low tillering values.

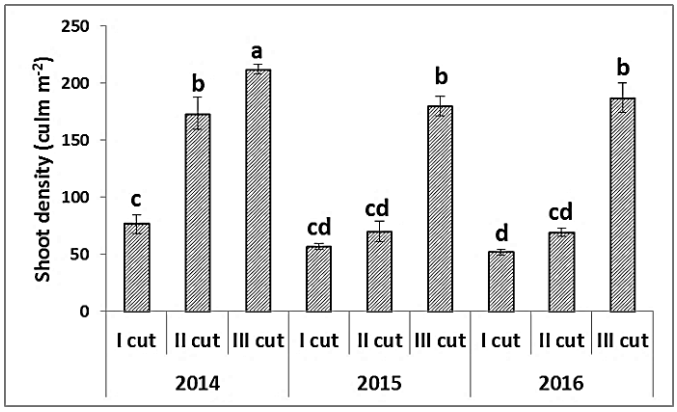


Figure 11 – Effect of the multiple cuttings on shoot density in sorghum during experimental years. Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

Dry biomass production as affected by AMF inoculation, species, experimental year and biomass cutting numbers

Comparing all the studied crops, the highest and lowest total aboveground dry biomass production was found in giant reed (42.7 ± 3.73 Mg ha^{-1}) and lolium (6.29 ± 0.39 Mg ha^{-1}). As regards the experimental years, giant reed and lolium showed a progressive increase over the years (Tab. 6), with the highest total dry biomass production in the third one (2016). In miscanthus however, no difference was observed between 2014 and 2015 (Tab. 6). On the contrary, for Jerusalem artichoke, maize and sorghum, the highest total aboveground dry biomass production was observed in the first year (2014) (Tab. 6). Considering Jerusalem artichoke biomass cutting numbers, in the average of the experimental years, no statistical differences were found between single and double

cutting on total aboveground dry biomass production (grand mean = 21.6 ± 1.46 Mg ha^{-1}). Focusing attention only on the double biomass cutting, Jerusalem artichoke showed significant ($p<0.001$) higher aboveground dry biomass production (+67.3%) at the first cut compared to the second one (Tab. 6).

In sorghum, it was observed a significant ($p<0.01$) decrease from the first to the third cut in dry biomass production, as shown in table 6.

Table 6. Main effects of experimental variables on dry biomass production (Mg ha^{-1})

Species	AMF treatment		Years			Biomass Cuttings		
	AMF-N	AMF-Y	2014	2015	2016	1st	2nd	3rd
A. donax	43.0 ± 5.96 ns	42.4 ± 4.75 ns	20.6 ± 1.10 c	45.6 ± 1.20 b	62.0 ± 3.17 a			
M. x giganteus	30.3 ± 3.68 ns	27.9 ± 3.76 ns	19.0 ± 0.71 b	22.3 ± 1.23 b	46.0 ± 1.36 a			
H. tuberosum (single cut)	18.2 ± 1.82 ns	21.9 ± 3.17 ns	26.1 ± 3.86 a	18.4 ± 1.16 b	15.6 ± 1.10 b			
H. tuberosum (double cut)	20.8 ± 3.61 b	25.4 ± 2.71 a	31.7 ± 0.73 a	15.9 ± 1.61 c	21.7 ± 3.36 b	14.5 ± 1.46 a	8.64 ± 1.05 b	
Z. mays	21.8 ± 2.08 ns	23.3 ± 2.14 ns	32.0 ± 0.61 a	17.7 ± 1.03 b	17.9 ± 0.79 b			
S. bicolor (multiple cut)	26.4 ± 1.45 ns	26.0 ± 1.30 ns	33.5 ± 1.61 a	20.2 ± 0.95 c	25.0 ± 1.24 b	11.6 ± 0.45 a	12.4 ± 1.04 a	2.23 ± 0.18 b
L. perenne	10.1 ± 0.90 ns	10.8 ± 0.97 ns	7.89 ± 0.33 c	9.05 ± 0.45 b	14.5 ± 0.55 a			

Among the studied crops, only in Jerusalem artichoke managed with double biomass cutting and in sorghum, AMF inoculation affected the total dry biomass production. In Jerusalem artichoke, in the average of the two cuts, AMF inoculation induced a significant ($p<0.05$) biomass production increase (+22.6%) than un-inoculated plots (Tab. 6) due to the heavy effect detected in 2016 (Fig. 12a). Considering the interaction between AMF inoculation and biomass cutting number, AMF significantly ($p<0.01$) effected biomass only at the first cut determining a dry biomass production increase of +39.7% at the first cut as compared to un-inoculated plots (Fig. 12b); no statistical difference in biomass was determined at the second cut by AMF treatments (grand mean = 8.64 ± 1.05 Mg ha^{-1}).

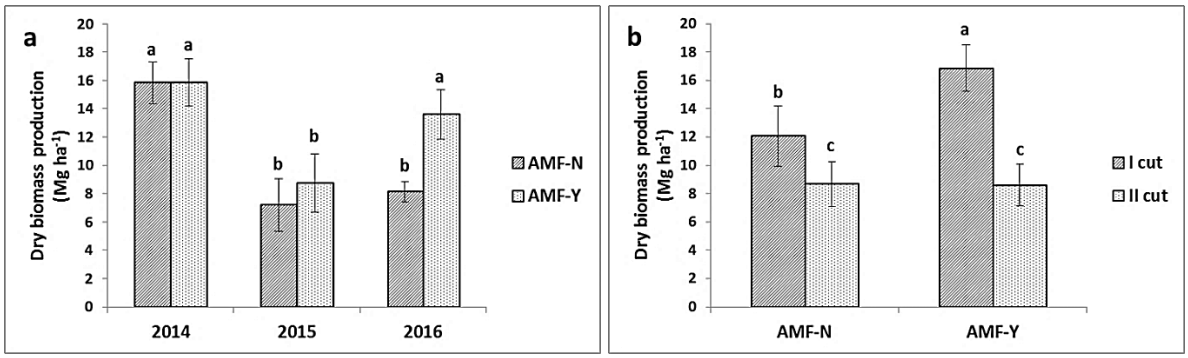


Figure 12 - Dry biomass production in Jerusalem artichoke managed with a double biomass cutting: a) AMF inoculation effect during the three years b) interaction between AMF inoculation x cuts. Different letters show statistical differences at $p<0.05$ and $p<0.01$ (LSD – Fisher Test).

In sorghum, analysing the interaction AMF inoculation x years on yearly cumulative aboveground dry biomass, emerged a significant ($p<0.05$) negative effect of AMF inoculation (-16.9 % compared to un-inoculated treatment) at the first year, whereas no effect was observed at the second and third year (Fig. 13a). The AMF negative effect in 2014 was mainly due to the noticeable reduction in the second cut biomass production (Fig. 13b).

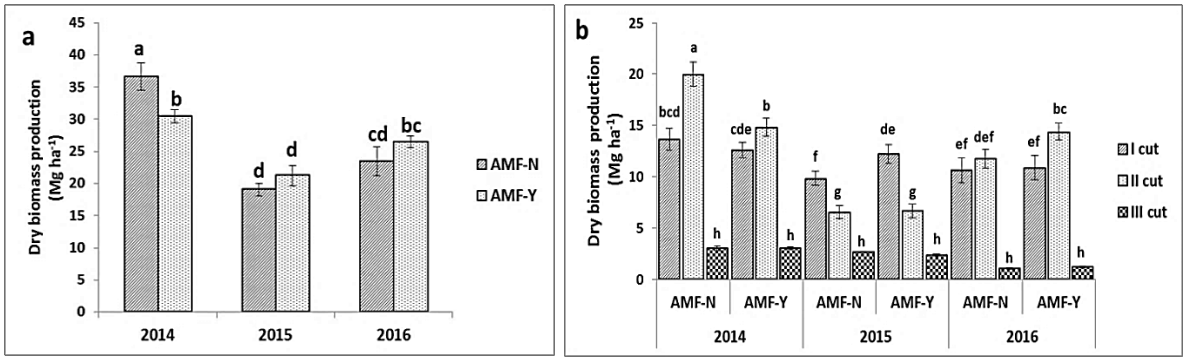


Figure 13 – Dry biomass production in sorghum: a) AMF inoculation effect during experimental years; b) interaction AMF x cuts x years. Different letters show statistical differences at $p<0.05$ (LSD – Fisher Test).

In lolium, the highest aboveground dry biomass production was detected at the third and the fourth cut, when it was well establishment in the growth boxes (Fig. 14). Considering the experimental years, when lolium was cut two times in the same year (first and third year), a significant ($p<0.01$) dry biomass production decrease was observed at the second cut as compared to the first one (-45.5% and -24.4% in 2014 and 2016, respectively) (Fig 14).

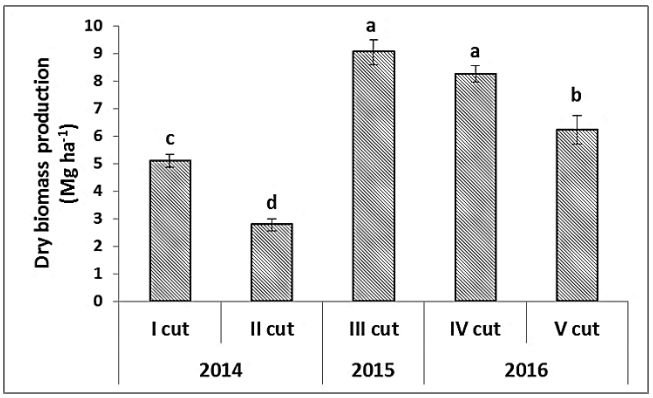


Figure 14- – Dry biomass production in *lolium* for each cutting during the experimental years. Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

In the average of the other studied factors, adding the cumulative aboveground dry biomass production in the three experimental years, the highest dry yield was observed in giant reed, followed by miscanthus, sorghum, Jerusalem artichoke, maize and lolium (Fig. 15).

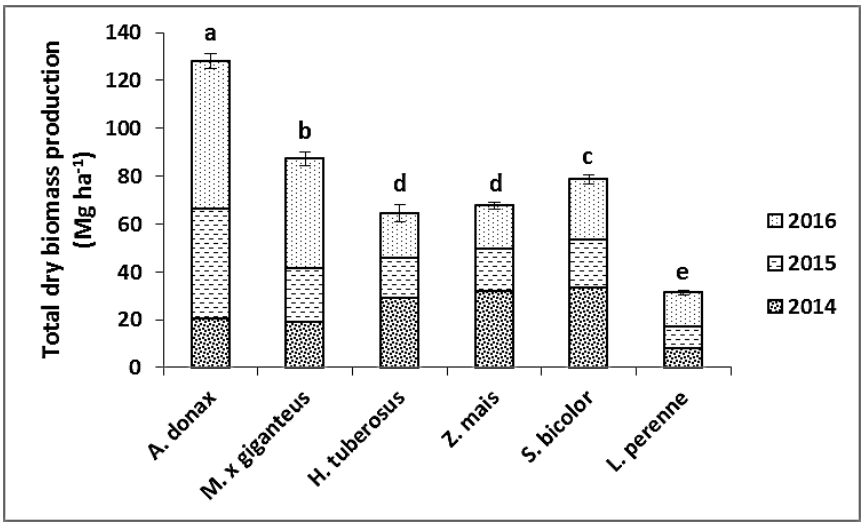


Figure 15 – Cumulative total dry biomass production in all the studied crops. Different letters show statistical differences at p<0.01 (LSD – Fisher Test).

Nitrogen and phosphorus concentrations in dry biomass tissues

AMF inoculation did not exert any effect on N and P biomass concentration in all the studied crops.

Regarding the biomass cutting numbers, AMF treatment did not shown any difference between single and double cut in Jerusalem artichoke for both nutrient concentrations. Analysing separately the two cuts, it was observed a positive effect of AMF on N biomass concentration only at the second cut, with a significant ($p<0.05$) increase (+27%) compared to un-inoculated plots (Tab. 7). In sorghum, AMF inoculation negatively ($p<0.01$) affected (-12.5%) the N biomass concentration only at the first cut (Tab. 7) whereas no effects were monitored at the second and the third cuts. An AMF significant ($p<0.001$) positive effect (+13.2%) on the P concentration in sorghum biomass was observed only at the third cut (Tab. 7).

Considering the cropping season effect on N and P concentration in the biomass dry matter (Tab. 7), giant reed biomass did not show any difference on N concentration among the three years; while, it showed a significantly ($p<0.01$) higher P concentration in 2016 compared to the previous years. Miscanthus, maize and lolium showed a significantly ($p<0.001$) higher N and P concentration in the first year (2014) compared to following ones. Comparing the Jerusalem artichoke biomass cutting numbers, the biomass harvested in the plots managed with double cutting showed a significant ($p<0.001$) higher N and P concentration (+58.2% and +114.8%, respectively) compared to single cut one (1.07% ± 0.09 for N and 0.084% ± 0.007 for P concentration). In particular, the interaction biomass cutting management x years, showed that the highest N concentration was monitored in the biomass of the first cut in 2014 (Fig. 16a); in the three years, the biomass harvested at the first cut showed significant ($p<0.001$) higher P concentration than the second cut (except 2016) and the single cut one (Fig 16b).

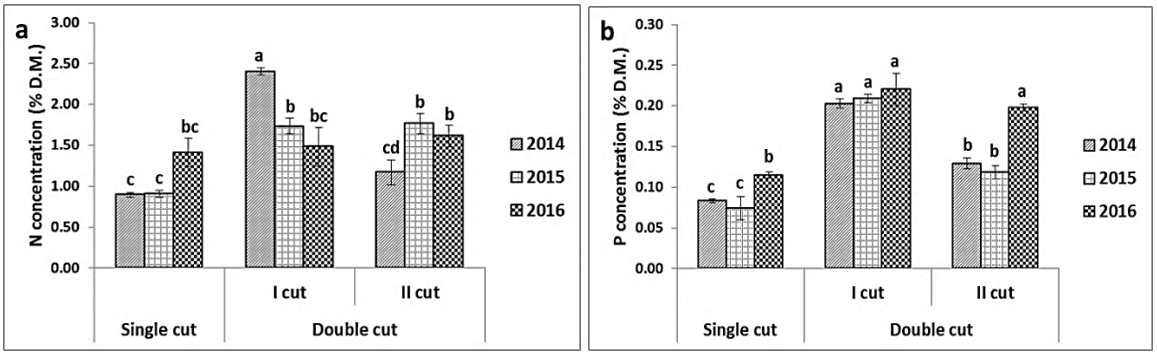


Figure 16– Effect of the interaction biomass cutting management x years in Jerusalem artichoke on N concentration (a) and P concentration (b). Different letters show statistical differences at $p<0.001$ (LSD – Fisher Test).

Table 7. Main effects of experimental variables on nitrogen and phosphorus concentration (% D.M.)

Species	Biomass Cutting	Concentration (% D.M.)	AMF treatment		Years		
			AMF-N	AMF-Y	2014	2015	2016
A. donax		N	1.230 ± 0.109 ns	1.249 ± 0.067 ns	1.317 ± 0.139 ns	1.161 ± 0.081 ns	1.241 ± 0.105 ns
		P	0.086 ± 0.008 ns	0.091 ± 0.011 ns	0.088 ± 0.009 b	0.063 ± 0.005 c	0.114 ± 0.012 a
M. x giganteus		N	0.692 ± 0.066 ns	0.794 ± 0.089 ns	1.014 ± 0.060 a	0.546 ± 0.034 b	0.670 ± 0.084 b
		P	0.066 ± 0.006 ns	0.074 ± 0.009 ns	0.093 ± 0.008 a	0.044 ± 0.002 c	0.073 ± 0.005 b
H. tuberosum (single cut)		N	1.109 ± 0.147 ns	1.035 ± 0.120 ns	0.896 ± 0.029 b	0.909 ± 0.046 b	1.411 ± 0.176 a
		P	0.085 ± 0.012 ns	0.096 ± 0.008 ns	0.083 ± 0.002 b	0.074 ± 0.014 b	0.115 ± 0.004 a
H. tuberosum (double cut)	1st	N	1.75 ± 0.19 ns	2.00 ± 0. 21 ns	2.406 ± 0.043 a	1.734 ± 0.096 b	1.485 ± 0.237 b
		P	0.21 ± 0.01 ns	0.21 ± 0.01 ns	0.203 ± 0.006 ns	0.209 ± 0.005 ns	0.220 ± 0.020 ns
	2st	N	1.34 ± 0.15 b	1.70 ± 0.11 a	1.170 ± 0.153 b	1.766 ± 0.124 a	1.616 ± 0.133 a
		P	0.15 ± 0.02 ns	0.15 ± 0.02 ns	0.129 ± 0.006 b	0.118 ± 0.008 b	0.198 ± 0.004 a
Z. mays		N	1.064 ± 0.040 ns	1.082 ± 0.035 ns	1.189 ± 0.019 a	1.001 ± 0.045 b	0.988 ± 0.039 b
		P	0.211 ± 0.018 ns	0.214 ± 0.020 ns	0.295 ± 0.004 a	0.149 ± 0.006 c	0.193 ± 0.004 b
S. bicolor (multiple cut)	1st	N	1.400 ± 0.061 a	1.225 ± 0.050 b	1.348 ± 0.096 ns	1.333 ± 0.076 ns	1.257 ± 0.050 ns
		P	0.159 ± 0.008 ns	0.164 ± 0.011 ns	0.150 ± 0.005 b	0.132 ± 0.004 c	0.201 ± 0.007 a
	2st	N	1.156 ± 0.095 ns	1.273 ± 0.071 ns	0.999 ± 0.025 b	1.343 ± 0.148 a	1.303 ± 0.043 a
		P	0.159 ± 0.011 ns	0.178 ± 0.013 ns	0.205 ± 0.014 a	0.130 ± 0.005 c	0.171 ± 0.009 b
	3st	N	1.064 ± 0.040 ns	1.082 ± 0.035 ns	2.379 ± 0.054 b	2.144 ± 0.029 c	2.993 ± 0.056 a
		P	0.319 ± 0.024 b	0.361 ± 0.030 a	0.397 ± 0.016 a	0.218 ± 0.004 b	0.405 ± 0.012 a
L. perenne		N	2.098 ± 0.143 ns	2.068 ± 0.138 ns	2.705 ± 0.063 a	2.041 ± 0.056 b	1.482 ± 0.012 c
		P	0.306 ± 0.010 ns	0.294 ± 0.014 ns	0.335 ± 0.010 a	0.299 ± 0.008 ab	0.265 ± 0.012 b

In sorghum, averaging the cuttings, there was not any differences on N concentration among years (grand mean = 1.68 ± 0.08); a significantly ($p<0.001$) lower (-36.1% and -38.2%) P concentration was found in 2015 as compared to 2014 and 2016, (0.251% ± 0.02 and 0.259% ± 0.02, respectively). Furthermore, the highest N and P concentrations were found at the third cut in all the experimental years (Fig. 17a and Fig. 17b).

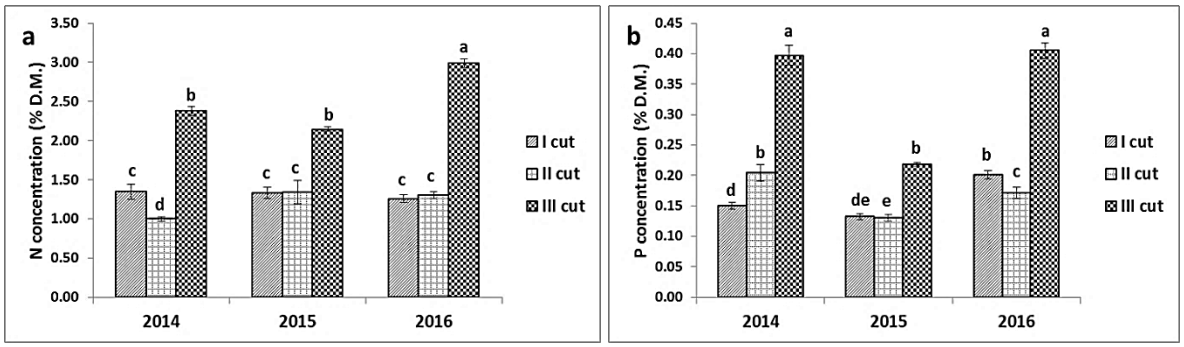


Figure 17 – Effect of the interaction between crops management x years in sorghum on N concentration (a) and P concentration (b). Different letters show statistical differences at $p<0.001$ (LSD – Fisher Test).

In lolium biomass, N concentration during the experimental years, showed a significant ($p<0.001$) constant decrease from the first to the fifth cut (Fig. 18a). Instead, the highest P concentration was found between the first and second cut whereas the lowest P concentration was found at the fourth cut (Fig. 18b).

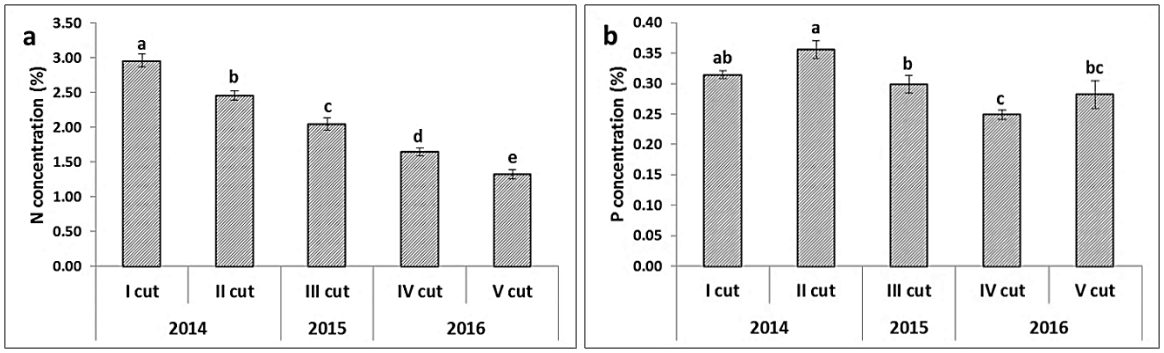


Figure 18 – Effect of the biomass cutting numbers in lolium during experimental years on N concentration (a) and P concentration (b). Different letters show statistical differences at $p<0.001$ (LSD – Fisher Test).

Total biomass nitrogen and phosphorus uptake per hectare

AMF inoculation did not exert any effect on N and P uptake in all the studied crops and cut managements. Despite the biomass cuttting management, in Jerusalem artichoke, AMF inoculation significantly ($p<0.05$) increase N (+31.2%) and P (+31.4%) uptake compared to un-inoculated plots which showed an uptake of 177.2 ± 25.0 Kg ha^{-1} for N and 17.6 ± 2.1 Kg ha^{-1} for P.

Regarding sorghum biomass cutting management, AMF inoculation negatively ($p<0.01$) affected (-25.3%) N uptake in the 2014, and positively ($p<0.01$) increased it (+20.6%) in the 2016 (Fig. 19a). Considering P, AMF inoculation significantly ($p<0.01$) increased (+26.1%) the sorghum uptake only in 2016 (Fig. 19b).

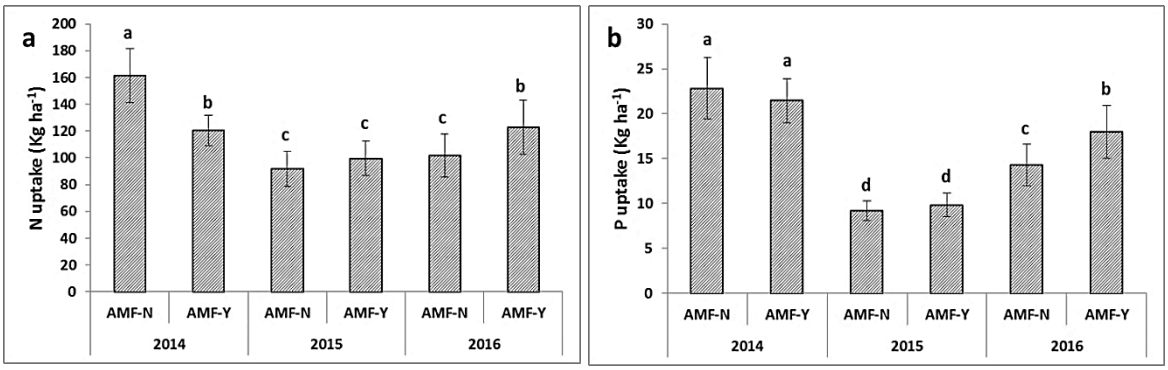


Figure 19 – Effect of the interaction between AMF treatments x years in sorghum on N uptake (a) and P uptake (b). Different letters show statistical differences at $p<0.01$ (LSD – Fisher Test).

The highest N and P uptakes were observed in 2016 for giant reed and miscanthus and in 2014 for maize (Tab. 8).

Table 8. Main effects of experimental variables on nitrogen and phosphorus uptake (Kg ha^{-1})

Species	Biomass Cutting	Uptake (Kg ha^{-1})	AMF treatment				Years					
			AMF-N		AMF-Y		2014		2015		2016	
A. donax		N	507.6 ± 71.6	ns	524.4 ± 63.0	ns	266.4 ± 25.2	c	530.2 ± 43.1	b	751.4 ± 46.4	a
		P	37.4 ± 7.34	ns	40.7 ± 8.48	ns	17.7 ± 1.68	b	28.5 ± 2.30	b	70.9 ± 7.96	a
M. x giganteus		N	196.1 ± 23.0	ns	218.2 ± 37.0	ns	191.3 ± 9.30	b	120.8 ± 8.73	c	309.4 ± 42.0	a
		P	19.6 ± 2.86	ns	20.9 ± 3.55	ns	17.5 ± 1.04	b	9.90 ± 0.84	c	33.4 ± 2.51	a
H. tuberosum (single cut)		N	195.6 ± 23.0	ns	216.9 ± 24.2	ns	233.1 ± 33.2	ns	168.0 ± 18.1	ns	217.6 ± 26.4	ns
		P	15.0 ± 1.90	ns	20.4 ± 2.26	ns	21.4 ± 2.82	ns	13.8 ± 3.31	ns	17.8 ± 0.77	ns
H. tuberosum (double cut)	1st	N	228.6 ± 63.4	ns	343.5 ± 56.4	ns	443.5 ± 12.0	a	189.2 ± 31.9	b	225.4 ± 86.7	b
		P	25.3 ± 4.29	ns	36.0 ± 4.92	ns	37.4 ± 1.36	ns	22.8 ± 3.60	ns	31.8 ± 9.16	ns
	2st	N	107.5 ± 15.4	ns	138.3 ± 14.8	ns	155.7 ± 22.2	a	90.3 ± 7.68	b	122.6 ± 10.6	ab
		P	12.5 ± 2.19	ns	12.9 ± 2.35	ns	17.1 ± 1.51	a	6.02 ± 0.41	b	15.0 ± 0.41	a
Z. mays		N	237.9 ± 28.9	ns	251.8 ± 30.9	ns	380.3 ± 7.04	a	176.7 ± 11.6	b	177.5 ± 11.3	b
		P	49.8 ± 8.68	ns	53.9 ± 9.89	ns	94.6 ± 2.61	a	26.3 ± 1.74	c	34.6 ± 1.82	b
S. bicolor (multiple cut)	1st	N	160.5 ± 14.7	ns	144.5 ± 7.59	ns	178.3 ± 18.1	a	145.3 ± 9.63	b	134.0 ± 9.90	b
		P	17.9 ± 1.21	ns	19.2 ± 1.47	ns	19.7 ± 1.11	a	14.5 ± 0.81	b	21.6 ± 1.73	a
	2st	N	140.8 ± 16.3	ns	145.5 ± 12.0	ns	172.5 ± 9.91	a	87.3 ± 10.1	b	169.7 ± 9.74	a
		P	21.6 ± 3.74	ns	22.2 ± 3.01	ns	34.7 ± 1.66	a	8.57 ± 0.58	c	22.3 ± 1.60	b
	3st	N	53.6 ± 5.79	ns	53.0 ± 4.59	ns	72.3 ± 3.67	a	54.2 ± 1.86	b	33.3 ± 1.71	c
		P	6.84 ± 0.88	b	7.87 ± 1.18	a	12.0 ± 0.60	a	5.52 ± 0.20	b	4.52 ± 0.28	c
L. perenne		N	119.2 ± 9.77	ns	129.5 ± 12.4	ns	109.8 ± 12.0	b	184.6 ± 12.5	a	108.7 ± 8.18	b
		P	17.9 ± 1.49	ns	18.1 ± 1.28	ns	12.9 ± 0.94	c	26.8 ± 1.24	a	18.7 ± 0.75	b

Jerusalem artichoke, in the average of single and double cut, showed the highest N uptake in 2014 while, the highest P uptake was found in 2014 and 2016 (Fig. 20a and 20b). Although there were not any differences on N and P uptake between single and double cut (grand mean = for 205.0 ± 19.2 Kg N ha^{-1} and 20.3 ± 1.85 Kg P ha^{-1}), considering the plots managed with two cuts, the highest N and P uptake were detected at the first cut (Fig 21a and 21b).

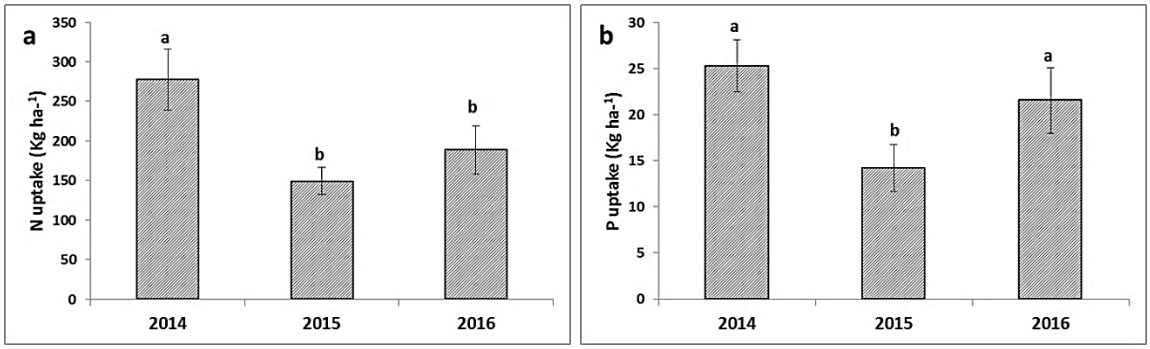


Figure 20– Effect of the years in Jerusalem artichoke in the average of the single and double biomass cutting N uptake (a) and P uptake (b). Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

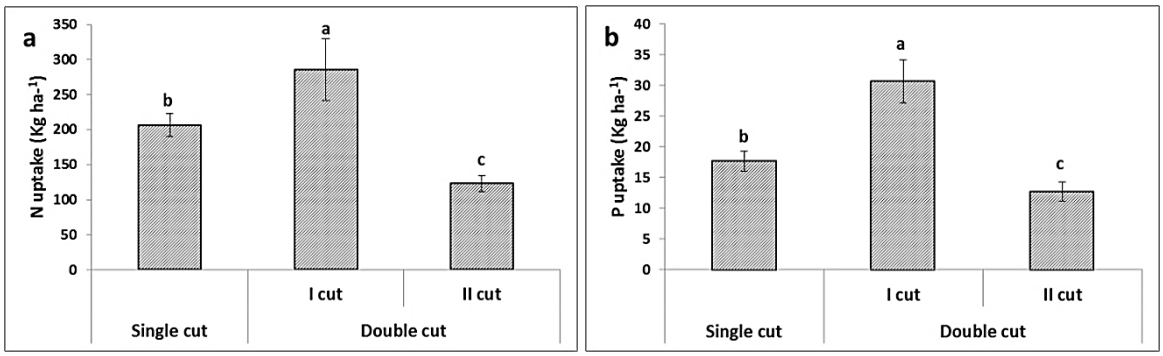


Figure 21 – Comparison single and double biomass cutting management in Jerusalem artichoke on N uptake (a) and P uptake (b). Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

In sorghum, the lowest N and P uptake was found at the third cut in all the three years. The highest N uptake were measured at the first and second cut in 2014, as well as to the second one in 2016 (Fig. 22a). Instead, the highest P uptake was found in the 2014 at second cut (Fig. 22b).

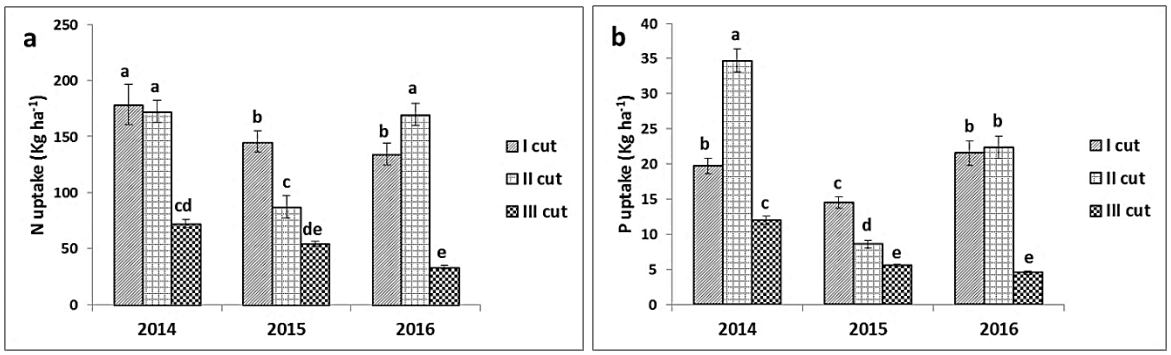


Figure 22 – Effect of the interaction between the three cutting x years in sorghum on N uptake (a) and P uptake (b). Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

Considering the biomass cuts during the experimental years in lolium, the same trend was observed for N and P uptake with a significant (p<0.001) strong decrease from the first to the second cut and from the third to the fifth one (Fig. 23a and 23b).

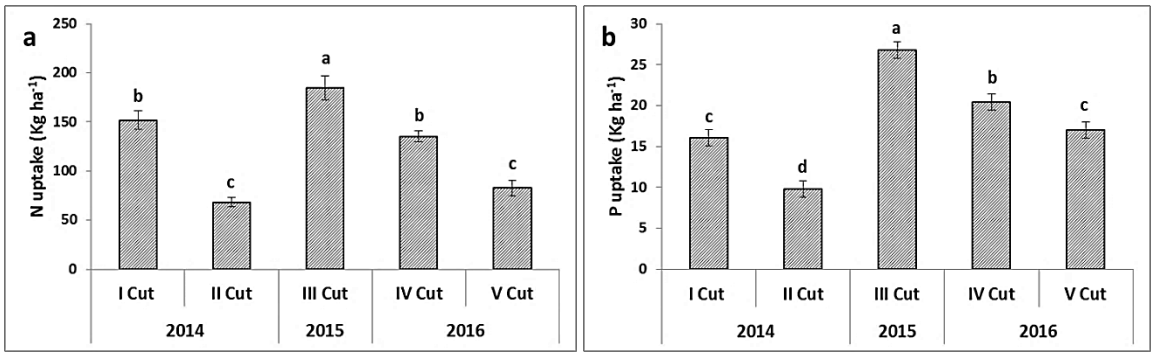


Figure 23 – Effect of the five cuts in lolium during experimental years onN uptake (a) and P uptake (b). Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

Nitrogen and phosphorus use efficiency (N_{ue} and P_{ue})

AMF inoculation did not exert any effect on N and P use efficiency. Analysing separately the three cut in sorghum, it was observed an AMF positive (p<0.05) effect on nitrogen use efficiency (N_{ue}) at the first cut and a significant (p<0.001) decrease on phosphorus use efficiency (P_{ue}) at the third cut (Tab. 9).

Considering the experimental years, in giant reed no statistical differences was found in relation to the years for N_{ue}, whereas, miscanthus and maize showed the highest efficiency in the 2015 and 2016 (Tab. 9). Instead, for the same species, the highest Pue was found in 2015 (Tab. 9).

Table 9. Main effects of experimental variables on nitrogen and phosphorus use efficiency (g g^{-1})

Species	Cut	NUE (g g^{-1})	AMF inoculation				Years					
			AMF-N		AMF-Y		2014		2015		2016	
A. donax		N	88.7 ± 7.98	ns	82.8 ± 4.86	ns	82.2 ± 8.74	ns	88.9 ± 5.88	ns	86.2 ± 9.75	ns
		P	1286.5 ± 133.3	ns	1286.1 ± 148.2	ns	1245.3 ± 157.3	b	1671.1 ± 145.2	a	942.4 ± 97.7	b
M. x giganteus		N	160.2 ± 15.7	ns	145.2 ± 17.5	ns	100.9 ± 5.51	b	189.7 ± 14.8	a	167.5 ± 21.5	a
		P	1671.4 ± 160.5	ns	1585.9 ± 212.3	ns	1115.8 ± 73.6	b	2318.7 ± 163.3	a	1451.4 ± 160.2	b
H. tuberosum (single cut)		N	97.0 ± 10.5	ns	101.7 ± 8.72	ns	111.9 ± 3.68	ns	110.8 ± 5.26	ns	75.3 ± 11.9	ns
		P	1197.7 ± 126.3	ns	1078.0 ± 88.2	ns	1210.5 ± 33.5	ns	1329.3 ± 154.3	ns	873.8 ± 27.8	ns
H. tuberosum (double cut)	1st	N	60.6 ± 6.18	ns	53.7 ± 7.53	ns	41.6 ± 0.75	ns	58.2 ± 3.19	ns	71.7 ± 9.29	ns
		P	478.6 ± 19.1	ns	480.8 ± 23.1	ns	494.4 ± 13.9	ns	478.9 ± 11.8	ns	465.8 ± 42.2	ns
	2st	N	80.9 ± 11.3	ns	60.3 ± 6.77	ns	91.1 ± 14.5	ns	57.6 ± 4.69	ns	63.2 ± 5.29	ns
		P	724.8 ± 76.3	ns	705.7 ± 71.9	ns	781.0 ± 0.75	ns	858.3 ± 3.20	ns	506.5 ± 9.29	ns
Z. mays		N	91.1 ± 3.83	ns	94.5 ± 3.78	ns	84.3 ± 1.34	b	101.3 ± 4.39	a	102.3 ± 4.24	a
		P	515.0 ± 43.4	ns	504.8 ± 45.1	ns	339.1 ± 4.46	c	680.7 ± 27.0	a	520.4 ± 12.1	b
S. bicolor (multiple cut)	1st	N	73.0 ± 3.38	b	83.3 ± 3.74	a	76.9 ± 5.66	ns	77.1 ± 5.21	ns	80.4 ± 3.29	ns
		P	644.9 ± 28.9	ns	644.7 ± 44.4	ns	670.7 ± 21.7	b	763.0 ± 27.9	a	500.9 ± 17.9	c
	2st	N	98.7 ± 15.0	ns	81.2 ± 4.39	ns	100.6 ± 2.50	ns	91.8 ± 23.5	ns	77.4 ± 2.83	ns
		P	659.5 ± 44.1	ns	594.7 ± 42.0	ns	505.1 ± 35.0	b	779.4 ± 33.6	a	596.9 ± 35.7	b
	3st	N	41.1 ± 1.69	ns	40.5 ± 1.82	ns	42.2 ± 0.99	b	46.7 ± 0.61	a	33.5 ± 0.63	c
		P	336.8 ± 28.9	a	304.5 ± 30.8	b	254.7 ± 10.7	b	458.7 ± 7.68	a	248.5 ± 7.89	b
L. perenne		N	52.1 ± 3.57	ns	53.1 ± 3.90	ns	37.5 ± 1.20	c	49.6 ± 2.00	b	69.1 ± 2.88	a
		P	333.6 ± 11.5	ns	354.0 ± 15.9	ns	301.6 ± 7.85	b	341.0 ± 19.9	b	387.4 ± 15.2	a

In relation to biomass cutting numbers, Jerusalem artichoke showed a significantly ($p<0.001$) higher N and P use efficiency in the single cut (+90.4% for N_{UE} and +55.5% for P_{UE}) compared to double cut (597.5 ± 35.4 g g^{-1} for N and 63.9 ± 4.21 g g^{-1} for P). In Jerusalem artichoke the interaction biomass cutting management x years, showed a significantly ($p<0.01$) higher N_{ue} in the single cut in the 2014 and 2015; while, in the double one only at the second cut in 2014 (Fig. 23a). The same trend of N_{ue} was also observed for P_{ue} in single cut in the 2014 and 2015, while in the double biomass cutting the highest P_{ue} was found in the 2014 and 2015 at the second cutting (Fig. 24b).

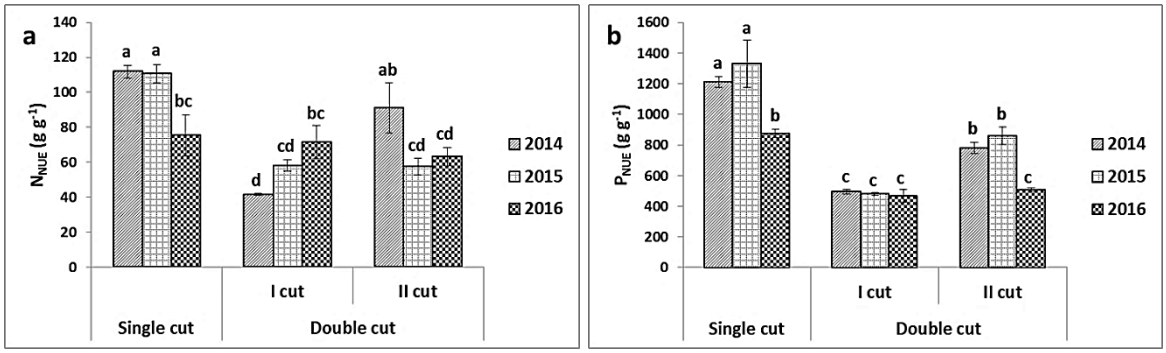


Figure 24 – Comparison between single and double cut in Jerusalem artichoke on: a) nitrogen and b) phosphorus use efficiency. Different letters show statistical differences at $p<0.01$ (LSD – Fisher Test).

In sorghum, the highest N_{ue} was observed at the second cut (Fig.25a). Focusing attention on P_{ue}, and considering the interaction biomass cutting x years, sorghum showed a significantly ($p<0.001$) higher P_{ue} in 2015 than in 2014 and 2016 for all the three cuttings (Fig. 25b).

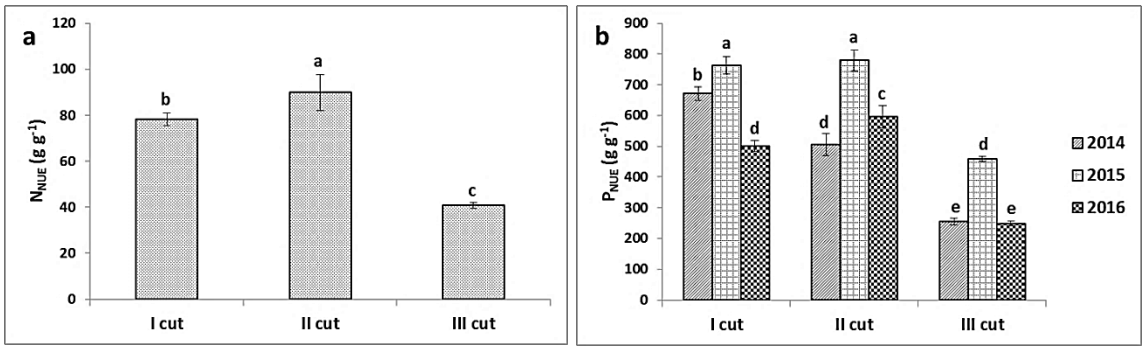


Figure 25– a) Effects of the three biomass cutting management on nitrogen use efficiency and b) interaction cuttings x years on phosphorus use efficiency in sorghum. Different letters show statistical differences at $p<0.001$ (LSD – Fisher Test).

In lolium a progressive increase from the first to the fifth cut in N_{ue} (Fig. 26a) was found; whereas, it was observed a lower P_{ue} between the first and the second cut, with an increase in the following cuttings (Fig. 26b).

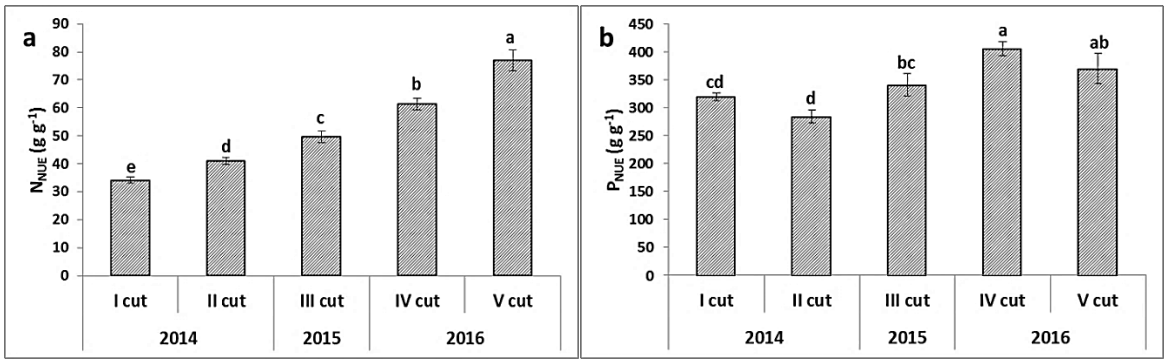


Figure 26 –Evaluation of the nitrogen (a) and phosphorus (b) use efficiency in lolium during the growing season. Different letters show statistical differences at p<0.001 (LSD – Fisher Test).

Overall, averaging the experimental years and cut managements, the highest Nue was observed in sorghum; while the highest Pue were found in sorghum and in miscanthus compared to the other crops (Fig. 27a and Fig. 27b).

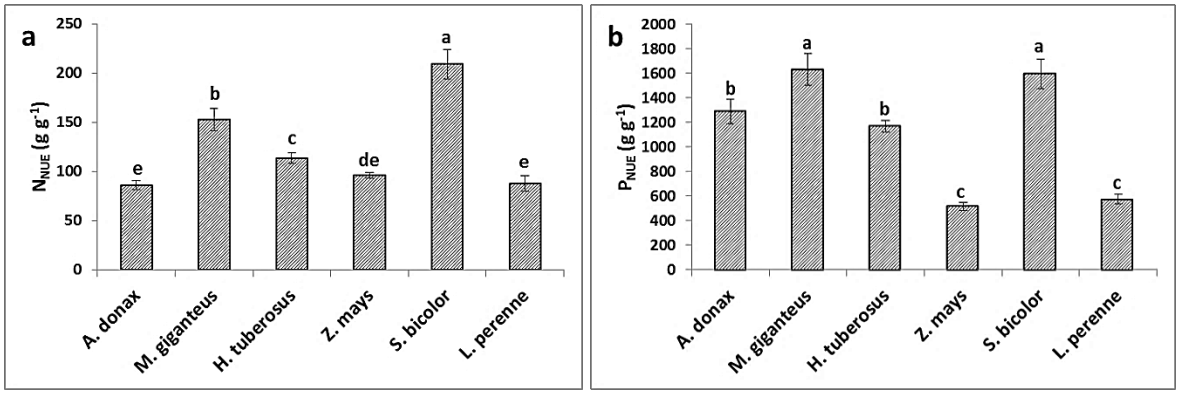


Figure 27 – Comparison of the nitrogen (a) and phosphorus (b) use efficiency in all crops. Different letters show statistical differences at p<0.01 (LSD – Fisher Test).

NH_4-N, NO_3-N and PO_4-P leaching

In the average of the studied crops, AMF inoculation exerted a positive environmental effect on the leaching of ammonium nitrogen, with a significant NH_4-N reduction of -32.8% and a negative effect on nitrate nitrogen with a significant NO_3-N leaching increase of +70.0% (Fig. 28a and 28b). No specific crop effect NH_4-N concentration in the percolation water was observed during experimental years with a concentration median value of 1.39 mg NH_4-N L^{-1}. On the contrary, crop species had significant ($p<0.001$) effect on NO_3-N leaching with the highest median value in presence of maize (30.0 mg NO_3-N L^{-1}) and the lowest median value in presence of lolium (1.21 mg NO_3-N L^{-1}) (Fig. 29). No differences were found between giant reed and sorghum (median value of 17.9 mg NO_3-N L-1), and between miscanthus and Jerusalem artichoke (median value

21.9 mg NO_3-N L^{-1}) (Fig.28). Also the TNK concentration was not affected by studied factors, with a median concentration of 3.74 mg TKN L^{-1}.

The P concentration in water percolation, ranged from 0.0 to 1.83 mg L^{-1} for TP and from 0.0 to 1.35 mg L^{-1} for PO_4-P.

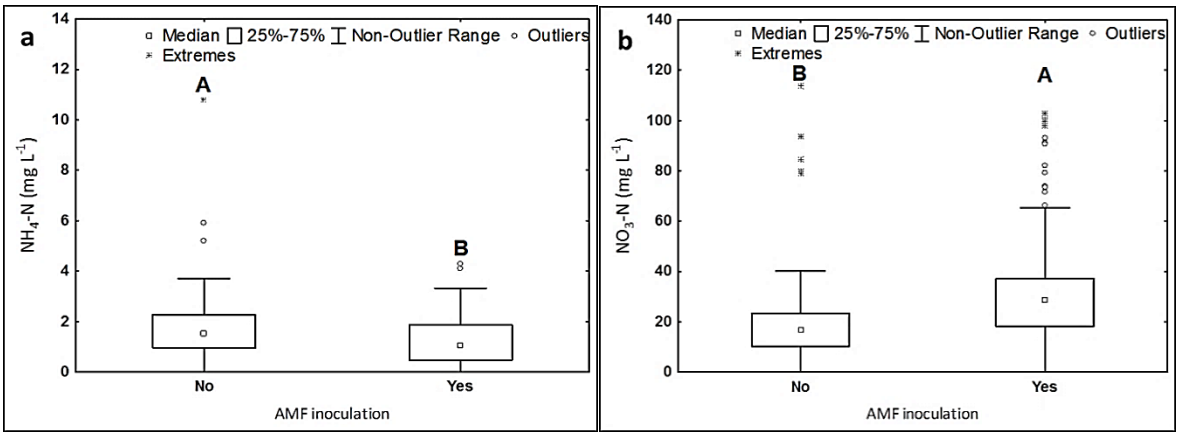


Figure 28– AMF inoculation effect on: a) NH_4-N and b) NO_3-N concentration in the water percolation. Different letters show statistical differences at $p<0.01$ and $p<0.001$(Test Mann-Whitney).

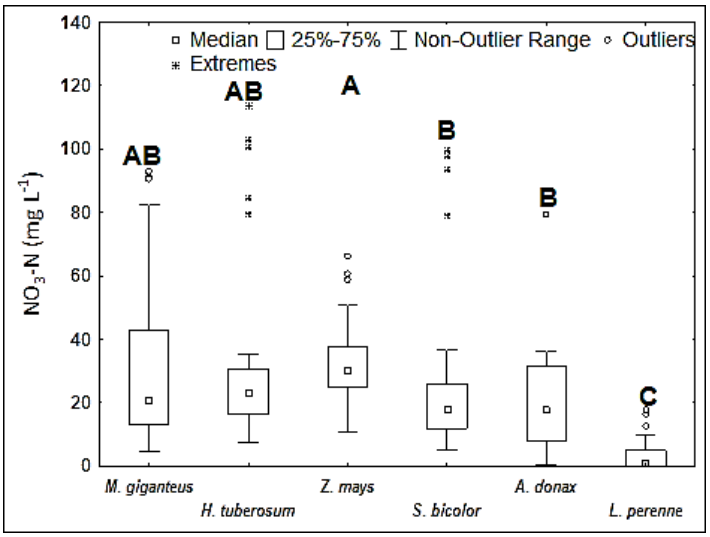


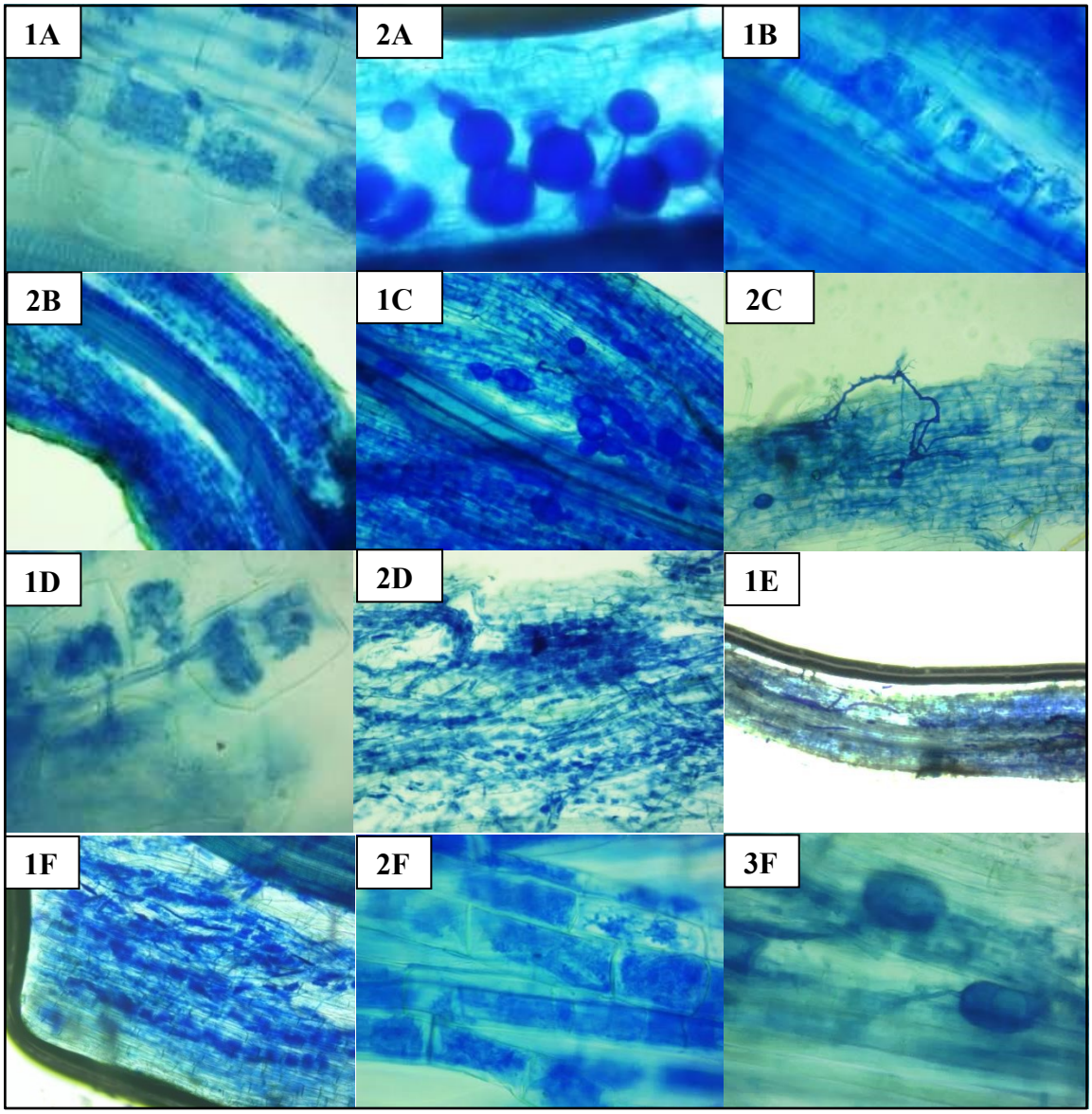
Figure 29 – Effect of the crops on NO_3-N concentration in water percolation. Different letters show statistical differences at $p<0.001$(Test Kruskal-Wallis).

AMF root colonization (%)

AMF root colonization was variable among the crop species and during the experimental years, with the lowest values measured at the first year for all species with the only exception of Jerusalem artichoke (Tab. 10). In fact, Jerusalem artichoke root colonization decreased from the first to third years; while an opposite trend was observed for the other crops (Tab. 10).

Table 10- AMF root colonization during the experimental years.

		Years														
		2014					2015					2016				
Species	AMF treatment	F%	m%	M%	a%	A%	F%	m%	M%	a%	A%	F%	m%	M%	a%	A%
A. donax	AMF-N	6.7	0.8	3.8	0.0	0.0	25.0	4.3	16.9	1.1	0.1	78.3	33.9	41.1	90.3	31.4
	AMF-Y	48.3	9.2	19.2	40.6	3.55	52.5	19.7	37.0	18.6	3.8	76.2	22.2	29.7	81.6	18.6
M. x giganteus	AMF-N	0.0	0.0	0.0	0.0	0.0	53.3	9.3	15.8	73.1	6.4	95.8	26.6	27.7	73.5	20.2
	AMF-Y	0.0	0.0	0.0	0.0	0.0	38.3	8.3	19.5	63.1	6.3	82.4	14.7	18.0	83.6	12.4
H. tuberosus	AMF-N	62.0	13.8	22.4	53.4	7.36	79.8	23.8	29.0	55.0	16.9	75.8	21.0	27.8	89.4	18.9
	AMF-Y	100	84.3	84.3	94.4	79.5	90.9	45.4	49.2	79.3	37.4	55.8	18.6	31.5	86.7	16.0
Z. mays	AMF-N	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	86.7	34.7	39.0	94.7	32.9
	AMF-Y	40.0	5.73	14.7	62.8	3.66	10.9	1.0	8.9	38.3	0.5	65.8	23.4	31.0	69.2	20.2
S. bicolor	AMF-N	0.0	0.0	0.0	0.0	0.0	40.0	1.2	3.0	38.8	0.47	28.3	3.6	14.4	73.7	3.2
	AMF-Y	0.0	0.0	0.0	0.0	0.0	20.0	0.6	2.9	8.2	0.05	34.2	3.3	11.1	79.0	2.4
L. perenne	AMF-N	0.0	0.0	0.0	0.0	0.0	31.7	8.4	25.8	59.6	4.4	82.1	36.1	43.5	86.0	30.5
	AMF-Y	36.7	3.8	10.0	59.4	2.43	66.7	22.5	25.8	41.9	15.0	82.7	27.0	31.6	90.3	23.9



Presence of AMF structures as arbuscules and vesicles at the third years (2016). Giant reed (1A and 2A); miscanthus (1B and 2B); Jerusalem artichoke (1C and 2C); maize (1D and 2D); sorghum (1E) and lolium (1F, 2F and 3F).

Soil moisture content

Considering the first two experimental years, for all crop species and soil layers the percentage soil moisture was significantly ($p<0.001$) higher during the first cropping season compared to the second one (Tab. 11).

The AMF inoculation determined a significantly ($p<0.05$) increase of soil moisture in the 0-30 cm depth soil layer in miscanthus (+22.3%), Jerusalem artichoke managed with double cut (+36.3%) and sorghum (+14.0%) compared to un-inoculated plots (Tab. 11). Instead, a significant ($p<0.05$) soil moisture decrease of -21.3% and -14.7% in presence of AMF inoculation was monitored for giant reed in the 0-30 cm layer and for Jerusalem artichoke managed with single cut in the 60-90 cm layer, respectively (Tab. 11).

Table 11 – Soil moisture content (%)

Plant species	Depth soil (cm)	Year			AMF inoculation		
		2014-2015	2015-2016	p-value	AMF-N	AMF-Y	p-value
A. donax	0-30	30.4	22.1	0.001	28.2	22.2	0.05
	30-60	33.4	18.4	0.001	25.4	23.3	ns
	60-90	32.8	15.0	0.001	22.4	20.8	ns
M. giganteus	0-30	32.1	18.0	0.001	21.8	26.6	0.05
	30-60	36.2	19.1	0.001	33.7	31.1	ns
	60-90	36.6	14.5	0.001	35.4	32.7	ns
H. tuberosus (SC)	0-30	28.9	17.1	0.001	22.7	18.2	ns
	30-60	35.5	16.5	0.001	22.1	22.0	ns
	60-90	32.2	13.8	0.001	18.4	15.7	0.05
H. tuberosus (DC)	0-30	26.3	17.7	0.001	18.9	25.8	0.05
	30-60	33.8	16.9	0.001	22.2	21.0	ns
	60-90	33.0	15.9	0.001	18.8	17.2	ns
Z. mays	0-30	28.2	13.8	0.001	19.8	22.6	ns
	30-60	33.9	17.0	0.001	29.7	28.5	ns
	60-90	34.4	18.0	0.001	32.8	29.4	ns
S. bicolor	0-30	29.0	18.4	0.001	24.3	27.7	0.05
	30-60	33.9	17.3	0.001	30.4	30.6	ns
	60-90	35.2	17.0	0.001	32.1	32.2	ns
L. perenne	0-30	26.5	12.0	0.001	18.42	16.10	ns
	30-60	30.7	16.2	0.001	25.50	28.40	ns
	60-90	32.9	16.6	0.001	29.57	31.69	ns

Soil CO_2 emissions

In 2014, in the average of the crop species, we observed a CO_2 peak emission (median value 7.2 g CO_2 m^{-2} h^{-1}) one hour after spreading with a significant decrease just after 24 hours (median value 0.8 g CO_2 m^{-2} h^{-1}), with emissions not significantly different from those measured before the spread (0.7 g CO_2 m^{-2} h^{-1}). Similarly, in 2015, we detected a CO_2 peak emission one hour after spreading (median value 4.3 g CO_2 m^{-2} h^{-1}) with a significant reduction after 24 hours (median value 0.5 g CO_2 m^{-2} h^{-1}).

During the first four measurements after DLF distribution (1-8 April 2014), in the perennial crops (giant reed, miscanthus and Jerusalem artichoke) transplanted about one mounth before digestate spreading (1 April 2014), there were no differences in soil CO_2 emissions (median value 0.40 g CO_2 m^{-2} h^{-1}) between AMF inoculated and un-inoculated plots . Also in first 6 measurements performed in 2015 (19-30 March 2015) and 4 ones of the 2016 (1-14 April 2016), after DLF distribution, no statistical differences were found in soil CO_2 emissions (median value 0.49 g CO_2 m^{-2} h^{-1} for 2015 and 0.73 g CO_2 m^{-2} h^{-1}) between AMF inoculated and un-inoculated plots in all the tested species.

Averaging all the species and considering all the measurements carried out during the crop growing season, AMF inoculation significantly ($p<0.001$) increase (+23.1%) soil CO_2 emissions as compared to un-inoculated plots (median value of 0.27 g CO_2 m^{-2} h^{-1}). Considering each crop, AMF inoculation determined a significant ($p<0.05$) soil CO_2 emissions increase for miscanthus (+40.0%) and for Jerusalem artichoke (+30.0%) compared to un-inoculated plots (Fig. 30a and Fig. 30b). No statistical difference was found in giant reed, maize, sorghum and lolium between AMF inoculated and un-inoculated plots (median values of 0.53, 0.28, 0.30 e 0.40 g CO_2 m^{-2} h^{-1}, respectively).

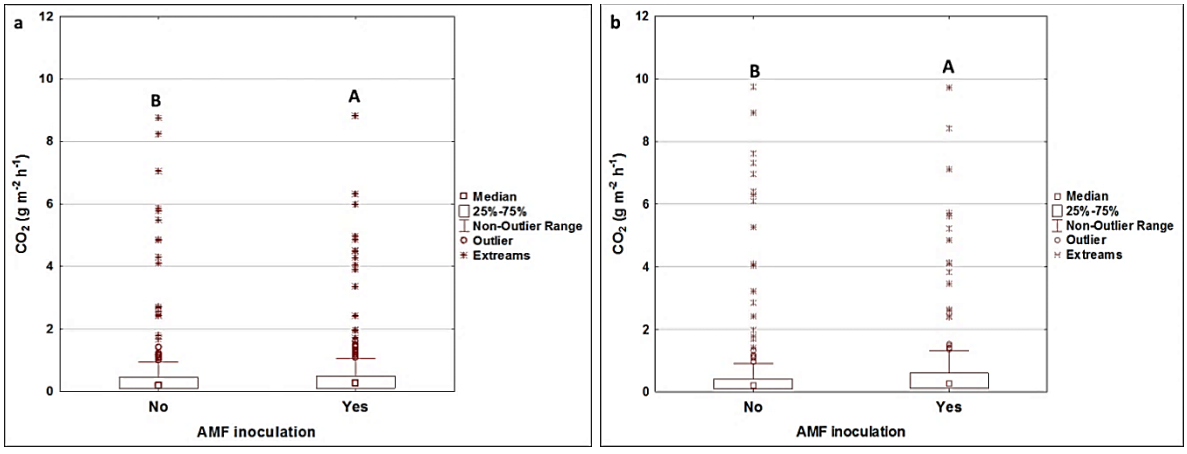


Figure 30 – AMF inoculation effect on soil CO_2 emission in a) miscanthus and b) Jerusalem artichoke. Different letters show statistical differences at $p<0.001$(Test Kruskal-Wallis).

For all crop species, except giant reed, soil CO_2 emissions were positively correlated (Spearman R) with soil temperature and negatively correlated (Spearman R) with soil moisture (Tab. 12).

Table 12 –Correlation between soil temperature and moisture and soil CO_2 emission.
Spearman's coefficients

Plant species	Soil temperature (°C)	Soil moisture (%)
A. donax	0.005^{ns}	0.028^{ns}
M. giganteus	0.5541^{***}	-0.1056^{**}
H. tuberosus	0.5142^{***}	-0.1513^{***}
Z. mays	0.5319^{***}	-0.0494^{ns}
S. bicolor	0.5905^{***}	-0.1183^{***}
L. perenne	0.6582^{***}	-0.2675^{***}

***= p<0.05; **= p<0.01; ***= p<0.001; ns= not significant**

Considering the cumulative CO_2 emissions at the end of the 25 monitoring months (05 May 2016), AMF inoculation determined a significant ($p<0.001$) cumulative CO_2-C increase (+17.7%) than un-inoculated plots (a grand median = 1619.3 g CO_2-C), with median values higher for giant reed and Jerusalem artichoke (+30.4%), and for maize (+24.5%), while, the lower median values were observed in miscanthus (+13.2), lolium (+9.70) and sorghum (+7.75%), compared to un-inoculated treatment (median values = 1134.2, 1399.8 and 1635.1 g CO_2-C for giant reed Jerusalem artichoke, and maize; and 1449.9, 2.258.8 and 1838.1 g CO_2-C for miscanthus, lolium and sorghum, respectively) (Fig. 31a and Fig. 31f).

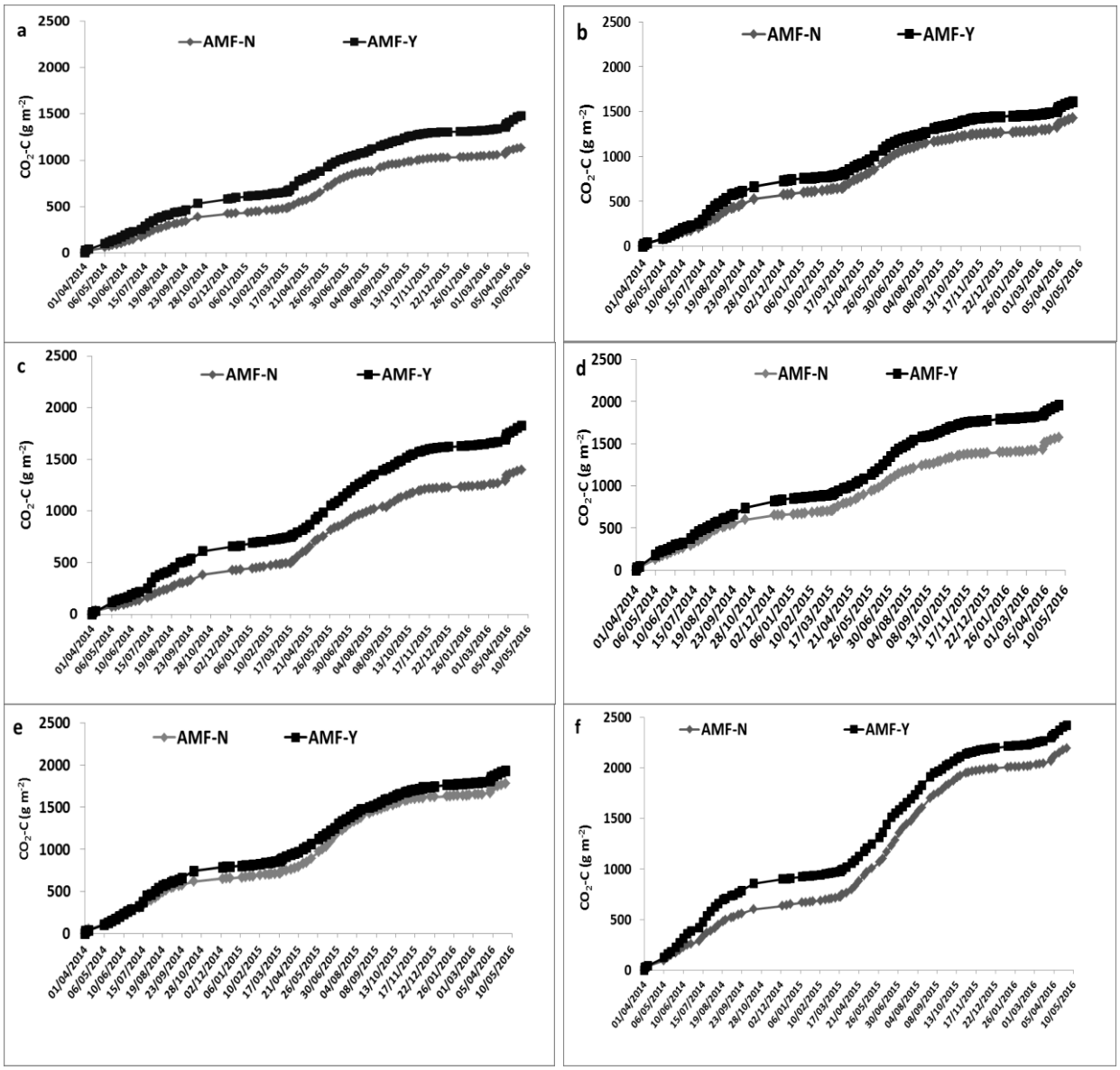


Figure 31 –Soil CO_2 emission cumulative in giant reed (a), miscanthus (b), Jerusalem artichoke (c), maize (d), sorghum (e) and lolium (f).

Biochemical Methane Potential (BMP)

A preliminary BMP analysis, performed only in the first year (2014), showed a CH_4 higher yield in absence of AMF inoculation for giant reed, miscanthus and Jerusalem artichoke managed with single cut whereas a CH_4 lower yield for maize, sorghum and lolium (Tab. 13).

Regarding to the cut biomass management, inside of the double cut in Jerusalem artichoke, the second cut showed higher CH_4 yield than the first one (Tab. 13). In sorghum and lolium the highest CH_4 yield was obtained at the second cut (Tab. 13).

Table 13 – Energy crops methane yield

Crops	cumulative CH_4 (mL)			
	AMF-N		AMF-Y	
	Mean	SD	Mean	SD
A. donax	222	5.9	214	9.4
M. x giganteus	215	1.8	213	2.2
H. tuberosus (SC)	211	8.9	189	9.0
H. tuberosus (DC) – I cut	6	2.3	7	0.9
H. tuberosus (DC) – II cut	137	87.6	199	11.0
Z. mays	5/3/71	-	115	97.6
S. bicolor (I cut)	5/96	-	169	25.5
S. bicolor (II cut)	180	9.2	214	5.6
S. bicolor (III cut)	6/131	-	209	18.0
L. perenne (I cut)	66/6	-	167/10	-
L. perenne (II cut)	192	16.0	226	6.0

***SC= single cut; *DC=double cut**

Discussion

In literature, the AMF positive effect on plant growth has been widely reported (Wu et al. 2005; Cho et al. 2009; Celebi et al., 2010; Abdel-Fattah et al., 2012; Tauler and Baranza, 2015). However, it is well known that the AMF effect is variable and dependent on plant mycorrhizal fungi interaction (Romero-Munar et al., 2017). According to this assumptions, AMF root colonization was variable during the experimental years, with different responses of the crops to the symbiosis, i.e. a positive AMF inoculation effect observed on Jerusalem artichoke managed with double biomass cutting and negative AMF inoculation effect on miscanthus. The lower AMF arbuscular abundance (A%) in the first experimental year, in all species excluding Jerusalem artichoke (with the highest A%), can be due to: 1) physical soil disturbance during the setting up of the experimental due to the boxes filling with soil in the first 50 cm depth. In fact, it is widely reported that the soil disturbance strongly negatively affects the success of the AMF inoculation and modify the AMF community (Jasper et al., 1989; Jansa et al., 2002; Verbruggen et al., 2013; Van der Heyde et al., 2017); 2) high nutrient contents in the soil used to fill the boxes. In a soil with high nutrient availability, the most of the plant nutrient uptake occurs from the rich circulating soil solution, neglecting the nutrient sources provided by AMF

symbiosis (Berruti et al., 2014); 3) high nutrients (especially N) input through DLF supply. It is known that AMF colonization decreases strongly with high fertilization input due to the reduction in carbon allocation from plant to mycorrhizas (Verbruggen et al., 2013; Berruti et al., 2014). This behavior of the plant can increase the AMF competition for limited C resources (Berruti et al., 2014).

In the second year, while Jerusalem artichoke showed an A% decline of about 53.0% compared to the first year, the other crops exhibited nil difference or slight increases of A% between the first and the second year. This result was probably due to the climate conditions recorded during 2015, that may have negatively affected AMF colonization. In particular, the lowest rains with an uneven distribution and the associated highest ET environmental demand during June and July, have led to a drastic soil moisture reduction. In nature, AMF spores must remain viable from one period of root growth to the next; however, under drought conditions, the decay in the spores viability is progressively correlated with the soil drought conditions and time of exposure which may be a critical factor in the success or AMF survival (Ruiz-Lozano et al., 1996). Few studies concern AMF sporulation under water stress conditions (Silva et al., 2015), with variable effects of soil water content on AMF spore germination in relation to fungi species and genera (Giovannetti et al., 2010). The AMF species spore's germination used in our research are strongly inhibited by osmotic potentials ranging from -0.50 to -2.20 MPa (Giovannetti et al., 2010). This latter result is in agreement with the percentage soil moisture recorded in 2015 in our study that ranged from 12.0 to 22.1% and corresponding to an osmotic pressure from about -0.50 to -0.10 MPa (Dal Ferro et al., 2016). The soil wetting and drying cycle actives the spores, determining an early and fast mycorrhizal colonization (Braunberger et al., 1996). It is well-known that the mycorrhizal spores must be well hydrate before starting their physiological activity (Tommerup, 1984). Consequently, the water stress inhibits the spores' germination and the hyphae network growing in the soil (Huang et al., 2011). The negative effects on AMF colonization registered during 2015, may be tied to the emergence of the spores germination tubes before the soil drying, and to their following damage determined by the following soil desiccation.

On the third year, the AMF inoculum persistence in the soil, taking into account that AMF inoculation was not performed also in all annual crops, was evaluated. A general AMF root colonization (A%) increase probably due to cropping systems stabilization (Berruti et al., 2014) was observed, except for Jerusalem artichoke.

In view of these finding, AMF inoculation was not efficient to promote dry biomass production in the studied crops, except in Jerusalem artichoke managed with double biomass cutting, probably due to high N input and indigenous mycorrhizal fungi presence in the plots.

Microscopic observations confirmed the presence of typical AMF structures such as arbuscules, vesicles or inter and intracellular hyphae in all studied plant species. Furthermore, the highest AMF root colonization in un-inoculated plots, could be explained by the presence of indigenous AMF communities in the soil used in the experimental trial. Indeed, it is known that AMF species introduced in a agro-ecosystem compete with local AMF communities presumably better adapted to edaphic conditions (Verbruggen et al., 2013).

In literature, the few studies on AMF effect for giant reed and miscanthus, reported a higher AMF root colonization for both perennial crops as compared to our results. In fact, Tauler and Baraza (2015), Baraza et al. (2016) and Romero-Munar et al. (2017) reported a giant reed mycorrhizal colonization ranging from 36.0 to 48.6%; whereas, for miscanthus Sarkar et al. (2015) and Firmin et al. (2015) found a mycorrhizal colonization ranging from 23.0 to 38.0%, up to maximum value of 70.0%. Although the sorghum is used as a trap culture for mycorrhizal propagation (Selvakumar et al., 2016), due to its capacity of establish AMF symbiosis and abundance sporulation, a lower AMF root colonization was found in our plants as compared to literature data, in which it ranges from 14.0 to 79.7% (Guo et al., 2013; Silva et al., 2015; Maucieri et al., 2016b). This latter results could be ascribed to the used sorghum genotype multi-cutting hybrid, that did not establish an effective AMF symbiosis. Instead, AMF root colonization in maize was in agreement with Celebi et al. (2010), Guo et al. (2013) and Tian et al. (2013), which found a mycorrhizal colonization ranging from 8.1% to 77.2%. To our knowledge there aren't specific studies on AMF root colonization of Jerusalem artichoke. Focusing on our first Jerusalem artichoke colonization data, during the three years we have found a range from 16.0% to 80.0%. In lolium our AMF root colonization results are in agreement with Hartwig et al. (2002) and Chen et al. (2007), which reported a colonization ranging from 3.2% to 25.7%.

It is a well-known that AMF improves plant nutrients (N and P) uptake efficiency (Smith and Read, 1997), particularly under limited macronutrients availability conditions (Cruz et al., 2004; Kannq et al., 2006; Li et al., 2006; Schreiner, 2007), by increasing the abilities of the host plants to explore a larger volume of soil than un-colonized plants

(Duponnois et al., 2008). Nevertheless, the carbon costs of AMF symbiosis can be high for the host plant (more than 20% of the C fixed by plant) (Bago et al., 2000). In an agriculture system under high nutrient input while the carbon costs remain the same for AMF symbiosis, the relative nutrient advantages for crops are reduced, and the mycorrhized plants performance can be lower compared to un-mycorrhized ones (Janos, 2007). However, the interaction between AMF symbiosis and fertilization remain complex and difficult to predict (Beauregard et al., 2008).

In Jerusalem artichoke managed with double biomass cutting, AMF inoculation determined the highest N biomass concentration and uptake only at the second cut. Instead, in sorghum AMF inoculation determined a reduction on N concentration biomass and uptake only in the first cut, while AMF inoculation exerted a positive effect on the P concentration biomass and uptake only in the third cut, showing the highest values than un-inoculated plots. Although Jerusalem artichoke and sorghum dry biomass productions after the first cut decreased, the highest nutrient biomass concentrations and uptakes in AMF inoculated plots as compared to un-inoculated ones, could be explained by the increase of the root absorptive surface area through the extra-radical hyphae (Amaya-Carpio et al., 2009; Kuzyakov and Xu, 2013).

Among the studied crops, only in sorghum AMF inoculation influenced positively N (at the 1st cut) and negatively P (at the 3rd cut) use efficiency, although it is widely reported that mycorrhizal symbiosis provides a major contribution to nutrient use efficiency in the crops due to increase of the plant root systems (Zhang et al., 2010).

In this study, in presence of AMF inoculation, a higher NO_3-N and lower NH_4-N leaching than un-inoculated plots was observed. This result could be explained by the AMF extra-radical hyphae preference to uptake nitrogen from soil as NH_4-N than NO_3-N, thus delivering it to the host plants (Frey and Schüepp, 1993; Johansen et al., 1993; Mäder et al., 2000; Govindarajulu et al., 2005; Tanaka and Yano, 2005). On the contrary, Bender et al. (2015) did not find any AMF effects on NH_4-N leaching losses and N plant uptake. The same authors at the end of the experimental period, reported high NH_4-N amounts in the soil, suggesting that NH_4-N was not AMF nitrogen favorite form for uptake or its translocation by AMF to plant is a slow process. Early paper showed that AMF can immobilize large nitrogen amounts in their hyphal biomass, Hodge and Fitter (2010), thus suggested that AMF hyphae could work as a sink for N, thus reducing N leaching (Bender et al., 2015). Another possible reason is that AMF presence may have promoted the microbial community that more efficiently immobilizes N (Bender et al., 2015).

Among the studied crops, the highest and lowest NO_3-N leaching losses were observed in maize and lolium, respectively. Although these crops belong to the same botanical family (Poaceae) with the same type of root system, but different spatial, density, length, hair and relative exploration of the topsoil soil volume by the roots, the lowest N leaching in lolium can be explained by its perennial nature.

AMF inoculation did not show significant effect on P leaching. The low P concentration found in percolation water, could be ascribed to the silty-loam soil used in our experiment, characterized by an alkaline pH and high Ca^{+} content. Phosphorus supplied with DLF, at contact with soil Ca carbonate, generates a calcium salt of phosphoric acid (di-calcium phosphate (DCP)) which could be partially precipitate and be absorbed by plant (Shen et al., 2011) or transformed to less available forms to plants at alkaline pH (such as octocalcium phosphate and hydroxyapatite) (Arai and Sparks, 2007) such as our soil pH.

As concerns soil CO_2 emission, AMF influence directly with their respiration and indirectly by influencing heterotrophic microorganisms the CO_2 soil flux (Cavagnaro et al., 2012). Considering all studied crops, AMF inoculation determined a soil CO_2 emission increase, in particular the highest CO_2 emission in miscanthus and Jerusalem artichoke could be ascribed to: 1) direct effect of AMF with their respiration; 2) indirect mycorrhizal effect due to the alteration of root exudation patterns that influence soil microbial activity (Lazcano et al., 2014) and the increase of roots respiration (Jones et al., 2004). In our study, the increase of soil CO_2-C emissions in presence of the AMF inoculation is in agreement with Cavagnaro et al. (2012) results who reported a higher soil CO_2 emission in plots containing mycorrhizal treatment.

Considering the above-ground biomass production in the studied crops, in giant reed and miscanthus the biomass harvested at the end of the first year was quite low due to the transplanting date, occurred in late February 2014. In fact, in the following two years, total above-ground dry biomass (DBP) produced by giant reed, was about 2.2 (2015) and 3.0 (2016) times higher as compared to the 2014, confirming the DBP rapidly increase from the young to mature crop (Angelini et al., 2005, 2009). Also shoot density followed the same trend of DBP. Nevertheless, the highest giant reed shoot dry weight per plant observed in the first year (172.0 g $shoot^{-1}$), compared to following years (134.0 and 163.0 g $shoot^{-1}$ in 2015 and 2016, respectively), can be ascribed to favorable climate conditions which have also significantly promote the culm diameter and leaf number. In miscanthus, although all bio-agronomic features and shoot density were higher on the first year, the

single shoot dry weight in 2014 was -30% and -50% lower as compared to 2015 (37.0 g shoot^{-1}) and 2016 (52.0 g shoot^{-1}); but despite this behavior, a quite low total above-ground dry biomass was observed at the end of the first and second year, and higher yield of about 2.2 time in 2016. Several studies carried out on the two above mentioned perennial crops, reported variable total above-ground dry biomass production ranging, for giant reed, from 22.0 to 47.0 Mg ha^{-1} (Angelini et al., 2005, 2009; Nassi o Di Nasso et al., 2011a, 2011b; Traina et al., 2015) up to maximum value of 99.0 Mg ha^{-1} under non-limiting water and N availability (Borin et al., 2013) and for miscanthus, ranging from 29.0 to 43.0 Mg ha^{-1} (Angelini et al., 2009; Nassi o Di Nasso et al., 2011a, 2011b; Traina et al., 2015). In both perennial crops, the highest N biomass concentrations was observed in 2014 and the lowest P biomass concentrations was in 2015. In giant reed, at the higher N biomass concentrations, corresponded the lowest N uptake due to the lowest total aboveground dry biomass; conversely at the lowest P biomass concentrations in 2015, a higher P uptake was observed probably due to the about 2.2-time higher total aboveground dry biomass than 2014. Despite the reduction in giant reed N biomass concentration in 2015 and 2016, we observed an opposite trend in the nutrient uptake, due to the higher total aboveground dry biomass. The high N biomass concentration in the 2014 for giant reed and miscanthus, could be attributed to predominance of young vegetative tissues (Kering et al., 2012).

In miscanthus although in the 2015, the N (-46%) and P (-52%) concentrations were lower compared to 2014, the slightly total aboveground dry biomass increase (+17.0%) was not able to compensate the lower nutrients concentration, determining the lowest N and P uptake. In miscanthus, Cosentino et al. (2007) reported a lower N concentration (values ranging from 0.53% to 0.63%) and N uptake (value ranging from 90.0 Kg ha^{-1} to 160 Kg ha^{-1}) compared to our study. For the same crop Cadoux et al. (2012) reported in P concentration (0.08%) and uptake values (22.5 Kg P ha^{-1}) in agreement with our results.

Jerusalem artichoke is a perennial rhizomatous grass (Heuzè et al., 2015). The favorable climate conditions of the first experimental year positively promoted all its bio-agronomics characteristic, in both biomass cutting managements, determining the highest above-ground dry biomass. Several studies indicated that Jerusalem artichoke is sensitive to water stress with negative effect on tubers yield and plant biomass production (Denoroy, 1996; Schittenhelm, 1999; Danuso et al., 2002; Monti et al., 2005). Our results are in agreement with these previous studies, confirming for this species, the above-ground biomass yield reduction in the dry cropping seasons. The shoot density increase

during the experimental years may be due to the formation of new tubers (or secondary tubers; Denoroy et al., 1996) that were not harvested during experiment. However, during experiment, the total above-ground dry biomass production decreased mainly in relation to the higher shoot intra-specific competition for water, nutrients and solar radiation availability.

In Jerusalem artichoke the dry biomass production, in the mean of the years and cutting managements (21.6 Mg ha^{-1}), was slightly lower compared to Baldini et al. (2004) (25.0 Mg ha^{-1}) and in line with Curt et al. (2006) (21.8 Mg ha^{-1}) and Matìas et al. (2013) (22.7 Mg ha^{-1}). Lower above-ground biomass production than our study was obtained by Liu et al. (2011) under drought soil and climatic condition (15.3 Mg ha^{-1}), cultivating 59 Jerusalem artichoke clones in 24 provinces of China.

The higher N and P biomass concentration of Jerusalem artichoke managed with double biomass cutting as compared to the single one is ascribed to the different phenological stage at which the harvest was carried out. It is well-known as the tissues nutrient concentrations change during the growing season, with higher values during the full growth activity (between July and August) as compared to late summer and winter period (Nassi o Di Nasso et al., 2011a). This explain because, in Jerusalem artichoke managed with a double biomass cutting, at the first cut, performed in June, the highest N and P biomass concentration obtained, combined with the highest dry biomass production, determined the highest N and P uptake values. The lower N and P uptake, observed at the second cut, may be explained by the short period between the first and second cut, which did not allow an efficient remobilization of the nutrients from the below-ground biomass to the above-ground one, resulting in a lower N and P concentration and also to the lowest above-ground biomass production.

Considering the annual crops, on the first year, the highest total above-ground dry biomass production in maize and sorghum, can be ascribed to the favorable climate conditions which promoted a higher leaf number culm height and diameter (except for sorghum). In the following two years, the total aboveground biomass production was drastically reduced in both crops, particularly during the driest second year. Comparing the annual biomass production in the three years, although maize and sorghum showed similar production in the 2014, in the following two years, sorghum showed a higher yield (+14.0% for 2015 and +40.0% for 2016) than maize. Unlike maize which is sensible to water stress (Pandey et al., 2000; Çakir, 2004) sorghum is a drought tolerant crop (Paes de Camargo and Hubbard, 1999), and its higher yields in the 2015 and 2016, can be

partially explained by its ability to capture and extract the water deep in the soil profile due to morphology of its root system (Wright and Smith, 1983; Singh and Singh, 1995), and osmotic adjustment at low levels of leaf water potential (Ludlow et al., 1990; Girma and Krieg, 1992).

The sorghum shoot density showed a progressive increase from the first cut to the third one during the three experimental years, with a significantly reduction in the second and third year comparing the first one, due to the lower tillering consequent to the low soil water availability. Sorghum shoot density increased as a result of the cuts. These results are confirmed by Duncan and Gardener (1984) that reported a shoot density increase (ranging from 4.0% to 22.0%) at the second cut in 10 sweet sorghum cultivars. The increase of shoot density has resulted in a progressive decrease of all bio-agronomic traits and aboveground biomass production according to with Iptas and Brohi (2003) who showed a decrease (ranging from -8.0% to -21%) from the first to the third cut in above-ground dry matter of sorghum-sudangrass hybrid.

Above-ground biomass production in maize, in the average of the study years (22.5 Mg ha^{-1}), was in line with the yields reported by Farrè and Faci (2006) (21.4 Mg ha^{-1}), Di Paolo and Rinaldi (2008) (23.5 Mg ha^{-1}), Kerckhoffs et al. (2012) (from 12.0 to 33.7 Mg ha^{-1}) and Ra et al. (2012) (20.1 Mg ha^{-1}). Instead the sorghum above-ground biomass production (26.2 Mg ha^{-1}) was higher than previous studies (Farrè and Faci (2006) (18.3 Mg ha^{-1}), Kerckhoffs et al. (2012) (24.9 Mg ha^{-1}) and Ra et al. (2012) (25.3 Mg ha^{-1}).

During the three trial years, in maize we have observed the same trend for N and P biomass concentrations and uptakes, with the highest values in the 2014 due to the highest total above-ground dry matter. In sorghum, at the higher N biomass concentration registered in the 2016 cropping season, corresponded a lower N uptake, due to the lower aboveground biomass production. The lowest P biomass concentration and uptake observed in maize and sorghum in the 2015, could be attributed at the lower soil moisture content (about -0.25 MPa and -0.20 MPa in deep soil 0-90 cm, respectively). Although few studies were carried out on this issue, it is well documented that low soil water content greatly reduces the diffusion rate of some ions, including phosphorus (Dunham and Nye 1976; Mackay and Barber 1985). Jupp and Newman (1987) in pots experiment on lolium reported a cessation of phosphorus uptake likely due to reduction in the diffusion rate of phosphorus to the root surface in the drying soil.

Considering the cuts management in sorghum, the highest N and P biomass concentration and the lowest N and P uptake were observed at the third cut. This result is due to a

higher nutrient absorption from soil promoted by the biomass cutting and at the same time to a lower above-ground dry biomass production.

Lolium, during the experimental activities, was managed with five cuts. In the 2014, in the first two cuts the lower total above-ground dry matter observed, can be explained by the crop establishment period. In fact, after this period, at the third (2015) and fourth (2016) cut, the highest total above-ground dry matter was found. Lolium is negatively affected by drought stress (Liu and Jiang, 2010) and requires a large amount of water to sustain its growth (Liu and Jiang, 2010; Sampoux et al., 2011, Turner et al., 2012). For this reason, after the third cut (2015), it was not possible schedule a further cut in October, due to the driest second year, which has determined a reduction and the end of the shoot growth, and subsequently the beginning of leaf lamina senescence (Blum, 1996).

In lolium, the decrease in N biomass concentration from first to fifth cut during the three experimental years could be attributed at DLF splash-plate distribution method that reduce full N crop availability as compared to the distribution in the soil layer. In fact, before sowing (in 2014), the DLF after spreading was incorporated in the soil through a minimum tillage, whereas in the following distribution (2015 and 2016), DLF was only spreaded on soil. Considering N losses from the soil surface, N volatilization after spreading must be taken in account, considering DLF chemical characteristics, especially high NH_4-N:TN ratio and high pH value. DLF with a high pH, directly in contact with the atmosphere, determined NH_4-N change into ammonia (NH_3) and its volatilization into air (Maurer and Müller, 2012; Nkoa, 2014). Pacholski et al. (2010) estimated a NH_3 volatilization between 7 and 24% of applied NH_4-H. Comparing the distribution methods, Wulf et al. (2002) quantified NH_3 gas emissions at about 350, 275, 160 and 50 mg NH_3-N m^{-2} h^{-1} in relation to splash plate, trailing shoe, harrow and injection methods respectively, within the first 10 h following DLF distribution. Nevertheless, the lower lolium N biomass concentration (-30%) and the highest N uptake at the third cut as compared to the first one, are explained by the higher aboveground biomass production.

It is widely reported that the P has poor mobility in the soil, with low concentrations in the soil solution and a large part of it is linked to diverse soil minerals. The adsorption/desorption and precipitation/dissolution equilibria, control the P concentration in the soil solution and, thereby, both its chemical mobility and bioavailability. In our study, in the three years, the lolium P tissue concentration was more or less stable, due to the loam-clay soil nature and/or alkaline pH soil. Despite, such as for N uptake, also the

highest P uptake found at the third cut, can be attributed to the higher aboveground dry biomass production.

Comparing the studied species, sorghum showed the highest N_{ue} followed by miscanthus, whereas considering P_{ue} both crops presented the highest values. Our N_{ue} values for both crops are in agreement with the data obtained by Olson et al. (2013), which reported values ranging from 111 to 370 g DM g^{-1} N^{-1} for sorghum and from 125 to 333 g DM g^{-1} N^{-1} for miscanthus. In our hybrid sorghum genotype for biomass production, the high N_{ue} could be explained by its long vegetative growth phase, due to the multiple cuts, efficient light interception, and radiation use efficiency (Mullet et al., 2014). The miscanthus N_{ue} could be ascribed by its low nutrient requirement during the crop life cycle (Cosentino et al., 2007; Cadoux al., 2012) due to nutrients translocation from aboveground biomass to the rhizome during the autumn/winter season and vice versa through spring growth (Lewandowski et al., 2000; Olson et a., 2013). Generally, the improvement of nutrient use efficiency is desirable, since it reduces the fertilizer input and negative environmental impacts, maintaining a good agriculture yield (Bender et al., 2015).

Considering the crop C3 and C4 physiological categories, the C4 species are more efficient convertors of sunlight into biomass and water use compared to C3 ones (Sage and Monson, 1998; Byrt et al., 2011). Nevertheless, in our study under the same environmental conditions and fertilization rate, the giant reed (C3 specie) compared to the other species, was more performant in term of dry biomass produced. However, this result could have been influenced by an uneven distribution of the rainfall during the experimental years. In fact, in the Mediterranean environment, when rarely water availability is not a limiting factor, a C4 species such as miscanthus would be able to optimize its biomass accumulation compared to C3 species such as giant reed (Nassi o Di Nassi et al., 2011b).

Considering CO_2 soil emission, the rapid flux during the first hour after DLF distribution, can be attributed to release of CO_2 dissolved in the digestate as well as to the rapid microorganism respiration of easily degradable C compounds (Bol et al., 2003; Fanguerio et al., 2010). Soil CO_2 emission trend after spreading is in line with previous studies carried out in laboratory conditions (Grigatti et al., 2011; Chen et al., 2012). After digestate distribution, these latter authors reported a very intensive soil CO_2 emission within of the first 24 h (Grigatti et al., 2011) and 48 h (Chen et al., 2012), which subsequently decreased as compared to the control. With the only exception for giant reed, soil CO_2 emission monitored in the plots cultivated with the other species was

positively correlated with temperature highlighting the positive effect of this parameter on microbial activity and negatively correlated with soil moisture, indicating the effect on organic material decomposition (Sänger et al., 2011) exerted by soil aerobic metabolism. In fact, high water content in soil profile reduce air permeability and gas diffusivity (Saggar et al., 2008; Ball, 2013) negatively influencing the oxygen availability for soil aerobic microbial population. However, the simultaneous effect of soil moisture and temperature on soil CO_2 emissions should be also taking into account (Maucieri et al., 2017) as reported in Suseela et al. (2012), who found that soil respiration proceeded fastest at the warmest temperatures when soil water content ranged from 20% to 30%.

Biochemical Methane Potential (BMP) is a procedure widely used to determine the methane yield of a given organic matter, including crop biomass, during its anaerobic degradation (Raposo et al., 2011). Di Girolamo et al. (2013) in a laboratory experiment evaluating the effect of hydrothermal pre-treated on giant reed methane yield, reported a CH_4 yield ranging from 273 to 337 mL g^{-1} VS for untreated and pre-treated without acid catalyst. Ragaglini et al. (2014), in the same crop, investigating the effect of different harvest times, reported a CH_4 yield ranging from 258.3 to 391.7 mL g^{-1} VS. In an experimental farm in North-Italy (Bologna), three perennial species (giant reed, switchgrass, and an inter-specific hybrid sorghum, known as sorghum Silk) and four annual crops (three sorghum genotypes, namely a fiber, sweet and forage hybrid and one maize hybrid) were evaluated for their methane yield (Barbanti et al., 2014). This latter study reported a CH_4 yield of 217 mL g^{-1} VS for giant reed, 271 mL g^{-1} VS for sorghum Silk, 316 mL g^{-1} VS for maize, and a range from 251 to 268 mL g^{-1} VS for the three sorghum genotypes. Chandra et al. (2012), in their review, among the major lignocellulosic crop biomass, showed an average approximate CH_4 yield in maize of 338 m mL g^{-1} VS. On *Miscanthus x giganteus*, Wahid et al. (2015) in mesophilic conditions, reported a CH_4 yield of 234.1 mL g^{-1} VS at 30 incubation days and 303.2 mL g^{-1} VS at 90 incubation days. Concerning *Lolium perenne*, Xie et al. (2011) studying the effects of alkal-termal pre-treatment, reported a CH_4 yield ranging from 325.8 (control) to 452.5 mL g^{-1} VS in NaOH treated samples . In our study, the CH_4 yields, measured only in the first experimental year, were widely variable in relation to crops biomass, AMF treatments and cutting management. Nevertheless, our CH_4 yield are lower than those reported in the literature. This finding can be attributed at the different analytical method used. In fact, we analyzed dry biomass, whereas, the reported studies the fresh one. In view of these finding, further and

more detailed analysis are needed to confirm the methanogenic potential of the six energy crops studied.

Conclusions

Our study showed that AMF inoculation was not able to enhance dry biomass production in the studied crops, with the only exception of the Jerusalem artichoke managed with double biomass cuts, probably due to the high N input and indigenous mycorrhizal present in all plots. Nevertheless, a positive environmental contribution was provided by AMF inoculation in relation to NH_4-N leaching reduction; conversely an increase in NO_3-N leaching and soil CO_2 emission were measured. Considering dry biomass production, giant reed (C3 plant) is confirmed to be the most productive among the studied energy crops, followed by miscanthus, sorghum, maize, Jerusalem artichoke, and lolium. On the other hand, giant reed showed a low nitrogen use efficiency and higher cumulative soil CO_2-C emissions (+30.4%). Instead, sorghum and miscanthus showed the best N and P utilization and the lower cumulative soil CO_2-C emissions. Considering the two annual crops (maize and sorghum), sorghum showed the highest biomass production, probably due to its drought tolerance and NUE.

In view of these finding, the use of DLF as organic fertilizer for biomass production and energy crops with NUE relatively high, can be considered a viable alternative to reduce mineral fertilization inputs and negative environmental impacts. Further researches are need to investigate the role of indigenous mycorrhizal community on crop production using organic fertilizer and their interaction with bio-fertilizer based on AMF.

Annex I

Considering all the annual and perennial crops studied, the root samples after begin washed with Tween 20 and rinsed several times in tap water, and subsequently they were cleared and stained with different procedures as follows:

Arundo donax L. (common name: Giant reed)

- ✓ Root samples were cleared in 10% KOH for 30 min, rinsed three time with tap water stained with 5% ink-vinegar (Pellikan Blue) for 1 hour and de-stained in tap water for 30 min.;

Miscanthus x giganteus Greef et Deu. (common name: Miscanthus)

- ✓ Root samples were cleared in 10% KOH for 10 min, rinsed three time with tap water stained with 5% ink-vinegar (Pellikan Blue) for 10 min., and de-stained in tap water for 5 min.;

Helianthus tuberosus L. (common name: Jerusalem artichokes)

- ✓ Root samples were cleared in 10% KOH for 20 min, rinsed three time with tap water stained with 5% ink-vinegar (Pellikan Blue) for 20 min., and de-stained in tap water slightly acidified for 10 min.;

Zea mays L. (common name: Maize)

- ✓ Root samples were cleared in 10% KOH for 10 min, rinsed three time with tap water stained with 5% ink-vinegar (Pellikan Blue) for 30 min., and de-stained in tap water slightly acidified for 15 min.;

Sorghum bicolor (L.) Moench (common name: Sorghum)

- Root samples were cleared in 10% KOH for 10 min, rinsed three time with tap water stained with 5% ink-vinegar (Pellikan Blue) for 30 min., and de-stained in tap water slightly acidified for 15 min.;

Lolium perenne L. (common name: Lolium)

- Root samples were cleared in 10% KOH for 3 min, rinsed three time with tap water stained with 5% ink-vinegar (Pellikan Blue) for 5 min., and de-stained in tap water slightly acidified for 5 min.;

Furthermore, all the root samples crops studied were also cleared and stained, as follow:

- Root samples were cleared with 10% KOH (45°C) for about 1 hour, rinsed three time with tap water, stained with 0.1% cotton blue in 80% lactic acid overnight, and then de-stained in 80% lactic acid for 48 hours.

Chapter III

Effects of mycorrhizal inoculation and digestate fertilization on triticale biomass production from fungicide-coated seeds

Abstract

Crop fertilization management using organic wastes and arbuscular mycorrhizal fungi (AMF) inoculation can play a crucial role in the agro-ecosystems sustainability. However, in the conventional agricultural systems, agrochemicals as fungicides can reduce the positive effect of AMF. The aim of this study was to evaluate the agronomic (biomass production) and environmental (soil CO_2 emission) effects of AMF inoculation and digestate spreading on triticale cultivation, using commercial seeds coated with fungicide. The field experiment was carried out in 2014-2015 at the University of Padua experimental farm (Italy), adopting a split-plot design, where the main plot was AMF inoculation (inoculated vs un-inoculated) and sub-plots were fertilization treatments (NF=no fertilization; DL=digestate liquid fraction; DS=digestate solid fraction; MF=mineral fertilization). Low AMF root colonization was observed, likely due to the effect of fungicide. AMF inoculation effect determined only a significant lower shoot density. Dry biomass yield was significantly higher in MF treatment (21.8 ± 1.04 Mg ha^{-1}) and lower in NF treatment (14.5 ± 0.73 Mg ha^{-1}), whereas, no significant difference was found between DS and DL treatments, with an average yield of 17.2 ± 2.10 Mg ha^{-1}. Soil CO_2 emissions, during cropping season, were not significantly different, if we consider both AMF inoculation and fertilization treatments with a median value of 447.3 mg m^{-2} h^{-1}.

Introduction

During the past century, agriculture industrialization induced a significant productivity increase, which led to a greater amount of food available to population (Pérez-Montano et al., 2014). On the other hand, in the last 30 years, the climate change caused by human activities has led, from year to year, to a significantly decrease of yield in the major cultivated crops (maize, soybean, rice and wheat) in the global harvest area (Iizumi and Ramankutty, 2016; Lesk et al., 2016). Furthermore, the unsuitable soil agronomic management practices, have promoted soil degradation and loss of organic matter and fertility, increasing production costs (to maintain high crops yield) and contributing to CO_2 emissions (Montemurro et al., 2007; Lal, 2008). Soil organic matter as well known, plays a significant role in preserve and improve soil fertility, by its positive effects on soil physical, chemical and biological proprieties (Montemurro et al., 2004), increasing soil carbon stocks (Raviv et al., 1998; Caravaca et al., 2002).

The formation of arbuscular mycorrhizal fungi (AMF) associations between the roots of many terrestrial plant species are widespread in the natural environment and can provide considerable benefits to the host plant (Gosling et al., 2006; Cavagnaro, 2014; Berruti et al., 2015). In particular, AMF play an important role in crop nutrition, by greatly increasing the absorptive surface of the root system. Moreover, the AMF rapid growth and high plasticity enable the mycorrhizal hyphae to exploit nutrient patches in the soil (Tibbett and Roots, 2000; Facelli and Facelli, 2002) and to increase uptake of nutrients in inorganic form, principally immobile phosphate (P) (Koide, 1991; George et al., 1995; Clark and Zeto, 2000) and nutrients from organic sources (Hodge et al., 2001; Hodge and Fitter, 2010). In addition, influencing soil microorganisms AMF indirectly affect soil biochemical reactions including organic matter mineralization and nitrification (Hamel, 2004). AMF association may also increase host plant resistance/tolerance against biotic (Hol and Cook, 2005; Akhtar and Siddiqui, 2008) and abiotic stresses, including salinity, drought and pollution (Franco-Ramírez et al., 2007; Giri et al., 2007; Sudová et al., 2007; Cartmill et al., 2008; Debiane et al., 2008, 2009; Campagnac et al., 2010). In return, the AMF receive carbon (C) from the host plant. Unsuitable agricultural practices, affect soil microorganisms activities; soil tillage, chemical fertilizers (Borriello et al., 2012; Berruti et al., 2014) and agro-chemical products, such as fungicides, used in coated seeds to control pathogens, can exhibit undesirable effects on non-target plant-beneficial microorganisms such as AMF (Campagnac et al., 2008), causing in the AMF communities a reduction in the number of individuals and species diversity (Gosling et

al., 2006). Several authors, report higher levels of AMF colonization, higher propagule numbers or higher diversity in organic farming (Bending et al., 2004; Oehl et al., 2003, 2004) and AMF is assumed that can compensate for the reduced use of P fertilizers (Galvez et al., 2001). However, the actual importance of AMF in the enhancing of resilience and functions of ecosystem and agro-ecosystems, in particular to crop performance, remains to be determined (Gosling et al., 2006).

The application of high quality organic materials as soil amendment and/or fertilizer, is the basis to support low-input sustainable agriculture, increasing or preserving soil organic matter content (Mäder et al., 2002) and improving fertility and optimizing crop production (Diacono and Montemurro, 2010).

The most important advantage of organic fertilizers is their participation in the natural nutrient cycle, while inorganic fertilizers are additional to it (Monnet, 2003). Digestate is the byproduct of the anaerobic digestion and due to process characteristics it is considered a good quality soil fertilizer and/or amendment (Nicoletto, 2013a, 2013b, 2014; Maucieri et al. 2016a). Its chemical composition depends on the feedstock and can therefore vary (Möller and Müller, 2012). The use of digestate (liquid and solid fractions) for soil fertilization and/or amendment, has led to an improvement of soil fertility with a decrease of the amount of chemical fertilizer used in cropping systems (Möller and Müller, 2012; Alburquerque et al., 2012).

Triticale (*x Triticosecale* sp. Wittmack ex A. Camus 1927) is an artificial hybrid cereal produced by a crossing between female parent wheat (*Triticum* spp. L.) with male parent rye (*Secale cereale* L.). This crossing has provided to triticale high agronomic features as yield of wheat and rusticity of rye. Interest for triticale utilization has steadily grown and triticale cultivars have been grown in more than 30 countries (McGoverin et al., 2011), because it is a suitable alternative to other cereals (Bassu et al., 2013). In Europe, in the last 13 years, the total area under cultivation for triticale has increased of about 45% (FAOSTAT, 2014). On the basis of its agronomic features, triticale is an interesting crop in Mediterranean optimal and marginal cultivation areas (Ehdaie et al., 2001; Giunta et al., 2003). Currently, this crop is grown for grazing, fresh forage, silage, and hay but even for human feed and bioethanol production. Furthermore, cereal straw is a main animal feed source and the use of triticale straw is in continuous expansion, especially in Mediterranean and semi-arid Countries (Cazzato et al., 2012).

The aim of this study was to evaluate the agronomic (biomass production) and environmental (soil CO_2 emission) effects of AMF inoculation and digestate spreading on triticale cultivation using commercial seed coated with fungicide.

Materials and Methods

Experiment description

The experiment was carried out in 2014-2015 cropping season at the University of Padua Experimental Farm "Lucio Toniolo", North-East Italy (45°20' N; 11°57' E) under field conditions. The climate of the site is sub-humid (Köppen climate classification), with a mean annual rainfall of about 850 mm, fairly uniformly distributed throughout of the year. The temperature increases from January (average minimum value: −1.5 °C) to July (average maximum: +27.2°C). According to the FAO-UNESCO classification the soil is a fulvi-calcaric Cambisol with a loamy texture. The adopted experimental design for the trial was a split-plot with three replications. AMF inoculation was the main plot (inoculated (AMF-Y) vs un-inoculated (AMF-N)), whereas fertilization treatments were the sub-plots (no fertilization (NF), mineral fertilization (MF), digestate liquid fraction (DL) and digestate solid fraction (DS)) randomly distributed. Each plot had a size of 16 m^2 (4x4m) for a total of 24 plots. Mineral fertilization has been distributed in three times as follows: i) before sowing: 40 kg N ha^{-1} as ammonium nitrate, 65 kg P ha^{-1} as superphosphate and 65 kg K ha^{-1} as potassium sulfate; ii) top-dressed: 50 kg N ha^{-1} as ammonium nitrate provided at 116 and 173 days after sowing (DAS) respectively. Organic fertilization with DL and DS were supplied manually before sowing, at rate equivalent to 140 kg N ha^{-1}. Due to their chemical composition (Tab. 1), 16 kg P ha^{-1} and 88 kg K ha^{-1} for DL and 105 kg P ha^{-1} and 109 kg K ha^{-1} for DS were also provided.

Triticale was sown on 28 October 2014, at a rate of 220 kg seeds ha^{-1}. In the trial were used seeds coated with fungicide (Celest® Trio Syngenta AG, Basel, Switzerland = 2.34% Fludioxonil, 2.34% Difenoconazoloe and 0.93% Tebuconazoloe). AMF inoculation was done with a commercial inoculum (MICOSAT F wp – CCS – Aosta, Italy: based on mycorrhizal fungi: *Funneliformis mosseae*, *F. caledonius*, *F. coronatus*, *Septoglomus viscosum*; saprophytic fungi: *Trichoderma harzianum*; and rhizosphere bacteria: *Pseudomonas fluorescens*, *Bacillus subtilis*, *Agrobacterium radiobacter*), mixed with seeds and at the sowing distributed at a dose of 1.2 kg ha^{-1}. After 149 DAS, a second inoculation was performed, distributing for each plots of AMF-Y treatment 1.6 g of commercial mix inoculum suspended in 10 liters of water.

Table 1 – Main chemical-physical characteristics of the digestate on dry weight basis. (DM = dry matter)

Parameter	Liquid fraction	Solid fraction
Dry Matter (%)	6.16	24.68
TKN (% DM)	9.09	2.23
NH_4-N (% DM)	5.10	0.94
NO_3-N (% DM)	0.08	0.03
P (% DM)	1.02	0.72
K (% DM)	5.41	1.53
Ca (% DM)	1.57	0.70
Mg (% DM)	0.94	0.62
Na (% DM)	0.46	0.12

Roots sampling and analysis

Root samples from three randomly selected plants per plot were collected during the growing season at three harvest time points (114, 177 and 223 DAS) with a hand-operated soil probe (5 cm diameter) at 20 cm depth. Roots were washed clean of soil with some drops of Tween 20 and then rinsed several times in tap water. Then, roots were cleared with 10% KOH, stained with 5% ink-vinegar and de-stained in distilled water (Vierheilig et al., 1998). AMF colonization percentages were estimated according to Trouvelot (1986), for each treatments as follows: F%= mycorrhization frequency (the percentage of root fragments showing fungal colonization), M%= AMF colonization intensity (the percentage of fungi structures referred to the whole root system), m%= AMF colonization intensity (the percentage of fungi structures referred to colonized root fragments), a%= abundance of arbuscules (percentage of arbuscules presence referred to the root fragments showing fungal colonization); A%= abundance of arbuscules (percentage of arbuscules presence referred to the whole root system).

Triticale bio-agronomic measurements

During growing season, four times from 143 to 178 DAS, the Normalized Difference Vegetation Index (NDVI) (APS1-CropCircle, Holland Scientific, Lincoln, NE, USA) was measured.

On 8 June 2015, the aerial biomass was harvested (at dough stage – BBCH scale (Hess et al., 1997) and the dry weight was determined by drying in a thermo-ventilated oven at 65°C until the constant weight was reached. Furthermore, at the harvest time, culm height and shoot density in each plot were detected.

Soil CO_2 emission

During growing season, seven times from 78 to 178 DAS, soil CO_2 emissions were measured in each plot. CO_2 flux was measured with the static non-stationary chamber

technique (Maucieri et al., 2016a) using a chamber with a volume of 5 L and 10 cm square base. Soil CO_2 flux was determined by measuring the temporal change in CO_2 concentration inside the chamber using a portable IR instrument (Geotech G150), detecting CO_2 concentrations at levels of parts per million.

CO_2 flux was calculated using the following formula:

$$CO_2 = \frac{V}{A} * \frac{dc}{dt}$$

where CO_2 flux is expressed in mg CO_2 m^{-2} s^{-1}; V (m^3) is the volume and A (m^2) the footprint of the flux chamber; 'c' is the CO_2 concentration (mg CO_2 m^{-3}) and 't' the time step (s).

In each CO_2 measurement point, soil temperature and moisture (TDR 100 Soil Moisture Meter) in the first 7.5 cm were also detected.

Cumulative $CO_{2(eq)}$ emission saving due to substitution of mineral fertilization with digestate fractions was calculated considering the quantity of macronutrients supplied (N, P_2O_5, K_2O), and using the specific emission factors reported in Capponi et al. (2012). Particularly, considering mineral fertilizers production, the estimated avoided $CO_{2(eq)}$ emissions for were of 3.26 kg $CO_{2(eq)}$) for each kg of N, 2.01 kg $CO_{2(eq)}$ for each kg of P_2O_5 and 1.41 kg $CO_{2(eq)}$ for each kg of K_2O.

Statistical analysis

AMF colonization percentage values were arccosine transformed; bio-agronomics and AMF colonization percentage values were subjected to a two-way analysis of variance (ANOVA) to assess the interactions between the two fixed factors (AMF inoculation and fertilization regimes). The data were post-hoc tested ($p<0.05$) using the Fisher LSD test.

Soil CO_2 emission data were not normally distributed so they were analyzed with Kruskal-Wallis and Mann-Whitney non-parametric tests. Correlation between soil temperature and moisture with CO_2 emissions were evaluated using Spearman Rank correlation.

Results

Root mycorrhization

Triticale roots did not show any mycorrhizal colonization at 114 and 177 DAS. Instead, at harvest time, which coincided with hard-dough stage (223 DAS), AMF root colonization was observed, without significant difference between inoculated and un-inoculated plots. AMF colonization values ranged from 5.0% to 85.0% for F%, from 0.1% to 19.2% for

M%, from 1.0% to 29.5% for m%, from 0.0% to 60.6% for a% and from 0.0% to 11.6% for A%.

Regardless the AMF inoculation, DS treatment showed significantly ($p<0.05$) higher values for F%, M% and A% compared to the other treatments (Tab. 2).

Table 2 - Fertilization treatment effects on AMF colonization at 223 days after sowing (mean ± SE).
MF = mineral fertilization, DL = digestate liquid fraction, DS = digestate solid fraction and NF = no fertilization. F% = mycorrhization frequency (the percentage of root fragments showing fungal colonization), M% = AMF colonization intensity (the percentage of fungi structures referred to the whole root system), m% = AMF colonization intensity (the percentage of fungi structures referred to colonized root fragments), a% = abundance of arbuscules (percentage of arbuscules presence referred to the root fragments showing fungal colonization); A% = abundance of arbuscules (percentage of arbuscules presence referred to the whole root system). Different letters indicate significant differences among fertilization treatments for Fisher LSD test at $p<0.05$. ns = not significant difference.

Mycorrhizal Index	Fertilization			
	MF	DL	DS	NF
F%	19.2 ± 5.4^{b}	13.3 ± 2.8^{b}	70.0 ± 7.5^{a}	17.5 ± 4.2^{b}
M%	2.8 ± 1.9^{b}	1.9 ± 0.6^{b}	11.3 ± 2.8^{a}	3.8 ± 1.7^{b}
m%	9.3 ± 4.3^{ns}	14.6 ± 4.1^{ns}	15.5 ± 3.7^{ns}	18.3 ± 7.8^{ns}
a%	36.4 ± 12.4^{ns}	20.4 ± 9.5^{ns}	50.5 ± 3.2^{ns}	30.4 ± 10.0^{ns}
A%	1.3 ± 0.8^{b}	0.5 ± 0.24^{b}	6.0 ± 1.7^{a}	1.7 ± 0.8^{b}

Triticale bio-agronomic traits and biomass yield

No statistical difference was determined by AMF inoculation on the culm height (Tab. 3) and NDVI index (Tab. 4), with a grand mean of 125 ± 6.9 cm plant^{-1} and 0.64 ± 0.07, respectively. The MF treatment has determined a significantly ($p<0.05$) greater culm height (+8.3%) than NF one (120.2 ± 6.8 cm plant^{-1}); whereas DS and DL treatments did not show statistical difference on culm height, with a grand mean of 125.3 ± 5.7 cm (Table 3). No significant interaction ($p=0.387$) showed fertilization and AMF inoculation tretments on culm height. In absence of AMF inoculation, shoot density showed a significant ($p<0.05$) increase of +16.4% as compared to AMF-Y treatment (396.0 ± 35.4 shoots m^{-2}). As expected, the shoot density was significantly ($p<0.05$) higher in the MF treatment than NF ones; among organic fertilization treatments, only DS was not statistically different from MF treatment (Tab. 3). No significant interaction ($p=0.371$) showed fertilization and AMF inoculation tretments on shoot density. NDVI index was significantly ($p<0.05$) influenced by fertilization treatments, DAS and their interaction, with the highest values always monitored for MF treatment and the lowest ones for NF treatment (Tab. 4).

Table 3 – AMF inoculation and fertilization treatments effects on bio-agronomic traits (mean ± SE).
MF = mineral fertilization, DL = digestate liquid fraction, DS = digestate solid fraction, NF = no fertilization, AMF-Y= inoculated and AMF-N=un-inoculated treatments. Capital letters denote significant differences among fertilization treatments using Fisher LSD test ($p<0.05$) whereas lower case letters denote significant differences between AMF treatments using Fisher LSD test ($p<0.05$). ns = no significant difference.

Bio-agronomic traits	AMF treatment	Fertilization				Mean
		MF	DL	DS	NF	
Culm height (cm plant^{-1})	AMF-Y	131.7±3.93^{ns}	122.3±3.48^{ns}	124.3±4.98^{ns}	116.0 ± 3.51^{ns}	123.6±2.40^{NS}
	AMF-N	128.7±3.93^{ns}	129.0±1.73^{ns}	125.7±2.73^{ns}	124.3 ± 2.96^{ns}	126.9±1.39^{NS}
	Mean	130.2±2.57^{A}	125.7±2.29^{AB}	125.0±2.56^{AB}	120.2±2.77^{B}	125.3±1.40
Shoot density (culms m^{-2})	AMF-Y	425.3±7.4^{cd}	388.0±13.9^{cd}	393.3±16.2^{cd}	377.3±33.8^{d}	396.0±10.2^{B}
	AMF-N	489.3±10.9^{ab}	440.0±17.4^{bc}	501.3±26.9^{a}	414.7±23.1^{cd}	461.3±13.8^{A}
	Mean	457.3±15.5^{A}	414.0±15.3^{BC}	447.3±27.9^{AB}	396.0±20.1^{C}	428.6±10.8

Table 4 - NDVI index at four different day after sowing (DAS) times (mean ± SE).

DAS	AMF treatment	Fertilization				Mean
		MF	DL	DS	NF	
143	AMF-Y	0.770±0.022	0.688±0.022	0.688±0.035	0.583±0.020	0.677±0.023
	AMF-N	0.730±0.009	0.695±0.018	0.671±0.022	0.591±0.051	0.672±0.020
	Mean	0.750±0.014	0.691±0.013	0.670±0.018	0.587±0.025	0.675±0.015
153	AMF-Y	0.713±0.010	0.652±0.016	0.651±0.017	0.573±0.021	0.647±0.017
	AMF-N	0.726±0.005	0.666±0.005	0.664±0.012	0.562±0.026	0.655±0.019
	Mean	0.720±0.006	0.659±0.008	0.658±0.010	0.567±0.015	0.651±0.012
162	AMF-Y	0.692±0.009	0.615±0.008	0.612±0.006	0.557±0.013	0.619±0.015
	AMF-N	0.727±0.001	0.605±0.013	0.632±0.012	0.527±0.018	0.623±0.022
	Mean	0.709±0.009	0.610±0.007	0.622±0.008	0.542±0.012	0.621±0.013
178	AMF-Y	0.740±0.013	0.639±0.022	0.637±0.039	0.513±0.015	0.632±0.026
	AMF-N	0.731±0.020	0.589±0.002	0.622±0.026	0.498±0.021	0.610±0.027
	Mean	0.736±0.011	0.614±0.015	0.629±0.021	0.506±0.012	0.621±0.018

ANOVA

	SS	DF	MS	F	Prob F	Sign. F	LSD ($p<0.05$)
DAS	0.0486	3	0.01618	32.684	0.00000	**	0.012916
AMF	0.0004	1	0.00039	0.2877	0.64540	**ns**	0.032315
Fertilization	0.3819	3	0.12729	37.749	0.00000	**	0.036523
DAS X AMF	0.0031	3	0.00105	2.1174	0.11032	**ns**	0.018266
DAS X Fertilization	0.0160	9	0.00178	3.5911	0.00175	**	0.025831
AMF X Fertilization	0.0012	3	0.00039	0.1171	0.94832	**ns**	0.051651
DAS X AMF X Fertilization	0.0071	9	0.00079	1.5969	0.14317	**ns**	0.036531
Residual	0.0238	48					
Total	0.5362	95					

Dry matter yield (DMY) was significantly ($p<0.05$) higher in the MF treatment with 21.8 ± 1.04 Mg ha^{-1} compared to organic fertilization which showed, on the average of the DS and DL, a decrease of -21.2%. The lowest DMY, as expected, was found in the NF sub-plots (14.5 ± 0.3 Mg ha^{-1}) with a significant ($p<0.05$) decrease compared to fertilization treatments of -33.5% (MF) and -15.5% (on the average of the DS and DL) (Fig. 1). No significant difference was showed ($p=0.464$) in relation to AMF inoculation and by interaction between fertilization and AMF inoculation ($p=0.128$) on DMY.

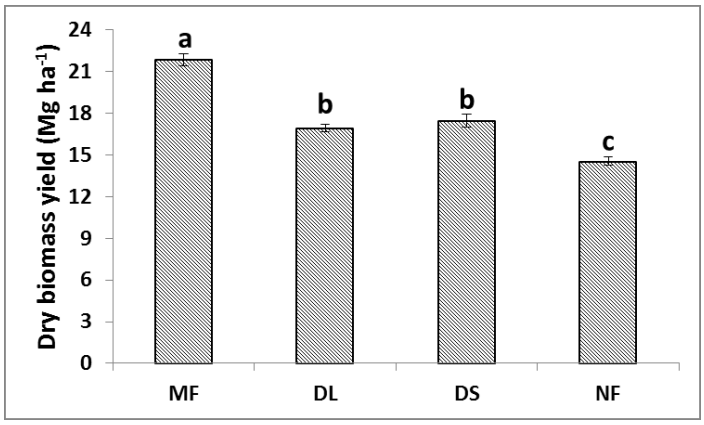


Figure 1 - Dry matter yield under different fertilization treatments (mean ± SE). MF = mineral fertilization, DL = digestate liquid fraction, DS = digestate solid fraction, NF = no fertilization. Different letters indicate significant differences among fertilization treatments for Fisher LSD test at $p<0.05$.

Soil CO_2 emission

No significant difference on soil CO_2 emission was detected among both AMF inoculation and fertilization treatments (Fig. 2), with an emission median value of 447.3 mg m^{-2} h^{-1}. During soil CO_2 emission measurements, in the upper 7.5 cm soil layer, moisture ranged from 23.8% to 57.4% and temperature from 4.8°C to 16.8°C. In the average of treatments, soil CO_2 emissions were positively correlated with soil temperature (Spearman R = 0.617; $p<0.001$), whereas no correlation were found with soil moisture (Spearman R = -0.015).

Considering the DL and DS macronutrients content (N, P_2O_5, K_2O), and using the $CO_{2(eq)}$ specific emission factors for mineral fertilizers production (Capponi et al., 2012), the avoided carbon emission in the atmosphere due to the substitution of mineral fertilizers with nutrients supplied through digestate was -674.4 and -1121.2 kg $CO_{2(eq)}$ ha^{-1} for DL and DS, respectively. On the contrary, MF determined a net in $CO_{2(eq)}$ emission in atmosphere (+863.8 kg $CO_{2(eq)}$ ha^{-1}).

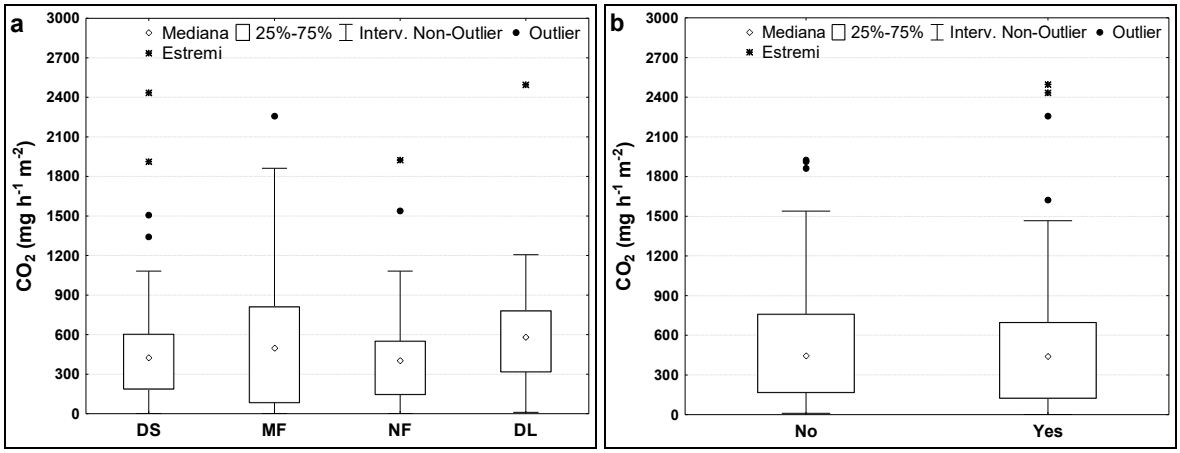


Figure 2 - Box-plot diagrams of soil CO_2 emissions in relation to fertilization (a) and AMF inoculation (b). MF = mineral fertilization, DL = digestate liquid fraction, DS = digestate solid fraction and NF = no fertilization.

Discussion

Root AMF colonization

Although the levels of arbuscular colonization found in triticale roots (A%) were lower than those reported in literature, which range from 25% to 66% (Pandey et al., 2005, Brito et al., 2012), it could be suggested that AMF inoculation was not effective, in relation to the seed coating with Celest® Trio, a fungicide containing *fludioxonil* and two sterol biosynthesis inhibitors (SBIs, *difenoconazole* and *tebuconazole*). When used alone, *fludioxonil* does not seem to affect AMF activity, as reported by Murillo-Williams and Pedersen (2008) that have observed on soybean seed where a fungicide (*fludioxonil*) was applied, a better AMF root colonization, potentially due to the lower competition by aggressive pathogens. However, if *fludioxonil* is used together with systemic fungicides, it can have a negative effect on AMF colonization (Jin et al., 2013). As a matter of fact, these latter authors have found a reduction (-9.4%) on mycorrhizal colonization in pea by indigenous AMF in response to the application of fungicides with systemic and non-systemic activities (Apron Maxx® RTA®). Interestingly, this suppressive effect on mycorrhizal colonization was more pronounced in inoculated plants. Moreover, the same authors have found that the suppressive effect is present only when commercial AMF inoculum were used in chickpea (-15.6%). Campagnac et al. (2008), Zocco et al. (2008), and Calonne et al. (2010), using various SBI fungicides at different concentrations, have reported drastic *Rhizophagus intraradices* development reductions (germination, germ tube elongation, colonization, extra-radical hyphal growth and sporulation). Therefore, in our study, the negative effect on AMF root colonization could have been determined by the mechanism of action of *difenoconazole* and *tebuconazole*. Considering that in the previous years, the soil was managed under conventional agronomic techniques using seeds coated with fungicide, the low root colonization could be due to the consequent detrimental effect also on the native AMF community.

Several authors have demonstrated that the application of high amounts of chemical fertilizers (particularly P and N) used in the intensive agrosystems to improve crop yield, negatively affect AMF root colonization and the number of AMF propagules in soil (Johnson, 1993; Liu et al., 2000; Kahiluoto et al., 2001; Burrows and Pfleger, 2002; Treseder and Allen, 2002). On the contrary, organic source of nutrients (manure, compost and crop residues), and slow-release fertilizers (such as rock phosphates) stimulate the activity of AMF (Gosling et al., 2006). A part of phosphorus contained in the DS is present in the form of struvite (formed during the anaerobic digestion process) which has

low P availability (Möller and Müller, 2012) and could have determined a higher percentage of AMF colonization. Moreover, Heydari and Maleki (2014) have observed significantly higher AMF colonization levels in inoculated barley supplied with rock-phosphate and struvite, compared to other fertilization treatments. In addition, the increased amount of organic matter present in the soil due to the DS supply could have reduced the effects of the hydrophobic fungicides (*difenoconazole* and *tebuconazole*) on AMF activity. This is supported by the work of Roy et al. (2000) that have observed an improved sorption of hydrophobic fungicides by humic substances in presence of low soil moisture.

Triticale bio-agronomic traits and biomass yield

In absence of AMF inoculation, the shoot density showed significant higher value than AMF-Y treatment in agreement with Hartnett et al. (1994) who found a similar response for others Poaceae species. Despite AMF inoculation the highest DMY was obtained in the MF treatment whereas the lowest one in NF one. Similar triticale DMY productions, using mineral fertilizers in Mediterranean condition, have been reported by Santiveri et. al. (2004) (24.3 Mg DMY ha^{-1} supplying 92 kg N ha^{-1}) and Giunta and Motzo (2004) (22.3 Mg DMY ha^{-1} supplying 97 kg N ha^{-1}), whereas, in the same cultivation area of this study (Po Valley), Delogu et al. (2002), supplying 170 kg N ha^{-1}, reported a production ranging from 8.3 to 19.2 Mg ha^{-1} of DMY at milk-dough stage. The digestate treatments lower DMY was probably due to the lower efficacy of N, since the other two macronutrients, P and K, applied with MF were lower compared to DS, and lower (P) and higher (K) as compared to DL. The lower efficacy of N applied with digestate can be due to: 1) the distribution period (all N in pre-sowing in DL and DS treatments, in three times in MF treatment), and 2) the possible ammonia volatilization losses (on average 15% NH_4^+-N applied) that occur manly within the first 10 hours after digestate distribution (Quakernack et al., 2012) especially for DL where NH_4^+-N represents about 56% of TKN.

Soil CO_2 emission

No significant difference on soil CO_2 emission among fertilization treatments is in agreement with our previous research (Maucieri et al., 2016a), where we observed, using only DL, a significant increase of soil CO_2 emission, only in the first days after distribution. The absence of significant difference in soil CO_2 emission among fertilization treatments, can be attributed to the characteristics of organic matter content in the digestate. In fact, the digestate used in this experiment came from a mesophilic (35-40

°C) anaerobic digestion plant, that had a substrate retention time of 88-92 days. According to Maucieri et al. (2017), it can be assumed that, considering anaerobic digestion process characteristics of digestate, except the easily available organic matter mostly degradable in the short term (Alburquerque et al., 2012), stabilized organic matter was supplied, which did not influence soil CO_2 emission during the monitored period.

Conclusions

To our knowledge, this is the first field study on triticale biomass production, which evaluates combined effects of AMF inoculation, seeds coated with fungicide and digestate fertilization.

The results obtained in this study, even if relative to one growing season, indicate that AMF inoculation determined only a reduction of shoot density without significant effect on biomass yield, suggesting that mycorrhizal inoculation increases plant's weight. All other parameters were not significantly affected by AMF inoculation. Mineral fertilization determined the highest DM yield (+27% respect to digestate treatments); even so, environmental (e.g. higher $CO_{2(eq)}$ emission) and economical (e.g. fertilizer costs) effects should be considered. In relation to soil CO_2 emissions, no significant differences were detected among treatments during cropping season.

On the basis of the biomass production, although lower than that obtained using chemical fertilizers, triticale fertilization with digestate could be an interesting agronomic practice in sustainable agriculture to reduce environmental and economic costs.

Chapter IV

Olive mill wastewater spreading and AMF inoculation effects in a low-input semi-arid Mediterranean crop succession

Abstract

The aim of this trial was to evaluate, in semi-arid marginal Mediterranean agro-ecosystem (Sicily-Italy), the effects of arbuscular mycorrhizal fungi (AMF) inoculation and olive mill wastewater (OMW) volumes (40 and 80 m^3 ha^{-1}) on forage (durum wheat-*M. scutellata* intercropping), and grain production of broad bean (*Vicia faba* L. minor) and chickpea (*Cicer arietinum* L.). AMF inoculation significantly increased (+13.6%) forage total dry biomass production and durum wheat nitrogen (+22.8%) and phosphorus (+32.5%) uptake. In broad bean, AMF inoculation significantly promoted phosphorus uptake (+11.5%) and root nodule number (+13.9%). Due to meteorological conditions, chickpea did not reach reproduction phase thus showing no production. OMW spreading reduced weed presence in the forage (-31.3%), broad bean root nodule number (-29.7%) and nodule dry weight (-22.7%). Furthermore, OMW spreading determined higher *M. scutellata* dry biomass production (+19.3%) compared to the control treatments (0, 40 and 80 m^3 H_2O ha^{-1}) which showed an average production of 361 g m^{-2}, and a significant increase in broad bean grain yield with a production of 2.46 ± 0.12 and 1.94 ± 0.09 Mg ha^{-1} in presence and absence of OMW, respectively. During the experiment AMF colonization was not affected by OMW volumes. The obtained results showed that the OMW spreading and AMF inoculum could be promising agronomic practices to valorize the Mediterranean marginal agro-ecosystem.

Introduction

Olive tree cultivation represents a significant part of the agricultural lands in the Mediterranean areas. The Spain is main producing country (2.5 million ha), followed by Italy (1.13 million ha), Greece (0.85 million ha) and Portugal (0.35 million ha) (FAOSTAT, 2013). From olive oil processing derive a huge amount of olive mill wastewater (OMW) (Kapellakis et al., 2008) with an annual production of about 30 10^6 OMW m^3 during a short period (from October to February) (Barbera et al., 2013). OMWs must be properly managed to avoid the negative environmental impacts associated with their disposal, due to the amount of phenolic compounds (Barbera et al., 2013; Di Bene et al., 2013) that exert phytotoxic and antimicrobial effects (Obied et al., 2005; Saadi et al., 2007). For this reason, olive oil producing countries have enact national directives to regulate the OMW spreading (according to d.lgs 152/2006 in Italy the legal limit is 80 m^3 OMW ha^{-1}). Considering OMW chemical composition (Paredes et al., 1999) it can be used in agriculture as soil amendment/fertilizer, especially to supply mineral nutrients such as K, P, Mg and Fe and organic matter (OM). Particularly taking into account their K and P content, OMW can be distributed on legume-based crop rotation to contribute to their nutrients requirements satisfaction.

Crop rotation and intercropping are important agronomic practice in semi-arid areas, increasing soil fertility and crop water use efficiency and enhancing environmental sustainability (Díaz-Ambrona and Mínguez, 2001; Scalise et al., 2015). The agro-ecological role of the crop rotation and intercropping between cereal and legumes becomes of crucial importance to support productions in low-input cropping systems of Mediterranean marginal land. In particular, legume crops play an important role for sustainability in cropping systems (Pala et al., 2007) through an improvement and yield stabilization due to the biologically fixed-N, and the reduction of the mineral nitrogen input (Bedoussac and Justes, 2010).

Arbuscular mycorrhizal fungi (AMF) are a widespread group of soil microbial community that form mutualistic symbiosis with about 90% of vascular plants (Berruti et al., 2014). This symbiosis is a key component of sustainable and low-input agricultural system; it is well- known to improve plant growth, nutrients uptake (Bücking and Kafle, 2015; Tarraf et al., 2015; Langeroodi et al., 2017), in particular phosphorus, and plant tolerance or resistance to abiotic and biotic stress (mainly, drought, salinity and soil-borne pathogens) (Ipsilantis et al., 2009). For these reasons, they are considered plant growth-promoting microorganisms.

So far, the OMW effects on soil microbial communities and in particular on AMF are poorly investigated (Mechri et al., 2008), and it is not yet clear whether OMW may have a negative impact on it. Ipsilantis et al. (2009) reported a significant AMF colonization decrease in broad bean just after OMW spreading and a colonization increase after 10 and 30 days after spreading. Di Bene et al. (2013) assessed the short- and long-term effects of long-lasting repeated OMW applications on mycorrhizal symbiosis, reporting that OMW applications decreased AMF colonization, but improved arbuscular abundance in both short- and long-term.

The aim of this experiment was to evaluate, in a three years crop succession in semi-arid low-input marginal Mediterranean agro-ecosystem, AMF inoculation and OMW volumes effects on forage (durum wheat-*M. scutellata* intercropping), broad bean (*Vicia faba* L. minor) and chickpea (*Cicer arietinum* L.) grain productions.

Materials and methods

Experiment description

The field experiment was carried out in Ispica, South Italy (Sicily) (36°44' N 14°58' E, 31 m a.s.l.) from October 2013 to June 2016 in a sandy soil (USDA, 1999) under Mediterranean conditions, according to Köppen climate classification (1936). The chemical-physical soil characteristics are reported in Table 1. A factorial split-plot design was adopted, with AMF inoculation (inoculated vs not inoculated) as split treatment (26.5 x 7.5 m) and OMW volumes (0, 40 and 80 m^3 h^{-1}) as the within-plot treatment (5.3 x 7.5 m). In addition, to highlight the effect of water quantity supplied with OMW, other two within-plots (40 and 80 m^3 H_2O ha^{-1}) have been included in the experimental design. OMW chemical composition, that was in line with literature data as reviewed by Roig et al. (2006), is reported in table 2.

In the first cropping season (October 2013 – April 2014) the effect of treatments (AMF inoculation and OMW) on *Triticum durum* Desf. sicilian old landraces (Timilia and Sicilia) intercropped with *Medicago scutellata* (L.) Mill. for forage production has been studied. Each within-plot was divided in six sub-plots (2.65 x 2.5 m), three for each wheat genotype, randomly distributed. The OMW and fresh water were distributed 22 days before sowing on 26th October 2013. Considering OMW macronutrients content (Tab. 2) at full dose (80 m^3 ha^{-1}), 32.8 Kg N ha^{-1}, 34.8 Kg P_2O_5 ha^{-1} and 413.5 Kg K_2O ha^{-1} were supplied to the soil. In the same day of OMW distribution, only in the first cropping season, 30 Mg ha^{-1} of cattle manure has been distributed in all plots. The OMW and

manure were buried by rotary hoeing in the first soil layer (10 cm) the day after distribution and a further rotary hoeing (about 20 cm depth) was carried out the day before sowing. To sustain the cereal crop, according to soil characteristics (Tab.1), and particularly the low soil N content and sandy texture, 40 kg urea-N ha^{-1} has been distributed at the durum wheat culm elongation phenological phase.

Table 1 – Soil physical-chemical characteristics

Parameters	Mean	(±SD)
Skeleton (%)	28.1	4.8
Sand (%)	73.6	1.5
Silt (%)	12.6	0.5
Clay (%)	13.8	2.0
Organic matter (%)	1.36	0.24
Total Nitrogen (%)	0.076	0.015
Total Phosphorus (%)	0.052	0.003
pH	7.3	0.1
EC (dS m^{-1})	0.320	0.096

Table 2 – Olive mill wastewater physical-chemical compositions

Parameters	2014	2015	2016	Mean	(±SD)	Literature data (Roig et al., 2006)
pH	4.34	5.21	4.87	4.81	0.44	4.2 – 5.17
Total Polyphenols (g L^{-1})	2.83	2.64	2.96	2.81	0.16	0.98 – 10.7
EC (dS m^{-1})	6.91	7.26	7.23	7.13	0.19	5.50 – 12.0
Dry matter (%)	6.30	6.60	6.58	6.49	0.17	6.33 – 7.19
Na^+ (g L^{-1})	0.18	0.23	0.21	0.21	0.03	0.11 – 0.30
K^+ (g L^{-1})	4.29	4.03	4.17	4.16	0.13	1.97 – 8.97
TP (g L^{-1})	0.19	0.27	0.28	0.25	0.05	0.14 – 0.31
TN (g L^{-1})	0.41	0.61	0.57	0.53	0.11	0.62 – 2.1

In the second cropping season (October 2014 – May 2015) *Vicia faba* L. minor (cv. Prothabon 101) was cultivated. In the split-plot design, the within-plots area (5.3 x 7.5 m) was divided in four subplots (2.65 x 3.75 m) instead of the previous six ones. The OMW and freshwater were distributed 60 days before sowing on 10th October 2014. Respect to the previous year, the macronutrients supply with OMW at full dose (80 m^3 ha^{-1}) was 48.8 Kg N ha^{-1}, 49.5 Kg P_2O_5 ha^{-1} and 388.4 Kg K_2O ha^{-1} due to the different composition (Tab.2). Nutrients supplied with OMW were the only fertilization. The OMW was buried by rotary hoeing in the first soil profile (10 cm) five days after spreading; a further rotary hoeing (about 20 cm) was carried out the day before sowing. After crop emergence, weeds were manually controlled until full crop development.

In the third cropping season (February 2016 – June 2016), *Cicer arietinum* L. (cv. Pascià) for grain production was cultivated, with the same experimental design of the second

year. The OMW and freshwater were distributed on 23rd October 2016, 4 months before sowing. OMW macronutrients supply at full dose (80 m^3 ha^{-1}) was 45.6 Kg N ha^{-1}, 51.3 Kg P_2O_5 ha^{-1} and 401.9 K_2O Kg ha^{-1}. The OMW was buried by rotary hoeing in the first soil profile (10 cm) three days after spreading; a further rotary hoeing soil tillage (about 20 cm) was carried on 23rd January and 1st February 2016. On the first month after crop emergence weeds were removed by hoeing.

Seeds evaluation and sowing dose choosing

Before sowing, for each crop, 1000 seeds weight and germination were evaluated to determine the amount of seed to be sown per unit area. Seed samples were randomly taken from seed stocks and 1000 seeds weight and percentage germination were assessed according to standard methods (ISTA, 1996). For wheat the 1000 seeds weight was 41.8 g for Sicilia and 29.0 g for Timilia with a germination of 92.8% and 97.5% for the two cultivar respectively. Durum wheat was sown at a dose of 400 germinable seeds m^{-2}. For *M. scutellata* the seeds used for sowing were collected from local population in the experimental area in the Spring 2013. The 1000 seeds weight was 1.52 g. Due to tegumental seed dormancy (Uzun and Aydin, 2004), germination was only 12.3%, so 2.5 g seeds m^{-2} were sown to reach 200 germinable seeds m^{-2}. For broad bean the 1000 seeds weight was 420.9 g and the germination was 86.7%; it was sown to obtain 35 germinable seeds m^{-2}. *C. arietinum* showed a 1000 seeds weight of 481.6 g and a germination rate of 82.4%; it was sowed at 30 germinable seeds m^{-2}.

AMF inoculation (based on *Rhizophagus intraradices* inoculum self-produced in laboratory) was carried out, for each cropping season, at the sowing (75 spore m^{-2}) distributing the inoculum along the row.

Meteorological variables

Over the experimental period, the main daily meteorological variables: maximum, minimum and average temperature, rainfall and potential evapotranspiration (ET_0) according to Penman-Monteith equation, were provided by the SIAS (Sicilian Informative Agro-Meteorological Service) though an agrometeorological stations located approximately 1.5 km from the experimental site.

Bio-agronomic parameters

For all cropping seasons bio-agronomic traits (culm height for wheat and scutellata; culm height and leaf number for broad bean and chickpea) were weekly monitored for all crop life cycle.

On the third study year, after the crop emergence, the driest climate conditions have reduced the plant growth until its stopped at the end of April 2016 and subsequently brought the plants to die in May 2016. For this reason, the data on the last research year are not discussed.

Biomass yield and analysis

In the first year forage was harvested on 15th April 2014 at the *M. scutellata* flowering in the inner area (0.5 x 0.5 m) of each plot. The biomass was subdivided in three categories (wheat, medicago, and weeds). For each biomass category, fresh weight was determined just after harvest in field with a portable balance whereas biomass dry weight was determined in a thermo-ventilated oven at 65 °C until constant weight.

In the second cropping season, broad bean grain was harvested on 30th May 2015 in the inner area (1 x 1 m) of each subplot. The following yield components: shoot density, plant^{-1} m^{-2}, pods number m^{-2} and pods weight were measured.

Forage dry biomass and broad bean grains were milled to determine the total Kjeldahl nitrogen (N) and phosphorus (P) content (Balthrop et al., 2011). Forage biomass fiber composition was determined in a pooled sample for each treatment according to Goering and Van Soest (1970).

Root analysis: AMF colonization and root nodule

At full flowering phase of legumes in both first (4th April 2014) and second (30th March 2015) experimental year, four wheat and broad bean plants per subplot were randomly selected. In these plants root samples were collected with a hand-operated soil probe (5 cm diameter) in the first 20 cm depth soil for total 240-sampled durum wheat plants and 160-sampled broad bean plants. In laboratory, the same procedure was used to remove the soil particles by root samples of the two corps. The root samples were washed with some drops of Tween 20 and then rinsed several times in tap water.

Durum wheat root samples were cleared with 10% KOH for 3 minutes, stained with 5% ink-vinegar for 5 minutes and de-stained in distilled water for 10 minutes (Vierheilig et al., 1998). Broad bean root samples were cleared with 10% KOH for 23 hours, (changing the KOH solution after 10 hours), rinsed three times in tap water and new cleared with hydrogen peroxide (36 vol.) for 30 minutes and again rinsed in tap water. Subsequently, root samples were stained with 5% dark ink-vinegar for 30 minutes and de-stained in tap water for other 30minutes. Later all roots samples were cut into small fragments (about 1 cm each) and mounted onto microscope slides with some drops of tap water.

Considering the high number of root samples in both years, they were stored at +4°C for later microscopic analysis.

AMF percentage colonization in the root cortex was estimated according to Trouvelot (1986) as follow: F%= mycorrhization frequency (the percentage of root fragments showing fungal colonization), M%= AMF colonization intensity (the percentage of fungi structures referred to the whole root system), m%= AMF colonization intensity (the percentage of fungi structures referred to colonized root fragments), a%= abundance of arbuscules (percentage of arbuscules presence referred to the root fragments showing fungal colonization); A%= abundance of arbuscules (percentage of arbuscules presence referred to the whole root system).

Moreover, additional four broad bean plants per treatment were sampled (total 40-sampled plants) with the whole root systems. Subsequently roots samples were washed and its nodules were manually separated from the roots to evaluate the nodule number and weight. Nodule dry weight was determined in a thermo-ventilated oven at 65 °C until constant weight.

Statistical analysis

All the percentage calculated were arccosine transformed before statistical analysis and the normality of data was checked using the Shapiro–Wilk test. Data were normally distributed and it subjected to analysis of variance (ANOVA). The data were post-hoc tested, using the Fisher's LSD test to determine significant differences.

Results

Meteorological variables

The experimental site is characterized by a typical Mediterranean climate, with an average annual temperature of 18.5 °C and average rainfall of 484 mm year^{-1} (1971-2000) (Servizio Metereologico dell'Aeronautica Militare). In our study, the average annual air temperature (+18.0°C) and the average annual rainfall (520 mm year^{-1}) was similar to the long-term average. Minimum and maximum temperatures during the three growing seasons ranged from +0.1°C to 37.9 °C recorded on January 1st 2015 and July 31th 2015, respectively.

The same trend was found for monthly average temperature and ET_0 with fairly uniform values during the three experimental years (Fig. 1a and Fig 1b), contrarily to the total amount rainfall that was quite different. In fact, in the first growing season (from

November 2013 to April 2014) was recoded 295 mm of rainfall fairly uniform distributed (Fig. 1b). A higher amount of rainfall (401 mm) was recorded during the second growing season compared to the first one (Fig. 1b), mainly concentrated (88%) between January and March 2015, during the broad been vegetative stage (Fig.1b). The lowest rainfall amount was measured during the third growing season (50.2 mm) mainly concentrate in the first eighteen days after sowing (72.1%). Furthermore, the remaining part of rainfall derived from 10 small events that were not useful for plant growth.

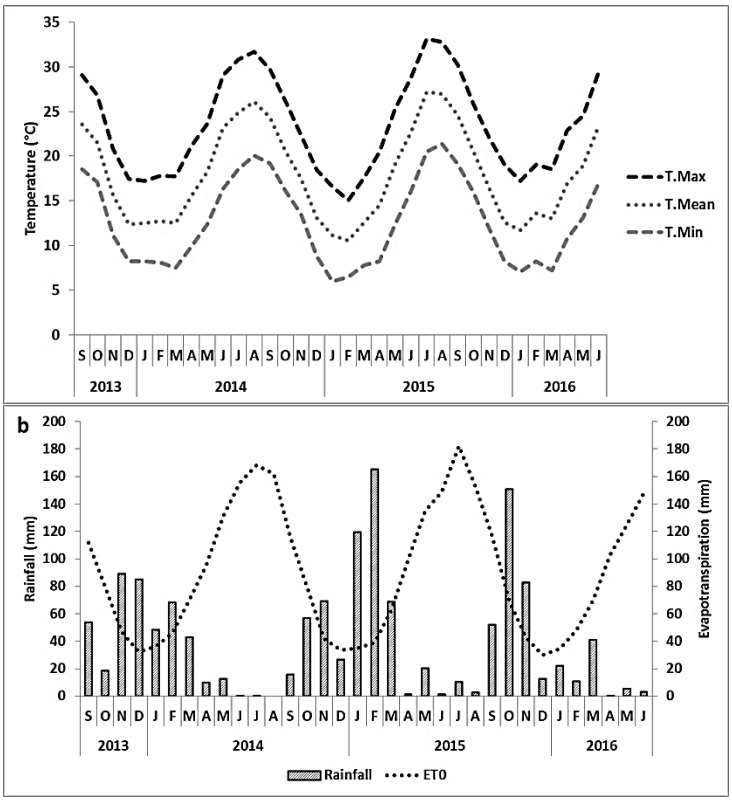


Figure 1 - Meteorological variables: a) temperature (maximum, minimum and average temperature) and b) rainfall and evapotranspiration (ET_0).

First growing season: Intercropping durum wheat-medicago

Bio-agronomic traits

In both wheat genotypes, crop emergence was observed 6 days after sowing (DAS), with higher uniformity in absence of OMW treatment. However, 19 DAS the durum wheat establishment in all the plots was uniform. The crop emergence of *M. scutellata* was observed during the first ten days in January 2014 and it was not affected by OMW treatment. Plants stature was not influenced by OMW distribution and AMF inoculation reaching an average value at the harvest time of 92.4 ± 1.8 cm and 68.9 ± 1.1 cm for wheat and scutellata, respectively. Considering wheat genotype, Sicilia reached a

significant ($p<0.01$) higher stature (+14%) than Timilia which showed an average culm height of 85.1 ± 2.1 cm.

Biomass Yield

At the harvest time, in the average of the studied treatments and only for durum wheat genotypes, the AMF inoculation determined a significant ($p<0.01$) dry matter yield increase (+30.8%) as compared to un-inoculated treatment (367.6 ± 22.6 g m^{-2}). No statistical differences in dry matter yield of durum wheat were found in relation to OMW volumes, with a grand mean of 423.5 ± 18.5 g m^{-2}.

M. scutellata and weeds dry matter was significantly ($p<0.05$) affected by OMW spreading volumes whereas no effect exerted the AMF inoculation. Spreading OMW at the dose of 40 m^3 ha^{-1} increased *M. scutellata* dry matter yield by +43.1% as compared to control (313.2 ± 16.6 g m^{-2}) whereas an opposite trend was observed for weeds dry matter production (Fig. 2). The control and 40 m^3 H_2O ha^{-1} showed the highest weed presence without significant differences between them (Fig. 2).

Considering total forage dry biomass yield, only AMF inoculation exerted a significant effect (+13.6%) as compared to un-inoculated treatment (842.5 ± 30.1 g m^{-2}).

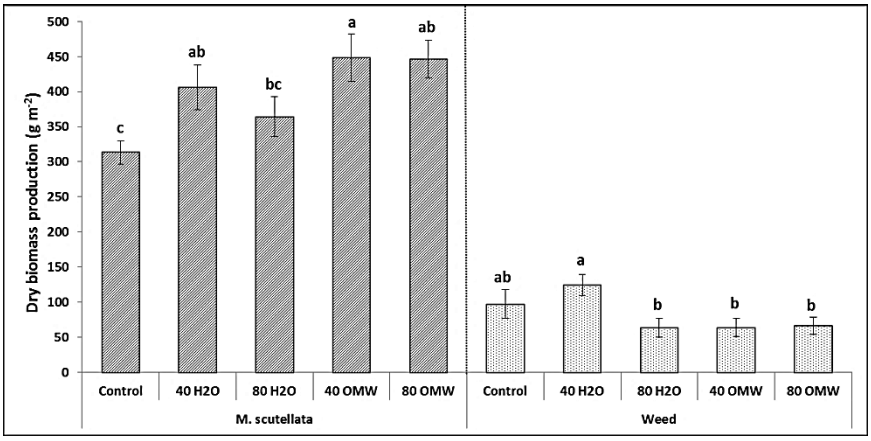


Figure 2 - Olive mill wastewater effects on *Medicago scutellata* and weeds dry matter production. Different letters show statistical differences of the treatment at $p<0.05$ (LSD – Fisher Test).

Nutrients uptake

Nitrogen and phosphorus uptake, in the average of the two crops for forage (durum wheat + scutellata) were significantly higher in presence of OMW volumes as compared to the control (Fig. 3a and 3b). Instead, AMF inoculation significantly ($p<0.01$) increase only forage P uptake (Fig. 4).

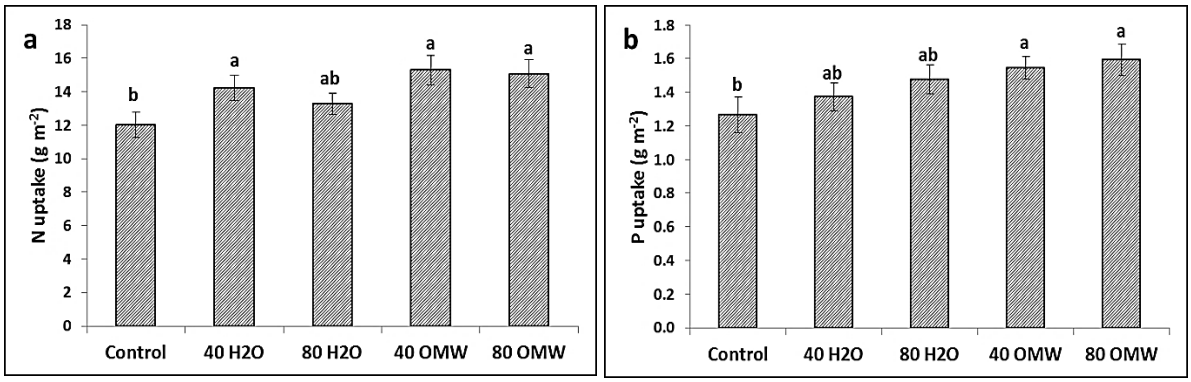


Figure 3a and 3b – Olive mill wastewater effect on uptake of N (a) and P (b) in forage. Different letters show statistical differences at p<0.01 (LSD – Fisher Test).

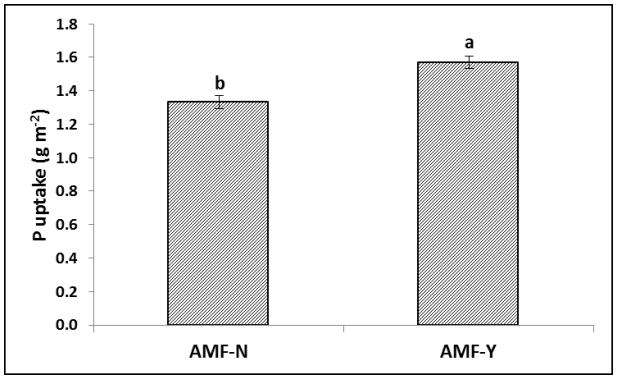


Figure 4 – Arbuscular mycorrhizal fungi effect on P uptake in forage. Different letters show statistical differences at p<0.01 (LSD – Fisher Test).

Considering the single crop, in durum wheat AMF inoculation significantly (p<0.01) increased wheat N and P uptake by +22.8% and +32.5% as compared to un-inoculated treatments (3.7 ± 0.2 g N m^{-2} and 0.69 ± 0.04 g P m^{-2}). In the average of the studied treatments, a significantly (p<0.01) higher N uptake was observed in Sicilia genotype (+19.2%) than in Timilia (3.7 ± 0.3 g N m^{-2}). In *M. scutellata*, no treatments effects were observed on N uptake, with a grand mean of 10.1 ± 0.34 g N m^{-2}. Independently of the spreading volumes, OMW distribution determined a significantly (p<0.01) higher (+41.8%) P uptake than control treatment (0.55 ± 0.03 g P m^{-2}).

Biomass fiber composition

Biomass fiber composition in both durum wheat genotypes intercropped with scutellata are reported in table 3. OMW treatments and AMF inoculation did not show any effect on biomass fiber components. Considering the two durum wheat genotype, Timila+scutellata forage showed a significant (p<0.05) higher ADL value (+11.6%) as compared to Sicilia+scutellata one (Tab.3).

Table 3 – Forage fiber composition. Data are expressed as percentage of dry matter. NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; AIA = acid insoluble ash.

Forage composition	AMF Treatment	OMW Treatment (m^3 ha^{-1})	NDF%	ADF%	ADL%	AIA%
Sicilia + ***Medicago scutellata***	No	Control	54.1	35.0	5.9	0.26
	No	40 H_2O	56.7	32.9	5.7	0.25
	No	80 H_2O	54.1	34.1	5.6	0.30
	No	40 OMW	54.0	33.5	6.4	0.33
	No	80 OMW	56.2	35.8	6.8	0.29
	Yes	Control	56.9	35.9	6.6	0.22
	Yes	40 H_2O	58.4	37.2	4.2	0.34
	Yes	80 H_2O	56.4	34.8	5.9	0.18
	Yes	40 OMW	57.5	33.6	6.3	0.30
	Yes	80 OMW	60.5	36.2	6.3	0.27
Timilia + ***Medicago scutellata***	No	Control	55.7	38.5	6.78	0.38
	No	40 H_2O	57.7	35.8	7.01	0.54
	No	80 H_2O	52.8	34.2	6.05	0.22
	No	40 OMW	57.6	38.4	7.39	0.25
	No	80 OMW	55.1	36.0	7.24	0.29
	Yes	Control	56.8	35.6	6.34	0.29
	Yes	40 H_2O	59.3	35.6	5.90	0.43
	Yes	80 H_2O	55.2	35.8	6.27	0.29
	Yes	40 OMW	54.0	34.6	7.36	0.27
	Yes	80 OMW	58.1	35.9	6.21	0.30

Root AMF colonization

Root AMF colonization in durum wheat was not significantly influenced by OMW treatments. In the average of the other studied factors, the percentage of AMF in the root system of durum wheat, was significantly ($p<0.01$) higher (F%=+38.3%, M%= +54.8% and A%=+67.8%) in the inoculated treatment compared to un-inoculated one which showed F% of 52.2% ± 2.94, M% of 16.1% ± 0.44 and A% of 12.1% ± 0.12. No statistical difference in durum wheat was observed for m% and a% with a mean value of 32.9% ± 1.11 and 78.1% ± 1.90, respectively.

Second growing season: *Vicia faba* L. cv. minor

Bio-agronomic traits

Climatic conditions delayed broad bean emergence that was observed 22 DAS. In fact: a) in the 30 days before sowing only 4.4 mm in 3 rain events (each lower than 3 mm) were recorded; b) in the two weeks after sowing only 14.8 mm in 5 rain events (each lower than 5 mm) were recorded. However, the rainfall recorded in all the growing season (401 mm) promoted a uniform crop development.

The maximum leaf number (123 DAS) and, stem height at harvesting (168 DAS) did not show significant differences among treatments (grand mean = 16.0 ± 0.16 leaf plant^{-1} and 149.0 ± 1.09 cm plant^{-1}, respectively).

Yield components

At the harvest, no statistical difference was found among treatments in shoot density, with a grand mean of 35.0 ± 0.84 plants m^{-2}. In the average of the AMF inoculation treatments, OMW spreading significantly ($p<0.01$) increased the number of pods per square meter and per plant (Fig. 5a and Fig. 5b), with maximum values at 40 m^3 OMW ha^{-1} (211 ± 27.5 pods m^{-2} and 6.3 ± 0.34 pods plant^{-1}). The distribution of OMW at 80 m^{-3} ha^{-1} did not determine significant differences as compared to the other treatments (Fig. 5a and Fig. 5b).

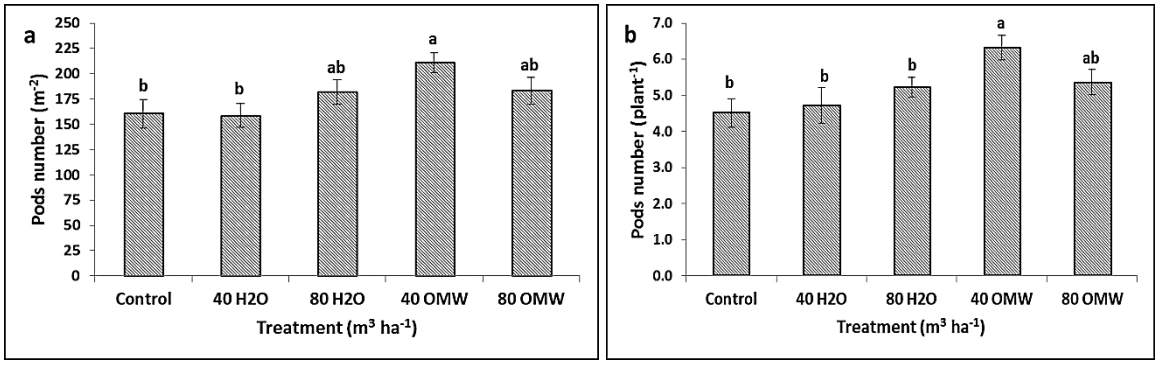


Figure 5 – Olive mill wastewater effect on a) pods number per square meter and b) pods number per plant. Different letters show statistical differences of the treatments at $p<0.01$ and $p<0.05$ (LSD – Fisher Test).

The grain yield was not influenced by AMF inoculation with an average yield of 2.15 ± 0.08 Mg ha^{-1} whereas a positive effect exerted the OMW spreading despite the volumes supplied with a mean value of 2.46 ± 0.12 Mg ha^{-1}. Control treatment (un-watered) was not statistical different as compared to the two treatments supplying fresh water, with an average yield of 1.94 ± 0.09 Mg ha^{-1} (Fig. 6).

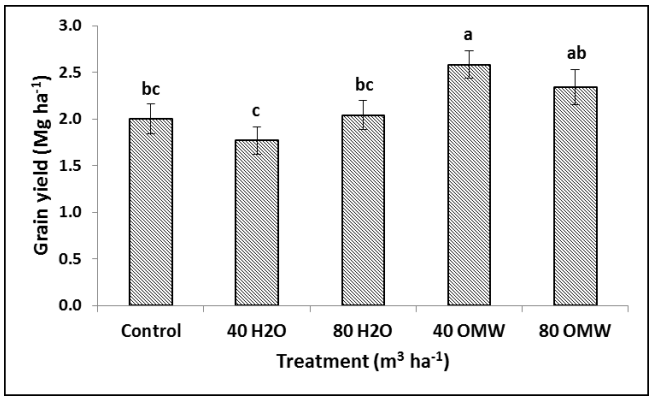


Figure 6 – Olive mill wastewater effects on broad bean grain yield. Different letters show statistical differences of the different treatments at $p<0.05$ (LSD – Fisher Test).

Broad bean grain nutrients content

Grain nitrogen content did not show statistical differences among treatments with grand mean of 4.25 ± 0.13% on dry matter base. Phosphorus grain content, on the average of the volumes distribution, was significantly ($p<0.05$) higher (+11.5%) in presence of AMF inoculation compared to un-inoculated treatment (0.37 ± 0.01% on dry matter base).

Root nodules

In the treatments without OMW distribution, AMF inoculation showed a positive effect on broad bean root nodule number for plants, with a significant ($p<0.05$) increase of 13.9% as compared to un-inoculated treatments (107.1 ± 1.0 nodule number plant^{-1} (Fig. 7a). The same trend was observed for nodule weight where AMF inoculation determined a significant ($p<0.05$) increase of +38.8% as compared to un-inoculated treatments (0.43 ± 0.03 g nodule plant^{-1}). No statistical difference was found between the two OMW levels (40 and 80 m^3 OMW ha^{-1}) for root nodule number and weight, with a mean value of 81.0 ± 4.24 nodule plant^{-1} and 0.39 ± 0.06 g plant^{-1}, respectively (Fig. 7a and Fig.7b). OMW supply however determined a lower nodule number and weight in un-inoculated treatments (Fig. 7a and 7b).

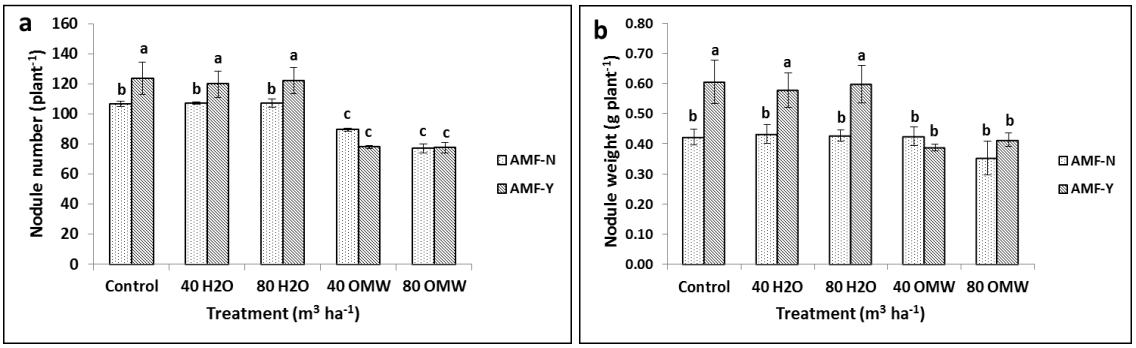


Figure 7 – Arbuscular mycorrhizal fungi and olive mill wastewater effects on: a) nodules number and b) nodules weight. Different letters show statistical differences of the different treatments at $p<0.05$ (LSD – Fisher Test).

Root AMF colonization

AMF inoculation did not show statistical difference on root percentage of colonization, ranging from 86.7% to 100% for F%, from 38.7% to 90.0% for M% and m%, from 83.3% to 100% for a% and from 38.3% to 90.0% for A%.

Discussion

It is widely believed that the agronomic use of OMW by direct spreading in agricultural lands represents one of the best solution for OMW management in Mediterranean countries due to the huge amount produced in only few months per year. However their

distribution is limited by some constraints, such as oil and greases (Amaral et al., 2008), high salinity, acidity and especially high phenolic compound concentrations (El Hadrami et al., 2004; Barbera et al., 2014; Cavallaro et al., 2014). Although polyphenols are the most limiting factor for spreading OMWs on soils because of their antimicrobial and phytotoxic effects especially during seed germination, they are rapidly degraded depending on environmental conditions and a delayed sowing after OMW spreading, is desirable to obtain good germination and emergence (Barbera et al., 2013). In our study, in the first growing season, the durum wheat sowing was performed 22 days after OMW spreading. This period allowed to reduce the OMW phytotoxic effect due to the phenolic compounds (Barbera et al., 2013). Thus, the lower wheat emergence uniformity in OMW treatments can be attributed to the OMW salinity. In fact, although the soil is not saline the particular meteorological trend recorded during the experiment, with a total of 4 mm of rainfall in the first 6 days after sowing, has probably caused a concentration of salts in the upper soil layer in which the seed was present reducing its imbibition and delaying germination processes. The uniform emergence observed, 19 DAS, confirms this interpretation, in fact the 83.8 mm of rainfall (44.4 mm in one event) recorded from 6^{th} to 19^{th} DAS, and the sandy texture of soil permitted the leaching of the salt added with OMW distribution, allowing a regular crop establishment.

In our study, despite low AMF root colonization (20.3%) in inoculated durum wheat, the AMF inoculum led to a higher dry matter yield than not inoculated treatment. Mycorrhizal symbiosis associated with plant roots was found to enhance durum wheat dry matter production under drought conditions. This effect may be referred to the increase of the soil mineral and water uptake through the extraction of soil water by the extra-radical hyphal network (Al-Karaki, 1998).

The increase in *M. scutellata* yield determined by OMWs may mainly be due to the positive effect of P and K supplied. In fact, these macronutrients have been shown exert positive effect on legume production (Mmbaga et al., 2015). On the contrary, OMW spreading reduced weeds dry matter production as a result of OMW phytotoxic properties as confirmed by Cayuela et al. (2008) that observed a reduction of more than 90% of weed seeds germination following the application of OMWs.

Crop production in soils with lower levels of N and P reduces the risk of environmental impacts from denitrification and leaching loss (Smith et al., 2008). Cereals are often intercropped with legumes for forage production to increase total yields and reduce N fertilizer input due to symbiotic nitrogen fixation. In fact, legumes release fixed N in the

soil through root exudates and it is subsequently mineralized and reabsorbed by the plant or taken up by a plant growing nearby. However, the AMF extra-radical hyphae may improve plant uptake, especially P (Johansen and Jensen, 1996). In our study, we observed a higher N uptake in Sicilia durum wheat genotype and in presence of AMF inoculation (1st year). These results suggest: 1) a genotype-specific response; and 2) an improve of N transfer from *M. scutellata* to durum wheat by AMF, in agreement with Johansen and Jensen (1996). AMF inoculation increased durum wheat P uptake in agreement with Li et al. (2006) and Smith et al. (2003) which report a significant P uptake increase in presence of AMF, especially in presence of *R. intraradices*.

The biomass fiber composition values measured in our study (Tab.3) are in agreement with scientific literature (Heuzè et al., 2015). Comparing the two wheat genotypes, the higher lignin content (ADL) found in Timilia+scutellata biomass as compared to Sicilia+scutellata one, indicate a low-quality forage (Short et al., 1974) suggesting that Sicilia should be preferred for forage production.

In second growing season, in broad bean a higher pod number was observed in presence of OMW with a higher grain yield compared to other treatments. This result could be explained by an improving in soil fertility determined by OMW application, mainly influencing K and P soil contents (Chaari et al., 2015; Steinmetz et al., 2015; Proietti et al., 2015). Furthermore, the OMW spreading has been demonstrated to be able to determine an improvement of the soil aggregate stability and soil water retention (Barbera et al., 2013). On the contrary, Mekki et al. (2006a) observed a decrease in all main yield components of broad bean, compared to the un treated control.

Although Mechri et al. (2008) reported that the abundance of AMF in soil was reduced due to the toxic effect exerted by OMW, in our study OMW distribution did not show any effects on AMF colonization in durum wheat and broad bean. Indeed, we found colonization also in un-inoculated plots, indicating that our experimental soil contains indigenous mycorrhizal community. Broad bean roots colonization was not significantly different comparing inoculated and un-inoculated treatments, but if we found a significantly higher P content in broad bean grain harvested in the inoculated treatment. This result may be attributed to the development and P uptake effectiveness of the extra-radical hyphae induced by the *Rhizophagus intraradices* present in the our inoculum, in agreement with Smith et al. (2004).

So far not much is known regarding the effects of OMW on the soil microbial community structure and more particularly on bacterial groups involved in important soil functions

(Karpouzas et al., 2010). Garcia-Barrionuevo et al. (1992) reported a stimulatory effect of OMW on nitrogen-fixing bacteria. Mekki et al. (2006b) found a significant reduction in the number of soil nitrifying bacteria at high OMW dose (400 m^3 ha^{-1}). Moreover, Di Serio et al. (2008) showed that high amounts of OMW increased the soil-denitrifying community and decreased slightly the population of nitrifying bacteria for the reductive effect of phenols.

In our study the nodulation was negatively affected by OMW distribution. The decrease in the nodules number and weight could be attributed to OMW toxic effect, determining a possible reduction of indigenous rhizobia population in our sandy in agreement with Ciafardini and Zullo (1998). In absence of OMW distributions, AMF inoculation promoted the nodulation in *V. fava* minor, as indicated by the highest nodule number and weight in agreement with Wang et al. (2011).

Conclusions

OMW spreading showed a positive effect on *M. scutellata* dry biomass production and broad bean grain yield highlighting the positive effect of this byproduct on legumes species. AMF inoculation significantly increased forage production whereas it did not affect broad bean grain yield because the abundance of indigenous AMF that have fully colonized the un- inoculated plants. Roots AMF colonization, in each experimental year, was not affected by OMW volumes and determined a significant higher N and P uptake by durum wheat biomass and P uptake by broad bean grain. AMF inoculation did not affect *M. scutellata* P and N uptake. Despite the inhibitory effect of OMW on broad bean nodulation, increased the broad bean yield (+21.1%) compared to without OMW plots. The obtained results showed that the OMW spreading and AMF inoculum could be promising agronomic practices to valorize the Mediterranean marginal low-input agro-ecosystem for animal feed production.

Chapter V

Effect of mycorrhizal inoculum, saline and water stress on *Panicum miliaceum* L. forage production in Mediterranean environment

Abstract

The aims of this study were to evaluate: i) in laboratory conditions, the effect of NaCl and mannitol at different osmotic pressures on germination of three proso millet (*Panicum miliaceum* L.) genotypes (VIR 9181, Unikum and Kinelskoje); ii) in a Mediterranean marginal soil, the effects of arbuscular mycorrhizal fungi (AMF) inoculation, irrigation water salinity and ETm restitution regimes on fresh biomass yield (FBY) at dough stage for forage production on the genotypes that showed the highest (Unikum) and lowest seed germination (Kinelskoje) in laboratory conditions. Germination was significantly reduced as osmotic pressure increased independently of the osmoticum. Regardless of the treatments, Unikum showed the highest germination rate (95.1%) and Kinelskoje the lowest one (80.4%). In open field, regardless of the studied factors, Unikum showed a higher FBY (620.4±126.3 g m^{-2}) than Kinelskoje (340.0±73.5 g m^{-2}). AMF inoculation did not influence FBY under salt condition whereas, in absence of the salt treatment, it significantly increased the Unikum FBY (+50.7%) as compared to the un-inoculated treatment (552.5±269 g m^{-2}). Increasing irrigation water salinity and ETm restitution respectively decreased and increased millet FBY. Our results suggest genotype-specific effects of AMF inoculation under freshwater irrigation, whereas, no effects were observed under saline water irrigation. The present study gives novel information about proso millet forage production using AMF inoculation under salt and drought stress in Mediterranean marginal area conditions, further investigations on the large-scale are needed to confirm our findings.

Introduction

In semi-arid and arid regions, salinity is one of the major causes of land degradation (Hasegawa et al., 2000; Zhu, 2003) and leads to huge economic losses due to reduction in total arable land area and crop productivity (Mahajan and Tuteja, 2005). Salts effects on agricultural soils are generally tied to soluble minerals present in irrigation water and high fertilization input (Villa-Castorena et al., 2003; Al-Karaki et al., 2006). In Europe, about 3.8 million ha are affected by soil salinization, particularly by naturally saline soils occur in Spain, Hungary, Greece and Bulgaria, or irrigation with salinity water induce soil salinization in significant areas of Sicily and the Ebro Valley in Spain and more locally in the other part of Italy, Hungary, Greece, Portugal, France, Slovakia, and Romania (Jones et al., 2012). Plants growing in saline soil are subjected to three distinct physiological stresses: 1) *toxic effects* of specific ions such as sodium and chloride, which disrupt the structure of enzymes and other macromolecules, damage cell organelles, reduce photosynthesis and respiration, inhibit protein synthesis, and induce ions deficiency (Juniper and Abbott, 1993; Ramoliya et al., 2004); 2) *osmotic effect* due to physiological drought because plants must maintain lower internal osmotic potentials to prevent water from moving from the roots into the soil (Aggarwal et al., 2012); 3) *nutrient imbalance* caused by depression in uptake and/or transport (Adiku et al., 2001; Marschner, 1995).

Among a wide array of rhizosphere microorganisms, arbuscular mycorrhizal fungi (AMF) are an essential component of sustainable and low-input agricultural systems (Ipsilantis et al., 2009). AMF symbiosis can exert positive effects on crop production (Candido et al., 2013; Sabia et al., 2015; Tarraf et al., 2015) and increase plant tolerance to abiotic stress such as drought (Ruiz-Lozano, 2003; Miransari, 2010) and salinity (Evelin et al. 2009; Miransari, 2010; Porcel et al., 2012). Possible AMF-mediated adaptation mechanisms inducing plant tolerance to saline conditions (Wu et al., 2010) include: 1) nutrient uptake improvement, especially phosphorus (P) (Al-Karaki 2000; Al-Karaki et al., 2001; Asghari et al., 2005); 2) accumulation of soluble sugars into the roots (Feng et al. 2002); 3) K+/Na+ ratio adjustment (Giri et al., 2007; Asghari, 2012); 4) antioxidant enzymatic activities (He et al., 2007). Although AMF can be found in saline soils, some of their features such as spore germination, fungal hyphae growth (Porcel et al., 2012), formation of mycorrhizal arbuscules (Tian et al., 2004; Sheng et al., 2008), and root colonization levels (Juniper and Abbott, 2006) may be negatively affected by high salinity. Despite these mycorrhizal behaviors, the positive influence on crop production exerted by AMF inoculum under salinity stress condition is not fully understood yet.

Proso millet (*Panicum miliaceum* L.) is a C4 annual plant, introduced from eastern and central Asia to Europe about 3000 years ago (Upadhyaya et al., 2011). It is a mycorrhizal plant that has previously been reported to strongly benefit from AMF inoculation (Channabasava et al. 2015). Moreover proso millet is the world's sixth most important cereal grain (Arab et al., 2013) and is mainly cultivated in Africa and Asia (Lèder , 2004). In Europe, it is cultivated on 61,233 ha with an average grain yield of 1.9 Mg ha^{-1} (FAOSTAT, 2014). This species has many desirable agronomic traits including short growing season (60-90 days), low nutrient and water requirements as well as excellent tolerance to salt, drought, high temperature, and other extreme conditions (Yue et al., 2016). For these reasons, proso millet can be also cultivated in marginal lands where other cereals do not fully succeed (Hunt et al., 2011). In several countries, it is harvested primarily for human consumption (Saleh et al., 2013; Morales et al., 2015), but it is also used as fodder (Morales et al., 2015). However, the specific scientific literature on proso millet use as forage crop, especially in the Mediterranean area, is lacking. In Iran, fresh biomass yields ranging from 16.1 Mg ha^{-1} (Jahansouz et al., 2014) to 43.6 Mg ha^{-1} (Mohajer et al., 2012) have been reported.

The semi-arid zones of Southern Italy are particularly prone to erosion due to a combination of climatic and edaphic factors, including soil salinity. In Sicily, marginal land reclamation programs are being now evaluated and sustainable practices, such as proso millet cultivation with AMF biofertilization, could represent a valid option.

The aims of this study on proso millet were to evaluate: i) in laboratory conditions, the effect of osmo-salinity stress on seed germination of different genotypes; ii) in a Mediterranean marginal soil, the effect of AMF inoculation under different saline water levels and crop evapotranspiration restitutions (ETm) on the milk-dough fresh biomass yield (FBY) for forage production.

Materials and methods

Experimental description

Laboratory experiments

The laboratory experiments were carried out at the Department of Agriculture, Food and Environment of the University of Catania (Italy). Three different genotypes of *Panicum miliaceum* L. (VIR 9181, Unikum and Kinelskoje) were chosen for seeds imbibition and germination tests. The three used seed lots had a 1,000 seeds weight of 4.85 g for VIR

9181, 5.63 g for Unikum and 6.60 g for Kinelskoje. The studied treatments are listed in table 1.

Table 1 – Osmotic pressure and agents of the germination solutions.

Treatment number	Osmotic agent	Osmotic pressure (MPa)
1	Distilled water	0
2	NaCl	-0.250
3	NaCl	-0.500
4	NaCl	-0.750
5	Mannitol	-0.250
6	Mannitol	-0.500
7	Mannitol	-0.750

Salinity stress was induced by adding NaCl at the concentrations able to give the same osmotic potentials of the mannitol solutions. Osmotic potential in NaCl solutions was verified using an automatic cryoscopic osmometer (Gonotec Osmomat 030 model, Berlin, Germany). Mannitol solutions were prepared, according to the required water potential, as described by Machado Neto et al. (2004).

Seed water uptake. For each treatment, seed water uptake at 2, 4, 17 and 21 hours of imbibition, was measured. For this purpose, 30 millet seeds of uniform size were hand-selected for each genotype and placed in Petri dishes (Ø 9 cm) with 9 ml of each studied solution under dark conditions at 25 ±1°C temperature. Each treatment was replicated three times. After initiation millet seeds were removed at each measuring time, drained, blotted with absorbent paper, weighed and placed again into the Petri dishes. Percentage seed water uptake was determined as:

Seed water uptake (%) = [(final weight−initial weight)/initial weight] × 100

Seed Germination. Hand-selected seeds of uniform size were surface sterilized with 5% (w/v) calcium hypochlorite for 5 min and rinsed four times with deionized water. These seeds were then transferred to Petri dishes (20 seeds per Ø 9 cm Petri dish) containing one Whatman® Filter Paper moistened with 9 ml of the studied solutions. Each treatment was replicated three times. Petri dishes were tightly sealed with Parafilm® to avoid water depletion. Seeds were allowed to germinate in a growth chamber at a temperature of 25 ± 1 °C in the dark. Seed germination was measured daily. Seeds were scored as germinated when a radicle extrusion ≥ 2 mm long was observed and subsequently they were removed

from Petri dishes. Germination percentage (GP) was calculated according to the International Seed Testing Association (ISTA) method (Ali & Idris 2015):

GP = Number of normally germinated seeds/ total seeds number × 100

Moreover, seedling vigor index (SVI) was calculated by the following formula according to Ali and Idris (2015):

SVI = (seedling length (cm) x germination percentage) / 100

Field experiment

The two millet genotypes that showed the highest (Unikum) and lowest (Kinealskoje) germination percentages in preliminary laboratory trial, were evaluated in an open field study (summer 2014) to test genotype-specific response under stress conditions. The experiment was conducted at the Experimental Farm "Cibali" of the Istituto Agrario Siciliano Valdisavoia (37°31' N, 15°04' E, 84 m a.s.l.) Catania (Italy) in a volcanic soil with sandy texture.

Over the experimental short-period, the main daily meteorological variables: maximum, minimum and average temperature, rainfall, solar radiation and potential evapotranspiration (ET_0), were provided by the INAF (Osservatorio Astrofisico di Catania) though an agrometeorological stations located approximately 500 m from the experimental site.

The adopted experimental design was a split-plot (Fig. 1) with the following treatments: AMF inoculation as the main factor (AMF-Y = inoculated plots and AMF-N = not inoculated plots), salt stress induced by irrigation with NaCl (0.5 dS m^{-1} = Salt-N and 5.0 dS m^{-1} = Salt-Y) as the second factor, water restitutions (25% ETm and 100% ETm) as the third one and millet genotypes (Unikum vs Kinelskoje) as the fourth one, replicated two times. Crop coefficient (Kc) and maximum evapotranspiration (ETm) were calculated in agreement with FAO-56 guidelines (Allen et al., 1998).

Sowing, weeding and thinning operations were done manually. The two genotypes were sowed on 10th June 2014, at 360 seeds m^{-2}, adopting an inter-row distance of 15 cm and a sowing depth of about 2 cm. At the sowing, AMF inoculation (based on *Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus* spp. and other microorganisms) was manually distributed at 600 propagules m^{-2} along the row. All plots received for the first 8 day after sowing (DAS) the same water volume (about 6 mm of fresh water every two days) for seed germination. Subsequently, at 10 DAS when the crop was at the first two leaf fully expanded, water restitution and NaCl treatments took place until harvest, distributing a total of 30.6 mm for 25% ETm and 159.3 mm for 100% ETm. After millet

plants emergence, 8 randomly selected plants per treatment were marked and weekly monitored for culm height, leaf number, and phenological phase in agreement with BBCH scale (Hess et al. 1997). Moreover, the leaf SPAD value, an indirect measure of chlorophyll content, was measured three times from 34 to 48 DAS (SPAD 502 Chlorophyll Meter - Spectrum Technologies, Inc).

On 4th August 2014, millet was harvested at milk-dough stage and fresh biomass yield and its components were detected. In four plants per subplot, the leaf area surface was measured using WinDias 2.0 (©DELTA-T Devices ltd 1995-2000).

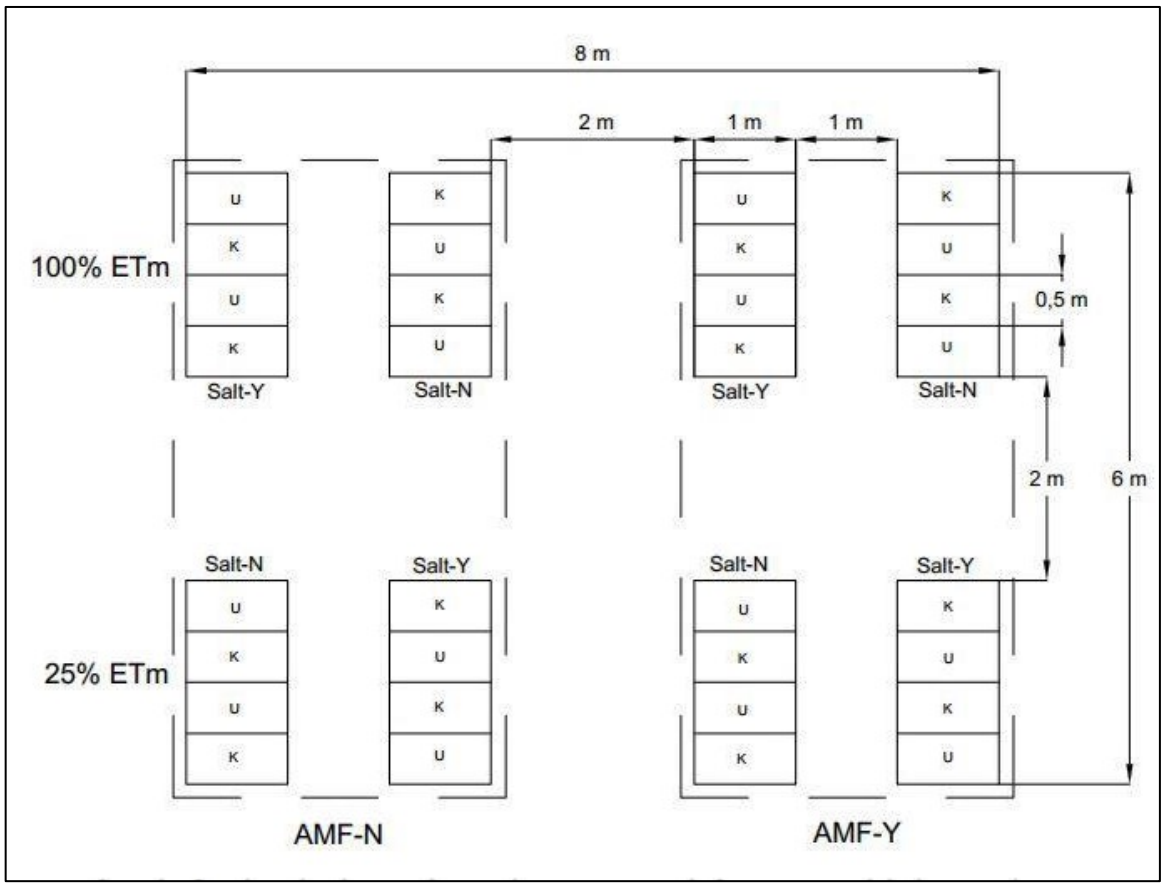


Figure 1 – Experimental design

Statistical analysis

Data were analyzed by ANOVA in order to evaluate the treatments effects. A post-hoc test was performed to compare means using the Fisher's LSD test (α=0.05). An arcsine transformation was applied to all data expressed as a percentage before performing ANOVA.

Results

Laboratory experiments

Seed water uptake: seed water uptake dynamics during the first 21 hours of imbibition were significantly ($p<0.001$) influenced by the osmotic pressure. Regardless of the genotype, significant higher water uptake was measured after 4, 17 and 21 hours in the control (0.0 MPa) compared to other osmotic pressure levels (Fig. 2a). Considering genotypes, after 2 and 4 hours, water uptake in Kinelskoje was significantly higher ($p<0.001$) than in the other two genotypes whereas no differences were found after 17 and 21 hours (Fig. 2b).

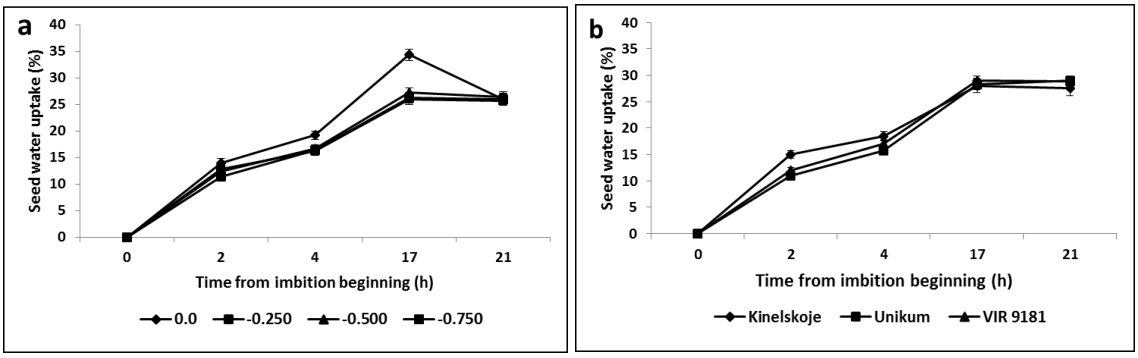


Figure 2 – Time course of water uptake as affected by: a) osmotic pressure and b) millet genotypes.

At 21 hours from the start of seed imbibition, water uptake was significantly ($p<0.05$) affected by the interaction between millet genotypes and osmotic pressure (Fig. 3a). Kinelskoje presented the highest water uptake at 0.0 MPa (38.7%) but was the most sensitive among the three genotypes to the decrease of water potential from -0.250 MPa to -0.500 MPa. At the lowest water potential (-0.750 MPa), all genotypes showed the same water uptake (on average 25.7%).

Seed germination: percentage germination of VIR 9181 and Kinelskoje was negatively affected by the decrease in water potential. In VIR 9181, significant differences in seed germination were observed from 0.0 to -0.250 MPa with a decrease of -4.72%; while no differences from to -0.250 to -0.750 MPa were found (Fig. 3b). In Kinelskoje, significantly ($p<0.05$) lower germination values were observed at -0.250 and -0.500 MPa (on average -8.0%), and at -0.750 MPa (-19.8%), compared to the 0 MPa (control-distilled water) (Fig. 3b). Unikum seed germination was not affected by osmotic pressure showing the highest germination (95.1% ± 0.75) (Fig. 3b) and SVI (3.65 ± 0.30) values. Reductions in SVI are observed in all the studied genotypes already al -0.250 MPa. However, a lower decrease was observed in Unikum as compared to the other cultivars (Tab. 2). As well as for water uptake and germination rate, the lowest water potential (-0.750 MPa) was the most detrimental for seedling growth and, regardless of the treatment

applied, determined the strongest significant ($p<0.01$) decrease of SVI (-70.9%) compared to the control (Tab. 2). No differences were found in seed water uptake, germination and seedling vigor index in relation to the used osmoticum (NaCl and mannitol).

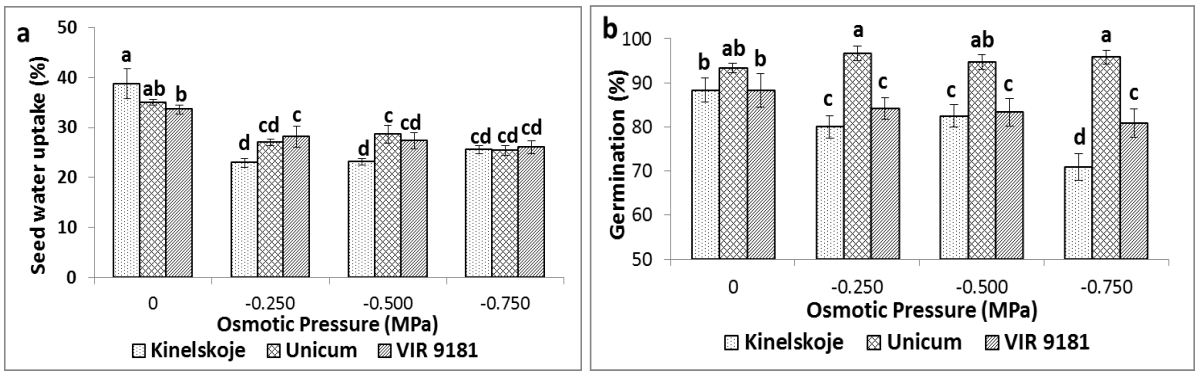


Figure 3 – Water uptake at the 21th hours (a) and germination percentage at the 96th hour (b) of millet genotypes under osmotic pressure. Different letters show statistical differences of the treatments at $p<0.05$ (LSD – Fisher Test).

Table 2 – Seedling vigor index (SVI) of millet genotypes in relation to osmotic pressure

Osmotic Pressure (MPa)	Genotypes			
	Kinelskoje	**Unikum**	**VIR 9181**	**mean**
0.0	3.93 ± 0.17	5.48 ± 0.07	5.35 ± 0.16	**4.92 ± 0.19**
-0.250	2.48 ± 0.29	4.08 ± 0.46	2.66 ± 0.23	**3.07 ± 0.25**
-0.500	2.45 ± 0.32	3.01 ± 0.36	2.93 ± 0.36	**2.79 ± 0.20**
-0.750	1.08 ± 0.13	2.04 ± 2.04	1.18 ± 0.10	**1.43 ± 0.13**
mean	**2.48 ± 0.24**	**3.65 ± 0.30**	**3.03 ± 0.33**	**3.05 ± 0.18**

ANOVA							L.S.D.	
	SS	**DF**	**MS**	**F**	**Prob F**	**Sign. F**	**p<0.05**	**p<0.01**
G	15.7309	2	7.8654	17.2022	2.33E-06	**	0.39247	0.52356
OP	108.764	3	36.256	79.2911	1.30E-18	**	0.45319	0.60456
S	0.0147	1	0.0147	0.0321	8.59E-01		0.32045	0.42749
G x OP	6.1833	6	1.0306	2.2539	5.37E-02		0.78495	1.04713
G x S	0.3383	2	0.1692	0.3700	6.93E-01		0.55504	0.74043
OP x S	0.5102	3	0.1701	0.3719	7.74E-01		0.64091	0.85498
G x OP x S	4.1026	6	0.6838	1.4954	2.00E-01		1.11009	1.48086
Residual	21.9472	48	0.4572					
Total	157.5909	71						

G= genotype; OP= Osmotic Pressure; S= Solute (NaCl and Mannitol)

Field experiment

Meteorological variables

The experimental site is characterized by a Mediterranean climate in agreement with Köppen and Geiger (1928), with an average annual temperature of 17.5 °C and average rainfall of 567 mm year^{-1} (Servizio Metereologico dell'Aeronautica Militare). During the trial period the highest temperature (+40°C) was recorded on June 26th 2014 and the lowest one (+11°C) on June 7th 2014 (Fig. 4a) and a fairly uniform solar radiation was observed during the experimental period (26.6 MJ m^{-2} d^{-1}) (Fig. 4b). In the first 16 DAS only 9.4 mm in 3 rain events (two of which lower than 1.0 mm) (Fig. 4a) were recorded between the first and fourth leaf phenological stage, according to BBCH scale (Hess et al., 1997). The evapotranspiration (ET_0) during the crop cycle, ranged from 3.4 (June 17th 2014) to 10.5 mm d^{-1} (June 26th 2014), with an total of 351.0 mm of evapotranspiration (Fig. 4b).

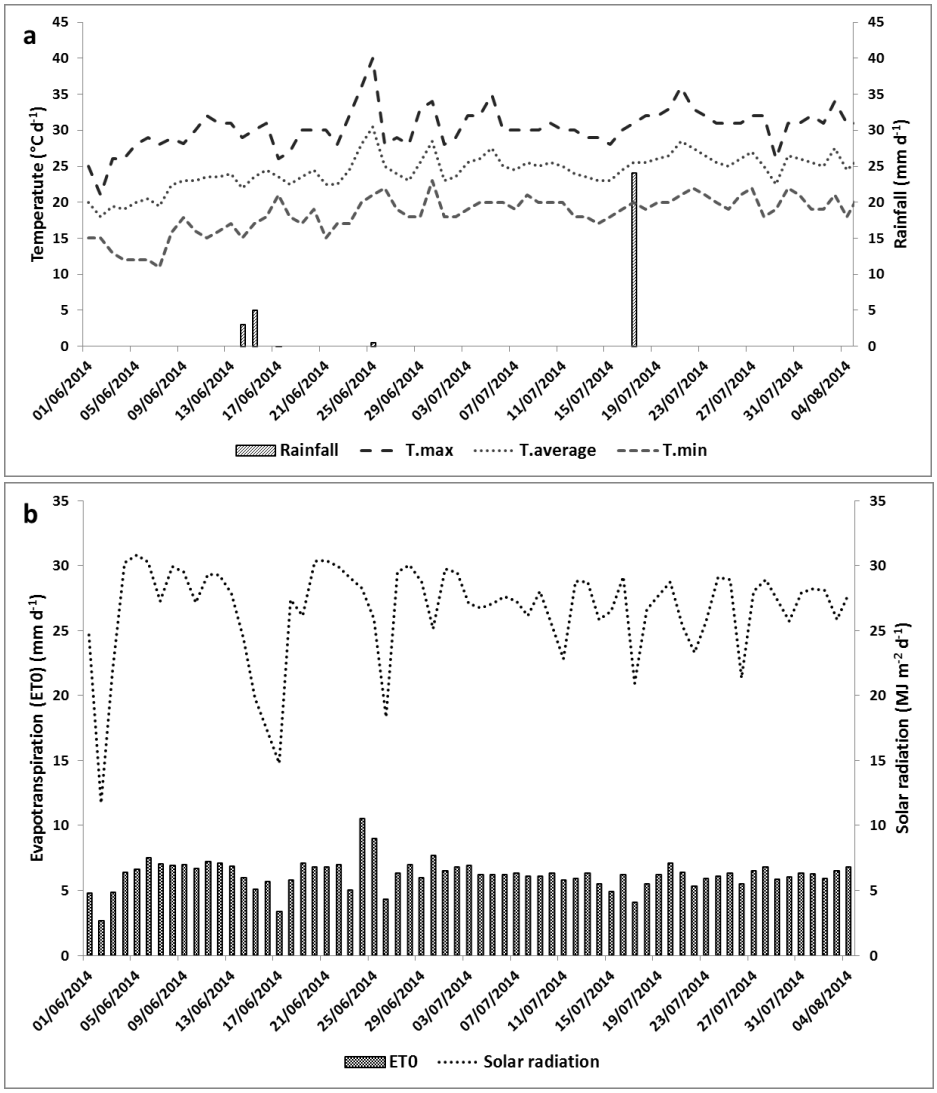


Figure 4 – Environmental condition site: a) rainfall, maximum, minimum and average temperature; b) evapotranspiration (ET_0) and solar radiation.

Fresh Biomass Yield (FBY)

FBY was significantly influenced by all the studied factors. In the average of the other studied factors, the main effects were: for genotype Unikum showed a significant higher FBY (+82.5%) than Kinelskoje (340.0 ± 73.5 g FBY m^{-1}); AMF inoculation induced a positive effect on FBY (+19.4%) as compared to un-inoculated plots (437.7 ± 112.3 g FBY m^{-1}); the lowest ETm restitution (25%) determined a significant decrease (-85.5%) on FBY as compared to 100% ETm (838.6 ± 77.5 g FBY m^{-1}); water salinity irrigation significantly reduced (-28.6%) FBY compared to the absence of salt stress (560.1 ± 116.1 g FBY m^{-1}).

Under salt stress conditions, AMF inoculation did not exert significant effects on FBY in both millet genotypes (Fig. 5). In the Salt-N treatment, only in Unikum the AMF-Y treatment determined a significant ($p<0.01$) FBY increase (+50.7%) compared to the AMF-N one (552.5 ± 269 g m^{-2}).

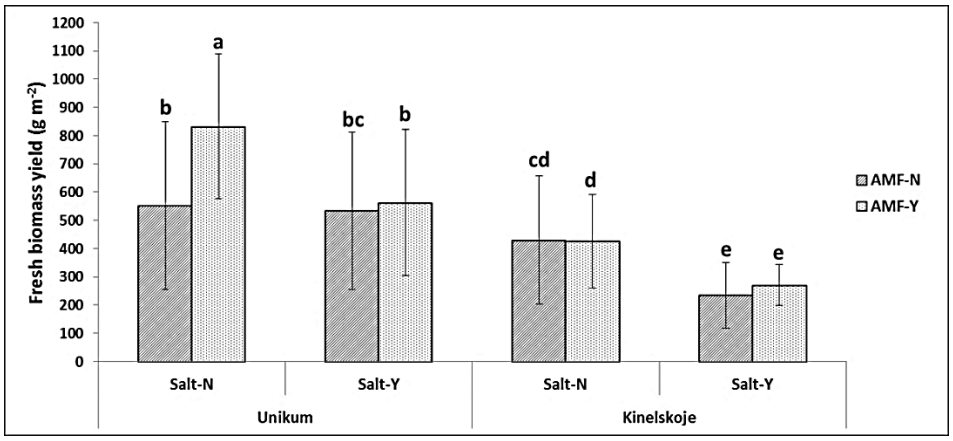


Figure 5 – Effect of arbuscular mycorrhizal inoculation on fresh biomass yield in two millet genotypes under salt conditions. Different letters show statistical differences of the treatments at $p<0.01$ (LSD – Fisher Test).

Considering water restitution, at 100% ETm, the Salt-Y treatment determined a significant ($p<0.01$) FBY decrease in both Unikum (-13.6%) and Kinelskoje (-45.9%) compared to the Salt-N treatments (1169.8 g m^{-2} in Unikum and 761.9 g m^{-2} in Kinelskoje). At 25% ETm in Unikum, the Salt-Y treatment determined a significant ($p<0.01$) reduction (-60.3%) of FBY compared to Salt-N one (215.4 ± 100.6 g m^{-2}), whereas, in Kinelskoje, no significant difference was observed between salt treatments with a mean value of 252 ± 63.8 g FBY m^{-2}. The 25% ETm significantly ($p<0.05$) reduced FBY in both genotypes (-86.2% and -84.1% for Unikum and Kinelskoje, respectively) compared to the 100% ETm treatments (1090.3 ± 49.7 g m^{-2} in Unikum and 587 ± 72.2 g m^{-2} in Kinelskoje). The AMF inoculation at 100% ETm restitution did not significantly affect the FBY compared

to the un-inoculated treatment (grand mean 838.6 ± 112.4 g m^{-2}). Conversely, at 25% ETm a significant ($p<0.01$) increase of FBY was observed in AMF inoculated plants (Fig. 6).

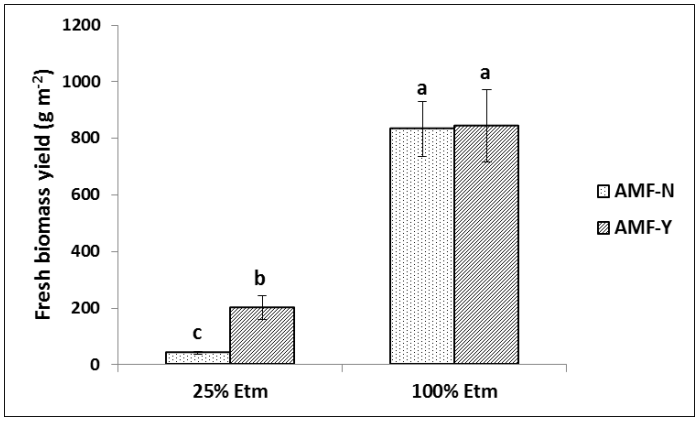


Figure 6 – **Effect of arbuscular mycorrhizal inoculation on fresh biomass yield under different water restitution levels.** Different letters show statistical differences of the treatments at $p<0.01$ (LSD – Fisher Test).

At harvest time, the relative moisture of FBY ranged from to 70.3% (25% ETm, AMF-Y, Salt-N) to 88.2% (100% ETm, AMF-N, Salt-Y) in Kinelskoje and from to 74.9% (25% ETm, AMF-N, Salt-N) to 89.3% (100% ETm, AMF-Y, Salt-N) in Unikum.

Shoot density

Shoot density was significantly ($p<0.01$) affected by genotype and water restitution. The main effects were: Unikum showed a higher shoot density (+35%) than Kinelskoje (131 ± 7.3 plants m^{-2}); the 25% ETm restitution determined a significant ($p<0.001$) decrease (-25.5%) in shoot density compared to 100% ETm restitution (176 ± 13 plants m^{-2}). The significant interaction ($p<0.01$) between water restitution and AMF inoculation, showed a positive effect explained by AMF inoculum at the lower ETm restitution and a negative one at full ETm restitution (Fig. 7).

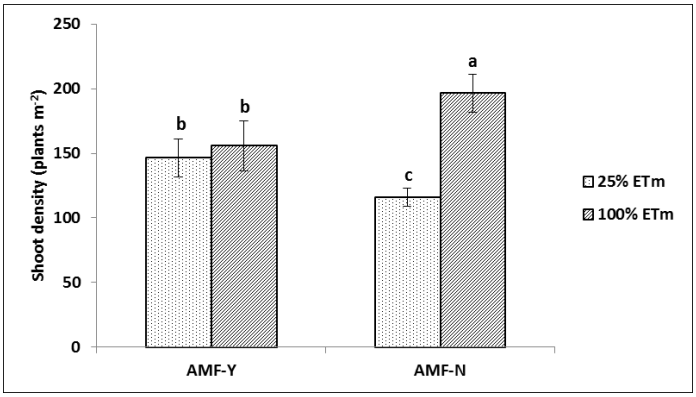


Figure 7 – **Effect of arbuscular mycorrhizal inoculation on shoot density under different water restitution levels.** Different letters show statistical differences of the treatments at $p<0.01$ (LSD – Fisher Test).

Culm height and fresh culm weight

The treatment effects on culm height is reported in table 3. Unikum showed a significantly ($p<0.05$) greater culm height (+23.6%) than Kinelskoje (20.7 ± 1.5 cm plant^{-1}). Only in Kinelskoje, AMF inoculation determined a significant culm height increase (+49.7%) compared to the AMF-N treatment. Regardless water restitution levels, in the Salt-N treatments, AMF inoculation significantly ($p<0.05$) increased culm height (+54.6%), whereas in the Salt-Y ones no differences were detected between AMF-Y and AMF-N (grand mean = 20.5 ± 1.60 cm plant^{-1}). Salt stress determined a significant ($p<0.05$) culm height decrease in Kinelskoje (-32.6%) compared to the Salt-N treatment (24.7 ± 2.44 cm plant-1), whereas no difference was observed in Unikum (25.6 ± 2.12 cm plant^{-1} on average). At 100% ETm water restitution, AMF inoculation determined a significantly ($p<0.05$) greater culm height than the un-inoculated treatments, whereas, at 25% ETm water restitution AMF inoculation did not determine a significant effect on culm height (14.6 ± 1.07 cm plant^{-1} on average). Considering the interaction between water restitution and genotype, Unikum showed a significantly ($p<0.05$) higher culm height (+28.3%) than Kinelskoje (27.8 ± 1.98 cm plant^{-1}) at 100% ETm water restitution, whereas, no difference was found at 25% ETm water restitution (14.6 ± 1.11 cm plant^{-1} on average).

The treatments effect on culm fresh weight is reported in table 4. The two genotypes did not show significant differences in culm fresh weight (grand mean = 1.17 ± 0.09 g plant^{-1}). Salinity stress, significantly affected culm fresh weight which was lowered by -39.5% (25% ETm) and -42.5% (100% ETm) as compared to the Salt-N treatments. At full ETm restitution and in absence of salt treatment, AMF inoculation determined a significant ($p<0.05$) culm fresh weight increase (+86.8%) compared to the un-inoculated control, whereas, under salinity stress, no AMF effect was detected. No significant effect of AMF inoculation was observed at 25% ETm restitution whatever the irrigation water salinity.

Panicle length and fresh panicle weight

The effect of the treatments on panicle length is reported in table 3. In absence of salt at both water restitution levels, significant positive effects ($p<0.05$) of AMF inoculation were observed on panicle length. In presence of the salt stress, AMF inoculation did not determine significant differences on panicle length, with grand mean values of 0.52 ± 0.35 cm plant^{-1} (25% ETm) and 13.7 ± 0.98 cm plant^{-1} (100% ETm). However, the panicle growth was completely inhibited by the lowest water restitution (25% ETm) in AMF-N treatment. At 100% ETm water restitution, Unikum showed a panicle length

significantly ($p<0.05$) higher (+43.0%) than Kinelskoje (11.9 ± 0.67 cm plant^{-1}), whereas no difference in this parameter was observed at 25% ETm (2.88 ± 0.92 cm plant^{-1} on average).

The effect of the treatments on panicle fresh weight is reported in table 4. At 100% ETm, AMF inoculation did not exert a positive effect on panicle fresh weight, since no difference was observed between the two AMF treatments with a grand mean value of 0.73 ± 0.08 g plant^{-1}. Comparing genotypes, the 100% ETm water restitution determined a significantly ($p<0.05$) higher panicle fresh weight (+77.2%) in Unikum than the Kinelskoje (0.53 ± 0.05 g plant^{-1}). No differences were found between the two millet genotypes at the lowest water restitution (25% ETm, with a grand mean = 0.15 ± 0.05 g plant^{-1}). Regardless of the other factors, Unikum showed a significantly ($p<0.05$) higher panicle fresh weight (+53.6%) than Kinelskoje (0.41 ± 0.05 g plant^{-1}). Salt-Y treatment negatively affected the panicle fresh weight with a significant ($p<0.05$) decrease (-29.9%) compared to the Salt-N one (0.51 ± 0.06 g plant^{-1}). AMF inoculation determined a significantly ($p<0.05$) higher panicle fresh weight (+41.5%) than the un-inoculated treatment (0.36 ± 0.06 g plant^{-1}).

Leaf number and leaves fresh weight

The effect of the treatments on leaf number and leaf fresh weight are reported in table 3 and table 4, respectively. Leaf number was significantly ($p<0.05$) higher in Unikum (+9.66%) than in Kinelskoje (4.20 ± 0.19 leaf culm^{-1}). In Kinelskosje, AMF inoculation determined a significant ($p<0.05$) increase in leaf number (+24.2%) and fresh weight (+76.2%) compared to the un-inoculated plants (3.75 ± 0.31 leaf culm^{-1} and 0.54 ± 0.08 g culm^{-1}), whereas, AMF inoculation did not show any effect in Unikum (4.60 ± 0.13 leaf culm^{-1} and 0.92 ± 0.09 g culm^{-1}). Regardless of the millet genotype, at 100% ETm restitution the AMF inoculation did not affect leaf number despite the salt treatments, whereas, at 25% ETm restitution a positive ($p<0.05$) effect of AMF inoculation was observed in absence of salt stress.

The two genotypes, at 100% ETm water restitution, did not show significant differences in the number of leaves. On the contrary, at the lowest water restitution level (25% ETm), a significant ($p<0.01$) reduction in the number of leaves was observed in both genotypes. In particular, Kineslkosje showed a -19.8% decrease compared to the Unikum (4.25 ± 0.13 leaf culm^{-1}).

Considering both stresses, salt irrigation and water restitution determined a significant ($p<0.01$) leaf fresh weight decrease at Salt-Y (-17.7%) and at 25%ETm (-51.1%) as

compared to Salt-N (0.91 ± 0.04 g culm^{-1}) and 100% ETm water restitution (1.11 ± 0.04 g culm^{-1}).

Table 3 – Bio-morphological proso millet characteristics.

Treatments				Culm height (cm)	Panicle length (cm)	Leaf number (plant^{-1})
Kinelskoje	**AMF-Y**	**Salt-Y**	**25% ETm**	13.3 ± 2.37	2.06 ± 1.35	3.75 ± 0.45
			100% ETm	25.1 ± 2.09	10.6 ± 1.11	5.50 ± 0.33
		Salt-N	**25% ETm**	18.1 ± 3.59	11.2 ± 0.64	4.88 ± 0.23
			100% ETm	42.8 ± 2.27	13.7 ± 0.39	4.50 ± 0.46
	AMF-N	**Salt-Y**	**25% ETm**	12.1 ± 1.43	0.00 ± 0.00	3.38 ± 0.26
			100% ETm	16.2 ± 1.02	10.9 ± 0.33	5.00 ± 0.19
		Salt-N	**25% ETm**	11.0 ± 0.58	0.00 ± 0.00	1.63 ± 0.63
			100% ETm	27.1 ± 2.54	12.5 ± 2.39	5.00 ± 0.27
Unikum	**AMF-Y**	**Salt-Y**	**25% ETm**	15.5 ± 1.39	0.00 ± 0.00	4.38 ± 0.18
			100% ETm	30.5 ± 1.79	16.0 ± 1.27	4.75 ± 0.25
		Salt-N	**25% ETm**	17.1 ± 2.79	9.81 ± 2.28	4.25 ± 0.25
			100% ETm	47.4 ± 1.68	21.2 ± 0.87	4.75 ± 0.25
	AMF-N	**Salt-Y**	**25% ETm**	17.5 ± 2.44	0.00 ± 0.00	4.00 ± 0.38
			100% ETm	33.6 ± 0.86	17.3 ± 0.60	5.13 ± 0.13
		Salt-N	**25% ETm**	12.0 ± 0.92	0.00 ± 0.00	4.38 ± 0.18
			100% ETm	31.1 ± 0.95	13.7 ± 1.15	5.25 ± 0.16

ANOVA

	Sign.	LSD	Sign.	LSD	Sign.	LSD
Genotype (G)	**	2.4764	**	1.4099	*	0.3142
Mycorrhizal inoculation (M)	**	2.4764	**	1.4099	ns	-
Water salinity (S)	**	2.4764	**	1.4099	*	0.3142
Water restitution (W)	**	2.4764	**	1.4099	**	0.4157
G x M	*	2.6469	ns	-	**	0.5879
G x S	**	3.5022	ns	-	ns	-
G x W	**	3.5022	**	1.9939	**	0.5879
M x S	**	3.5022	**	1.9939	ns	-
M x W	**	3.5022	**	1.9939	**	0.5879
S x W	**	3.5022	**	1.9939	ns	-
G x M x S	ns	-	ns	-	ns	-
G x M x W	ns	-	*	2.8198	ns	-
G x S x W	ns	-	ns	-	ns	-
M x S x W	ns	-	*	2.8198	ns	-
G x M x S x W	ns	-	ns	-	ns	-

***= p<0.05; **= p<0.01; ns = not significant**

Table 4 – Proso millet fresh weight yield components of main culm yield.

Treatments				Culm (g)	Panicle (g)	Leaves (g)
Kinelskoje	AMF-Y	Salt-Y	25% ETm	0.37 ± 0.11	0.16 ± 0.11	0.63 ± 0.23
			100% ETm	1.36 ± 0.29	0.42 ± 0.11	1.10 ± 0.16
		Salt-N	25% ETm	0.96 ± 0.17	0.47 ± 0.07	0.93 ± 0.08
			100% ETm	2.82 ± 0.24	0.57 ± 0.06	1.13 ± 0.15
	AMF-N	Salt-Y	25% ETm	0.22 ± 0.04	0.00 ± 0.00	0.22 ± 0.04
			100% ETm	0.81 ± 0.09	0.54 ± 0.08	0.67 ± 0.12
		Salt-N	25% ETm	0.34 ± 0.04	0.00 ± 0.00	0.15 ± 0.08
			100% ETm	1.74 ± 0.27	0.59 ± 0.11	1.10 ± 0.18
Unikum	AMF-Y	Salt-Y	25% ETm	0.41 ± 0.18	0.00 ± 0.00	0.58 ± 0.17
			100% ETm	1.69 ± 0.29	0.84 ± 0.15	1.04 ± 0.23
		Salt-N	25% ETm	0.54 ± 0.12	0.53 ± 0.11	0.79 ± 0.06
			100% ETm	3.38 ± 0.44	1.12 ± 0.16	1.40 ± 0.12
	AMF-N	Salt-Y	25% ETm	0.40 ± 0.07	0.00 ± 0.00	0.61 ± 0.23
			100% ETm	1.61 ± 0.20	0.94 ± 0.25	1.18 ± 0.15
		Salt-N	25% ETm	0.47 ± 0.10	0.00 ± 0.00	0.47 ± 0.10
			100% ETm	1.58 ± 0.22	0.85 ± 0.17	1.34 ± 0.11

ANOVA

	Sign.	LSD	Sign.	LSD	Sign.	LSD
Genotype (G)	ns	-	**	0.14837	*	0.140
Mycorrhizal inoculation (M)	**	0.24871	**	0.14837	*	0.140
Water salinity (S)	**	0.24871	**	0.14837	**	0.185
Water restitution (W)	**	0.24871	**	0.14837	**	0.185
G x M	ns	-	ns	-	*	0.198
G x S	ns	-	ns	-	ns	-
G x W	*	0.26583	**	0.20983	ns	-
M x S	**	0.35172	**	0.20983	ns	-
M x W	**	0.35172	*	0.15859	ns	-
S x W	**	0.35172	ns	-	ns	-
G x M x S	ns	-	ns	-	ns	-
G x M x W	ns	-	ns	-	ns	-
G x S x W	ns	-	ns	-	ns	-
M x S x W	ns	-	ns	-	ns	-
G x M x S x W	ns	-	ns	-	ns	-

***= p<0.05; **= p<0.01; ns = not significant**

Leaf SPAD

In Kinelskoje, AMF inoculation determined a significant ($p<0.01$) increase (+13.7%) in leaf SPAD value compared to the AMF-N treatment (37.8 ± 1.21), but not in Unikum (grand mean SPAD value = 40.9 ± 0.78) (Fig. 8a). At 25% ETm restitution, Kinelskoje showed a significant ($p<0.01$) decrease (-18.1%) in leaf SPAD value as compared to full water restitution (44.4 ± 0.45), whereas, no statistical difference was found in Unikum with an average leaf SPAD value of 40.9 ± 0.78 (Fig. 8b). No effect was found due to irrigation water salinity levels, regardless of other studied factors.

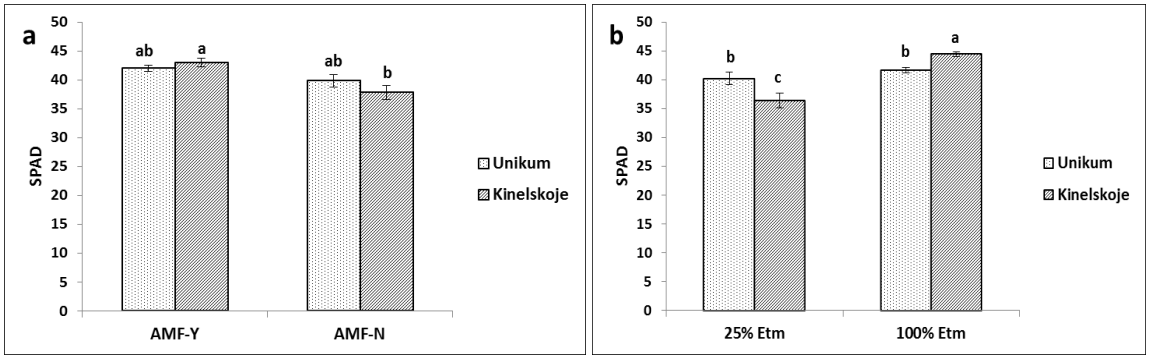


Figure 8 – Effect of the studied factors on the SPAD value. a) genotype x AMF inoculation; b) genotype x ETm restitution. Different letters show statistical differences of the treatments at p<0.01 (LSD – Fisher Test).

Leaf area surface

Salt treatment did not determine any different leaf area in AMF-N plots. AMF inoculation determined a significant (p<0.05) increase (+80.5%) in leaf area in Salt-N compared to Salt-Y (26.6 ± 5.3 cm^2 $plant^{-1}$) (Fig. 9a). Regardless of the genotypes, without AMF inoculation, at 25% ETm water restitution, we observed a significant (p<0.05) decrease in leaf area (-68.1%) compared to 100% ETm restitution (47.2 ± 3.67 cm^2 $plant^{-1}$). At both water restitution levels, no significant difference was found in presence of AMF inoculation (Fig. 9b).

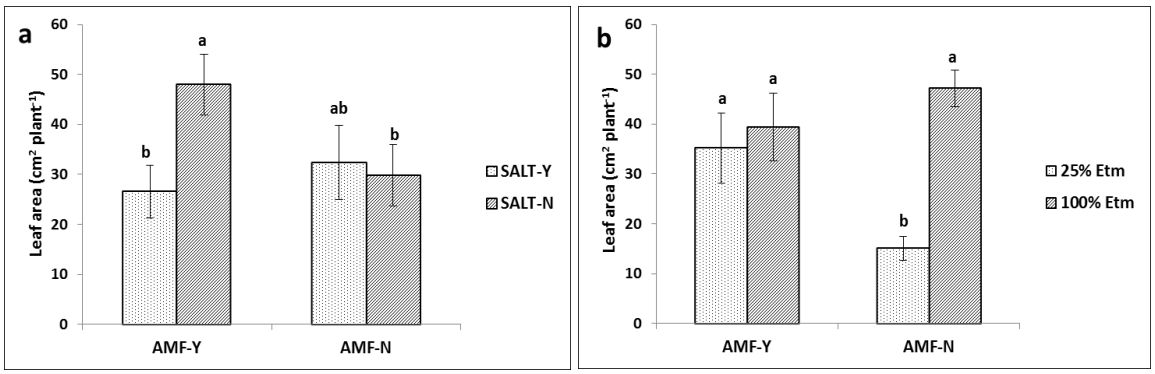


Figure 9 – Effect of the studied factors on leaf area. a) Salt x AMF inoculation; b) ETm restitution x AMF inoculation. Different letters show statistical differences of the treatments at p<0.05 (LSD – Fisher Test).

Discussion

Laboratory experiments

In Mediterranean areas, germination process is affected by abiotic stresses such as high salinity and drought which determine a lower seed water absorption (Dodd & Donovan 1999) and affect the mobilization of stored reserves or directly the synthesis of proteins in germinating embryos (Almansouri et al. 2001), thus jeopardizing growth processes. Almansouri et al. (2001), in a study on durum wheat, have shown a reduction or inhibition in germination in response to the decrease in water potential. However, these authors, at an osmotic pressure similar to the one tested in our study (-0.580 MPa), did not find any effect on final germination percentage in iso-osmotic solution of NaCl and mannitol. In our case, seed water uptake was negatively affected by the external water potential decrease, due to the reduced diffusivity of water to the seed coats, but the seeds moisture was sufficient to ensure a good germination in all genotypes.

Generally, seed germination decreased with increasing salt concentrations and salt specific effects (Ryan et al. 1975; Cavallaro et al. 2016). Sabir et al. (2008) on 18 millet accessions collected from different Pakistan areas, demonstrated an inter-cultivar variation for NaCl tolerance (0.0, -0.300, -0.600 and -0.900 MPa).These authors reported a general significant salt-induced depression on seed germination in all the accessions, except for the four ones which showed the highest percentage at -0.900 MPa. In our experiment, although salt stress range was more limited (up to -0.750 MPa) as compared to Sabir et al. (2008) study, the three genotypes were not affected by salt levels, suggesting their salt tolerance.

The lack of differences in seed water uptake, germination and seedling vigor index obtained in our study comparing osmotica (NaCl or mannitol) can be explained by a similar penetrating behavior of NaCl and mannitol in the plant tissue that contributes to adjust the internal osmotic potential decrease in the germinating seeds, thus allowing to maintain a sufficient water uptake under a high external water potential.

Field experiments

The FBY showed genotype and salinity specific responses indicating Unikum, between the studied genotypes, as the best performing under salinity and water stress conditions. In presence of salt treatment, regardless the genotype, the effect of AMF inoculation was inhibited, resulting that mycorrhizal inoculum did not promote FBY. This finding is not in line with Daei et al. (2009) that reported, in durum wheat cultivated under salinity

condition (7.41 dS m^{-1}), a significant increase on growth and grain yield of mycorrhized plants, due to enhanced nutrient uptake. Juniper and Abbott (2006) showed that the germination of spores of all the AMF fungi tested was delayed and the hyphal growth from propagules was reduced in presence of NaCl. This latter result could explained the absence of a significant AMF effect on FBY at the end of crop cycle. AMF inoculation determined a better FBY response under water-stress conditions whereas no effects were seen in well-watered plots. This finding is in agreement with the results obtained by Porcel et al. (2004) and Saia et al. (2014) in different pedo-climatic conditions on sorghum and berseem clover. They reported a positive AMF effect under water shortage and not AMF effect at full ETm restitution. At 100% ETm restitution, AMF inoculation significantly decreased shoot density and increased culm height. On the contrary, under water stress conditions (25% ETm) AMF inoculation increased shoot density but did not influence culm height. These results suggest that: i) under water-stress conditions (25% ETm), in presence of AMF inoculation the increase in FBY can be attributed to higher shoot density; ii) under well-watered condition (100% ETm), the absence of significant difference on FBY between AMF treatments, can be attributed by the increase of mycorrhized plant weight nevertheless the lower shoot density. Considering the panicle emission and development, a positive effect was exerted by AMF inoculation in both ETm restitution levels, especially in absence of salt stress, suggesting that the mycorrhizal fungi promoted the transition from vegetative to reproductive phenological phase (Oladele and Awodun 2014). All experimental factors exerted significant influences on leaf characteristics. AMF inoculation promoted a positive effect on leaf size and fresh and dry (data not show) weight in agreement with Augé et al. (2015) and Fagbola et al. (2001) which reported respectively an improve leaf water status and leaf dry weight in mycorrhized plants. Considering SPAD value, AMF inoculation increased this parameter, confirming the mycorrhizal positive effect on plant physiological activity (Zhou et al., 2015).

Saline water irrigation negatively influenced the leaf fresh weight without significant effects on leaf number, dry weight and surface, supporting that salt stress mainly reduces leaf water content (Fidalgo et al. 2004). Moreover, salinity stress did not exert any significant effects on SPAD values in both genotypes, despite of this stress determines a chlorophyll damaging, with a reduction on photosynthetic activity and leaf senescence acceleration (Shi and Guo, 2006; Beltrano et al. 2013). Our results show that proso millet withstand at the salinity level adopted in this study, without any reduction in chlorophyll content. This

could be due to the relatively brief exposition to stress that millet underwent during its short growing season. Under water stress conditions (25% ETm) exerted highly negative effects on leaf characteristics; in this conditions, AMF inoculation could partially overcome such negative effects, confirming their beneficial role on plant water status under drought stress (Beltrano and Ronco 2008; Grümberg et al. 2015).

AMF inoculation determined a better FBY response under water-stress conditions whereas no effects were seen in well-watered plots. This finding is in agreement with previous results obtained by Porcel et al. (2004) and Saia et al. (2014) in different pedo-climatic conditions on sorghum and berseem clover, reporting a positive AMF effect under water shortage and not AMF effect at full ETm restitution.

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Conclusions

Osmotic stress determined a genotype-specific response with the strongest reduction in seed water uptake, germination and seedling vigor index observed at –0.750 MPa. Unikum germination (95.3%) was not affected by osmotic pressures with a higher SVI as compared to Kinelskoje and VIR 9181. Unikum was the best genotype in the laboratory trial and confirmed this finding also in open field conditions producing more than double FBY than Kinelskoje. Although the AMF inoculation effect was negatively influenced by irrigation with saline water, it exercised a positive effect (+377.3%) on FBY under water stress. The present study gives novel information about proso millet forage production, highlighting the AMF potential role as bio-fertilizers under low input sustainable agriculture in semi-arid Mediterranean marginal land, but further large-scale researches are needed to confirm our findings.

Chapter VI
General Conclusions

During the Ph. D. study, the research activities were focused to evaluate the effects of arbuscular mycorrhizal fungi (AMF) on annual and perennial herbaceous crops, mainly cultivated for biomass production. These crops were managed through the use of olive mill wastewater (OMW) and digestate liquid or solid fraction (DLF or DSF) as fertilizer and soil amendment, or irrigated with poor quality water. The trials were carried out in two Italian regions (Veneto and Sicily), with different pedo-climatic conditions.

Experimental trials in Veneto region:

The research has focused on the agronomic role of AMF in relation to: i) energy crops biomass production using DLF as fertilizer; ii) triticale biomass production under different fertilization rates (mineral fertilizer, DLF, DSF and not fertilizer), using seed-coated fungicides; considering also the environmental impacts in terms of soil CO_2 emissions and nitrogen leaching. The most relevant findings are the following:

- on energy crops, AMF inoculation was not effective to promote the biomass production, due to the high N fertilization rate provided with DLF application and presence of indigenous mycorrhizal community in experimental plot soils; moreover, AMF root colonization in energy crops was negatively affected by soil disturbance, induced by tillage, chemical fertilization, and dry climate condition;
- in the triticale trial, the AMF root colonization was mostly inhibited by the presence of seed-coated fungicide. The higher AMF colonization percentage, measured in presence of organic matter (DSF), could be due to: i) the reduction of fungicide negative effects as a result of the hydrophobic absorption of fungicide molecules by organic matter; ii) the presence in DSF of phosphorus in a low available form (struvite);
- a beneficial environmental contribution was provided by AMF inoculation only for NH_4-N leaching reduction; however, AMF inoculation increases NO_3-N soil loss;
- the AMF inoculation increased soil CO_2 emission only in *M. x giganteus* (+42.1%) and *H. tuberous* (+27.6%) compared to the un-inoculated plots;
- *A. donax* was the most productive energy crop in terms of biomass production among the studied crops, followed by *M. x giganteus*, *S. bicolor*, *Z. mais*, *H. tuberous* and *L. perenne*, with a lower nitrogen use efficiency (NUE) and higher cumulative soil CO_2-C emissions (+30.4%) as compared to the other species;
- *M. x giganteus* and *S. bicolor* showed the best N and P use efficiency, with a moderate NO_3-N leaching and lower cumulative soil CO_2-C emissions than to the other crops;

- comparing the two annual energy crops (sorghum and maize), sorghum showed the highest biomass production probably due to its drought tolerance and better NUE;
- in *x Triticosecale* sp. Wittmack trial, the mineral fertilization determined the highest dry biomass production (+27% respect to DLF and DSF treatments), even if the environmental (e.g. higher $CO_{2(eq)}$ emission) and economical (e.g. fertilizer costs) aspects should be considered.

Experimental trials in the Sicily region:

The AMF inoculation effects were studied in: i) a three years legume-based succession producing forage (*durum wheat-M. scutellata* intercropping), followed by a second year broad bean and chickpea the last year for grain production, using different OMW volumes as the main fertilization source; ii) a yearly evaluation of millet genotypes for forage production using irrigation water at two salinity levels and two water restitution regimes. The most relevant findings are the following:

- AMF inoculation increased the durum wheat biomass production (+30.8%), but not affected the *M. scutellata* biomass yield;
- AMF root colonization was not influenced by OMW volumes, with a higher N (+22.8%) and P (+32.5%) uptake observed in inoculated durum wheat than in un-inoculated plots; while, no statistical effect of AMF was found on N and P uptake in *M. scutellata*;
- AMF inoculation did not affect the broad bean grain yield due to the root full colonization by indigenous mycorrhizal community which was also present in un-inoculated soil;
- a higher P uptake in broad bean grain was measured with AMF inoculation treatment, probably due to the better uptake efficiency of *R. intraradices* (present in our inoculum) as compared to indigenous mycorrhizal;
- root nodule number and weight of the nitrogen fixing bacteria of broad bean were promoted by AMF inoculation;
- OMW spreading promoted the fabacee productions with a higher biomass in *M. scutellata* and grain yield in broad bean, whereas it did not show any effect on durum wheat biomass production;
- OMW spreading reduced the weed biomass and broad bean nodulation. This effect can be ascribed to OMW allelopathic effects on weed seed germination and toxic effect on indigenous rhizobia community.

- salt stress conditions negatively influenced AMF inoculum effects on millet forage production, without any difference between the two millet genotypes (Unikum and Kinelskoje); whereas, in absence of the salinity treatment, AMF inoculation increased the forage production only in Unikum;
- under water stress condition, AMF inoculation promoted millet forage production, whereas no significant difference was observed in well-watered condition;
- Unikum proved to be the best millet genotype in open field conditions with more than double forage production compared to Kinelskoje.

Thus, it can be concluded that in Veneto trials conditions, AMF inoculation exerted poor or nil effects. In view of sustainable agriculture in our agroecosystems, a promising agronomic practice can be considered the use of digestate liquid or solid fraction as organic fertilization, thus reducing environmental and economic costs and maintaining a good crop biomass production, but under our experimental conditions adopted, AMF fertilizers are ineffective.

In Sicilian experimental conditions, even if OMW spreading and AMF inoculum could be encouraging agronomic practices to valorize the Mediterranean marginal low-input agro-ecosystem for animal feed production, the inoculation should be well considered in conditions of saline water irrigation since salinity stress negatively affect AMF inoculum.

In conclusion, further researches should consider that AMF inoculation is more efficient in low nutrient soil condition and its effect can be inhibited by high nutrient input and fungicides use. Moreover, the role of indigenous mycorrhizal community on crop production and its interaction with non-native AMF introduced in agro-ecosystem by bio-fertilizers, should be considered especially in organic agriculture.

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