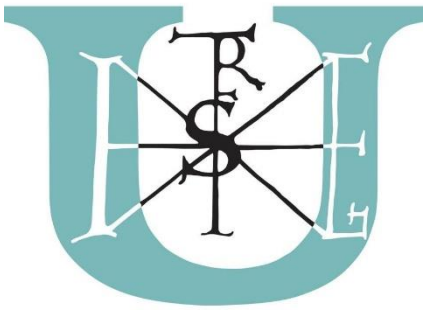

SZENT ISTVÁN UNIVERSITY

Impact of arbuscular mycorrhizal fungi on plant tolerance to some abiotic stresses and phytopathogens

PhD dissertation

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ABBREVIATIONS

ABA	Abscisic acid
AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
AOC	Allene oxide cyclase
CAT	Catalase
Cmm	*Clavibacter michiganensis* subsp. *michiganensis*
DAT	Days after transplanting
ET	Ethylene
ETI	Effector-triggered immunity
HR	hypersensitive response
ISR	Induced systemic resistance
JA	Jasmonates
LOX	Lipoxygenase
MAMPs	microorganism-associated molecular patterns
MDA	Malondialdehyde
MIR	Mycorrhiza-induced resistance
PAL	Phenylalamine ammonia-lyase
PPO	Polyphenol Oxidase
POD	Peroxidase
PR	Pathogenensis-Related
Pse	*Pseudomonas fluorescens*
PSI	Photosystem I
PSII	Photosystem II
PAMPs	pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
Tri	*Trichoderma*

1. INTRODUCTION

The negative impacts of climate change and other stress factors on yields of crops have already occurred on a global scale in agriculture. Under field cultivation conditions, on average, obtained crop yield is only approximately 50% of their potential productivity owing to abiotic factors (Hatfield and Walthall, 2015). Beside heat stress, one of the primary abiotic stressors, salinity, water deficit, nutrient deficiency and heavy metals can seriously affect plant growth their productivity. Biotic stressors also cause remarkable yield losses, the damage reaching up to 50–100% unless crop protection practices are applied. Thus, the frequency of plants that are confronted with abiotic and/or biotic stress could be greater, with more complex interactions of multiple stresses.

In planta, a variety of responses at cellular and molecular level are activated under stress conditions leading to direct reflection in plant growth, development and yield. For example, under abiotic stresses, disturbance of stomatal opening, water and nutrient relations in plants, curtailed photosynthetic rate, disruption of osmotic and ionic homeostasis, damage of functional and structural proteins and membranes, alterations in the expression patterns of a group of genes could occur, resulting in plant responses affecting plant growth rates and productivity. However, plants have actively evolved a wide range of protective strategies to fight against unfavourable environmental conditions. To deal with stresses and curtail their harmful effects, some alterations in plant morphology (Ruiz-Lozano et al., 2006; Fusconi and Berta, 2012), cell metabolic reprogramming may occur in plants to promote regular processes of biochemistry and physiology (Massad et al., 2012) and stress-tolerance mechanisms may be activated in plants for stress adaptation.

Noticeably, under natural conditions plants are frequently associated with microbes, which directly modulates plant responses to stresses. Some plant-microbe interactions result in alleviating stress-related damages, enhancement of plant tolerance to environmental stresses (Turner et al., 2013; Ngumbi and Kloepper, 2014). As an important element of soils, microorganisms are an integral component of the agricultural system. Arbuscular mycorrhizal (AM) fungi, a ubiquitous soil microbe, can associate with the roots of most terrestrial plant species. These beneficial fungi have been reported to significantly contribute multiple benefits to its host plants (Bonfante and Genre, 2010). Enhancement of mineral nutrients, water supply, improved seedling survival, increased growth and yield, uniformity of horticultural crops, and earlier and increased flowering (Azcón-Aguilar and Barea, 1997; Vosátka and Albrechtová,

2008; Gaur et al., 1998; Kaya et al., 2009; Russo and Perkins-Veazie, 2010) were observed in AM colonized plants. The exploitation of AM symbiosis is one of the most effective practices to improve plant tolerance to abiotic stress (Birhane et al., 2012). Additionally, root colonization by AMF (arbuscular mycorrhizal fungi) enhances the plant's resistance to biotic or abiotic stresses (Birhane et al., 2012, Jung et al., 2012) through the remarkable reprogramming of plant functions, significant alterations in the hormonal balance and transcriptional profile, primary and secondary metabolism inside plants during AMF colonization of their host (Pozo et al., 2009).

Early studies demonstrated the considerable contribution of AMF to enhanced stress tolerance of the host plants by several AM-induced mechanisms of host tolerance to abiotic stresses such as more effective antioxidative systems, defense enzymes; modifications in host physiology, e.g. osmotic adjustment, gas exchange, photosynthesis; remarkable alterations of sugars, proline, polyamines, stress phytohormones, expression patterns of stress-responsive genes (Abdel Latef, 2013; Abdel Latef and Chaoxing, 2011a; 2014; Hajiboland, 2013; Abdel Latef and Miransari, 2014). To pathogens, AM-induced resistance in their hosts consists of plant nutrition and damage compensation, competition for photosynthates or colonization sites between AMF and phytopathogens and induction of systemic resistance as a result of AM colonization process. The purpose of the present study was to explore the impact of AMF on plant tolerance to some abiotic stresses and phytopathogens. Our further aim was to investigate some mycorrhiza-induced mechanisms of stress tolerance in the host plants.

Objectives

Our aims were to

Assess any mycorrhiza-induced protection against *Clavibacter michiganensis* subsp. *michiganensis* in tomato plants using 7 different AMF isolates. If so, examine the possible role of ethylene (ET) signalling pathway in mycorrhiza-induced resistance (MIR)

Investigate the impact of AM colonization with two different AM fungi species on tomato plant response to drought, heat, combined drought and heat stress. Subsequently, to explore AM-induced mechanisms of stress tolerance in the host tomato plants.

Examine the potential of AM and its combinations with other beneficial microbes *Trichoderma*, *Pseudomonas fluorescens* for improvement of plant growth, fruit yield and inducing defense enzymes in different pepper genotypes during the plant growth stages under field conditions.

2. LITERATURE REVIEW

2.1 Abiotic and biotic stresses in plants

2.1.1 Abiotic stresses

In nature, plants are frequently coped with various environmental stresses caused by abiotic and biotic factors. Abiotic stresses are overwhelming challenges to agricultural productivity causing yield losses of crops, which have been more profound due to climate change and industrialisation. The principal abiotic stressors including drought, high salinity, cold, and heat have detrimental effects on the survival, plant growth and yields of primary crops up to 70% (Kaur et al., 2008; Thakur et al., 2010; Ahmad et al., 2012; Mantri et al., 2012), thus menacing the food security throughout the world. In fact, drought has influenced 64% of the world's total land area while the cold and salinity stresses have by 57% and 6%, respectively (Mittler, 2006; Cramer et al., 2011). Riadh et al. (2010) calculated that saline and other soil problems such as erosion, soil degradation affect 3.6 billion ha (approximate 69%) of world dryland agriculture whereas 50% of total irrigated land on the globe is adversely impacted by salt-affected soils (Ruan et al., 2010). Irrigation and application of fertilizers in agriculture, together with low rainfall and overuse of groundwater resources also contribute considerably to salinization (Cantrell and Linderman, 2001; Al-Karaki, 2006). Qadir et al. (2014) calculated that world's annual loss of US$ 27.3 billion in crop production is due to land degradation caused by salinity in irrigated areas. More seriously, ever-increasing salinization of agricultural land will cause a 30% loss of agricultural areas in the next 25 years, even up to 50% loss by 2050 and salt-affected soils have almost extended to 34 million irrigated hectares (FAO, 2012), exerting a significant global impact. Drought is the single most destructing stress, reducing crop productivity more than any other stressor (Lambers et al., 2008). Climate change will increase the global temperature from 1.8 to 4.0^{o}C higher than the present temperature by 2100 and risks from extreme weather events (IPCC, 2007). More serious and frequent occurrences of drought and extreme temperatures under global warming scenarios have been predicted (IPCC 2007; Walter et al., 2011). Plants are more vulnerable to rising high-temperature variation while remarkably, extreme heat stress occurs during the plant reproductive stage, severely devastating the crop production in many areas on the globe (Sato et al., 2006, Lobell et al., 2011). Similarly, cold stress limits the growth, production, geographical distribution of crops and negatively influences their quality and post-harvest life (Kumar, 2013; Thakur and Nayyar, 2013). While a majority of temperate crops acquire chilling and freezing tolerance, numerous agronomically

important plants are unable to obtain cold acclimation (Yadav, 2010). In addition, plants in the field are often confronted with not only a single stressor but a variety of stress combinations at distinct physiological stages and in different growth stages. Negative effects of these primary abiotic stresses on plants are presented in Figure 1.

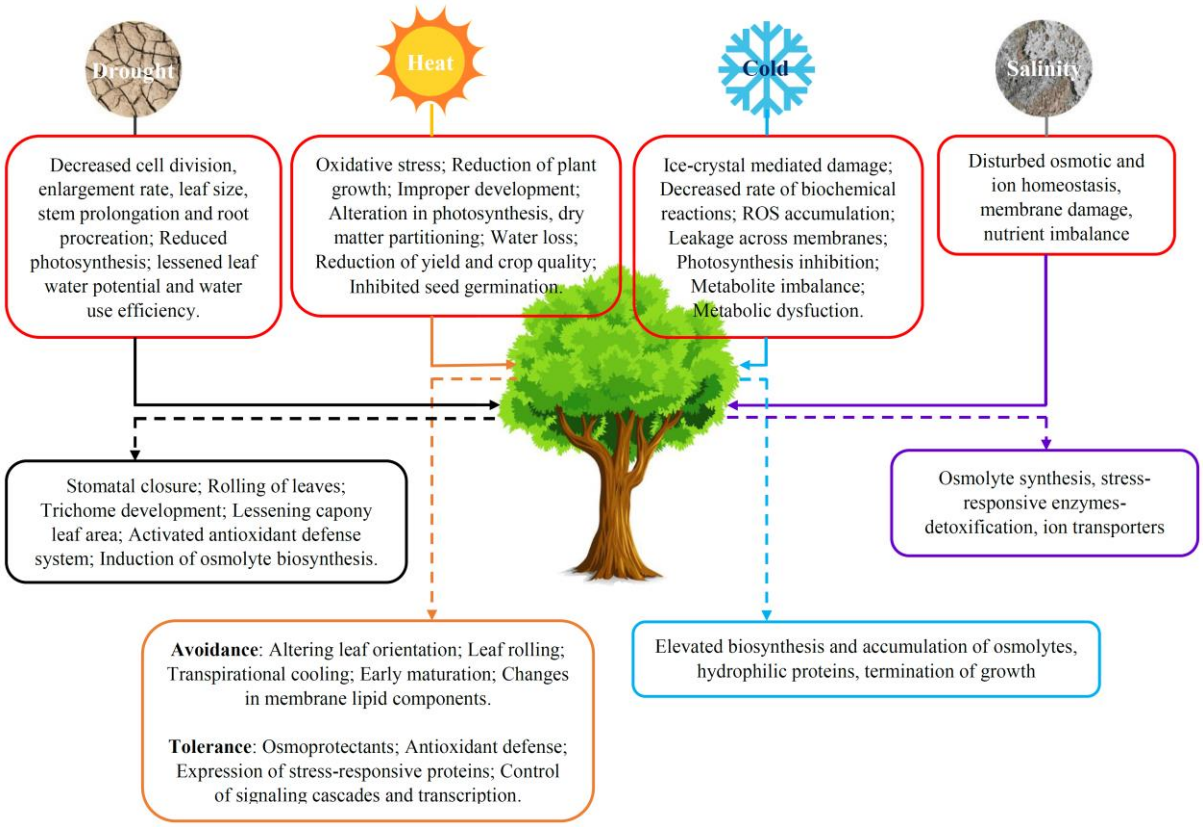


Figure 1. Negative effects (red rounded rectangle) of primary abiotic stresses on plants and plant responses to drought (black rounded rectangle), heat (orange rounded rectangle), cold (blue rounded rectangle), salinity stress (purple rounded rectangle).

2.1.1.1 Drought stress

Water deficit stress seriously diminishes normal growth with a substantial curtailment of plant growth rate as well as biomass accumulation. As a result of dehydration stress, decreased cell division and enlargement rate, leaf size, stem prolongation and root procreation, disturbance of stomatal opening, water and nutrient relations in plants, lowered leaf water potential and water use efficiency, eventually leading to the reduction of crop productivity (Li et al., 2009; Farooq et al., 2009). In addition, drought triggers higher biosynthesis of abscisic acid (ABA), reducing stomatal conductance to lower transpiration (Yamaguchi-Shinozaki and Shinozaki, 2006). Photosynthetic rate is curtailed by closed stomata, membrane damage, disrupted activities of different enzymes, in particular, those playing a role in ATP synthesis (Farooq et al., 2012).

However, plants respond to dehydration stress by different mechanisms to gain drought tolerance, such as minimized water loss by improved diffusive resistance (Farooq et al., 2012), rolling leaves to depress light absorbance (Ehleringer and Cooper, 1992), development of trichomes (Larcher, 2000), lessening canopy leaf area through growth reduction and discarding of older leaves (Chaves et al., 2003), simultaneously inclined water uptake by enhanced root abundance and depth (Farooq et al., 2012). Proline, glycine betaine, fructans, trehalose, polyols which act as major osmoprotectants are accumulated at high levels to maintain cell turgor pressure and normal physiological activities (Bartels and Phillips, 2010). Antioxidant defence system of plants exposed to drought conditions is triggered to protect cells from oxidative damage through strengthened non-enzymatic and enzymatic antioxidants (Carvalho, 2008).

2.1.1.2 Salt stress

Salt stress characterized by a high concentration of soluble salts in soils limits crop growth and productivity by ionic toxicity, reduced uptake of phosphorus, potassium, nitrate and calcium and osmotic stress (Vahdati and Lotfi, 2013). The osmotic concentration of the salty soil solution is greater than that in cells, hence, inhibiting plant uptake of water and minerals such as K^+ and Ca^{2+} whereas abundant and available Na^+, Cl^- ions can penetrate plant cells and directly toxify membranes, interfere normal metabolic activities in cells (Hasegawa et al., 2000; Munns, 2002; Munns and Tester, 2008). Furthermore, some secondary effects of saline stress include changes in assimilation, decreased cell enlargement, and membrane function, depressed cytosolic metabolism and ROS overproduction (Kumar, 2013). Ionic toxicity, osmotic stress, and nutritional deficiencies under salt stress altogether generate metabolic imbalances and oxidative stress in plants. Therefore, main plant mechanisms against ionic stress under salinity conditions comprise of the lowered uptake of toxifying ions like Na^+, Cl^- into cells and compartmentalization of these ions in the vacuole (Kumar, 2013). Simultaneously, osmotic adjustments in plants are implemented by generating compatible solutes such as proline and glycine betaine while production of ROS scavengers is necessary to prevent destructive ROS (Parihar et al., 2015).

2.1.1.3 Temperature stresses

High temperature causes various, detrimental effects on growth, development, physiological aspects of plants and crop yield as well as quality (Hasanuzzaman et al., 2012, 2013). Subsequently, oxidative stress, a secondary stress, is induced by heat stress due to over-generation of ROS in plants (Hasanuzzaman et al., 2012, 2013). Plant reactions to heat stress

are various, dependent on temperature degree, stress duration and plant species. Particularly, a fatal collapse of cellular organization might occur after minutes of plant exposure to extremely high temperature, resulting from damaged and dead cells (Ahuja et al., 2010). Heat stress affects all plant growth stages due to negative impacts on protein stability, membranes, RNA and cytoskeleton structures, metabolic balance in cells (Pagamas and Nawata, 2008; Ruelland and Zachowski, 2010; Suzuki et al., 2011, 2012). Under high-temperature stress, prohibition of seed germination or abnormal seedlings were observed in many plant species (Toh et al., 2008; Kumar et al., 2011; Piramila et al., 2012) while photosynthesis is considerably affected, in particular in C_3 plants (Yang et al., 2006). Noticeably, a short exposure to heat stress during reproduction can afflict substantially decreased floral buds and greater flower abortion, devastating whole crop productivity (Sato et al., 2006, Lobell et al., 2011). Plants have evolved adaptation mechanisms to high-temperature stress consisting of (1) tolerance: biosynthesis of osmoprotectants, antioxidant defense, expression of stress-responsive proteins, control of signaling cascades and transcription; (2) avoidance: altering leaf orientation, leaf rolling, transpirational cooling, early maturation, changing membrane lipid components (Hasanuzzaman et al., 2013).

Cold stress causes different detrimental effects consisting of poor seed germination, retarded seedlings, chlorosis (Yoshida et al., 1996), lessened leaf enlargement and growth (Sowinski et al., 2005; Rymen et al., 2007), wilting (Bagnall et al., 1983), withering, low tillering and probably results in necrosis (Yadav, 2010), seriously affects development of plant reproduction (Kaur et al., 2008; Ohnishi et al., 2010; Kumar et al., 2011), ultimately dropping the grain yield (Suzuki et al., 2008). All aspects of cellular function in plants under low-temperature stress are impacted adversely (Yadav, 2010). Fundamental negative influence of cold stress is an induction of serious membrane damage mainly due to the intensive dehydration linked with freezing during the stress (Yadav, 2010). Chilling also leads to disturbed DNA strands, RNA secondary structure stabilization, protein complex destabilization, lowered enzymatic activity, disintegrated membranes, over-produced ROS, inhibition of photosynthetic capacity, solute leakage (Nayyar et al., 2005a, b, c, d; Nayyar and Chander, 2004), metabolite imbalance and metabolic dysfunction (Yadav, 2010).

In other words, abiotic stressors trigger remarkable modifications of morphological, biochemical and molecular aspects *in planta*, eventually resulting in lessened plant growth and yield (Wang et al., 2001). Perturbation of synthesis, level, and storage of primary as well as secondary metabolites in plants are induced by stresses. Drought, salt stress, extreme temperatures are interconnected and mainly manifested as osmotic stress, which leads to the

disturbance of cell homeostasis and ion dispersal (Serrano and Rodriguez-Navarro, 2001; Zhu, 2001), denaturation of functional and structural proteins of plant cells (Smirnoff, 1998). Consequently, these environmental diversities often activate same signalling pathways in stressed plants (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001; Zhu, 2001, 2002) and plant responses, for example, the generation of stress-responsive proteins, antioxidants and accumulation of osmoprotectants (Zhu et al.,1997; Cushman and Bohnert, 2000). The complex plant response to major abiotic stresses, involving many genes and biochemical-molecular mechanisms, is presented in Figure 2. Molecular mechanisms of abiotic stress tolerance include the activation and control of specific genes related to the stress signalling, transcriptional factors, protection of cell membranes and proteins, uptake and transport of water, ion (Wang et al., 2003).

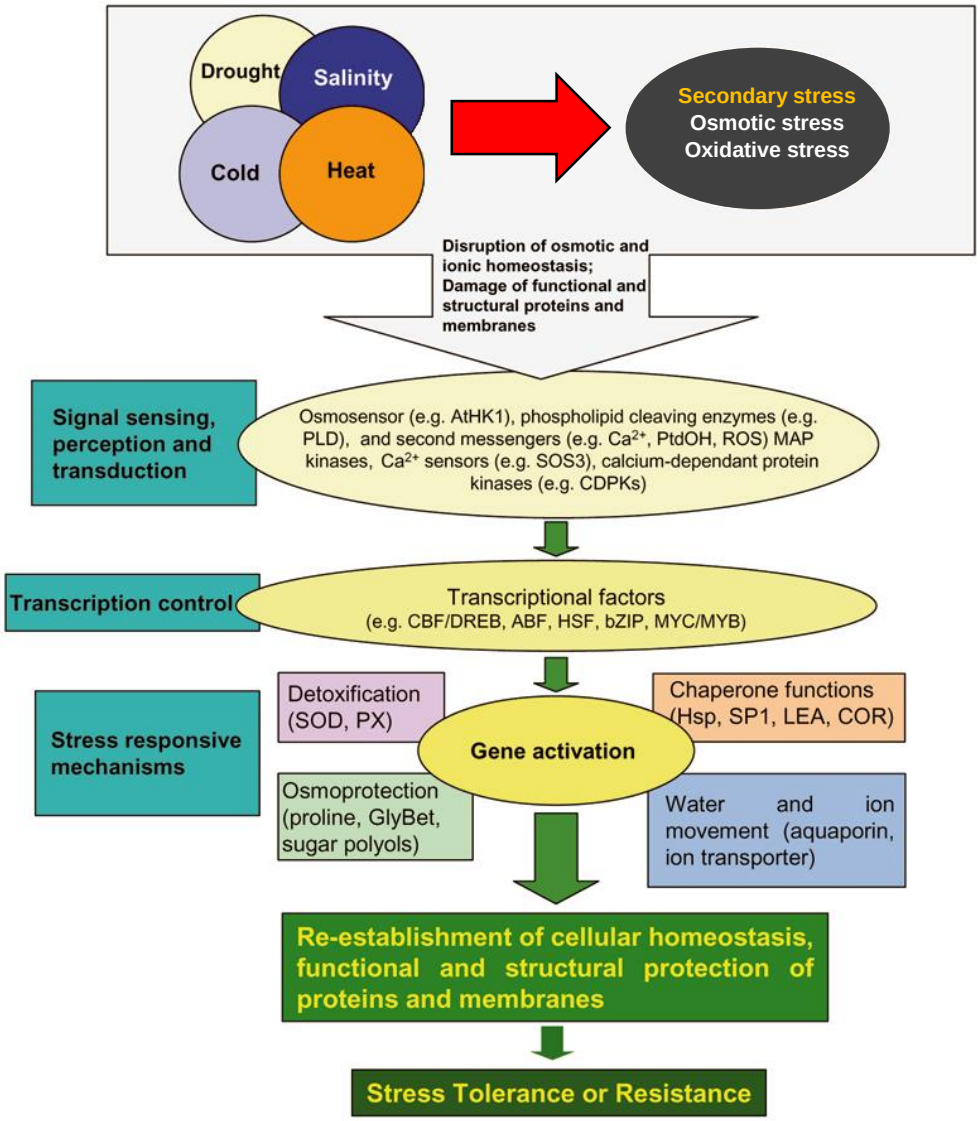


Figure 2. The complex response of plants to drought, heat, salinity, cold stress (Wang et al., 2003). These abiotic stresses often share similar stress signalling pathways and provoke membrane, protein damages as well as osmotic and oxidative stress. The initial stress signals are sensed, perceived and transduced by plant cells through osmosensors, secondary messengers, mitogen-activated protein kinases (MAP kinases), calcium-dependent protein kinases (CDPKs) and other components which subsequently activate transcription factors and stress-responsive mechanisms to maintain cellular homeostasis. One or few processes in the plant response to stresses do not operate effectively, which may cause the plant unable to withstand detrimental effects of the stresses.

2.1.2 Biotic stresses

Biotic stresses caused by pathogenic bacteria, fungi, nematodes, protists, insects, viruses and viroids decrease the yield of most crops on farmer's fields, particularly yield losses could be up

to 100%. Extremely severe examples of biotic stresses included the potato blight *Phytophthora infestans* in Ireland in 1845 that resulted in a loss of 80 % of potato yields and over 2 millions of people died of starvation; Southern Corn Leaf Blight by *Cochliobolus heterostrophus* in the United States in the 1970s; coffee rust in Brazil in 1967; the great Bengal famine in 1943 due to outbreak of brown leaf spot *Helminthosporium oryzae* fungus in rice (Hussain, 2015). Moreover, Soybean Asian Rust accounted for 37–67 % of yield loss in Brazil (Kumudini et al., 2008), causing $4 billion income loss for soybean farmers in Brazil from 2006 to 2011 whereas, in Asia, the yield reduction by this fungal pathogen was up to 80% (Miles et al., 2003).

The diverse range of biotic stresses makes situations more complicated. For instance, in common beans, 45 pathogenic viruses, bacteria, fungi, and nematodes in various regions and cases were reported (Vieira, 1983). The majority of phytopathogens are able to express pathogenic races or biotypes, creating further hindrances to plant breeding, where a new resistant cultivar is successful against a specific pathogen race but might be susceptible to other races (Borém and Fritsche-Neto, 2012). In addition, genetic adaptability, short life-span of pathogens and change in existing common pathogen races in an area can evolve resistance to pesticides and shorten the life-span of resistant cultivars. Insects are definitely the most versatile and diverse group, therefore, various insects feed on plant parts such as leaves, stems, pods, fruits, and roots at different plant growth stages and even postharvest period (for example, borer, weevils), posing significant crop losses. In fact, it is said that herbivorous insects are accountable for wiping out one-fifth of the global crop production yearly. In some regions, pests might not be major stresses in a certain year, however; they may outbreak in the latter years.

Climate changes further influence the current genetic polymorphism in old phytopathogenic agents and insect communities, leading to the evolution of aggressive strains or biotypes (Anderson et al., 2004), bringing new pathogens and pests to crop production. World's food production is also estimated to endure more serious incidences of insects, diseases due to global warming. Hence, outbreaks of disease or insect pests are predicted to continue to damage food production or even exacerbated by extending to the regions they were not prevailing before (Ijaz and Khan, 2012) and some present secondary biotic stresses in many crops will be main threats under effects of climate change.

Plant resistance mechanisms against a variety of phytopathogens and insect pests are involved with various processes at morphological, physiological, genetic, biochemical and molecular level (Howe and Jander, 2008). There are two main resistance mechanisms including constitutive resistance that exhibits continuously as basal resistance to prevent from pests attack

and inducible resistance showing more rapid and effective responses to combat against 'alien organisms' upon plants being attacked (Onaga and Wydra, 2016). Constitutive defenses are involved in creating morphological and structural barriers (thicker cell walls, leaf surface waxes, trichomes, etc.), generation of chemical compounds (phenolics, nitrogen compounds, glucosinolates, saponins, metabolites, terpenoids and steroids), proteins and enzymes (Ferreira et al., 2007; Freeman and Beattie, 2008; Dahal et al., 2009) conferring resistance against pest invasion as well as strengthening plant rigidity. The inducible defence responses are linked with the generation of toxic compounds, pathogen cell-degrading enzymes such as chitinases, glucanases, even a hypersensitive response in plants (Onaga and Wydra, 2016). These inducible compounds might be stored under inactive precursors and transformed into their active forms once plants are under attack.

Operation of innate immune system in plants highly relies on pattern recognition receptors (PRRs) to sense conserved pathogen-associated molecular patterns (PAMPs)/microorganism-associated molecular patterns (MAMPs), for instance, pathogen-specific flagellin, lipopolysaccharides, peptidoglycans, nucleic acids (Akira et al., 2006) or herbivore-associated molecular patterns (HAMPs) such as insect-specific components of oral secretions, saliva, oviposition fluid (Mithöfer and Boland, 2008) to detect attackers, then activate PRR-mediated immune responses, PAMP-triggered immunity (PTI) in pathogen-plant interaction (Monaghan and Zipfel, 2012). On the opposite side, some pathogen or insect invaders have evolved the ability to suppress or overcome PTI due to survival pressure. Another innate immunity of plants called effector-triggered immunity (ETI), where the protective immune response in the plant is activated by bacterial toxins or secreted proteins (effectors) that function as hijacking cytoskeletal machinery, halting transcription and repressing the immune system in plants (Rajamuthiah and Mylonakis, 2014).

Inducible resistance constitutes induced systemic resistance (ISR) and systemic acquired resistance (SAR). SAR is associated with SA signalling pathway defense, obtained as a result of necrotizing plant tissues by pathogens, either as a part of a hypersensitive response (HR) (Ross, 1961) and provoke long-lasting protection against diverse pathogens and insects (Ryals et al., 1999; Kuc, 1987). ISR is promoted by beneficial microbes, for example, plant growth promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF) and linked to JA-signaling pathway defenses. Both SAR and ISR play a crucial role in protecting plants against biotic stresses. Additionally, plants apply the strategy of RNA interference to prevent against exotic nucleic acids from viruses. A summary of plant resistance mechanisms of resistance to phytopathogens is described in Figure 3.

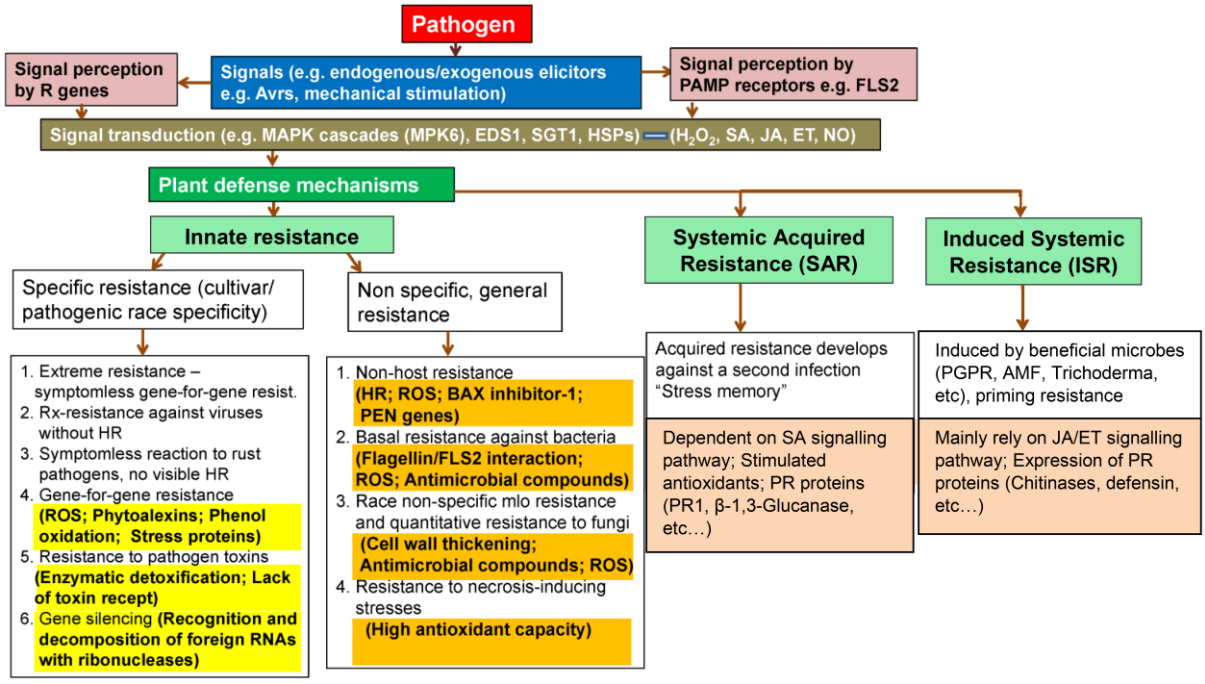


Figure 3. Plant resistance mechanisms of resistance to phytopathogens (modified from Onaga and Wydra, 2016). Pathogenic signals such as PAMPS/DAMPs and effectors are perceived by PRRs or R-genes of attacked plants, respectively. In the cells, PTI/ETI mechanism activates defense signalling pathways including reactive oxygen species (ROS), defense hormones (for example, SA, JA and ET), mitogen-activated protein kinases (MAPK), and transcription factor families, which provokes innate resistance or acquired/induced resistance or even both.

2.1.3 Stress combinations

Under natural conditions, the frequency that plants face multiple stresses which have additively detrimental effects could be high. Noticeably, plant responses to combined stresses are unique and different from responses of plants exposed to single stresses (Mittler, 2006; Suzuki et al., 2014). Occurrences of stress combinations lead to highly complicated responses *in planta* since these reactions are mostly regulated by diverse signalling pathways which may prohibit or synergize each other (Suzuki et al., 2014). In fact, in field conditions stress combinations of stressors such as drought with cold or heat stress, drought/heat and salt stress are common to many agricultural regions of the world and could limit crop productivity. Negative and positive interactions of stress combinations can occur, impacting on crop growth, physiology, productivity, and yield (Mittler, 2006; Mittler and Blumwald, 2010). Different combinations of abiotic and/or biotic stresses with a negative or positive interaction of stress effects on crops are summarized in Figure 4.

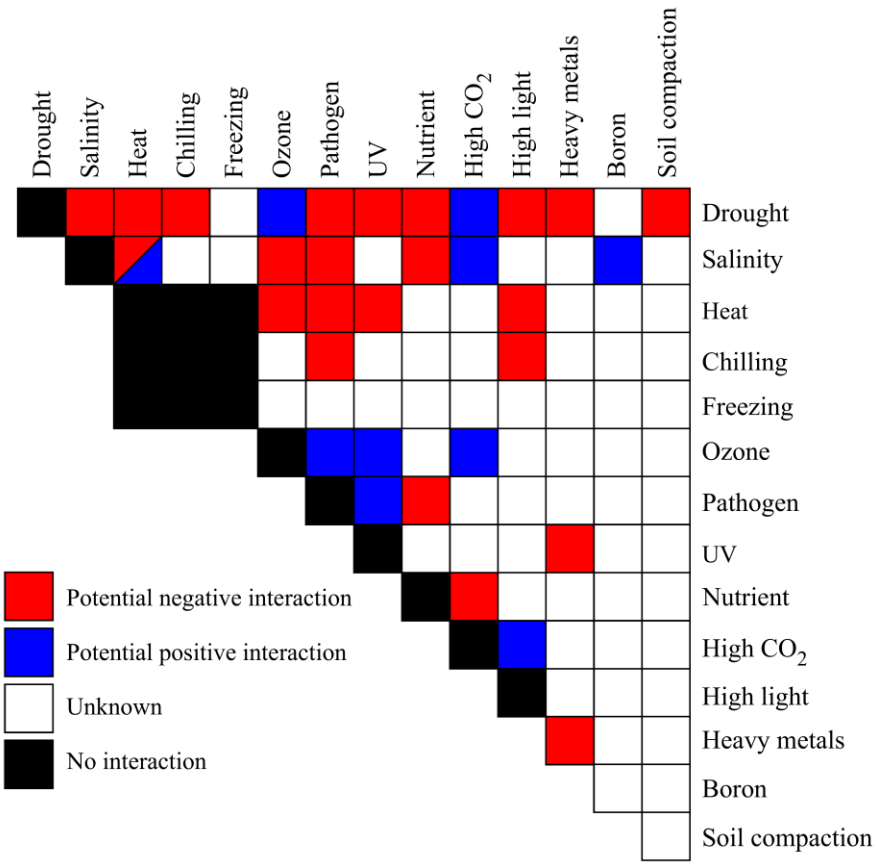


Figure 4. The stress matrix (Suzuki et al., 2014). Diverse combinations of environmental stresses which have positively (blue), negatively (red), unknown (white) or no interaction (black) between individual stresses, affecting plants under field conditions are described in the form of a matrix.

2.2 Arbuscular mycorrhizal fungi

AMF are obligate symbionts in roots of 80% of terrestrial plant species. AMF are probably the most pervasive fungi in soils due to constituting 5-36% of the total soil biomass and 9-55% of the soil microbe biomass (Olson et al. 1999). Both partners benefit from the mutual association: AMF get carbohydrates from the host plant while in turn the plant is supplied with more mineral nutrients and water from the AM partner.

2.2.1 Biological characteristics of AMF

Main structures of AMF include extraradical hyphae, intraradical hyphae, arbuscules, auxiliary cells, spores and vesicles (Souza, 2015). The extraradical hyphae are a massive mycelial network with unlimited growth, which is able to expand beyond the rhizosphere to take up nutrients and water, then transfer it to the host plant through another hyphal network with limited growth inside root cells called intraradical hyphae (Bonfante and Genre, 2010). The

intraradical hyphae are highly branched to form tree-shaped structures, arbuscules in the inner cortex of roots, thus establishing the symbiotic interfaces for nutrient exchanges between the plant and AMF (Gutjahr and Parniske, 2013). Vesicles can be formed from intraradical hyphae at their terminal or intercalary position in the root cortex to contain a high quantity of lipids and function as the endophytic storage organs (Goltapeh et al., 2008). Spores originated from extraradical, intraradical hyphae (Ramos et al., 2008a, b, c) are asexual spherical structures, serve as main survival units of AMF (Souza et al., 2015) while auxiliary cells produced from the extraradical hyphae in only some species of Order Diversisporales (Redecker et al., 2013) with probably reproductive function (Souza et al., 2015) or nutritional and storage functions (Morton and Benny, 1990). The mycorrhizal colonization process can be separated into distinct steps characterized by a series of complicated morphogenetic changes in the fungus, including germinating spores, differentiating hyphae, appressorium formation, penetrating host roots, formation of intraradical hyphae, intercellular growth along with developed external mycelium, forming arbuscules, then exchanging nutrients and carbohydrates between the fungus and host (Goltapeh et al., 2008). Photos of arbuscular mycorrhizal fungi taken under a microscope is in Figure 5.

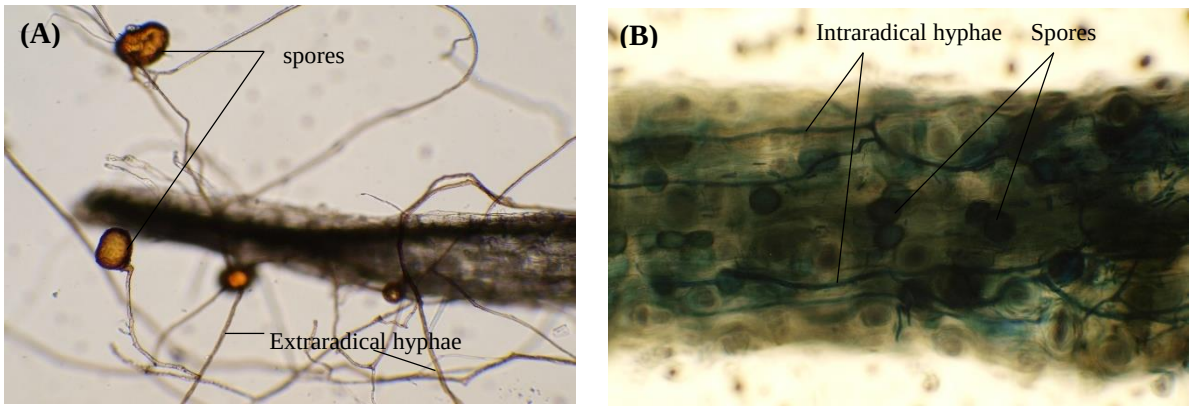


Figure 5. Arbuscular mycorrhizal fungi outside (A, light orange colour) and inside (B, dyed by Trypan blue) a piece of tomato root under a stereomicroscope at × 100 magnification.

2.2.2 Taxonomy of AMF

At present, AMF are arranged in the Phylum Glomeromycota which is divided into four orders, eleven families, twenty-five genus, and over 200 species (Redecker et al., 2013) (Figure 6).

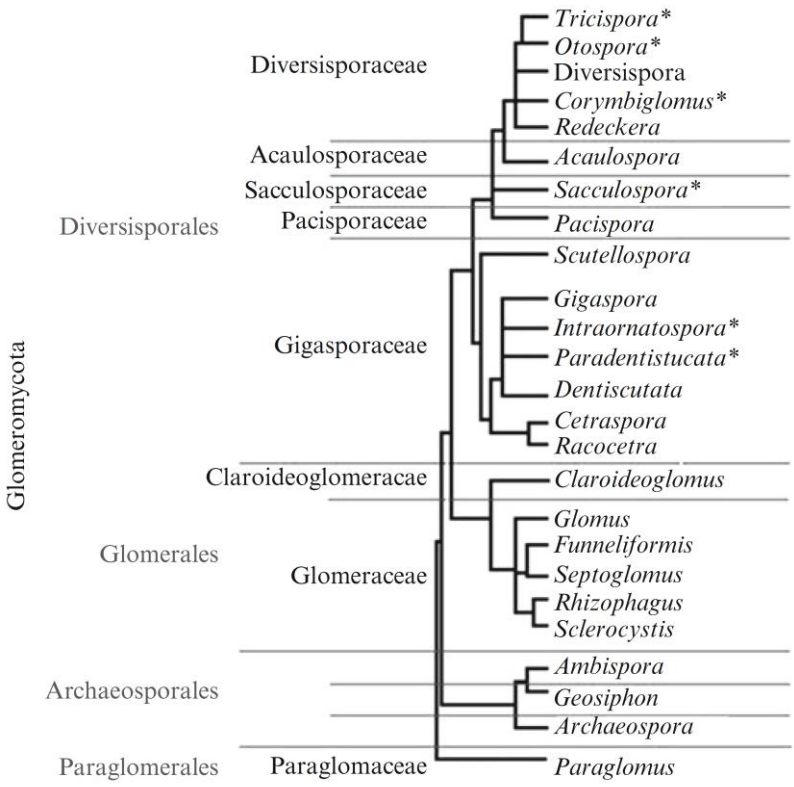


Figure 6. Taxonomy of AMF (Redecker et al., 2013; Krüger et al., 2012; Oehl et al., 2011). *Insufficient evidence, but no formal action taken.

2.2.3 Beneficial effects of AMF

Mycorrhizal colonization provokes improvement of plant nutrient, water status and plant growth (Smith and Read, 1997) and reproductive capacity (Lu and Koide, 1994), and enhanced plant resistance to environmental adversities (Jung et al., 2012; Birhane et al., 2012).

The fungal symbiont contributes significantly to plant nutrient uptake through the acquisition of mineral nutrients such as nitrogen (N), potassium (K), sulphur (S) (Allen and Shachar-Hill, 2009), in particular phosphorus (P) and micronutrients from the soil solution by the extraradical mycelial network (Caris et al., 1998; George, 2000; Linderman, 1992). In fact, the host root system is extended by widespread extraradical mycelia, which enables colonized roots to reach more nutrient pools unavailable to uncolonized roots because similarly to fine root hairs, the fungal hyphae can function as nutrient uptake organ. Hyphae diameters are quite smaller than those of fine root hairs (3–7 μm versus 5-20 μm) (Bago, 2000; Dodd et al., 2000; Wulfsohn and Nyengaard, 1999), however, the hyphal densities are ten-hundred times higher than root densities (Miller et al., 1995; Ravnskov et al., 1999; Schweiger et al., 1999; Dodd et al., 2000), thus effective absorption surfaces of the host plant is enhanced considerably. In the other hand,

AM production of extracellular phosphatase mineralizing organic P for uptake was reported (Koide and Kabir, 2000; Joner et al., 2000).

In addition to facilitating the acquisition of micronutrients, AMF store these elements in order to prevent their concentrations to reach toxic levels to the host plant (Goltapeh et al., 2008). Indeed, the fungal symbiont could serve as a sink for copper, zinc and cobalt (Bowen et al., 1974; Cooper and Tinker, 1981). Cress et al. (1979) revealed siderophore activity associated with four AMF species, indicating that siderophores production that specifically chelate iron may be partly the reason for increased iron uptake by AMF (Goltapeh et al., 2008). Although most benefit to the host plant is higher phosphorus uptake, improved micronutrients by AM play a necessary role in enhanced growth and yield of the host. Also, due to large and extensive hyphae system in the soil, AMF could create a favourable environment for the development of many beneficial organisms or synergize with many of them, for example, phosphate solubilizing bacteria (Toro et al., 1998), resulting in promoting nutrient cycling and plant nutrition. Synergism between AMF and other soil microbes involved in fixing atmospheric nitrogen had been reported. For example, Xavier and Germida (2002) indicated that Rhizobium nodulation in legume hosts could be improved through co-inoculation with AMF.

Regarding soil properties, glycoprotein glomalin produced copiously by AMF hyphae (Rillig et al., 2001) act like a glue, binding carbon, nitrogen and other biological components of soil to the mineral components, clay and sand, therefore increasing soil carbon storage as well as soil organic matter (Six et al., 2000). The soil glomalin content associates highly with water stability of aggregates (Wright and Upadhyaya, 1998). Glomalin production is proposed as a mechanism that AMF facilitate plant growth in the natural environment (Rillig and Steinberg, 2002). Hence, the fungal symbiont can improve remarkably soil texture and water relations (Bethlenfalvay and Shuepp, 1994).

The utilization of AMF inoculum as a biofertilizer in agriculture, in site remediation, horticulture or landscape restoration had been described. The fungi have critical contributions to maintaining and enhancing soil quality, nutrient uptake, fertility, regulation and functioning of plant communities and plant biodiversity, productivity in microcosms, in particular under phosphorus-limited conditions (Vander Heijden et al., 1998; Smith and Read, 1997). In terms of environmental aspects, use of AMF can reduce the input of inorganic fertilisers, especially phosphorus ones, and pesticides in agriculture due to bio-protection by AM against various detrimental environmental adversities and enhancement of plant production and growth. Under water-deficit conditions, mycorrhizae can improve water status of the host plant by direct water

uptake through interlacing external hyphae in soil and transfer it to the host (Auge, 2004) and plant tolerance against detrimental effects of drought by many mechanisms such as altering display of stomatal conductance (Auge, 2001), and osmotic adjustment (Wu et al., 2006).

2.3 AM plant resistance to phytopathogens

Numerous reports on enhanced resistance to soil-borne pathogens such as fungal pathogens *Rhizoctonia, Fusarium* or *Verticillium*, oomycetes *Phytophthora*, *Pythium, Aphanomyces,* and bacteria *Erwinia carotovora* in mycorrhizal plants were published (reviewed by Whipps, 2004; Hage-Ahmed et al., 2013; Chang et al., 2012; Ozgonen and Erkilic, 2007; Gallou et al., 2011; Zhang and Franken, 2014; Nair et al, 2015b; Liu and Matsubara, 2015; Ismail and Hijri, 2012). Furthermore, AMF can protect the host plant against parasitic nematodes *Pratylenchus* and *Meloidogyne* (Pinochet et al., 1996; de la Peña et al., 2006; Li et al. 2006; Westphal et al., 2008; Zhang et al., 2008; Lax et al., 2011; Vos et al., 2012), root rot *Thielaviopsis basicola* in *Petunia hybrida* (Hayek et al., 2014), white rot *Sclerotinia sclerotiorum* in sunflower (Bán et al., 2017) and melon root rot *Monosporascus cannonballus* (Aleandri et al., 2015). Thus, AMF are able to protect from a broad range of soil pathogens. However, the degree of protection largely depends on specific AMF isolates (Kobra et al., 2009) where *Glomus mosseae* seems to be more effective than other AMF (Pozo et al., 2002; Utkhede, 2006; Ozgonen and Erkilic, 2007) and specific varieties (Bán et al., 2017).

AMF protection of its host plants from soil pathogens consists of many mechanisms which may be operative simultaneously at multiple levels (Azcón-Aguilar and Barea, 1996). In addition to improved plant nutrition and damage compensation, competition for photosynthates or colonization sites between AMF and pathogens has been reported (Pozo et al., 2010). Mycorrhization modifies root system architecture and morphology (Schellenbaum et al., 1991; Norman et al., 1996), enhance biological activity in the host rhizosphere, a phenomenon called ‘mycorrhizosphere effect’ (Linderman, 1988), which may have negative influences on the development of soil pathogens and nematodes. AM colonization exerts alterations in components of root exudates that considerably decrease the sporulation of *Phytophthora fragariae* (Norman and Hooker, 2000). As root exudates determine to shape microbial communities in soil (Badri and Vivanco, 2009), the changes in exudation into the mycorrhizosphere may attract antagonistic microbes against soil phytopathogens. Furthermore, mycorrhiza-induced resistance (MIR) in the host plant due to mycorrhization process is another important mechanism to protect host plants from attackers, nevertheless; it requires full AM colonization (Slezack et al., 2000; Khaosaad et al., 2007). Fritz et al. (2006) revealed that

although nutrient enhancement in hosts could be highlighted, AM plant tolerance or resistance to pathogens cannot be regarded as a mere consequence of enhanced phosphorus mineral.

AMF can repress above-ground phytopathogens through two mechanisms: (1) alterations in nutrient status of host plants and modifications of the source-sink relation within it (Pozo et al., 2010) and/or (2) exerting MIR in AM-inoculated plants (Jung et al., 2012). Powdery mildew and rust fungi (*Blumeria*, *Oidium*, *Uromyces*), biotrophic phytopathogens, might develop better in mycorrhizal plants though augmented AM plant tolerance was often seen (Gernns et al., 2001; Whipps, 2004). Conversely, reduced development of *Blumeria graminis* f. sp. *tritici* in *Funneliformis mosseae* pre-colonized wheat was demonstrated (Mustafa et al., 2014, 2017). In terms of hemibiotrophic pathogens, findings from Lee et al. (2005), Saldajeno et al. (2013 a,b) and Chandanie et al. (2006) revealed that AM plants were against *Colletotrichum orbiculare*, the causal agent of anthracnose in cucumber whereas the decrease in wilting caused *Colletotrichum* cf. *gloeosporioides* in two cultivars of St. John's wort pre-inoculated with *Glomus intraradices* (Richter et al., 2011). Moreover, other studies indicated enhanced resistance of AM plants to shoot pathogens such as phytoplasmas (Batlle et al., 2011; D'Amelio et al., 2011), *Spiroplasma citri* in *Madagascar periwinkle* (Tahat et al., 2014), *Alternaria solani* in tomato (Song et al., 2015; Nair et al., 2015a), *Magnaporthe grisea* in *Oryza sativa* plants (Campos-Soriano et al., 2012), *Botrytis cinerea* in roses and tomato (Møller et al., 2009; Pozo et al., 2010; Fiorilli et al., 2011), *Pseudomonas syringae* in tomato leaves (García-Garrido and Ocampom, 1989), *Xanthomonas campestris* pv. *alfalfae* in *Medicago truncatula* (Liu et al., 2007), and *Tomato yellow leaf curl Sardinia virus* (Maffei et al., 2014). By contrast, Shaul et at. (1999) uncovered leaves of AM tobacco plants infected by *Botrytis cinerea* or tobacco mosaic virus expressed a higher incidence and severity of necrotic lesions in comparison to uncolonized plants.

2.3.1 AM modulation of the host plant defence system

During AM colonization, a remarkable transcriptional reprogramming occurs in the host root (Güimil et al., 2005; Liu et al., 2007; López-Ráez et al., 2010b), which modifies the primary and secondary metabolism in host plants (Hause et al., 2007; Toussaint, 2007; Schliemann et al., 2008). Noticeably, these secondary metabolites such as strigolactones, phenolic and allelopathic compounds play a critical role in complex interactions between the plant and soil microbes consisted of phytopathogens in the rhizosphere (Zeng, 2006; López-Ráez et al., 2010a, 2011b; Cipollini et al., 2012). Additionally, the contents of defence-related phytohormones, salicylic acid (SA), jasmonates (JAs), ethylene (ET) and abscisic acid (ABA)

fine-tuning plant defence responses to biotic stresses (López-Ráez et al. 2010b; Pieterse et al., 2009) are also altered in mycorrhizal plants, influencing the host plant defence mechanisms (Pozo et al., 2010).

In fact, mycorrhizal colonization seems to be negatively affected by SA-signalling pathway defences (de Román et al., 2011; Herrera-Medina et al., 2003), therefore; prohibition of SA-mediated reactions is necessary for mycorrhizal establishment (Dumas-Gaudot et al., 2000). Campos-Soriano et al. (2010) and Kloppholz et al. (2011) found that AMF can actively secrete effector proteins in order to actively repress SA-dependent defenses. In the other hand, the control of JA contents has a key role in the AM functions of the symbiosis when the colonization progresses (Hause et al., 2002, 2007; Hause and Schaarschmidt, 2009; Jung et al., 2012). A considerable rise in JAs in AM tomato roots and leaves was observed (López-Ráez et al., 2010b; Nair et al., 2015ab). Remarkably, substantially increased expression of marker genes for JA responses was found in the host plants, which might show an enhanced sensitivity to the hormone (Pozo et al., 2009). JA is widely known as a major regulator of plant defences against necrotrophic pathogens (Peña-Cortés et al., 2004; Pozo et al., 2005), thus an activation of the JA signalling pathway in mycorrhizal plants leads to be more resistant to necrotrophic phytopathogens (Pozo and Azcón-Aguilar, 2007).

Cumulation of defense compounds in AM colonized plants has been described to a much lower extent than in plant–pathogen interactions (Gianinazzi-Pearson et al., 1996). Increased ROS, activation of phenylpropanoid metabolism and induction of different plant defence-related enzymes, specific isoforms of hydrolytic ones, accumulation of PR proteins and the expression of defence genes in mycorrhizal plants have been detected (Pozo et al., 1999; Pozo et al., 2002).

As soon as AM formation, the plant actively controls the development of AMF within the roots to avoid excessive invasion and carbon drainage, maintaining the mutualistic interaction, therefore; plant defense system are tightly activated to moderate the fungal partner during symbiosis periods, called ‘autoregulation’ of mycorrhization, which may directly affect root pathogens as a side effect (Jung et al., 2012).

Noticeably, AM modulation of plant defences during AM establishment takes place not only in the roots but also in the shoots. Liu et al. (2007) uncovered a complex pattern of alterations in gene expression both in roots and shoots linked with mycorrhizal colonization in *Medicago truncatula* plants, leading to enhanced resistance to shoot pathogens *Xanthomonas campestris*. Conversely, Shaul et al. (1999) indicated that a suppression of certain defences might occur

such as a postponement in the systemic accumulation of PR1 as treatment with SA or analogs in shoots of mycorrhizal tobacco plants. This modulation might influence the interaction with shoot pathogens.

2.3.2 Mycorrhiza-induced resistance

Priming could be an important mechanism in MIR, shown by faster and stronger defence reactions triggered in the AM plants being attacked by pathogens in comparison to non-AM plants (Pozo and Azccón-Aguilar, 2007). Systemically primed defence responses in above-ground tissues and marker genes of JA signalling defences upregulated in AM tomato plants in the presence of pathogen were illustrated, emphasizing a crucial role of the JA-regulated pathway in MIR (Pozo et al., 2009). Even the fact that JA signaling pathway is necessary for MIR has been confirmed by several studies using either tomato mutants such as JA biosynthesis mutant (spr2), prosystemin-overexpressing 35S::PS plants, JA biosynthesis inhibitor salicylhydroxamic acid (SHAM) or *PvLOX2* silenced common bean (*Phaseolus vulgaris* L.) when AM plants were infected by *Alternaria solani* (Song et al., 2015; Nair et al., 2015a), *Sclerotinia sclerotiorum* (Mora-Romero et al., 2015), *Fusarium oxysporum* f. sp. *lycopersici* (Nair et al., 2015b).

Song et al. (2015) also confirmed priming of MIR in *Funneliformis mosseae* pretreated tomato plants with higher activities of enzymes β-1,3-glucanase, chitinase, phenylalamine ammonia-lyase (PAL), lipoxygenase (LOX) post infection of *Alternaria solani*. Moreover, upregulation of genes encoding PR1, PR2, PR3 proteins as well as defense-responsive genes LOX, allene oxide cyclase (AOC), PAL in mycorrhizal plants were found upon the pathogen attack as compared to non-AM plants although most of these genes were not affected in plants inoculated by AMF alone (Song et al., 2015). By using *Asparagus officinalis* plants in a split root system, Liu and Matsubara (2015) described that MIR enhanced antioxidative capacity, for instance, higher SOD, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, and total contents of polyphenol and ascorbic acid in both mycorrhizal roots (pre-treated by *Glomus intraradices* + 50 mM NaCl) and non-mycorrhizal roots in the presence of *Fusarium oxysporum* f. sp. *asparagi*.

Richter and co-workers (2011) demonstrated that MIR against *Colletotrichum* cf. *gloeosporioides* (Cfg) of the two *Hypericum perforatum* genotypes resulted from elevated levels of ascorbic acid activities of ROS scavengers, monodehydroascorbate reductase (MDHAR), decreased lipid peroxidation and induction of antioxidants after a challenge of the

pathogen in mycorrhizal plants. Regarding nematode - AM symbiosis interaction, *Meloidogyne incognita* was substantially reduced in the mycorrhizal roots (*G. mosseae*) in relation to the non-mycorrhizal roots, and greater expression of specific defense genes in plants was observed, suggesting that the phenylpropanoid pathway and ROS metabolism are crucial in the MIR against nematodes (Vos et al., 2013).

However, Nair et al. (2015a) showed non-priming MIR in tomato plants pre-colonized by *Glomus fasciculatum* were challenged with *Alternaria alternata*. In fact, mycorrhizal plants increased LOX activity three times than control plants, but this did not incline further upon the pathogen attack while the enzyme activity doubled in non-AM plants. Besides, more elevated LOX activity together four times higher MeJA in leaves of AM plants than those in non-AM plants were recorded whereas at molecular level, JA-biosynthetic gene (*OPR3*) and JA-responsive gene (*COI1*) expressed six- and 42-fold higher, respectively, in leaves of mycorrhizal plants in comparison to controls (Nair et al., 2015a). Together with this, upregulation of SA marker gene PR1, gene encoding wound-inducible polypeptide prosystemin were also found in AM plants (Nair et al., 2015a). Similarly, Mustafa et al. (2017) illustrated that MIR against *Blumeria graminis* f. sp. *tritici*, a foliar biotrophic pathogen in wheat pre-inoculated by *Funneliformis mosseae* were not linked to priming in wheat leaves rather than modulation of plant defences during mycorrhization. Instead, the authors observed locally accumulated polyphenolic compounds and hydrogen peroxide at penetration sites of the powdery mildew in epidermal cells of leaves of mycorrhizal wheat in 48h post infection. Indeed, no higher activities of peroxidase (POX) and LOX enzyme in AM wheat than non-AM ones in response to pathogen application were observed and genes of defence markers POX, PAL, Chitinase 1 (*CHI1*), nonexpressor of pathogenesis-related proteins 1 (*NPR1*) were not upregulated in leaves of mycorrhizal plants upon the pathogen attack while the upregulations of these genes were found in non-AM plants infected by the powdery mildew (Mustafa et al., 2017).

In summary, AMF are able to confer resistance against a wide range of different phytopathogens. However, AM protection ability differs among AMF isolates, depends on specific host plants/varieties and is modulated by environmental conditions. In addition to nutrient improvement and local changes in the host plant, modulation of plant defences by the symbiotic fungi may play a crucial role in MIR which could be operative under priming or non-priming mechanism.

2.4 AM plant tolerance to abiotic stresses

AMF are renown to have positive effects on crop growth and production under abiotic stresses, for instance, heat, cold, drought, salinity and nutrient-deficiency and heavy metal stress (Abdel Latef et al., 2016). Diverse mechanisms of AMF-induced tolerance against the environmental adversities are proposed (Rivera-Becerril et al., 2005; Smith and Read, 2008), consisted of (1) improved water and nutrient status of the host plant; (2) significant alterations of sugars, polyamines, proline, lipids, stress hormones and signaling and stress-related genes expression (López-Ráez et al. 2010); (3) changes in plant physiology, e.g. osmotic adjustment, gas exchange, photosynthesis, relative permeability and electrolyte leakage; (4) improved antioxidative systems to effectively alleviate oxidative stress caused by ROS; (5) root and fungal chelation and inactivation or elimination of polluting substances.

2.4.1 Drought stress

Water depletion in the root zone, very high transpiration rate or excessive ROS production resulting in oxidative stress *in planta* can be major causes of adverse influences of water deficit on plants. Mycorrhization has been described to improve drought tolerance in many plant species such as maize, wheat, barley, soybean, onion, lettuce, tomato (Augé, 2001). This is virtually attributed to mycorrhiza induced changes in plant phenology (Augé, 2004), root morphology and the capacity of widespread extraradical mycelium to access tiny pores that root hairs are unable to reach (Smith and Read, 2008), stabilization of the soil aggregates, increasing soil moisture retention and water absorption (Bethlenfalvay and Shuepp, 1994). Indeed, greater water use efficiency in mycorrhizal plants during water deficit has been demonstrated (Ruiz-Lozano and Aroca, 2010; Borde et al., 2012). AM inoculation also increases water uptake, leaf water potential and stomatal conductance, then greater water contents, and improved gas exchange, transpiration and photosynthesis in droughted mycorrhizal plants (Lee et al. 2012; Gholamhoseini et al. 2013). Significantly higher content of chlorophyll in AM plants has been detected in comparison to that of non-AM ones (Mathur and Vyas, 1995; Gemma et al., 1997) and during drought stress, mycorrhizal colonization alleviated the decrease in this parameter (Asrar and Elhindi, 2011; Zhu et al., 2012; Abdelmoneim et al., 2014). It is believed that photosynthetic rate correlates with chlorophyll content and stomatal conductance which have been enhanced by AMF. Mycorrhization has been revealed to mitigate harmful influences of water deficit on photochemical efficiency and photosystem (PS) II reaction center (Baker, 2008; Zhu et al., 2012).

AMF are able to change water relation of the host via regulation of hormones or by the production of compatible solutes in colonized plants (Ruíz-Sánchez et al., 2010; Fan and Liu, 2011). ABA is the most substantial stress hormonal signal, modulates transpiration rate (Zhang et al., 2006), hydraulic conductivity of roots (Aroca, 2006), aquaporin expression (Aroca et al., 2006). The level of this hormone is elevated in plant tissues under drought stress to limit stomatal opening for minimized water loss and activate different stress-responsive genes, resulting in better drought tolerance (Zhang et al., 2006). Doubkova et al. (2013) showed that mycorrhizal plants had a lower content of ABA than non-mycorrhizal ones under drought conditions, illustrating that colonized plants are confronted with less intensity of water stress. Higher osmolyte accumulation such as sugars, proline, glycine betaine lower the cell osmotic potential in AM plants subjected to water stress, allowing increased water retention (Abbaspour et al., 2012; Baslam and Goicoechea, 2012; Yooyongwech et al., 2013). By contrast, several studies have indicated that AM-induced decline in soluble sugars in some droughted mycorrhizal plants such as *Erythrina variegata* (Manoharan et al., 2010) and *Casuarina equisetifolia* (Zhang et al., 2010) suffered less drought stress. The greater content of amino acids in mycorrhizal roots and shoots uncovered a higher osmotic protection through accumulated amino acids in AM colonized plants (Kapoor et al., 2013). Drought tolerance as a result of improved proline content in AM plants has been described in a variety of studies (Manoharan et al., 2010; Rapparini and Peñuelas, 2014), nonetheless, others revealed that under water shortage, a declined accumulation of proline was detected in AM plants relative to non-AM counterparts (Asrar et al., 2012; Doubková et al., 2013). In addition, *Funneliformis mosseae* pre-inoculated *Poncirus trifoliate* plants subjected to water deficit decreased tissue proline content and enhanced the host plant growth and biomass, which may be attributed to the prohibition of glutamate biosynthetic pathway of proline with improved proline degradation (Zou et al., 2013). AM plants under drought conditions also showed higher levels of soluble nitrogenous compounds and free polyamines (Rapparini and Peñuelas, 2014).

Insufficiency of necessary nutrients consisting of Ca, Fe, K, Mg, P, and Zn caused by drought stress may be mitigated in AM plants (Bagheri et al., 2012; Gholamhoseini et al., 2013). Mycorrhizal colonization is well known to enhance P nutrient in the host plants, leading to better plant tolerance against water stress (Gholamhoseini et al., 2013). Trifoliate orange seedlings inoculated by *G. versiforme* substantially inclined the leaf K, Ca and root P, Ca, Fe contents under ample water and water deficit, respectively (Wu and Zou, 2010). *Pistachio* plants pretreated by *G. intraradices* and *G. mosseae* augmented considerably levels of P, K, Zn and Mn when plants were subjected to various soil moisture conditions (Bagheri et al., 2012).

Hydraulic conductivity of AM roots was improved to absorb more N, P, K, resulting in strengthened protein concentration in host plants under water stress (Gholamhoseini et al., 2013).

Drought leads to unbalanced homeostasis, subsequently over-generation of ROS, such as $O_2^{\bullet-}$, $^1O_2$, $^{\bullet}OH$ and H_2O_2 in plants, destroying plant cells or even causing the death of cells (Smirnoff, 1993). AM plants can improve contents of main antioxidants or ROS scavengers in enzymatic as well as non-enzymatic defense system (Rapparini and Peñuelas, 2014). The AM-induced alleviation of detrimental drought effects is involved to the increment of antioxidant concentrations and higher antioxidative enzyme activities *in planta* (Wu and Zou, 2010; Ruíz-Sánchez et al., 2010; Baslam and Goicoechea, 2012). Previous studies indicated that elevated levels of flavonoids (Abbaspour et al., 2012), isoprenoids (Rapparini et al., 2008; Rapparini and Peñuelas, 2014), specific isoprenoid-derived apocarotenoids (Walter and Strack, 2011) and strigolactones (Lopez-Ráez et al., 2008) in mycorrhizal plants also contribute to a defensive system against water stress. Mechanisms of AMF-induced tolerance in plants exposed to salinity and drought stresses are presented in Figure 7.

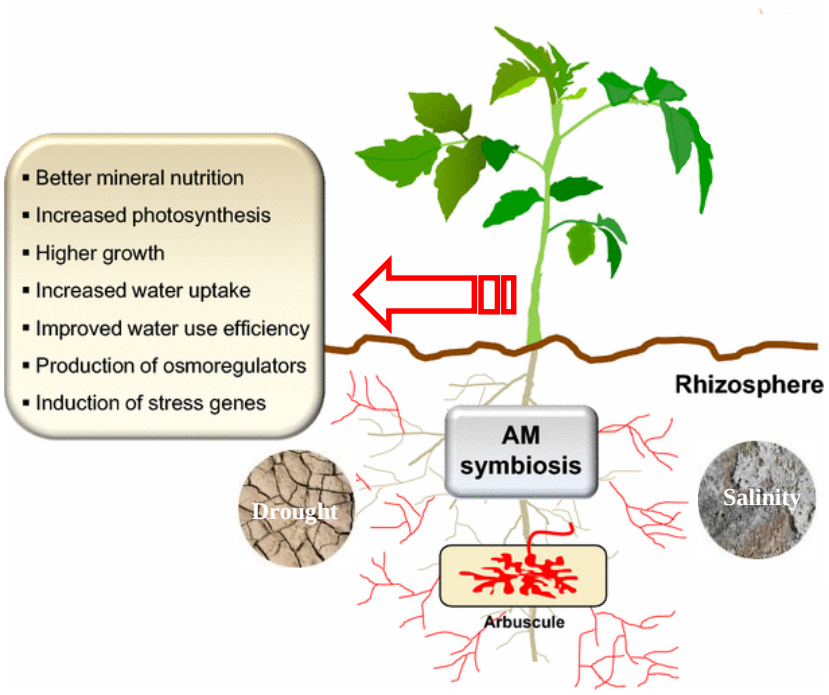


Figure 7. Mechanisms of AMF-induced tolerance in plants exposed to salinity and drought stresses (López-Ráez, 2016).

2.4.2 Salt stress

A wide range of reports indicated that AMF can enhance the host growth and yield under salinity stress due to augmented nutrient and water uptake, increased photosynthesis (Evelin et al., 2009; Abdel Latef and Chaoxing, 2011a; Aroca et al., 2013; Kapoor et al., 2013; Abdel Latef and Chaoxing, 2014; Talaat and Shawky, 2014; Hashem et al., 2014). AM plants subjected to salinity conditions had improved root hydraulic conductivity (Kapoor et al., 2008), modified root morphology (Kothari et al., 1990) and elevated stomatal opening (Sheng et al., 2008), increased chlorophyll content, greater uptake of Nitrogen and Magnesium, interestingly limited Na-transport under salt conditions (Borde et al., 2010; Abdel Latef and Chaoxing, 2011a; Çekiç et al., 2012; Talaat and Shawky, 2014). Improvement in cytokinin level and translocation of photosynthates in mycorrhizal plants subjected to saline stress were reported (Hameed et al., 2014). Talaat and Shawky (2014) observed an enhancement of PSI, PSII performance and carbonic anhydrase concentration in colonized plants. AM plants under salt stress exhibited also decreased oxidative stress due to curtailed lipid peroxidation of membranes (Abdel Latef and Chaoxing, 2014; Talaat and Shawky, 2014; Yang et al., 2014) with lowered malondialdehyde (MDA) content and strengthened antioxidative defense responses (Ahmad et al., 2012a,b; Anjum et al., 2015). Remarkably, AM inoculation enhanced plasma membrane integrity, decreasing lipid peroxidation and mitigating electrolyte leakage (Garg and Manchanda, 2009; Ahmad et al., 2012a,b; Evelin et al., 2012; Abdel Latef and Chaoxing, 2014). AM symbiosis presented lower levels of H_2O_2 and MDA relative to non-mycorrhizal plants, suggesting less accumulated ROS and lower membrane damage in colonized plants than uncolonized ones (Talaat and Shawky, 2014; Abdel Latef and Chaoxing, 2014; Yang et al., 2014). Mycorrhization decreased destructive ROS by improving the antioxidative enzymatic system (Abdel Latef and Chaoxing, 2011a; 2014; Evelin and Kapoor, 2014).

High contents of sugars accumulated in AM plants has been found as a tolerance mechanism against saline (Sheng et al., 2011; Talaat and Shawky, 2011, 2014; Abdel Latef and Chaoxing, 2014). Increased soluble sugars in host plants were revealed as a result of AM-induced improvement in photosynthesis (Abdel Latef and Chaoxing, 2014). Nevertheless, detrimental influences of the mycorrhizal colonization and accumulation of sugar in AM associated plants during salt stress were observed (Beltrano et al., 2013). *Glomus mossaea* associated pepper plants under salinity stress had an elevated content of soluble protein and total free amino acids (Abdel Latef and Chaoxing, 2014). Higher proline concentration in AM inoculated plants than in non-inoculated plants have been described (Sharifi et al., 2007; Kaya et al., 2009; Sheng et al., 2011; Evelin and Kapoor, 2014). By contrast, some authors indicated that proline content is

also curtailed in AM plants (Rabie and Almadini, 2005; Jahromi et al., 2008). AM plants subjected to salinity conditions exhibited higher betaine production, which plays a significant role in osmotic protection, and photosynthetic processes (Sheng et al., 2011). The mycorrhizal application was demonstrated to induce higher organic acids accumulation in maize plants under saline conditions (Sheng et al., 2011), which is attributed to cation balance and pH homeostasis (Hatzig et al., 2010). These organic acids might function as compatible solutes in vacuoles and prevent toxic chloride accumulation in cells (Hajiboland, 2013).

An increase in nitrate assimilation strengthening nitrate uptake and activity of nitrate reductase, enhancement of K^+ accumulation, sustained K^+/Na^+ ratio and ultimately avoided disruptions in a variety of enzymatic processes and prohibition in protein synthesis occur in AM symbiosis (Talaat and Shawky, 2014). Stimulation of nutrient absorption like N, P, K, Ca, Mg, Cu, Fe, Zn and restriction of Na and Cl uptake in colonized plants were demonstrated (Evelin et al., 2012), decreasing ion toxicity by salinity stress whereas mitigating nutrient insufficiency and harmful effects in the host cells (Kapoor et al., 2013) and maintaining higher K^+/Na^+, Ca^{2+}/Na^+ and Ca^{2+}/Mg^{2+} ratios by the host plant compared to their non-mycorrhizal ones (Evelin et al., 2012). Nonetheless, conflicting reports state that AM inoculation occasionally elevates Na^+ absorption. Improved nutrition, in particular, P and sustaining higher Ca^{2+}/Na^+ ratio are the critical determinants to bring beneficial impacts on membrane integrity in AM plants (Evelin et al., 2012; Abdel Latef and Miransari, 2014).

2.4.3 Temperature stresses

Temperature is one of the main environmental factors remarkably influencing all processes in plants (Źróbek-Sokolnik, 2012). Cold stress causes various negative effects on plant growth and development. AM inoculation was illustrated to induce higher plant tolerance against cold stress in numerous reports (Liu et al., 2011; Birhane et al., 2012; Chen et al., 2013; Liu et al., 2013). Mycorrhizal plants exposed to low temperature were reported to have improved growth as compared to non-mycorrhizal ones (Zhu et al., 2010a; Abdel Latef and Chaoxing, 2011b; Liu et al., 2011; Chen et al., 2013), which is attributed to enhancement of photosynthesis (Gamalero et al., 2009; Birhane et al., 2012), water status of host plants (Zhu et al., 2010a) and increased metabolites such as soluble sugars and proteins, proline (Abdel Latef and Chaoxing, 2011b). Nonetheless, mycorrhizal maize plants had shoot and root dry weights unchanged in comparison to the non-AM plants exposed to cold stress conditions (Zhu et al., 2015).

Indeed, AM plants subjected to low-temperature stress showed a greater water conservation, water holding capacity, relative water content and water-use-efficiency (Zhu et al., 2010a; Liu et al., 2014b), may indicate as a result of higher root hydraulic conductivity and mycorrhizal extraradical hyphae assisted water uptake (Zhu et al., 2010a) although few studies proved that AMF did not change water content in plants under low-temperature stress (Aroca et al., 2007; Liu et al., 2014a). In addition, Zhu et al. (2010a) demonstrated that colonized maize plants had enhanced stomatal conductance and transpiration rate than those of non-AM plants exposed to cold stress, suggesting that AM inoculation could elevate the gas exchange capacity via increased stomatal conductance and plant transpiration rates. Under cold stress, AMF improve water transport in the host plant through not only mediation of their own aquaporin activities but also regulation of aquaporin gene expression in the host plants such as higher expression of *PvPIP1;3* gene and PIP protein abundance (Aroca et al., 2007), increased expression of *OsPIP1;1*, *OsPIP1;3*, *OsPIP2;1* and *OsPIP2*;*5* gene (Liu et al., 2014b).

The improved plant water status contributes indirectly to better osmotic adjustment, elevated gas exchange, effective photochemistry of PSII and nutrient absorption (Zhu et al., 2012). Higher chlorophyll contents, net photosynthetic rate (Pn) in AM-colonized plants under cold stress were observed (Zhu et al. 2010a, 2015; Abdel Latef and Chaoxing, 2011b), therefore, mycorrhizal inoculation often induce higher plant growth. Besides, Chen et al. (2013) indicated that mycorrhization in roots of cucumber plants under unstressed temperatures significantly promoted phenolics, flavonoids and lignin accumulation in leaves, and substantially dropped H_2O_2 production. Zhu et al. (2010b) and Abdel Latef and Chaoxing (2011b) illustrated that AMF also triggered higher activities of antioxidative enzymes such as SOD, CAT, POD, APX in their hosts in relation to non-colonized plants exposed to cold stress. Mechanisms of AMF-induced tolerance to temperature stresses in plants are presented in Figure 8.

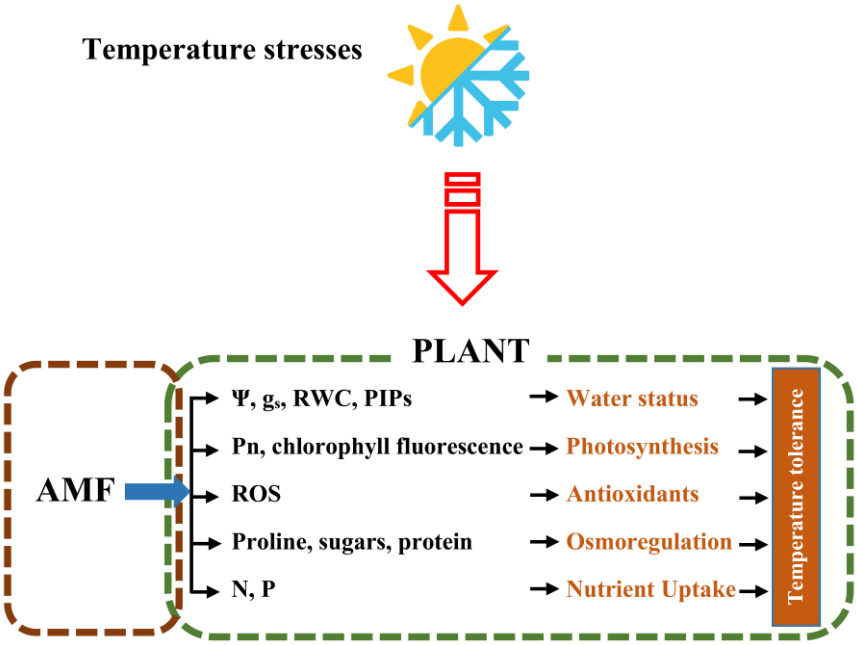


Figure 8. Possible mechanisms of AMF-induced tolerance to temperature stresses in plants.

Similar to cold stress, heat stress severely affects plant growth and development. There is still limited information about AM plants treated by heat stress, however, few studies showed that AMF could enhance plant growth under heat stress (Gavito et al., 2005) whilst other work showed that biomass of maize plants pretreated by *G. etunicatum* was similar to non-AM ones under heat stress (Zhu et al., 2012). AM plants exhibited improved net photosynthetic rate, higher stomatal conductance, transpiration rate, and maximum quantum efficiency of PSII photochemistry in relation to non-AM counterparts under high-temperature stress (Zhu et al., 2012), proving that mycorrhizal inoculation reduced the heat stress damages to PSII reaction center and photosynthetic apparatus. AMF were described to enhance water conservation and protect against dehydration and metabolic disruption, improve water use efficiency (WUE), water holding capacity (WHC), water absorption capacity as the main AM-induced mechanisms of heat stress tolerance (Zhu et al., 2012).

3. MATERIALS AND METHODS

3.1 Target plants

Tomato seeds (*Solanum lycopersicum* L.) cultivar MoneyMaker, Never ripe (Nr, ethylene-insensitive mutant), Pearson and three hybrids of sweet pepper (*Capsicum annuum* L.), Karpia, Karpex and Kaptur were used in our experiments.

3.2 Arbuscular mycorrhizal fungi inocula

The inocula including 8 different AMF species were propagated separately in open-pot cultures with *Zea mays* and *Medicago truncatula* as host plants for 9 months. The basic AMF samples originated from different national mycorrhizal collections, *Funneliformis mosseae* BEG 12 (Fm), *Funneliformis geosporum* BEG 11 (Fg), *Rhizophagus irregularis* MUCL43194 (DAOM197198) (Ri), *Rhizophagus* sp. MUCL43204 (Rs), *Septoglomus constrictum* (formerly *Glomus constrictum* Trappe.) (Sc), *Septoglomus deserticola* BEG 73, *Claroideoglomus claroideum* BEG 23 (Cc), *Gigaspora margarita* BEG 34 (Gm). Mycorrhizal inocula consisted of sand, peat, mycelia, infected root fragments and spores. Mycorrhizal inoculations were implemented before transferring germinated seeds to pots.

Symbivit®, a commercial mycorrhizal product (a mixture of *Glomus intraradices, G. mosseae, G. etunicatum, G. claroideum, G. microaggregatum, G. geosporum*) (Symbiom Ltd., Lanskroun, Czech Republic; www.symbiom.cz) was utilized in the field experiment.

3.3 Other beneficial microbes

Trichoderma harzianum isolate (SzIE35) in the collection of Szent István University was cultured on Potato Dextrose Agar (PDA) at 25^{o}C for 4 days, and the inocula were produced in potato-dextrose broth shake culture at (150 rpm) for 1 week at 25^{o}C ± 2^{o}C. The cultures were filtered through a double layer of sterilized gauze, and a clean conidial suspension was prepared at the concentration of 10^7 per ml.

Pseudomonas fluorescens isolate (PK17) originated from the collection of the Szent István University was prepared by growing in liquid R2A medium (Difco) at 25°C for 36 h, suspended in 0.1 M $MgSO_4$ buffer, washed twice and re-suspended in distilled water at 10^8 CFU per ml.

3.4 Bacterial Pathogen

Clavibacter michiganensis subsp. *michiganensis* (B.01778) from National Collection of Agricultural and Industrial Microorganisms (Hungary) was grown for 72 h in LB medium at 27°C. Bacterial suspension was concentrated by centrifugation at 5,400 g for 20 min, washed twice and diluted to 10^9 CFU/ml using sterile 10 mM $MgCl_2$. An equal quantity of bacterial suspensions (50 µl) injected into the stem region between the cotyledons of 7-week-old plants with a syringe fitted with a 30-gauge needle and 50 µl sterile 10mM $MgCl_2$ solution was used for mock-infected plants (non-Cmm plants).

3.5 Plant growth and experiment design

3.5.1 Mycorrhizal tomato plant tolerance to Cmm

3.5.1.1 Effect of different AMF isolates on tomato plant resistance against Cmm

This experiment was carried out from July to October 2015. Tomato seeds (*Solanum lycopersicum* L. cv. MoneyMaker) were treated with 2.7% sodium hypochlorite containing 0.02% (v/v) Tween-20 for 30 min, then washed with sterilized distilled water several times, and germinated on wet filter paper in Petri dishes at 26°C for 3 days. Pre-germinated seeds were sown in each pot containing 0.5 kg of sterile sand: peat (4:1, v/v) mixture. Before planting the seeds, different mycorrhizal inoculations, representing different treatments were prepared. There were eight treatments including plants inoculated separately with one of seven different AMF isolates altogether with non-AM plants. Thirty grams of inoculum was placed at 3 cm below pre-germinated seeds in the pots at the time of transferring seeds. Non-AM plants received thirty grams of autoclaved mycorrhizal inoculum and 3 ml aliquot of a filtrate (< 20 µm) of the AM inoculum to supply a general microbial population free of AM propagules. Thirty replicates of each treatment settled in a growth chamber. Pots were randomly distributed and cultivated at 23/28°C with 16/8 hours of photoperiod, the light intensity of 600 µmol/m^2/s and 60% humidity. Watering twice and fertilizing once a week with Long Ashton nutrient solution (Hewitt, 1966), adjusted to 3.2 µM $Na_2HPO_4.12H_2O$. After 7 weeks of growth, bacterial pathogen Cmm injection was performed, as described below. When plants reached 10 weeks of growth, plant biomass and mycorrhizal colonization, disease severity index were determined.

3.5.1.2 Role of ethylene in *Rizophagus irregularis*-induced resistance against Cmm

This experiment was implemented between April and July 2016. Tomato seeds (*Solanum lycopersicum* L.) of Never ripe (Nr), ethylene-insensitive mutant and its background Pearson kindly provided by Tomato Genetics Resource Center (University of California, Davis) were used. Before planting the seeds, inoculation with *Rhizophagus irregularis* (MUCL43194) and non-inoculation was implemented in each genotype. Fourteen replicates of each treatment were distributed randomly in a growth chamber. All growth conditions were the same as the description in 3.5.1.1. Cmm injection performed after 7 weeks of plant growth, as described below. Shoot fresh and dry weight, mycorrhizal colonization and disease severity index were examined at 10 weeks of plant growth.

3.5.2 Mycorrhizal tomato plant tolerance to drought, heat stress, combined drought and heat stress

This experiment was set up from November 2015 to January 2016. *Solanum lycopersicum* var. MoneyMaker seeds were disinfected in 2.5% sodium hypochlorite containing 0.02% (v/v) Tween-20 for 30 minutes, then rinsed with distilled water several times and put on Petri dishes to germinate for 3 days at room temperature. Subsequently, the germinated seeds were placed in 0.5-lit plastic pots filled with an autoclaved mixture of sand and soil (4:1, v/v). The loamy soil was collected at the experimental station of Szent István University, Gödöllő, Hungary. The soil properties were pH 7.1, 1.61% organic matter, N 15.6 mg kg^{-1}, available P 36 mg kg^{-1}, available K 60 mg kg^{-1}.

The experiment consisted of three groups: non-AM plants, plants inoculated with AM fungi, *Septoglomus deserticola* BEG 73 or *Septoglomus constrictum* (formerly *Glomus constrictum* Trappe). Mycorrhizal inoculums were harvested in an open-pot culture of *Zea mays* L. after 4 months of cultivation and included infected root fragments, sand, spores and mycelia. Thirty grams of inoculum were used for AM inoculation in each seedling while plants without AM inoculation were supplemented with 3 ml aliquot of a filtrate (< 20 µm) of the AM inoculum and thirty grams of autoclaved inoculum. Plants were distributed randomly and grown in a growth chamber (EKOCHIL 1500) at 26/20°C with 16/8 hour photoperiod, light intensity of 800 µmol m^{-2} s^{-1} and 60% humidity, and watered two times and fertilized one time a week with Long Ashton nutrient solution (Hewitt, 1966) with phosphorus concentration adjusted (3.2 µM $Na_2HPO_4.12H_2O$) until stress treatments. When plants reached 6 weeks of age, the stress treatments were carried out.

All plants at this point were divided into twelve treatments, then arranged in Randomized Complete Block Design with two factors: (1) plants without or with mycorrhizal fungi (*Septoglomus deserticola* or *Septoglomus constrictum*) and (2) stress applications. In detail, twelve treatments included mycorrhizal and nonmycorrhizal plants in normal conditions (well-watered, 26/20°C with 16/8 hours photoperiod and 60% relative humidity, 100% field capacity), drought conditions, heat conditions and combined heat and drought conditions. Drought stress was imposed by watering plants at 50% field capacity for 7 days, followed by withholding water for the next 3 days while the temperature, relative humidity and light regime were maintained at a level found in normal conditions. Heat treatment was accomplished by transferring well-watered plants kept in normal conditions to high temperature (42°C for 6h) (Zhou et al., 2014) at the very end of the harvest. The combined heat and drought stress were applied to drought-stressed plants (with and without mycorrhizal fungi) by exposing them to high temperatures (42°C for 6h) at the very end of drought period as described. Each treatment had 10 replicates. After 10 days of treatment, all plants were measured by equipment to determine the stress status of the plants, then harvested simultaneously. Fully expanded leaves (excluding petioles) and root samples were immediately frozen in liquid nitrogen and stored at -80^{o}C until further analysis.

3.5.3 Field experiment

The field experiment was conducted from May to September 2014. Three sweet pepper (*Capsicum annuum* L.) hybrids, Karpia, Karpex and Kaptur were used for this study at the experimental station of Szent István University, Gödöllő, Hungary (47.59^{o}N and 19.35^{o}E). The soil of the experimental station had brown forest soil, sandy loam in texture, consisting of 69% sand, 22% silt, and 9% clay, having chemical properties presented in Table 1. A moldboard plough to 25 cm depth was used for soil tillage after each harvesting time and conventional seedbeds were prepared by chisel plowing followed by disking.

Table 1. Chemical properties of the soil in the experiment

pH	EC (mS cm^{-1})	Organic matter (%)	NO_3 (N)	P_2O_5	K_2O	Ca	Mg	Fe	Cu	B	SO_4 (S)	Cl	HCO_3	Na
			mg kg^{-1}											
7.1	0.254	1.61	15.6	36.0	60	140	24.3	208	7.6	0.5	4.0	0	534	10

The average daily temperatures and precipitation were 12.8^{o}C and 4.04 mm in May; 16.8^{o}C, 1.2 mm in June; 18.7^{o}C, 4.27 mm in July; 15.7^{o}C, 4.26 mm in August; 12.6^{o}C, 2.24 mm in September 2014, respectively.

Seedlings of pepper varieties were propagated at the beginning of April in a greenhouse using special horticulture substrate [Klasmann TS3: 80% white sphagnum peat and 20% frozen black sphagnum-peat, slow-release 14N–16P–18K (w/w/w) fertilizer, pH 6.00] for 7 weeks. Then the seedlings were transplanted on 16th May, arranged in double rows with a distance of 0.8 m between beds, 0.3 m between the rows and 0.3 m between the plants. 72 g of NPK and 36 g of $Ca(NO_3)_2$ per square meter were applied for the whole season and drip irrigation was used to maintain substrate moisture content close to field capacity values (20% w/w) during the growth period.

All treatments including seven microbial inoculations and three cultivars were arranged in a randomized complete block design. The seven microbial inoculations were arbuscular mycorrhizal fungi (AM), *Trichoderma* (Tri), plant growth promoting bacteria (Pse) and their combinations (AM+Tri; AM+Tri+Pse; AM+Pse) and non-inoculation (control) plants with 30 replications per treatment each cultivar.

Before transplanting mycorrhizal fungi in the field, the commercial product Symbivit® was applied at 25 g of inoculum per pepper seedling into the planting hole and seedlings were planted immediately (AM treatment).

Trichoderma harzianum (SzIE35) treatment: 10 ml freshly prepared conidia with the volume of 0.2-liter sterile water were implemented to the seedlings 27 days after transplanting (Tri treatment).

Pseudomonas fluorescens (PK17) treatment: 10 ml fresh bacterium suspension was applied to the respective treatments (10^8 CFU ml^{-1}) 27 days after transplanting (Pse treatment).

Leaves at the same level from five different plants per treatment were collected at 29, 49, 69 days after transplanting (DAT) and kept in the -80^{o}C until enzyme assays. Roots from five plants per treatment were taken before harvesting for the estimation of mycorrhizal colonization rate.

3.6 Assessment of mycorrhizal colonization

Samples for estimating root colonization were collected before harvesting. Five randomly chosen pepper plants from the same treatment were dug out with a soil core of 25 × 25 × 25 cm. The roots and the soil were stored in separate plastic bags at 4°C until processing within 24h. Approximately, 500 mg of fine roots from each plant were transferred to separate tubes and were subjected to the staining technique of Vierheilig et al. (1998). Internal fungal

structures (hyphae, arbuscules, vesicles) were examined under a stereomicroscope at × 100 magnification and the percentage of root length colonized calculated using the gridline intersect method (Giovannetti and Mosse, 1980).

3.7 Measurement of tomato plant biomass

In Cmm experiment, four different plants of each treatment were harvested at 21 days post the infection of Cmm (dpi) to measure fresh plant biomass, then dried in a hot-air oven at 70°C for 2 days to obtain their dry weight.

In drought and heat stress experiment, shoot fresh weight from five different plants per treatment were weighed. Afterwards, their dry weight was measured.

3.8 Determination of pepper plant biomass and yield

In the field experiment, shoot and root fresh weight from 5 random pepper plants per treatment were collected to weigh at 70 days after transplanting, then shoot and root dry weight were determined. The pepper harvesting was performed randomly by hand at the biological maturity stage and evaluated for plants in each treatment.

3.9 Estimation of leaf water potential

Leaf water potential was examined in the individual fifth tomato leaves from the shoot apex of five different plants each treatment using a pressure chamber, following the description of Boyer (1995).

3.10 Measurement of relative water content

Relative water content (RWC) was measured as follows: leaves of five different plants in each treatment were weighed (fresh weight, FW), then saturated with water, re-weighed to record turgid weight (TW), finally dried at 70^{o}C for 2 days to have dry weight (DW). RWC was calculated as: $RWC\ (\%) = [(FW-DW)/(TW-DW)]\times 100$.

3.11 Disease severity index measurement

Disease severity was assessed after 7, 14, 17, 21 dpi, based on a 0-5 arbitrary scale as follows: 0, leaves expressing no wilting; 1, ≤ 10% of leaves expressing wilting; 2, 11-25% of leaves with wilting; 3, sectored wilting, 26-49% of leaves expressing wilting associated with chlorosis; 4, pronounced collapse as leaf extended, 50-74% of leaves expressing wilting; 5, whole plant

wilted. A mean disease severity index (DSI) was calculated from each treatment by adding the score of the 30 plants, and presenting the value as a percentage using the formula described by Raupach et al. (1996): DSI (%) = [(∑rating no. x no. of plants in rating) x 100%]/(total no. of plants x highest rating).

3.12 Determination of stomatal conductance

Measurement of stomatal conductance was implemented at the third leaves from the shoot apex of five different plants per treatment using a porometer system (Delta-T AP4, UK).

3.13 Chlorophyll fluorescence determination

Chlorophyll fluorescence parameter, the maximum efficiency of PSII photochemistry after 30 minutes of dark-adaption (F_v/F_m) was determined using Walz – PAM 2500. The measurement was made at the third leaves from the shoot apex of five different plants in each treatment.

3.14 Determination of hydrogen peroxide accumulation

The concentration of H_2O_2 was measured according to the description of Alexieva et al. (2001). 0.5 g of leaf sample was homogenized with 5 ml of cold 0.1% (w/v) trichloroacetic acid (TCA), then centrifuged at 12,000×g for 15 min (4°C). The reaction mixture included 0.5 ml of 100 mM potassium phosphate buffer (pH 7.0), 1 cm^3 of 1 M KI and 0.5 ml of the leaf extract supernatant. The reaction occurred for 1 h in darkness and absorbance was measured by spectrophotometer at 390 nm.

3.15 Determination of oxidative damage to lipids

The lipid peroxidation level in leaves was determined according to Heath and Packer (1969). Briefly, 0.2 g of leaf sample was homogenized in 5 ml 0.1% TCA, centrifuged at 10,000×g for 5 min. The mixture was prepared with 4 ml of 20% TCA containing 0.5% TBA and 1 ml aliquot of the leaf supernatant, then heated at 95°C for 15 min and cooled shortly. MDA was calculated by subtraction of absorbances from the mixture at 532 nm and 600 nm, expressed as nmol of MDA with using extinction coefficient of 155 mM^{-1} cm^{-1}

Four leaf samples from 4 different plants per treatment were used for the measurements and each sample had three technical replicates.

3.16 Determination of antioxidant enzymatic activities

0.5 g of frozen (-80^{o}C) leaf material of each sample from treatments was homogenized in liquid N_2 with 3 ml of 50 mM Tris-HCl buffer (pH 7.8) containing 7.5% (w/v) polyvinyl-pyrrolidone K25 and 1 mM Na_2EDTA, and centrifuged at 10,000 x g for 20 minutes at 4^{o}C. The supernatants were used for measuring peroxidase, polyphenol oxidase, superoxide dismutase and catalase activities, the protein concentration of all leaf extracts was estimated according to the method of Bradford (1976).

Polyphenol oxidase (PPO, EC 1.10.3.1) activity was measured by modified Fehrmann and Dimond (1967) method. The 2.2 ml of reaction mixture made up of 0.1 M sodium phosphate buffer (pH 6.0), 1 mM Na_2EDTA, 20 mM catechol with 200 µl of the crude leaf extract was used to assay the enzyme activity at 400 nm in 10 minutes. Changes in absorbance per protein concentration per unit time were estimated.

Peroxidase (POD, EC 1.11.1.7) activity was determined by Rathmell and Sequeira (1974) method. Briefly, 10 µl plant extract was added to 2.2 ml of reaction mixture consisting of 0.1 M sodium phosphate buffer (pH 6.0), 100 µl of 50 mM Guaiacol, 100 µl of 12mM H_2O_2. The absorbance was recorded at 436 nm in 5 minutes. The enzyme activity was calculated by the changes of absorbance per mg protein per minute.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured spectrophotometrically at 560 nm according to the method of Beyer and Fridovich (1987). Shortly, 20 µl of the crude extract and 20 µl of 1 mM riboflavin were added to 2 ml of reaction mixture composed of 50 mM phosphate buffer (pH 7.8) consisting of 2 mM EDTA, 0.025% Triton X-100, 55 µM Nitroblue tetrazolium (NBT), and 9.9 mM L-methionine. One unit of SOD activity (U) was defined as the required enzyme volume to result in 50% inhibition of the reduction of NBT as recorded at 560 nm.

Catalase (CAT, EC 1.11.1.6) activity was determined following the method of Aebi and Lester (1984). The 3-ml reaction mixture consisting of 2 ml of leaf extract diluted (x200) in the buffer of 50 mM potassium phosphate (pH 7.0) and 10 mM of hydrogen peroxide. The absorbance decrease at 240 nm of the reaction was recorded as deposition level of H_2O_2. The enzyme activity was expressed as the changes in absorbance per protein concentration per unit time.

3.17 RNA Isolation and Quantitative real-time PCR (qRT-PCR) analysis.

RNA from samples were isolated using the Origene kit (USA) following the manufacturer's protocol, and the quantity of RNA was recorded by Nanophotometer (IMPLEN). 3.0 µg total RNA in a 20 µl reaction volume was used to synthesize cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to manufacturer's instruction. Quantitative real-time PCR reaction were implemented in a total volume of 20 µl, containing 2.0 µl cDNA, 1 µl of 10 µM of each primer, and 10 µl of VeriQuest SYBR Green qPCR Master Mix (2X) (Affymetrix) using qPCR Corbett RG-6000 programmed with an initial step of 10 min at 95^{o}C followed by 40 cycles alternating between 15s at 95^{o}C and 1 min at 60^{o}C. Aquaporin gene (*SlPIP2.7*) and the biosynthetic gene of Jasmonate (*SlLOXD*), Abscisic Acid (*SlNCED*) were determined with primers listed in Table 2. At least three technical replicate qRT-PCR reactions were carried out per primer pair. The relative expression levels were normalized with the expression data of tomato Actin gene by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Three samples of leaves and roots from three different plants were examined in each treatment.

Table 2. List of primers used in this study

Target gene	GenBank Accession (ID)	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Reference
SlLOXD (JA biosynthesis)	U37840	GACTGGTCCAAGTTCACGATCC	ATGTGCTGCCAATATAAATGGTTCC	178	Uppalapati et al. (2005)
SlNCED (ABA biosynthesis)	Z97215	ACCCACGAGTCCAGATTTC	GGTTCAAAAAGAGGGTTAGC	139	López-Ráez et al. (2010)
SlPIP2.7 (plasma membrane aquaporin)	Solyc01g111660.2.1	CAGCAGTGACATTTGGGTTG	CCAACTCCACAAATTGCACC	100	This study
SlActin (Housekeeping)	U60480.1	GGTGTGATGGTGGGTATGG	GCTGACAATTCCGTGCTC	108	Oirdi et al. (2011)

3.18 Statistical analysis.

SAS 9.1 (SAS Institute, Cary, North Carolina) package for Windows was used for statistical analysis. Data were evaluated by either two-way factorial analysis of variance (ANOVA) with inoculation treatment and stress treatment, microbial treatment and cultivars or one-way analysis of variance. Mean values were compared by Tukey posthoc test at $P < 0.05$.

4. RESULTS AND DISCUSSION

4.1 Mycorrhiza-induced alleviation of plant disease caused by *Clavibacter michiganensis* subsp. *michiganensis* and role of ethylene in mycorrhiza-induced resistance in tomato

4.1.1 *Rhizophagus irregularis* induces plant resistance against Cmm effectively among AMF isolates tested.

After 10 weeks of growth, mycorrhizal colonization rate among AM treatments was significantly different (Figure 9A), while no substantial differences in growth response among them could be observed (data not shown). The highest colonization level (64.5%) was gained by *Rhizophagus irregularis* (MUCL43194) which is the most widespread and most frequently studied AM fungal species while the lowest colonization was *Gigaspora margarita* (37.4%).

Besides different responses to mycorrhizal inoculation on colonization processes, three levels of responses on disease sensitivity are also recognized at 17 and 21 dpi although no significant differences in DSI among treatments were found at 7 and 14 dpi (Figure 9B). Tomato plants inoculated with *Rhizophagus irregularis* showed both highest colonization and induced resistance to Cmm after 21 days of bacterial infection (DSI 54.5%), while the effect of other isolates (*Funneliformis mosseae*, *Gigaspora margarita* and *Claroideoglomus claroideum*) were intermediate on colonization and high on induced resistance. Surprisingly, plants inoculated with *Gigaspora margarita* showed lower colonization than other tested isolates while a high resistance to Cmm (DSI 62.5%). Together no significant differences in plant biomass of all treatments was observed, the MIR was not related to enhanced plant growth due to AMF.

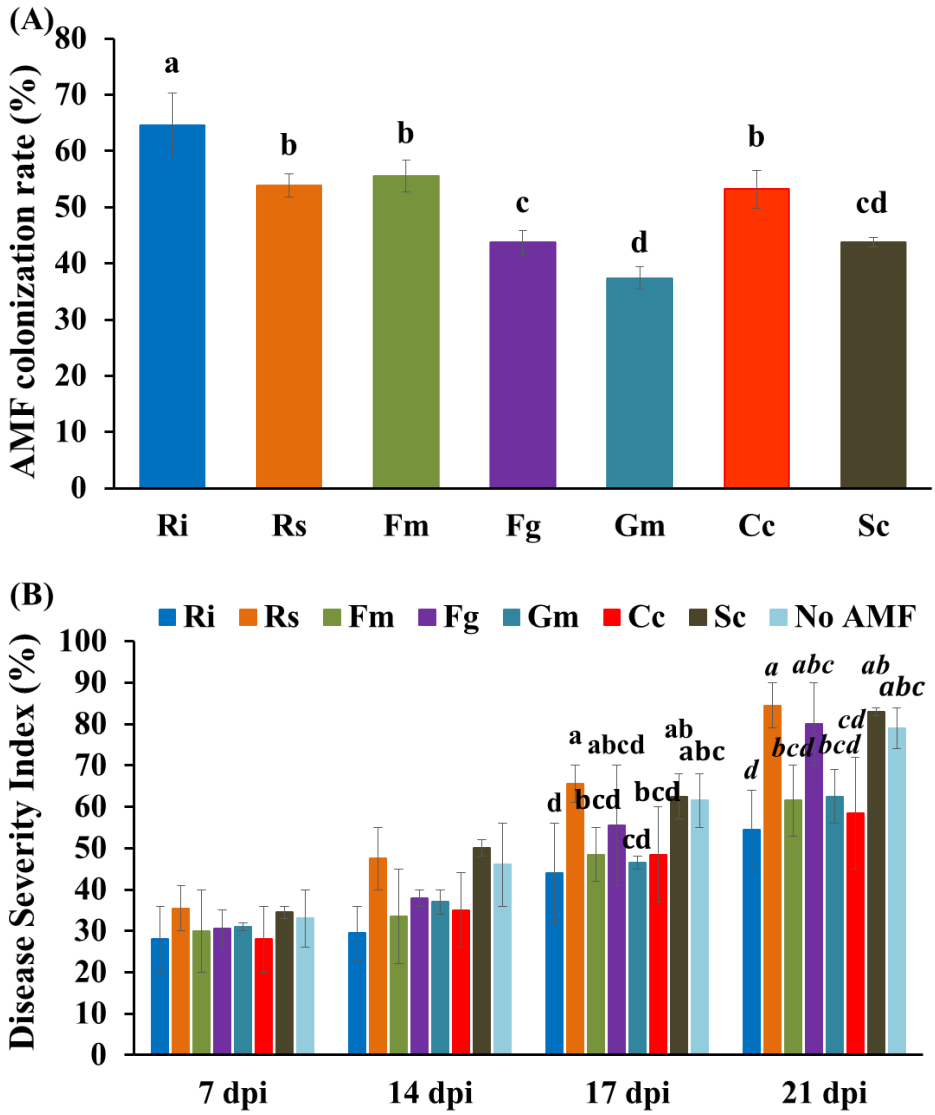


Figure 9. Arbuscular mycorrhizal fungi colonization rate (A) after 10 weeks of growth and disease severity index (DSI) (B) in AM and non-AM plants at 7, 14, 17, 21 days of post inoculation of Cmm (dpi). Ri, *Rizophagus intraradices* MUCL 43194; Rs, *Rhizophagus* sp. MUCL 43204; Fm, *Funneliformis mosseae* BEG 12; Fg, *Funneliformis geosporum* BEG 11; Gm, *Gigaspora margarita* BEG 34; Cc, *Claroideoglomus claroideum* BEG 23; Sc, *Septoglomus constrictum*. Bars present means ± Standard Error. Different regular and italic letters denote significant differences in DSI among treatments at 17 and 21 dpi, respectively.

4.1.2 *Rhizophagus irregularis* induced resistance is dependent on ethylene

In order to explore the involvement of ET in mycorrhiza-induced resistance, we used Never ripe (Nr), whose one member of the ET receptor gene family is mutated, resulting in ET insensitivity in tomato plants (Lanahan et al., 1994) and its corresponding background (Pearson) while *Rhizophagus irregularis* was chosen as AMF inoculation based on the result of our

previous experiment. Ri-induced resistance was also observed in the background plants inoculated by Ri at 7, 14, 17, 21 dpi, confirming the result of our previous experiment (Figure 10). In addition, ET insensitivity limited disease development of Cmm due to the fact that DSI of Nr plants was considerably lower than that of the Pearson background during three weeks of Cmm infection. Remarkably, insensitivity of ET in Nr plants colonized with Ri eliminated the MIR against Cmm when its DSI was similar to that of Pearson plants without Ri inoculation over the course of Cmm infection, suggesting that ET plays a key role in Ri-induced resistance against Cmm.

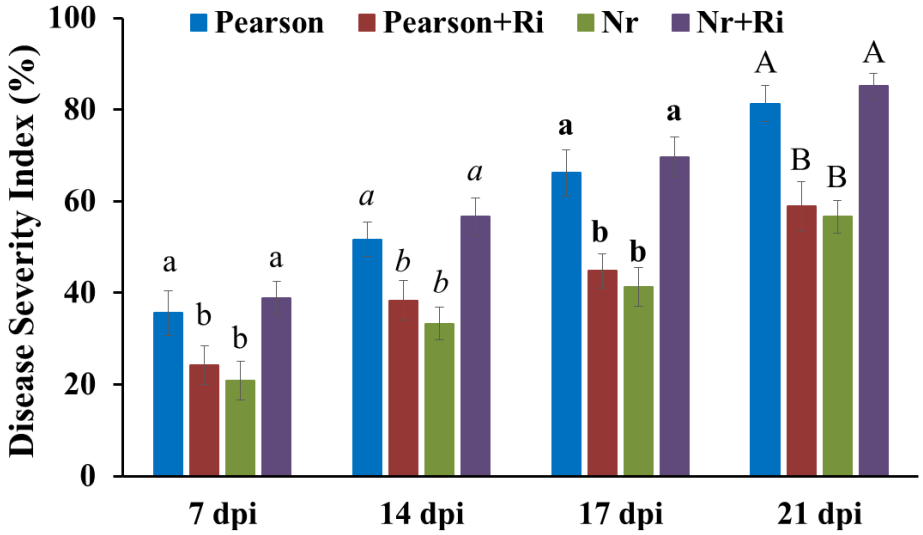


Figure 10. Disease severity index (DSI) of AM and non-AM plants at 7, 14, 17, 21 days post inoculation (dpi) of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in ethylene insensitive mutant (Nr) and its wild-type (Pearson). Ri, *Rizophagus irregularis* MUCL 43194. Bars present means ± Standard Error. Different regular, italic, bold and capital letters denote significant differences in DSI among treatments at 7, 14, 17 and 21 dpi, respectively.

Noticeably, AM colonization failed to increase shoot fresh and dry weight in plants in our experimental conditions, where no remarkable differences in shoot fresh and dry weight between Pearson and Pearson+Ri, Nr and Nr+Ri were detected (Table 3). Cmm significantly decreased shoot fresh by 34% and dry weight by 24% in Nr mutant and its background but the more pronounced reduction in shoot dry weight (52%) was in the treatment Nr+Ri+Cmm. Interestingly, AM colonization rate in Nr+Ri was increased by 17%, as compared to Pearson+Ri whilst this value was most severely reduced (28.7%) in Nr+Ri+Cmm.

Table 3. Shoot fresh and dry weight, AM colonization rate in Never ripe (Nr) mutant and its background Pearson with or without *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) infection.

Treatment	Shoot fresh weight (g)	Shoot dry weight (g)	AM colonization (%)
Pearson	26.35 ± 2.6 ab	1.97 ± 0.1 a	0
Pearson + Cmm	13.95 ± 1.7 b	0.93 ± 0.2 b	0
Pearson + Ri	28.76 ± 1.9 a	2.10 ± 0.2 a	54.0 ± 0.7 **b**
Pearson + Ri + Cmm	14.40 ± 2.2 b	1.04 ± 0.2 b	52.5 ± 1.1 **b**
Nr	20.74 ± 1.5 A	1.44 ± 0.2 A	0
Nr + Cmm	13.69 ± 1.0 B	1.09 ± 0.1 B	0
Nr + Ri	20.11 ± 1.4 A	1.52 ± 0.1 A	65.1 ± 0.3 **a**
Nr + Ri + Cmm	10.06 ± 1.3 B	0.75 ± 0.1 C	38.5 ± 2.1 **c**

AM, arbuscular mycorrhizal. Ri, *Rhizophagus irregularis*. Parameters were shown as the mean value of four replicates ± Standard Error. Different regular and capital letters each column express significant differences in shoot fresh and dry weight of the background Pearson and Nr mutant, respectively. Different bold letters indicate significant differences in AM colonization of all plants pretreated by Ri.

4.1.3 Discussion

Different AM colonization rate of the isolates found in our results reflects various AM colonization strategies. *Rhizophagus isolates*, high colonization in the present study, produce an extensive hyphal network providing a competitive advantage over other isolates (Silvani et. al., 2014) whereas *Gigaspora* sp. shows lower capacity in that process as we also recognized it, moreover, its re-establishment of mycelium though their anastomoses offer also smaller capacity to colonize the roots compared to other species (de la Providencia et al., 2005). Other tested strains in our work, namely *Rhizophagus* sp. (MUCL 43204), *Claroideoglomus claroideum* (BEG 23) and *Funneliformis mosseae* (BEG 12) have an intermediate level of colonization confirming that tomato is intermediate in mycorrhizal dependency (Plenchette et al., 1983).

It is known that AM symbiosis could enhance plant resistance against a wide range of viral, bacterial and fungal pathogens (Fiorilli et al., 2011; Fritz et al., 2006; Liu et al., 2007; Maffei et al., 2014; Song et al., 2015) but our report is the only one regarding bacterial disease caused by Cmm. The present study shows Ri induced the most effective systemic resistance against Cmm among seven AMF isolates tested. Different mechanisms are proposed to interpret the role of AMF in plant protection. During mycorrhization, AMF-induced modulation of plant defence responses takes place to achieve a functional symbiosis, leading to activated host immunity locally as well as systemically, called primed state of the plant allowing trigger of defence responses quicker and more effectively upon being attacked by potential enemies (Jung

et al., 2012). Furthermore, a remarkable transcriptional reprogramming, significant alterations in the hormonal balance, primary and secondary metabolism occur in the host plants during mycorrhization (Jung et al., 2012; López-Ráez et al., 2010b) Transcriptional changes in both roots and shoots in *Medicago truncatula* plants inoculated by *Rhizophagus irregularis* (syn. *Glomus intraradices*), *Gigaspora gigantea*, *Glomus versiforme*, resulting in increased resistance to shoot pathogen *Xanthomonas campestris* pv. *alfalfae* (Liu et al., 2007). Even, common mycorrhizal networks underground among tomato plants pretreated by *Funneliformis mosseae* (syn. *Glomus mosseae*) induced resistance of neighbours against *Alternaria solani* (Song et al., 2010). More recently, AMF primed resistance to *Alternaria solani* has been proved in tomato plants treated with *Funneliformis mosseae* that showed enhanced expressions of important defense genes and higher activities of defense-related enzymes as compared to non-AM plants (Song et al., 2015).

It is believed that AMF can enhance its host plant nutrient and growth but improved mycorrhizal plant growth was not observed in both of our experiments, perhaps due to the fact that plants were cultivated in pots under suboptimal conditions. Thus, the protective role of AMF was associated with mechanisms other than a better plant fitness, most probably linked to plant defenses.

AM colonization rate in the ET insensitivity mutant was increased in relation to the Pearson background, which is in line with the results of several studies revealing that ET has detrimental effects on mycorrhizal development in the symbiosis (Fracetto et al., 2017; Santos et al., 2016). It should be noted that the colonization was most profoundly decreased (28.7%) in Never ripe plants with Ri and Cmm inoculation, suggesting that Cmm negatively affected AM development in the ET-insensitive mutant. This may be owing to the fact that Cmm weakened plant fitness, leading to decreasing photosynthate source for the mycorrhizal symbiont and/or Cmm activated the plant defense system that consequently inhibits the mycorrhizal development. Similarly, aboveground pathogen *Colletotrichum gloeosporioides* decreased belowground AM colonization in *Phaseolus vulgaris* due to its activation of plant defense responses (Ballhorn et al., 2014).

ET is the main component of plant defense signals that are generated during a wide range of microbe-plant interactions and acts as a crucial regulator of plant immunity (Broekaert et al., 2006; Van Loon et al., 2006). Our analyzed results indicated that on the one hand, impaired perception of ET decreased significantly the development of wilt symptoms caused by Cmm, which is in accordance with earlier studies revealing that ET in the host plant is crucial in the

regulation of the susceptibility to Cmm in tomato plants (Balaji et al., 2008; Savidor et al., 2012; Savidor et al., 2014). On the other hand, ET is required for MIR against Cmm due to the fact that Nr plants pretreated by Ri did not induce resistance against this bacterial pathogen. Previous reports have emphasized a necessary role of the Jasmonate-regulated pathway in MIR against different pathogens in several plants (Mora-Romero et al., 2015; Nair et al., 2015a,b; Pozo et al., 2010; Song et al., 2015). Nonetheless, it is worth noting that there is little information about the involvement of ET signalling pathway in MIR. Our result is the first observation of MIR against Cmm mediated by ET. Numerous studies demonstrated that together with Jasmonate, ET plays a central role in control of induced resistance by various beneficial microbes such as *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61 (Yan et al., 2002) in tomato plants, *Trichoderma harzianum* T39 (Korolev et al., 2008), *Pseudomonas fluorescens* Q2-87 (Weller et al., 2012), *Pseudomonas protegens* CHA0 (Iavicoli et al., 2003), *Penicillium* sp. GP16-2 (Hossain et al., 2008) in Arabidopsis. In fact, ET is able to stimulate the production of distinct pathogenesis-related (PR) proteins or phytoalexins derived from the phenylpropanoid pathway leading to rigidification of cell walls in a wide range of plant species (Abeles et al., 1992; Arshad and Frankenberger, 2002), thus enhancing plant resistance against pathogens. Additionally, JA- and ET-signalling pathway often operate synergistically to induce the effector genes of induced defense responses (Ellis and Turner, 2001; Pieterse and Van Loon, 1999; Schenk et al., 2000).

4.2 Arbuscular mycorrhizal fungi alleviate negative effects of drought, heat stress, combined drought and heat stress in tomato plants

4.2.1 Plant growth and symbiotic development

Stress treatments considerably reduced the shoot biomass in all plants, with a decrease more pronounced in the combined stresses. Although AM applications did not increase the dry or fresh weight of shoot significantly in the non-stress treatments when exposed to drought and drought + heat stress, plants pretreated by *S. constrictum* showed a significant rise in growth parameters as compared to the corresponding plants without AM. However, the beneficial impact of AM colonization failed to be recorded in the heat treatment (Table 4).

Colonization was not found in non-AM plants, while it reached about 70% in plants inoculated with either *S. deserticola* or *S. constrictum* at harvest time without the effects of stress treatment. Moreover, no significant differences in the colonization rates between two AM inoculums were observed after stress applications, whereas drought and drought + heat stress decreased the

proportion of plants inoculated by *S. deserticola* by 19.3% and *S. constrictum* by 20.3% in relation to unstressed counterparts (Table 4).

Table 4. Shoot fresh and dry weight and AM colonization rate in non-inoculated plants and plants inoculated by either *S. deserticola* or *S. constrictum* under non-stress, drought, heat stress and the combined stress conditions.

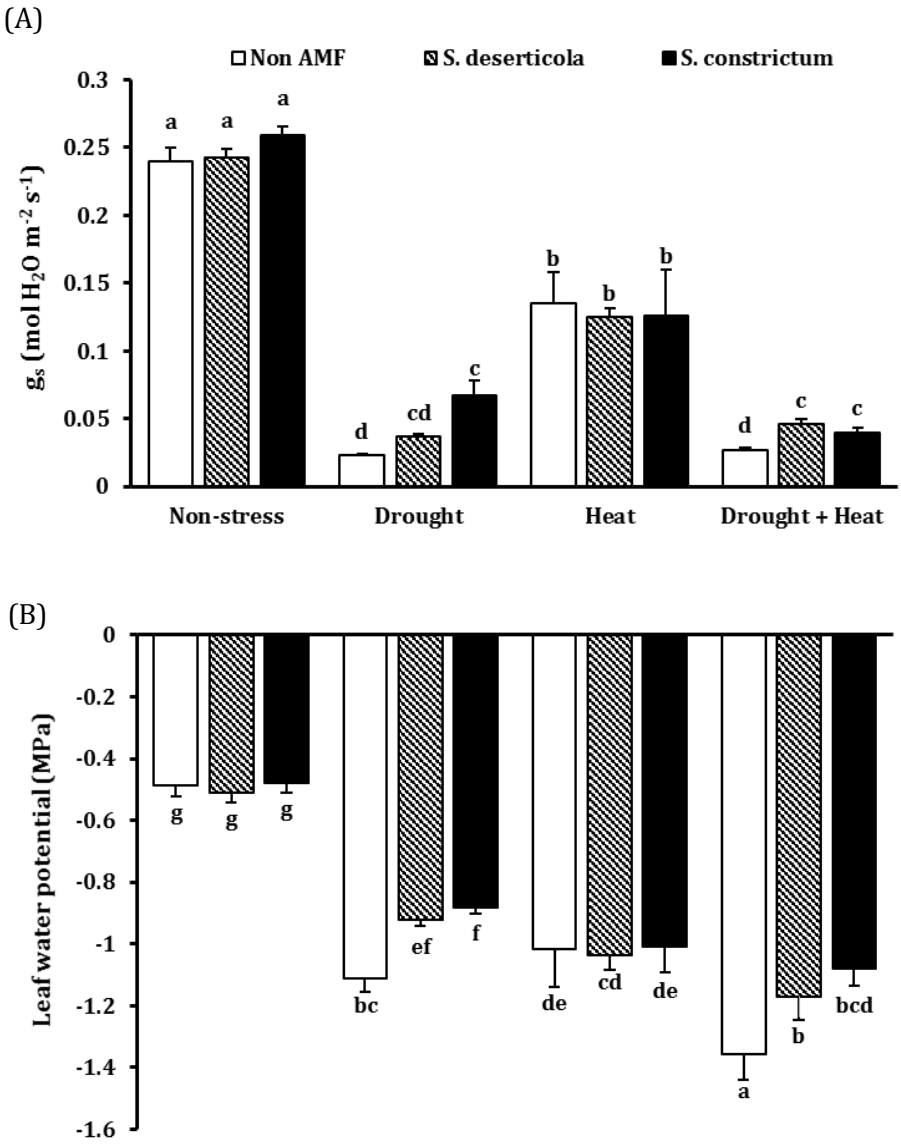
Treatments		Shoot Fresh Weight (g plant^{-1})	Shoot Dry Weight (g plant^{-1})	AM colonization (%)
Non-stress	Non AMF	28.98 ± 0.76 a	1.82 ± 0.15 ab	0 ± 0.00 d
	S. deserticola	28.93 ± 1.97 a	1.84 ± 0.08 ab	67.53 ± 7.94 ab
	S. constrictum	29.15 ± 1.65 a	1.91 ± 0.14 a	73.60 ± 8.14 a
Drought	Non AMF	18.70 ± 1.51 ef	1.46 ± 0.11 de	0 ± 0.00 d
	S. deserticola	20.10 ± 0.74 de	1.53 ± 0.14 de	54.73 ± 5.83 c
	S. constrictum	21.24 ± 1.81 bcd	1.73 ± 0.03 bc	58.10 ± 1.45 bc
Heat	Non AMF	22.48 ± 1.20 bc	1.52 ± 0.15 de	0 ± 0.00 d
	S. deserticola	21.10 ± 1.21 bcd	1.39 ± 0.13 ef	67.93 ± 7.12 ab
	S. constrictum	23.35 ± 1.69 b	1.62 ± 0.19 bcd	67.87 ± 5.55 ab
Drought + Heat	Non AMF	18.64 ± 1.01 ef	1.27 ± 0.09 f	0 ± 0.00 d
	S. deserticola	17.59 ± 1.54 f	1.38 ± 0.09 ef	54.47 ± 7.28 c
	S. constrictum	20.90 ± 1.35 cd	1.58 ± 0.10 cd	58.63 ± 3.94 bc

For each parameter the means ± standard errors are presented. Different letters within a column indicate significant difference among treatments by Tukey's post hoc test at $P \leq 0.05$ (for shoot fresh and dry weight n = 5, AMF colonization n = 4).

4.2.2 Stomatal conductance, leaf water potential and relative water content

No significant variations in stomatal conductance (gs), relative water content and leaf water potential between AM and non-AM plants in non-stress conditions were recorded (Figure 11). However, these physiological parameters were reduced sharply as a consequence of stresses, the reductions being particularly pronounced in the combination of drought and heat stress. Under heat stress the decline in gs in both non-AM and AM plants was less than that in other stresses although there were no significant differences among heat-stress treatments. Importantly, colonized plants heightened gs dramatically in their leaves under drought and drought+heat stress, with values nearly twice as high on average as those of the uncolonized ones, even as high as threefold values when plants were inoculated with *S. constrictum* under drought stress. Similarly, the effectiveness of AM colonization in alleviating the decrease in leaf water potential and relative water content was not detected under heat stress alone. Noticeably, AM-plants tended to enhance these parameters when subjected to the drought and

drought + heat stress in comparison with the corresponding uninoculated plants, with the higher values being obtained in those inoculated with *S. constrictum.*



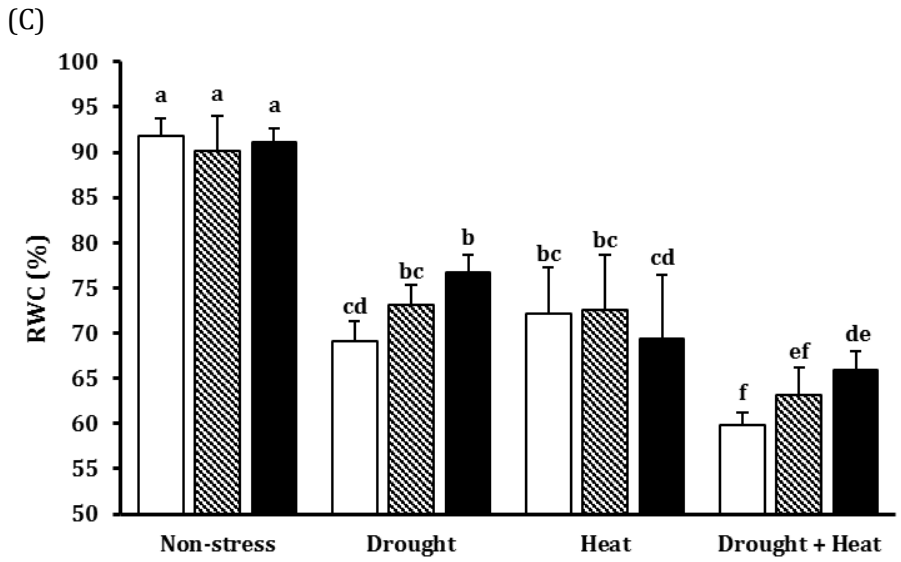


Figure 11. Stomatal conductance (A), leaf water potential (B) and relative water content (RWC) (C) in non-AM plants and plants inoculated by either *S. deserticola* or *S. constrictum* subjected to non-stress, drought, heat and combined stress conditions. Each bar represents mean ± standard deviation (n = 5). Different letters indicate significant difference among treatments by Tukey's post hoc test at $P \leq 0.05$.

4.2.3 Chlorophyll fluorescence

Maximal photosystem II photochemical efficiency (F_v/F_m) of AM and non-AM plants decreased significantly in relation to non-stressed plants subjected to stresses (Figure 12). Heat stress resulted in no significant differences in F_v/F_m between uncolonized and colonized plants, whereas under drought and heat+drought stress conditions AM symbiosis considerably increased F_v/F_m in tomato plants, particularly when inoculated with *S. constrictum*.

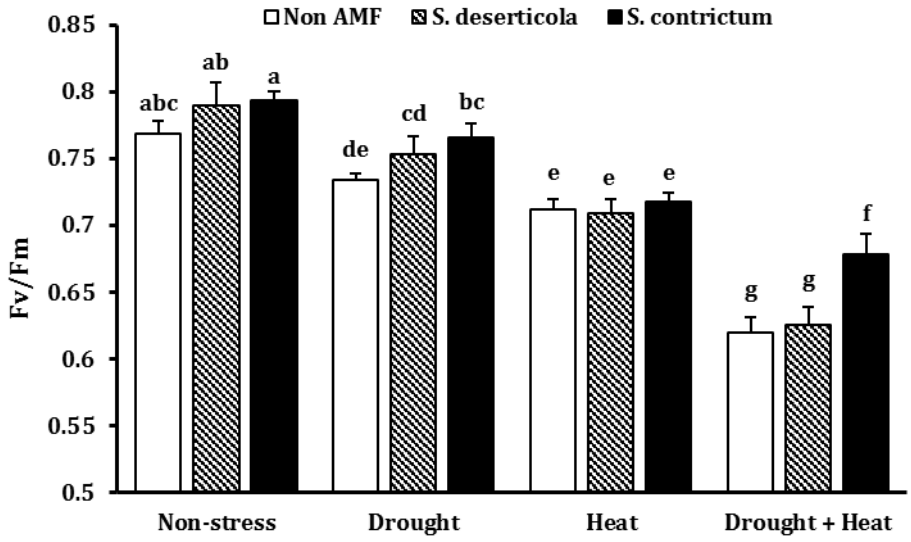


Figure 12. Maximal PSII photochemical efficiency (F_v/F_m) in non-AM plants and plants inoculated by either *S. deserticola* or *S. constrictum* subjected to non-stress, drought, heat and combined stress conditions. Each bar represents mean ± standard deviation (n = 5). Different letters indicate significant difference among treatments by Tukey's post hoc test at $P \leq 0.05$.

4.2.4 Accumulation of hydrogen peroxide and lipid peroxidation

Both AM and non-AM plants showed similar values of MDA and H_2O_2 in non-stress conditions (Figure 13). Nonetheless, stresses caused significantly higher MDA and H_2O_2 contents in leaves of tomato plants, in which these values were most significantly affected after plants were subjected to heat + drought stress. In non-AM plants, the levels of H_2O_2 were induced twofold, sixfold and ninefold in drought, heat and the combined stresses, respectively, while mycorrhizal plants, especially the ones inoculated with *S. constrictum* showed substantially reduced levels of oxidative damage to lipids under stress treatments and decreased the level of H_2O_2 accumulation by 31.5% under drought stress, 40.3% under heat stress and 59.5% under the combined stress, relative to non-AM ones.

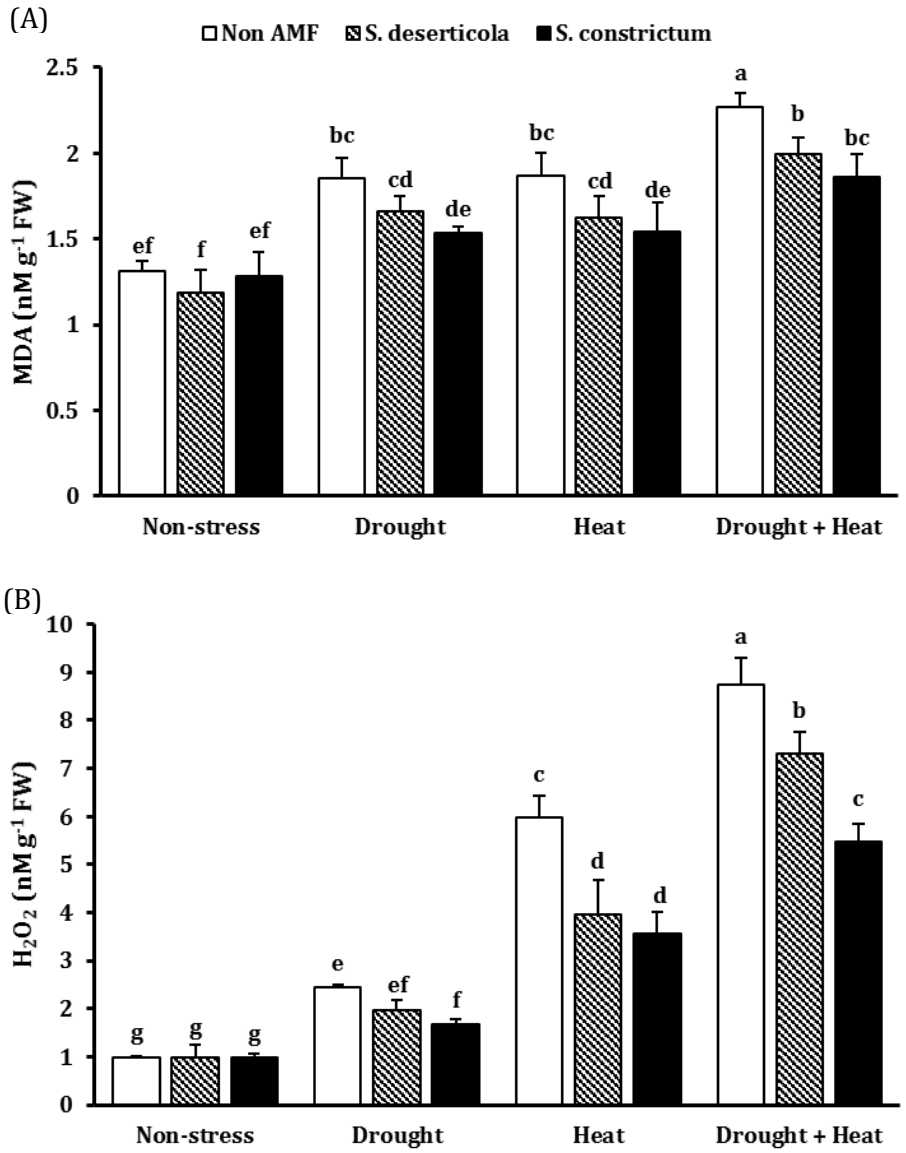


Figure 13. Malondialdehyde (MDA) (A) and H_2O_2 (B) accumulation in leaves of non-AM plants and plants inoculated by either *S. deserticola* or *S. constrictum* subjected to non-stress, drought, heat and combined stress conditions. Each bar represents mean ± standard deviation (n = 4). Different letters indicate significant difference among treatments by Tukey's post hoc test at $P \leq 0.05$.

4.2.5 Antioxidant enzyme activities

Activities of antioxidant enzymes like POD, SOD, CAT in the leaves of uninoculated and inoculated plants were not significantly different in normal growing conditions, but their levels increased in colonized plants under stress conditions (Figure 14A, B, C). Non-AM plants exhibited considerably lower levels of POD activity than AM plants in stress treatments, although, no significant differences between the two AM species were detected, except for the better enhancement in plants colonized with *S. constrictum* in drought + heat stress. Similarly,

the inoculation with *S. constrictum* considerably improved SOD activity under drought and drought + heat stress while AM colonization did not change enzyme activities under heat-stress conditions. CAT activity in both AM-plants increased in the similar fashion as plants were subjected to all stresses.

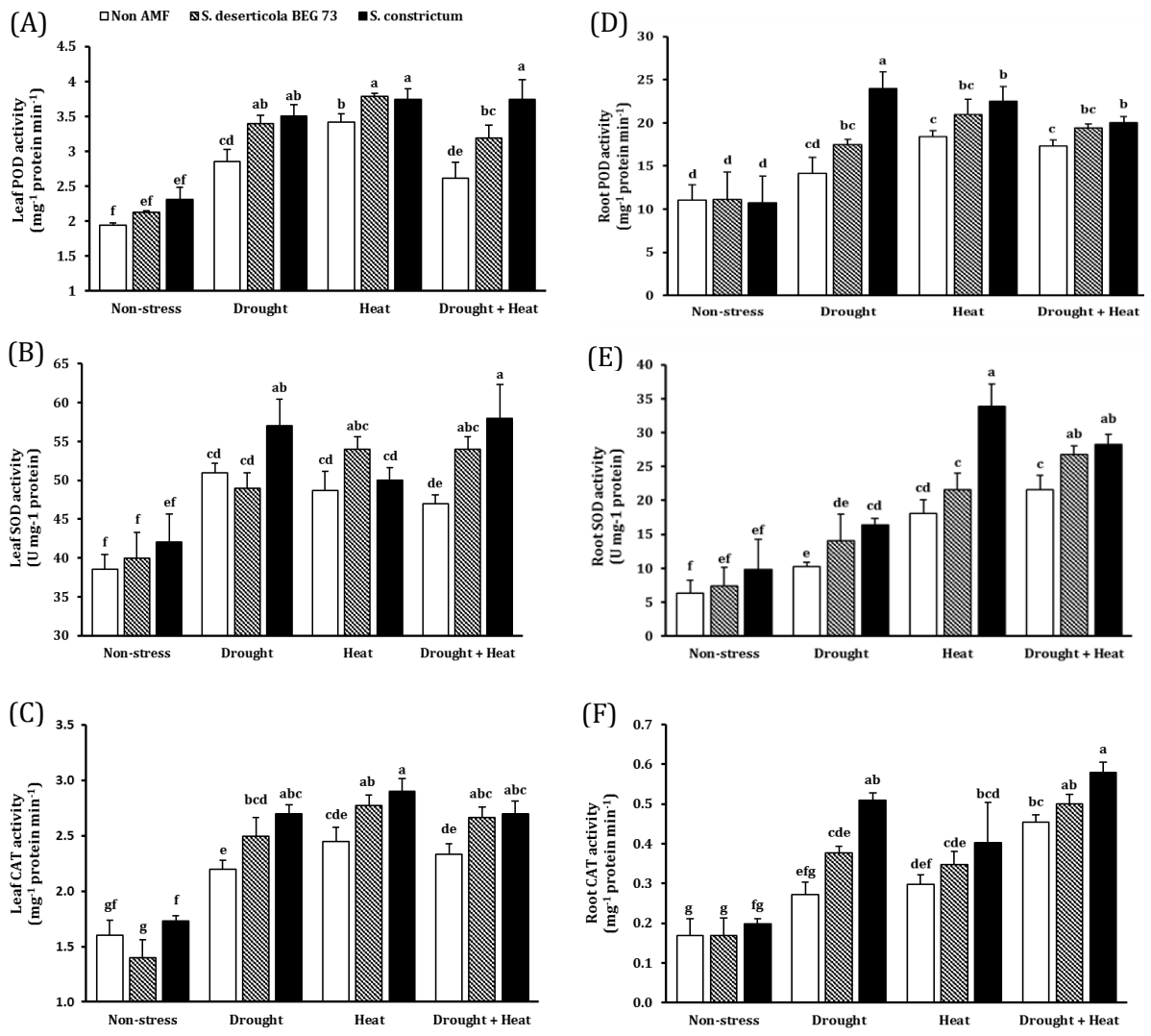


Figure 14. Activity of Peroxidase (POD) (A), Superoxide Dismutase (SOD) (B), Catalase (CAT) (C) in leaves and POD (D), SOD (E), CAT (F) in roots of non-AM plants and plants inoculated by either *S. deserticola* or *S. constrictum* subjected to non-stress, drought, heat and combined stress conditions. Each bar represents mean ± standard deviation (n = 4). Different letters indicate significant difference among treatments by Tukey's post hoc test at $P \leq 0.05$.

In roots, the similar increase in POD, SOD, CAT activities were also observed in AM plants subjected to drought, heat and the combined stresses with *S. constrictum* being a more efficient enhancer than *S. deserticola* (Figure 14D, E, F). POD levels were induced 70% more effectively

in plants treated with *S constrictum* under drought stress than non-AM plants. Also, the same inoculant nearly doubled SOD activity in colonized plants in comparison with uninoculated plants in the heat stress treatment. CAT activity was substantially strengthened in colonized roots, except in heat-stress treatments.

4.2.6 Expression of biosynthetic gene of ABA, JA and aquaporin genes

Based on the physiological performances of AM plants under stress conditions tested, only samples of *S. constrictum* pretreated plants were chosen for the analysis of the expression of ABA, JA biosynthetic gene and an important aquaporin gene. Drought treatment significantly upregulated *SlNCED* gene in roots of non-AM plants in relation to non-stress plants (Figure 15A). Remarkably, the gene expression was lowered in roots colonized by *S. constrictum* as compared with the non-inoculated ones under drought stress while no significant differences in the expression of root *SlNCED* gene between AM and non-AM plants were found under normal growing conditions and other stresses. Root *SlLOXD* gene in both AM and non-AM plants was upregulated by stresses (Figure 15B). Application of *S. constrictum* significantly increased *SlLOXD* gene expression under all conditions except heat stress in comparison with their counterparts in the non-AM plants.

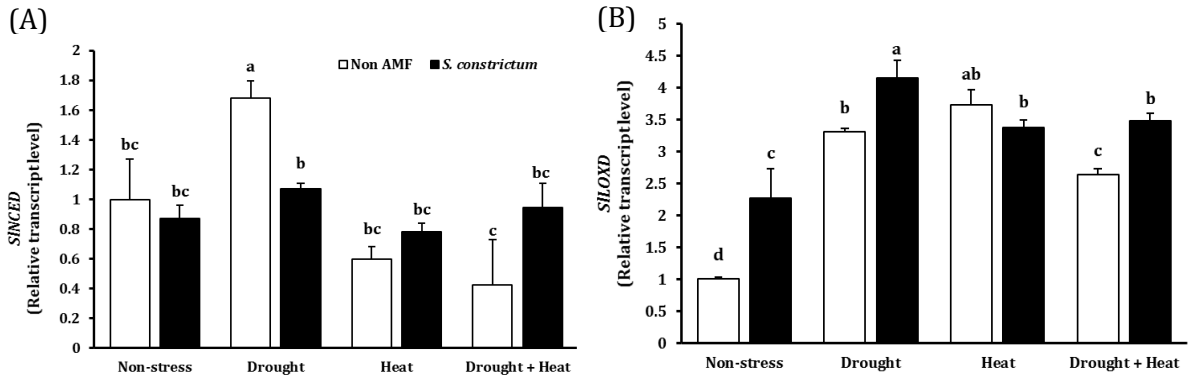


Figure 15. Expression of ABA-biosynthetic gene *SlNCED* (A) and JA-biosynthetic gene *SlLOXD* (B) in roots of non-AM and *S. constrictum* inoculated plants under non-stress, drought, heat and combined stress conditions. Each bar represents mean ± standard deviation. Different letters indicate significant difference among treatments by Tukey's post hoc test at $P \leq 0.05$.

SlPIP2.7 proved one of the important aquaporin genes conferring drought tolerance in tomato plants (Li et al., 2016) and was chosen to examine whether *S. constrictum* influence expression of aquaporin gene of the host plant. Although inoculation of *S. constrictum* enhanced the expression levels of root *SlPIP2*.7 in normal growing conditions, drought and heat stress lessened it, while its transcript levels decreased under the combined stress (Figure 16).

Nonetheless, no significant differences in root *SlPIP2.7* expression between AM and non-AM plants were found under heat and drought+heat stress.

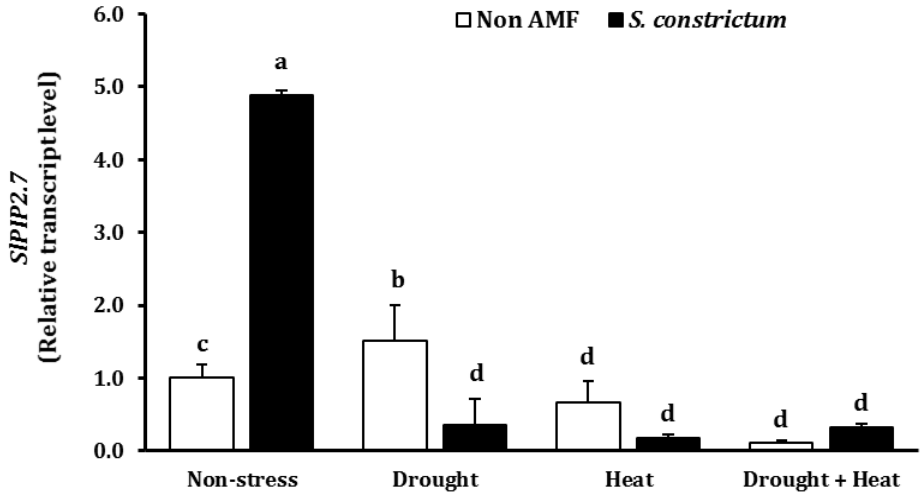


Figure 16. Expression of aquaporin genes *SlPIP2.7* in roots of non-AM and *S. constrictum* inoculated plants under non-stress, drought, heat and combined both stress conditions. Each bar represents mean ± standard deviation. Different letters indicate significant difference among treatments by Tukey's post hoc test at $P \leq 0.05$.

4.2.7 Discussion

AM symbiosis was reported to enhance tolerance to single abiotic stresses in tomato plants (Abdel Latef and Chaoxing, 2011b; Chitarra et al., 2016). In our present study, two AM isolates, *S. deserticola* and *S. constrictum* were used to examine their beneficial effects on plant tolerance to water deficit, heat stress and the integrated stresses.

Our results showed that no significant differences in size between non-AM and AM plants were found under non-stress conditions regardless of the AM isolates (Table 4), thus different tolerance to stress treatments in the present study was not related to improved plant growth owing to AM application. Plants inoculated with *S. constrictum* grew better than those pretreated by *S. deserticola* and the non-mycorrhizal ones under drought and drought + heat stress. Similarly, enhanced plant biomass by AM was reported in several studies under drought stress (Ruiz-Lozano et al., 2016) and under heat stress (Maya and Matsubara, 2013) although discrepant observations were also described (Calvo-Polanco et al., 2016; Matsubara et al., 2014) as a result of specific combination of AM isolates and genotypes. In the present study, root colonization of both fungal species was affected by the drought and combined stress but did not change in heat stress (Table 4), which is in accordance with earlier studies conducted under

deficient water (Chitarra et al., 2016) and heat stress (Maya and Matsubara, 2013; Matsubara et al., 2014).

Mycorrhization had a marked influence on the stomatal behaviour in the leaves of host plants, which determined the exchange of CO_2 gas, unavoidable efflux of water vapour, thus influenced photosynthetic processes and eventually biomass production and crop yield (Augé et al., 2015). Furthermore, stomatal conductance altered by AM inoculations is associated closely with leaf water potential and relative water content in host plants. Our findings pointed out that the values of stomatal conductance, leaf water potential and relative water content under heat stress conditions did not differ among treatments, indicating that mycorrhization might not mitigate the adverse effects of heat stress on the host water status. In contrast, AM plants under drought or drought + heat stress strengthened greatly stomatal conductance in comparison to non-AM plants. In addition, leaf water potential and relative water content were higher in the presence of mycorrhiza, suggesting that AM plants enhanced water status under water deficit and combined stresses. Previous studies with conflicting results of these parameters under drought (Augé et al., 2001, Chitarra et al., 2016; Bárzana et al., 2012) might be due to variations in experimental designs, growth and stress conditions as well as specific plant-fungus associations. The enhanced water status and physiology in AM plants under water-related stresses might be explained by the fact that AM-induced changes occur in root morphology or enhance root fitness (Kothari et al., 1990; Price et al., 1989) and fungal external hyphae can penetrate pores that are beyond the root zone or inaccessible for the root systems, thus allowing colonized roots to access additional water reservoir in the soil (Augé, 2004; Augé et al., 2015).

Aquaporins are membrane intrinsic proteins facilitating and regulating the passive movements of water by osmosis via membranes (Kruse et al., 2006). Mycorrhizal symbiosis may mediate the expression of aquaporin genes to increase hydraulic conductivity of roots and plant tolerance under osmotic stresses (Bárzana et al., 2014). Indeed, in our experiment upregulation of *SlPIP2*.7 in roots colonized by *S. constrictum* under non-stress conditions was observed. Interestingly, the same in AM plants was down-regulated only under drought conditions. The lower expression of the aquaporin gene in colonized roots under drought conditions is supported by observations in mycorrhizal maize subjected to sustained drought (Bárzana et al., 2014). Lower expression of PIP genes in AM-plants might be part of a mechanism to reduce water permeability of cell membranes and to conserve cellular water under water deficit (Smart et al., 2001). Nonetheless, no significant expressions of *SlPIP2*.7 were found under the combined stress, which may indicate that AM symbiosis is likely to regulate other aquaporin genes to influence the water status of host plants.

Damage to photosystem II (PSII) under abiotic stresses is most often reflected by a decrease in maximum quantum efficiency of PSII, Fv/Fm (Baker, 2008). In the present study heat stress had a more pronounced negative effect on Fv/Fm of plants than drought stress and Fv/Fm decreased the most in plants exposed to the combination of both stresses (Figure 12). Our results may suggest that the abiotic stresses tested affected the photosynthetic system by reducing the photosynthetic efficiency of AM and non-AM plants and that damage to PSII was most severe in plants after exposure to drought+heat stress. However, application of *S. constrictum* triggered higher Fv/Fm in the colonized plants compared with non-AM and *S. deserticola* colonized plants under drought and drought + heat stress, which supports the results of Ruiz-Lozano et al. (2016) obtained under moderate and severe drought conditions. Similarly, two-hour treatment of heat stress (42°C) (Camejo et al., 2005) and three-hour treatment (Camejo et al., 2006) decreased F_v/F_m values considerably in cultivated tomato but not in wild thermotolerant tomato. Under water-stressed conditions, an increased PSII efficiency stimulated by AM symbiosis was described in stressed plants (Bárzana et al., 2012; Ruiz-Lozano et al., 2016; Chitarra et al., 2016). A positive correlation between abiotic stress tolerance in colonized plants and maintaining PSII efficiency was proved, which, in turn, sustained (Porcel and Ruiz-Lozano, 2004) or even enhanced (Ruiz-Sánchez et al., 2010) plant productivity. Plants inoculated by *S. constrictum* showed greater stomatal conductance, and better functioning of the photosynthetic machinery, which may explain why these plants were less affected by drought and drought + heat stress (higher shoot biomass).

ABA, a well-known stress phytohormone, plays an important role in forming and maintaining symbiotic relationships (Herrera-Medina et al., 2007; Martín-Rodríguez et al., 2010). ABA production promotes stomatal closure, limits water loss when plants are exposed to salinity and water stress (Lim et al., 2015). Involvement of ABA in the mechanisms by which mycorrhizal symbiosis impacts stomatal conductance under water-deficit stress was proposed (Ludwig-Müller, 2010; Ruiz-Lozano et al., 2016). In the present study, expression of ABA-biosynthetic gene *SlNCED* in roots under drought stress (Figure 15A) of non-AM plants was more upregulated than that in plants inoculated with *S. constrictum*, which may suggest that AM plants tolerated less stress than non-AM ones. The finding is in disagreement with the report of Ruiz-Lozano et al. (2016) and Chitarra et al. (2016), which may be due to different AMF isolates and experimental conditions applied.

Jasmonates are involved in the formation and development of AM symbiosis (Wasternack and Hause, 2013; Bucher et al., 2014). Nonetheless, there is little information on the exact role of this hormone in abiotic stress responses in AM plants. Our results showed significantly higher

expression levels of JA-biosynthetic gene *SlLOXD* in roots colonized by *S. constrictum* under drought and combined stress conditions (Figure 15B), while in heat stress the expression level decreased. Upregulation of *SlLOXD* gene was observed in AM tomato plants under drought conditions (Chitarra et al., 2016). High expression levels of *SlLOXD* in AM plants in our study may help the plant to respond to water stress by inducing a LOXD-mediated pathway because LOXD functions as a component of the octadecanoid defence-signalling pathway, and it may act as a regulator in response to various stresses (Hu et al., 2013). Nevertheless, the reason why this gene was down-regulated by heat stress in the presence of AM symbiosis remains unknown.

Drought and extreme temperatures result in the loss of equilibrium between scavenging and generation of ROS such as $O_2^{\bullet-}$ and H_2O_2, which causes ROS levels to increase thereby inducing destructive oxidative stresses such as membrane lipid peroxidation and protein oxidation leading eventually to the damage of plant cells (Miller et al., 2010). Plants also have evolved different antioxidative strategies to detoxify harmful ROS components by non-enzymatic and enzymatic antioxidant defence systems in which POD, SOD and CAT are main enzymatic ROS scavengers (Wu et al., 2014). MDA is a reliable indicator of peroxidation of lipid membranes in higher plants, reflecting free radical-induced oxidative damages at cellular level under abiotic stresses (Paradiso et al., 2008). In the present work, there was an increase in MDA and H_2O_2 accumulation in the leaves of stressed plants, although the levels of MDA and H_2O_2 were lower in AM plants than in non-AM plants according to the different stresses. Our findings are in accordance with observations made in tomato plants subjected to low temperature (Abdel Latef and Chaoxing, 2011b), drought (Chitarra et al., 2016), as well as in maize under temperature stress (Zhu et al., 2010b). However, the levels of oxidative damage to lipids and H_2O_2 that remained unchanged or became even higher in AM plant under water deficit (Calvo-Polanco et al., 2016). The MDA amounts in AM plants remained lower than those in non-AM plants, indicating that AM symbiosis could mitigate the peroxidation of membrane lipids. The reduction in MDA and H_2O_2 levels in AM plants under the different stress conditions imposed in relation to non-AM plants could be explained by the significantly improved POD, SOD, CAT enzyme activities in roots as well as leaves with respect to all treatments, which is in agreement with the results obtained in tomato plants exposed to low temperature (Abdel Latef and Chaoxing, 2011b), drought (Wang et al., 2014) and in cyclamen plants (Maya and Matsubara, 2013) and maize (Zhu et al., 2010b) under heat stress. The mycorrhizal inoculants used apparently play a crucial role in orchestrating antioxidant activities in the shoot and roots of colonized plants in the process of the abiotic stress tolerance, which may be one of the important mechanisms of AM-induced tolerance to environmental stresses. The analyzed results

demonstrated that mycorrhizal plants were able to prevent oxidative damage induced by drought, heat stress, and drought+heat stress, with a more effective antioxidant machinery particularly when inoculated with *S. constrictum*.

Overall, AM inoculations had significant influences on several physiological and molecular processes in the host plants under drought and the combined stress conditions. Indeed, these processes could be distinctively regulated depending on the specific mycorrhizal inoculants, leading to increased physiological performances in plants enabling them to exhibit adaptations and tolerance to stress conditions. In case of heat stress, lower levels of H_2O_2 accumulation and reduced oxidative damage to the lipid components were observed along with enhanced antioxidative enzyme activities in AM plants, suggesting that they had better stress tolerance, although water relations and other physiological aspects of AM plants showed no improvement at the time of measurements.

4.3 Arbuscular mycorrhizal fungi and its combinations with *Trichoderma*, *Pseudomonas fluorescens* positively influence plant growth, yield and modulate defense enzymes during the plant growth stages in three pepper genotypes.

4.3.1 Mycorrhizal colonization

AM colonization in mycorrhizae-inoculated plants was remarkably higher than in others without AM inoculation although no significant differences could be found among mycorrhizal treatments (Figure 17). Interestingly, the combination of three inoculants reached maximum colonization percentage in roots, up to 59% in Karpia cv. whereas the rates still gained approximately 30% in no microbial treatment under field conditions. No substantial differences in mycorrhizal colonization rates among pepper cultivars and no interaction between microbial inoculations and cultivars were recorded.

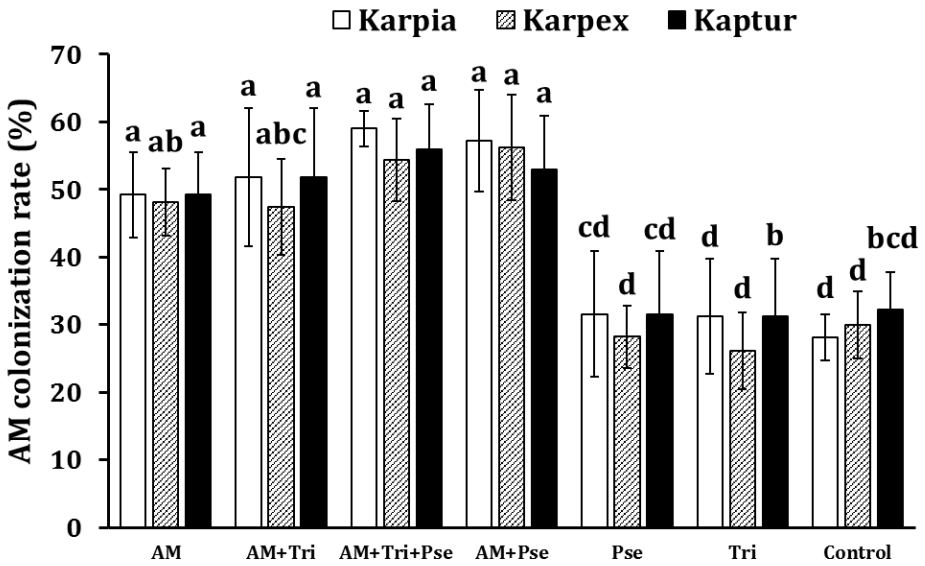


Figure 17. Mycorrhizal colonization of microbial inoculations of three pepper cultivars, Karpia, Karpex, Kaptur. AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Each bar presents mean ± standard deviation. Different letters denote significant differences among treatments according to Tukey's post hoc test ($P < 0.05$).

4.3.2 Biomass production

Triple inoculation (AM+Tri+Pse) elevated root fresh weight in pepper plants, particularly in Karpex and Kaptur cv. when compared with corresponding control plants (Figure 18A). Unexpectedly, other microbial applications with single inoculant or combined ones in Karpia and Kaptur cv. had declined or remained unchanged the values while the root fresh weight was improved in Karpex cv. pretreated by microbes. Similarly, root dry weight in AM+Tri+Pse treatment reached highest values in each pepper variety whilst in other treatments using beneficial microbes singly or together this parameter only was higher in Karpex cv. in comparison to their control plants (Figure 18B).

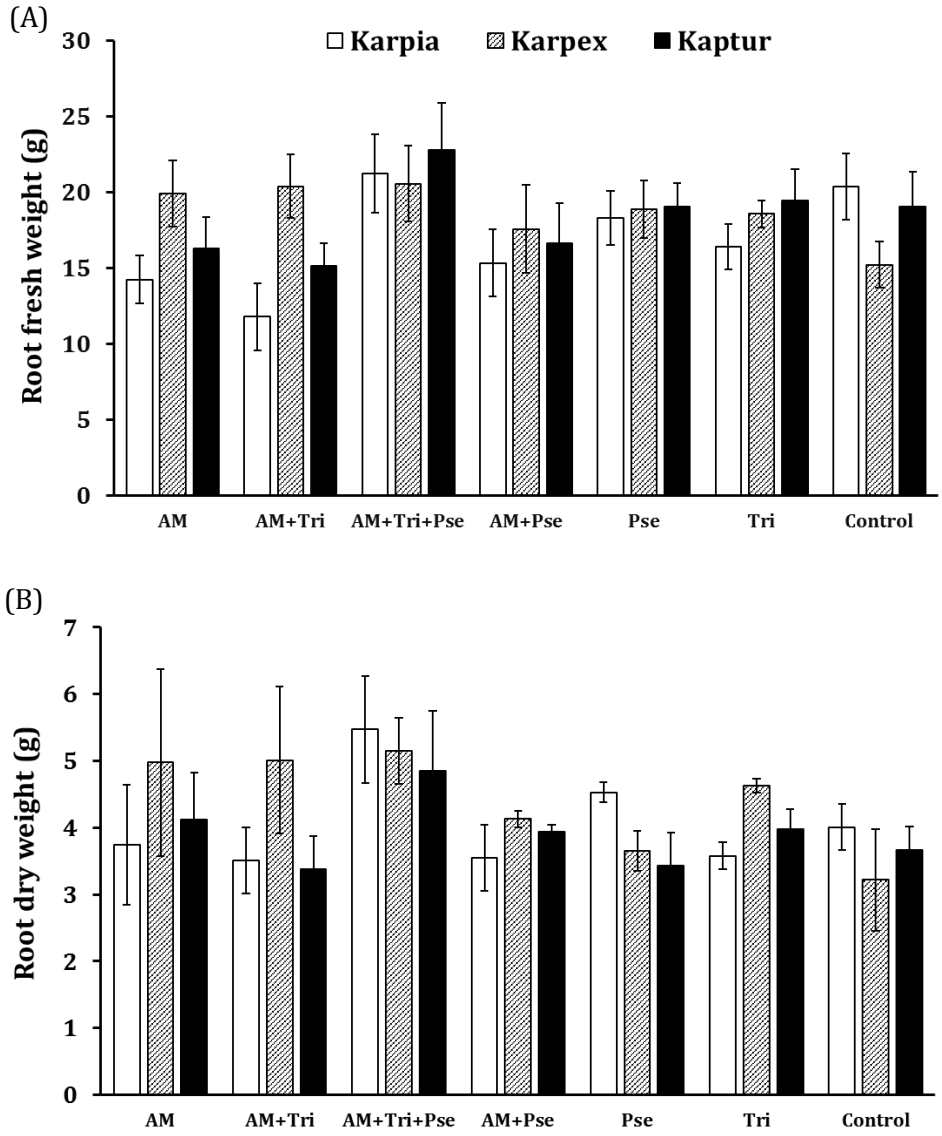


Figure 18. Root fresh weight (A) and dry weight (B) in microbial treatments of three pepper cultivars, Karpia, Karpex, Kaptur at 70 days after transplanting. Each bar presents the mean ± standard deviation. AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*.

Pepper plants inoculated with three inoculants exhibited greatest shoot fresh weight in three cultivars with highest values in Karpia cv. (Figure 19A). In detail, triple inoculation increased this parameter by 91.5% in Karpia, 88.6% in Karpex and 65.2% in Kaptur, as compared to their control. In addition, application of AM alone, Tri singly or combined AM+Pse also gained higher fresh weight of shoots in comparison to their counterparts in each variety. Plants pretreated by Pse presented the greater value in Karpia cv but not in other cultivars. Overall, microbial applications increased shoot dry weight in three pepper varieties, particularly in Karpex cv. However, some combinations between microbes and cultivars leading to no changes in this value were AM treatment in Karpia and Kaptur, AM+Tri in Kaptur, Pse in both Karpex

and Kaptur (Figure 19B). Most noticeably, combined AM+Tri+Pse application induced highest increases of shoot dry weight in each pepper cultivar, in which the shoot dry weight in Karpia cv. gained greatest.

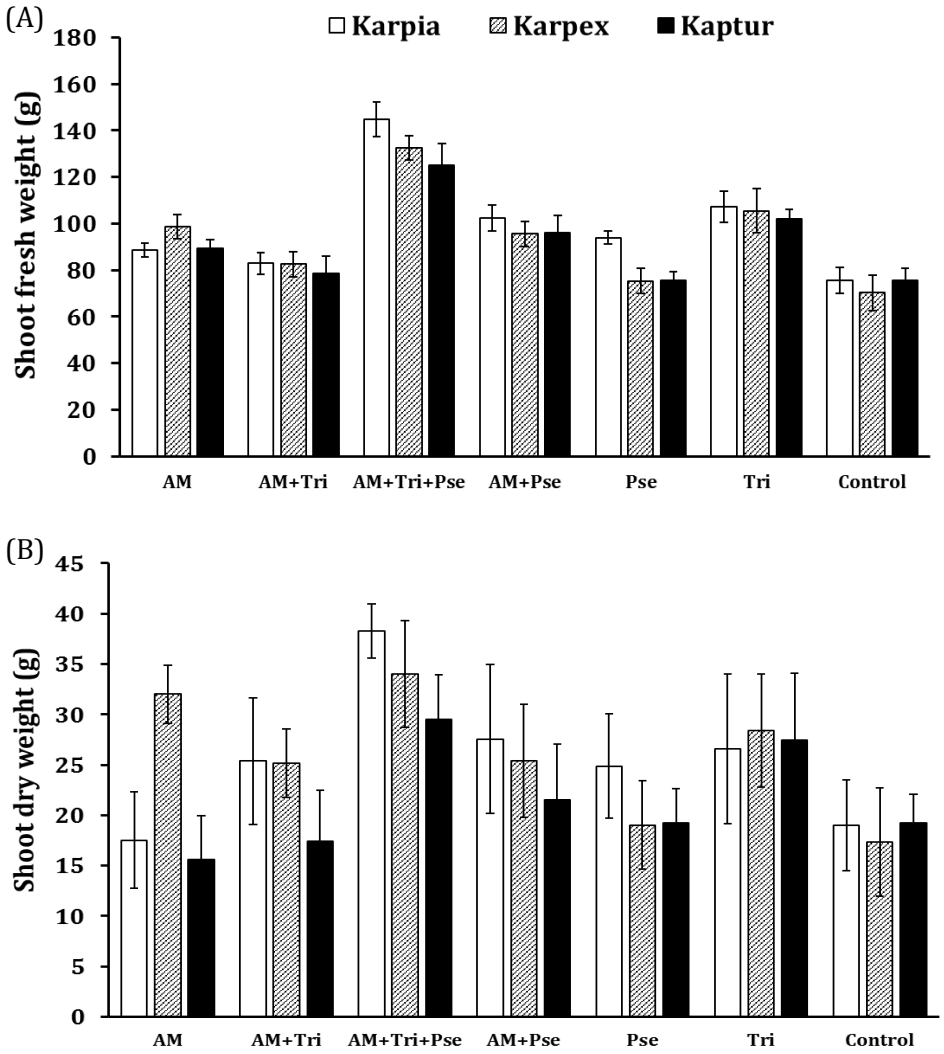


Figure 19. Shoot fresh weight (A) and dry weight (B) in microbial treatments of three pepper cultivars, Karpia, Karpex, Kaptur at 70 days after transplanting. Each bar presents the mean ± standard deviation. AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*.

4.3.3 Fruit Yield

Inoculation with different microbes alone or together with others altered fruit yield of pepper plants in all pepper cultivars although significant differences depended on specific microbe-cultivar combinations (Table 5). The highest yield was recorded in AM+Tri+Pse combination as the best inoculation in Karpia and Karpex cv., while in Kaptur, the value was highest in

plants pretreated by AM+Pse as the most enhancing application. Obviously, application of three inoculants gained highest fruit yield when the main effect of microbial inoculation was compared statistically, however, microbial applications had the greater effect on yield in Karpia and Kaptur (on average, increased 46% and 51%, respectively, in comparison to their non-inoculation treatment) (Table 5). No interaction between microbial treatment and cultivar in fruit yield was recognized.

Table 5. Fruit Yield (g) of microbial inoculations of three pepper cultivars (Karpia, Karpex, Kaptur).

Treatment	Karpia	Karpex	Kaptur	Means of microbial inoculations
AM	3438 ± 370 ab	4267 ± 934 ab	3952 ± 837 ab	3885 AB
AM+Tri	4068 ± 195 ab	4844 ± 518 ab	3264 ± 144 ab	4058 AB
AM+Tri +Pse	5310 ± 619 a	5382 ± 229 a	4066 ± 291 ab	4919 A
AM+Pse	3844 ± 465 ab	4094 ± 551 ab	4775 ± 581 a	4487 AB
Pse	4430 ± 902 ab	4136 ± 401 ab	3856 ± 327 ab	4085 AB
Tri	3826 ± 534 ab	4125 ± 168 ab	4089 ± 137 ab	4013 AB
Control	2846 ± 118 b	4279 ± 951 ab	2647 ± 545 b	3257 B
Means of cultivars	3882 ns	4445 ns	3799 ns	
% increase due to microbial inoculation	46%	4.6%	51%	
M x C	ns			

AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different regular letters denote significant differences among combinations between microbial inoculation and cultivar. Different capital letters present significant differences among means of microbial inoculations. ns, non-significant differences among means of cultivars. All comparisons were followed by Tukey's post hoc test ($P < 0.05$).

4.3.4 Impact of AMF and its combinations with *Trichoderma* and *Pseudomonas fluorescens* on defense enzymes

The two-factor analyses of ANOVA (Table 6) illustrate a highly significant impact of the beneficial microbe applications ($P<0.001$) on the activities of all four defense enzymes, PPO, POD, SOD and CAT. Different pepper cultivars also had the considerable difference (at least $P<0.01$) in PPO, POD, CAT activity (at 29, 49 DAT) but not in SOD. In addition, the interaction between microbial inoculation and cultivar was significant ($P<0.001$) on tested enzymes, PPO, POD, CAT.

Table 6. Significance of two main effects (microbial inoculation, M and cultivar, C) and their interaction between M and C on defense enzymes activities during the course of the experiment.

Enzyme activities	Microbial inoculation (M)	Pepper cultivar (C)	M x C
PPO			
29 DAT	***	***	***
49 DAT	***	**	**
69 DAT	***	***	***
POD			
29 DAT	***	***	***
49 DAT	***	***	***
69 DAT	***	***	***
SOD			
29 DAT	***	ns	ns
49 DAT	***	ns	ns
69 DAT	***	ns	ns
CAT			
29 DAT	***	***	***
49 DAT	***	***	***
69 DAT	***	ns	ns

DAT, days after transplanting; ns, non significant; ***, **, significant at $P \leq 0.001$, 0.01, respectively.

4.3.4.1 PPO activity

Overall, application of beneficial microbes dramatically decreased PPO activity in leaves, when compared to the non-inoculated from 29 DAT to 49 DAT except that using the combination of AM, Tri, Pse doubled this enzyme level at 29 DAT (Table 7). Intriguingly, until 69 DAT, this trend was converted, the PPO levels from microbial inoculations were raised significantly, especially in AM treatment. However, the single application of Tri constantly decreased it during the plant growth.

Table 7. Main effects of microbial inoculation on PPO activity in leaves of three pepper varieties during the plant growth

PPO activity (mg^{-1} protein min^{-1})	AM	AM+Tri	AM+Tri +Pse	AM+Pse	Pse	Tri	Control
29 DAT	0.21 d	0.81 b	1.72 a	0.26 d	0.40 c	0.23 d	0.88 b
49 DAT	0.75 *b*	0.50 *cd*	0.36 *d*			0.56 *c*	0.93 *a*
69 DAT	1.25 **a**	0.79 **c**	1.04 **b**			0.18 **e**	0.52 **d**

DAT, days after transplanting. AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among microbial inoculations at 29, 49, 69 DAT, respectively.

There were significant differences in the PPO activity among three pepper varieties over time (Table 8). Karpia and Karpex cultivars were more sensitive to the PPO activity improvement in

leaves from microbial applications than Kaptur one. Furthermore, the interaction between the microbial application and cultivar in all time points of measuring this enzyme was shown in Table 6 ($P<0.01$).

Table 8. Main effects of cultivar on PPO activity in leaves of various microbial inoculations during the plant growth

PPO activity (mg^{-1} protein min^{-1})	**Karpia**	**Karpex**	**Kaptur**
29 DAT	0.62 b	0.74 a	0.57 b
49 DAT	0.69 *a*	0.54 *b*	0.63 *ab*
69 DAT	0.73 **b**	1.14 **a**	0.40 **c**

DAT, days after transplanting. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among pepper cultivars at 29, 49, 69 DAT, respectively.

The most striking feature is that leaf PPO activity of three-microbe combined treatments (AM, Tri, Pse) was risen sharply in three varieties at 29 DAT, in which the PPO levels in leaves of Karpia were most strengthened (Figure 20). Indeed, multiple application with three inoculants increased the enzyme activity by 87.1% in Karpia, 85.1% in Karpex and 124% in Kaptur, relative to their control. Other inoculations had the trend of decreasing PPO activity in pepper leaves when compared to the controls while dual inoculation of AM and Tri made no changes on the PPO activity of each cultivar ($P < 0.05$). When the pepper plants reached 49 DAT, all microbial treatments had lowered PPO level. At the final measurement (69 DAT), the highest enzyme activity was recorded in Karpex cultivar pretreated by AM, followed by combined treatments with three microbes in Karpia and Karpex, and dual inoculation of AM and Tri in Karpex. It is worth noting that the others declined significantly or remained unchanged as compared to their non-inoculated control in each cultivar. Intriguingly, using only AM enhanced increasingly PPO activity in three varieties whereas its combination with Tri or Tri + Pse decreased it at 49 DAT, then recovered but not completely at 69 DAT (Table 8). By contrast, the pattern of PPO changes in Tri treatment and the control were increased at the middle phase (49 DAT), then declined at the end (69 DAT).

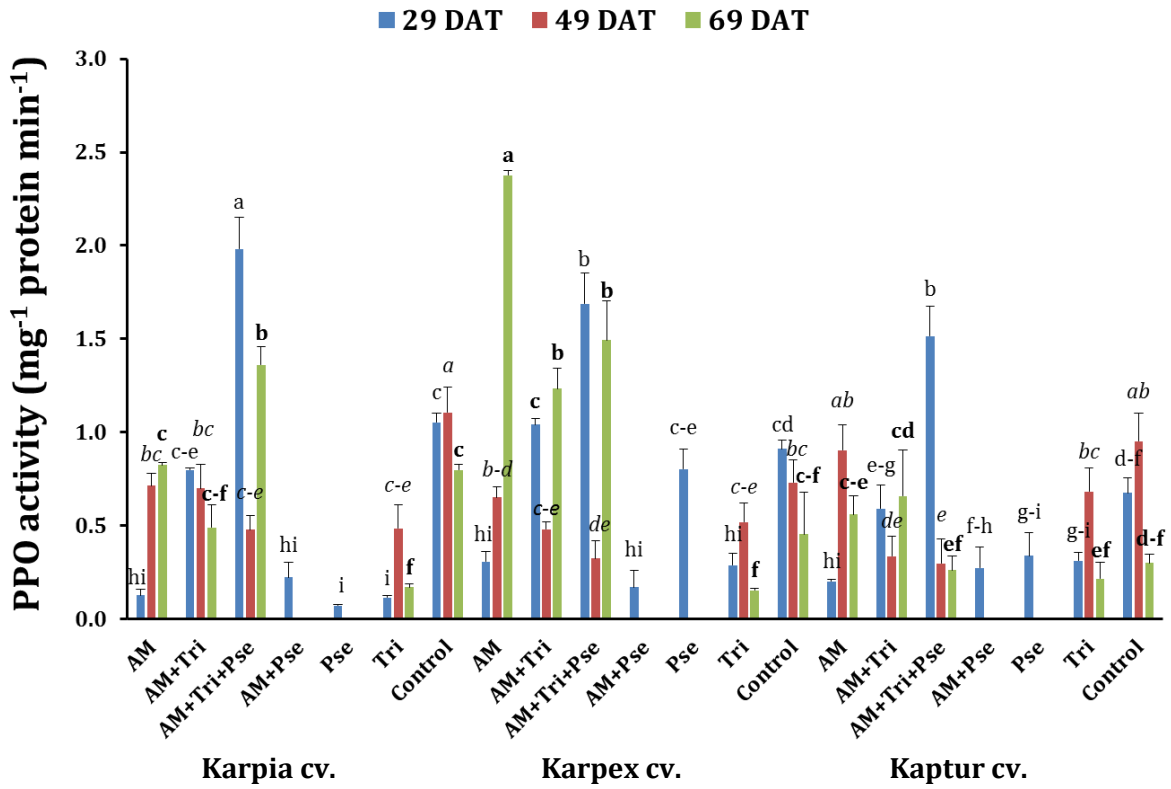


Figure 20. Polyphenol oxidase (PPO) activity of microbial inoculations in leaves of three pepper cultivars, Karpia, Karpex, Kaptur at 29, 49, 69 days after transplanting (DAT). AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among treatments at 29, 49, 69 DAT, respectively.

4.3.4.2 POD activity

There was an interaction between microbial treatment and pepper cultivar on the POD activity (Table 6). As shown in Table 9, the leaf POD activity was raised substantially in AM application (increased by 48.7%), followed by the mixed use of AM and Tri (increased by 16.7%) at 29 DAT, in relation to the control. At the next period, inoculation of AM alone and three-microbe combined treatment led to significantly higher enzyme activity than other treatments including the control. It is interesting that all microbial inoculations boosted this enzyme activity, compared to the control at 69 DAT, in which AM treatment was the most effective enhancer (increased more than triple). Our results also indicated a substantial difference among three pepper genotypes in the duration of plant growth (Table 10) and Karpia variety had the highest POD activity at the later fruiting phase.

Table 9. Main effects of microbial inoculation on POD activity in leaves of three pepper varieties during the plant growth

POD activity (mg^{-1} protein min^{-1})	**AM**	**AM+Tri**	**AM+Tri +Pse**	**AM+Pse**	**Pse**	**Tri**	**Control**
29 DAT	1.16 a	0.91 b	0.84 bc	0.73 cd	0.83 bc	0.67 d	0.78 cd
49 DAT	1.79 *a*	1.52 *b*	1.86 *a*			1.53 *b*	1.40 *b*
69 DAT	4.16 **a**	2.44 **c**	2.09 **c**			3.10 **b**	0.96 **d**

DAT, days after planting. ns, non-significant. AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among microbial inoculations at 29, 49, 69 DAT, respectively.

Table 10. Main effects of cultivar on POD activities in leaves of various microbial inoculations during the plant growth

POD activity (mg^{-1} protein min^{-1})	**Karpia**	**Karpex**	**Kaptur**
29 DAT	0.90 a	0.76 b	0.87 a
49 DAT	1.46 *b*	1.72 *a*	1.67 *a*
69 DAT	4.08 **a**	2.27 **b**	1.30 **c**

DAT, days after transplanting. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among cultivars at 29, 49, 69 DAT, respectively.

At 29 DAT, using AM alone in Karpex and Kaptur variety induced 158.4% and 72.2% higher POD activity in leaves; however, the POD level of AM treatment in Karpia was not changed statistically, compared to their control (Figure 21). Other microbial treatments declined or made no change of POD level in three cultivars in relation to their controls except for Pse inoculation in Karpex, the integrated inoculation of AM and Tri in Karpex or AM+Tri+Pse in Kaptur. At 49 DAT, utilizing AM alone or Tri singly did not change this enzyme activity in Karpia and Karpex but conversely, in Kaptur their POD activity rose significantly. Combination of three beneficial microbes, AM, Tri, Pse promoted a higher POD level in each variety (40.7%, 28.4% and 30.5% higher than that of the control in Karpia, Karpex and Kaptur, respectively). In addition, this enzyme activity from the co-inoculation of AM and Tri remained unchanged in three cultivars, when compared to their control in each genotype. At 69 DAT, all treatments enhanced substantially the POD level in both Karpia and Karpex variety whilst only the application of AM increased considerably POD level in Kaptur. Obviously, AM treatment gained the highest enzyme activity in Karpia. Overall, all inoculations had an increasing trend of POD level during the pepper plant growth in Karpia and Karpex variety; nevertheless, this trend only occurred in AM treatment in Kaptur whereas the pattern of POD activity changes in

the control plants of all cultivars peaked at 49 DAT, dropped at the final stage (Table 9 and Figure 21).

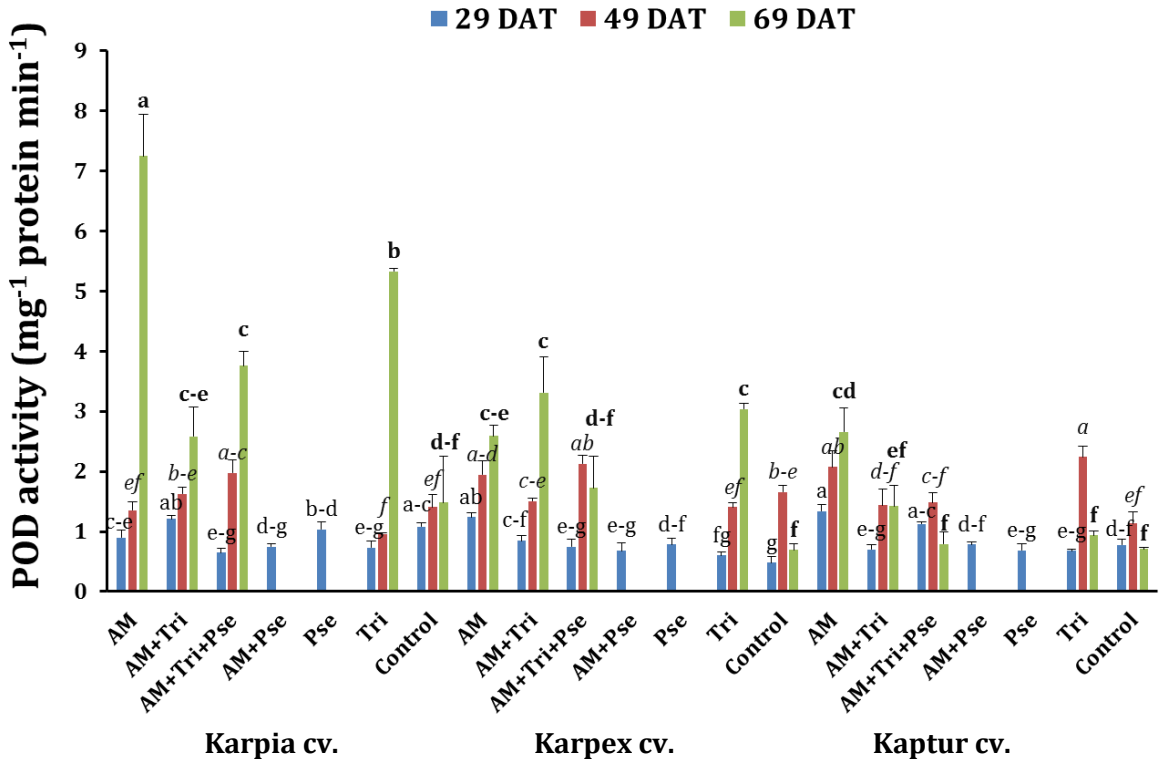


Figure 21. Peroxidase (POD) activity of microbial inoculations in leaves of three pepper cultivars, Karpia, Karpex, Kaptur at 29, 49, 69 days after transplanting (DAT). AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among treatments at 29, 49, 69 DAT, respectively.

4.3.4.3 SOD activity

Our results illustrated that the application of beneficial microbes influenced significantly the SOD activity in leaves during the plant growth ($P < 0.05$) (Table 11). The leaf SOD level in AM treatment was lowest while utilizing Pse alone and three-microbe combination stimulated the highest leaf SOD level at 29 DAT. At 49 DAT, the only inoculation of Tri had a lower SOD activity than others. In the final stage (69 DAT), treatments inoculated by AM alone or AM + Tri or the three microbes (AM+Tri+Pse) enhanced sharply the SOD level compared to the control. In terms of varieties, there were no significant differences of SOD level among the three (Table 6).

Table 11. Main effects of microbial inoculation on SOD activity in leaves of three pepper varieties during the plant growth

SOD activity (U mg^{-1} protein)	**AM**	**AM+Tri**	**AM+Tri +Pse**	**AM+Pse**	**Pse**	**Tri**	**Control**
29 DAT	7.22 c	12.22 b	18.35 a	12.93 b	18.67 a	13.85 b	14.60 b
49 DAT	24.09 *a*	21.33 *a*	20.96 *a*			14.73 *b*	21.82 *a*
69 DAT	10.90 **a**	12.01 **a**	4.77 **b**			3.57 **bc**	2.67 **c**

DAT, days after transplanting. AM, Arbuscular mycorrhizal fungi; *Tri*, *Trichoderma*; *Pse*, *Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among microbial inoculations at 29, 49, 69 DAT, respectively.

AM treatment in all pepper varieties decreased substantially or had unchanged SOD activity in leaves while the combination of AM, Tri, Pse in Karpex and Kaptur cultivar increased the enzyme by 60.6% and 32.4%, respectively, in relation to their control at 29 DAT (Figure 22). Utilizing Pse alone in Karpia and Karpex also showed 41.7% and 57.3% higher SOD activity than the controls at this stage, respectively. Besides that, other treatments did not change significantly compared to their control in each variety. At 49 DAT, only the combination of three beneficial microbes in Karpia promoted 47.6% higher SOD activity than its control whereas other treatments had the trend of no change or decreasing its counterpart. However, the application of AM in three cultivars, AM and Tri combination in Karpex and Kaptur enhanced considerably the enzyme activity at 69 DAT. Moreover, other treatments had no significant influences on this enzyme in each variety at this stage. During the plant growth, the overall pattern of SOD activity for all treatment peaked at 49 DAT and finally declined (Table 11). Most noticeably, AM and its combinations alleviated this drop at the final stage, especially AM and AM+Tri which had four-fold and five-fold of SOD level compared to the control, respectively.

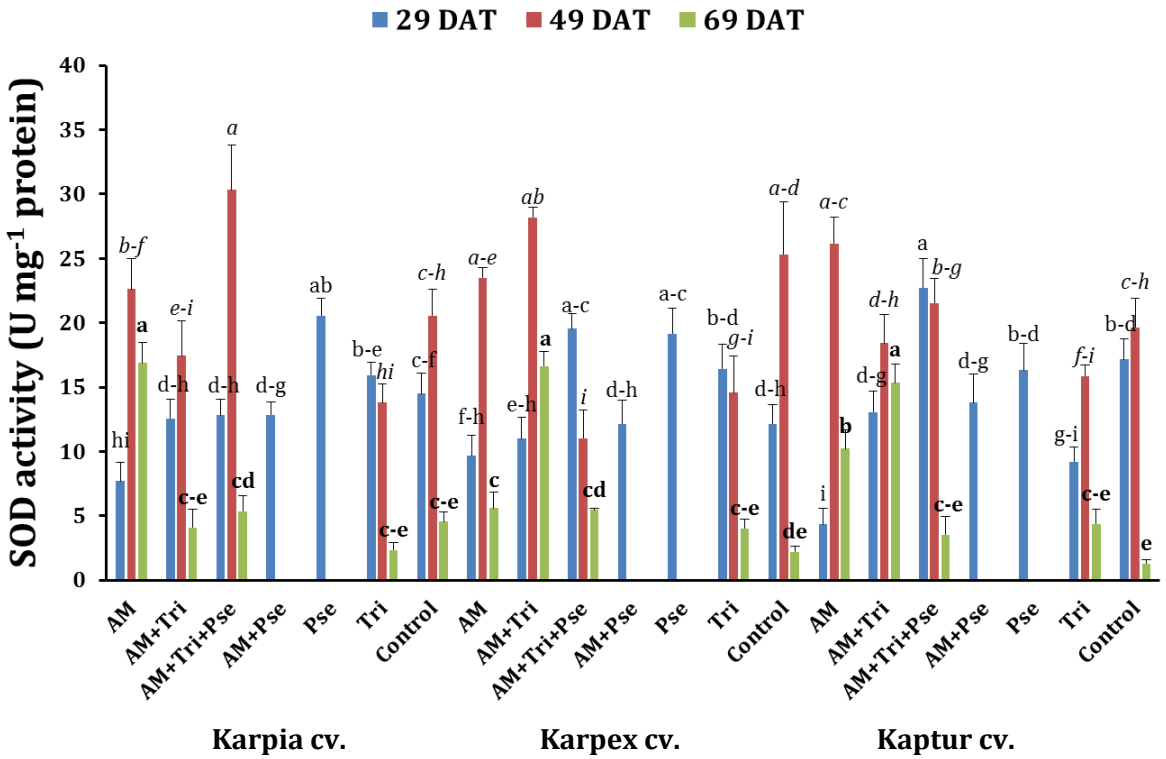


Figure 22. Superoxide Dismutase (SOD) activity of microbial inoculations in leaves on three pepper cultivars, Karpia, Karpex, Kaptur at 29, 49, 69 days after transplanting (DAT). AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among treatments at 29, 49, 69 DAT, respectively.

4.3.4.4 CAT activity

As presented in Table 12, all microbial treatments decreased considerably the CAT activity in leaves at 29 DAT. At 49 DAT, the inoculation of AM alone or AM, Tri, Pse combination did not influence the CAT activity while inoculation of Tri continued to drop this enzyme level. Intriguingly, at the final stage (69 DAT), all microbial treatments significantly increased this enzyme level. Furthermore, there were significant differences of CAT enzyme activity among three pepper varieties at 29 and 49 DAT but no considerable differences of CAT level were observed at 69 DAT (Table 6). Kaptur cultivar showed the highest CAT level compared to others (Table 13).

Table 12. Main effects of microbial inoculation on CAT activity in leaves of three pepper varieties during the plant growth

CAT activity (mg^{-1} protein min^{-1})	**AM**	**AM+Tri**	**AM+Tri +Pse**	**AM+Pse**	**Pse**	**Tri**	**Control**
29 DAT	1.49 c	1.15 d	1.49 c	1.45 c	1.96 b	1.60 c	2.25 a
49 DAT	0.83 *ab*	0.75 *b*	0.86 *ab*			0.41 *c*	0.90 *a*
69 DAT	0.86 **b**	1.45 **a**	1.46 **a**			0.98 **b**	0.40 **c**

DAT, days after transplanting. AM, Arbuscular mycorrhizal fungi; *Tri, Trichoderma*; *Pse, Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among microbial inoculations at 29, 49, 69 DAT, respectively.

Table 13. Main effects of cultivar on enzyme activities in leaves of various microbial inoculations during the plant growth.

CAT activity (mg^{-1} protein min^{-1})	**Karpia**	**Karpex**	**Kaptur**
29 DAT	1.63 b	1.50 c	1.75 a
49 DAT	0.67 *b*	0.82 *a*	0.76 *a*
69 DAT	1.05	0.98	1.06

DAT, days after transplanting. ns, non-significant. Different regular, italic letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among cultivars at 29, 49 DAT, respectively.

At 29 DAT, all microbial treatments reduced significantly the CAT activity in leaves except in the case of Pse inoculation in Karpex variety (Figure 23). In the next period of plant growth (49 DAT), AM inoculation improved the CAT activity in Karpia while its counterpart lowered and had no significant change in Karpex and Kaptur cultivar, respectively. Combination of AM with Tri or three beneficial microbes in Karpex increased the enzyme activity by 31.1% or 35.1%, respectively. In contrast, other treatments decreased the CAT level compared to its control in each variety. At the final stage (69 DAT), AM application elevated CAT activity by 98% in Karpia and 285.1% in Kaptur but had no change in that of Karpex. Integrated inoculation of three microbes and inoculation of Tri increased nearly fourfold and twofold this enzyme, respectively, in Karpia whilst all combinations of microbes in Karpex and Kaptur also induced the CAT level. In general, CAT activity was on the downward trend during the pepper plant growth (Table 12), however, few microbial combinations, triple application in Karpia or dual inoculation of AM and Tri in Karpex and Kaptur produced the upward trend of CAT activity. Apparently, application of beneficial microbes alleviated the decreased trend in the pepper plants.

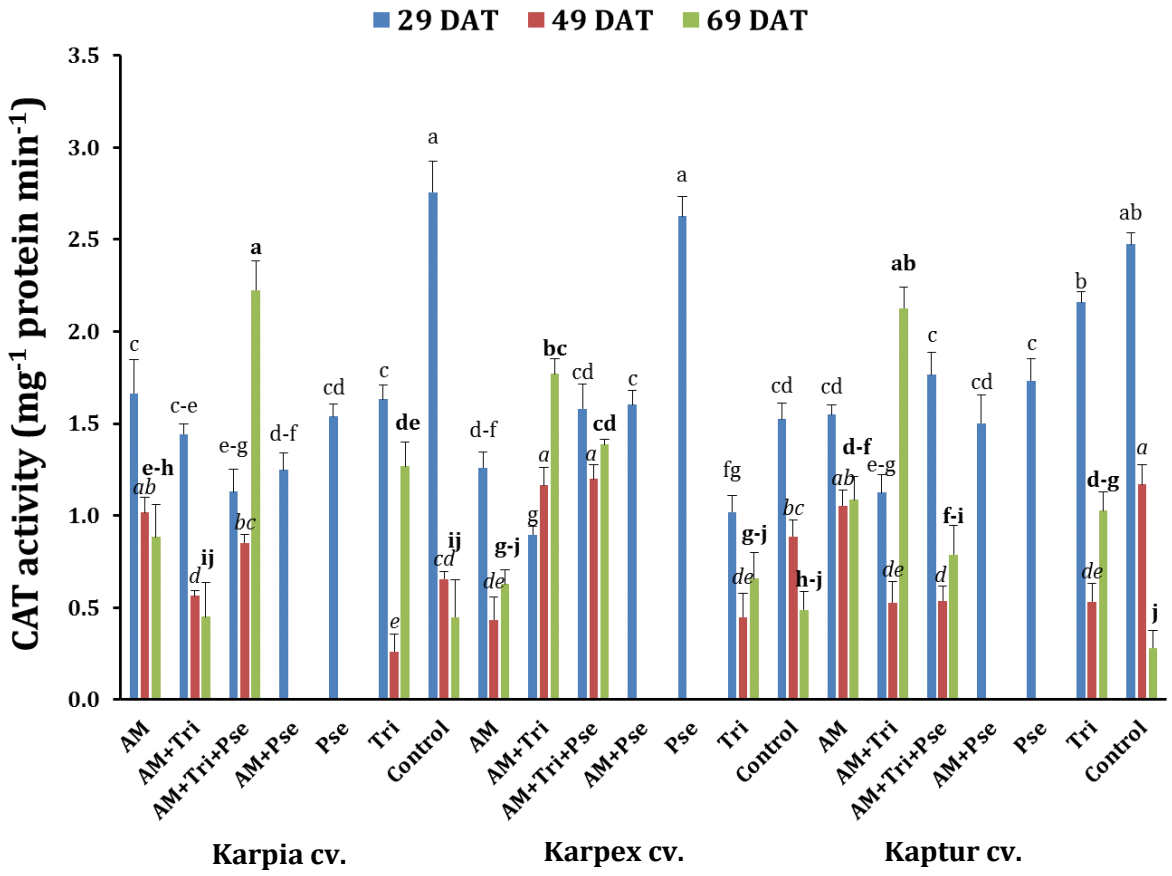


Figure 23. Catalase (CAT) activity of microbial inoculations in leaves on three pepper cultivars, Karpia, Karpex, Kaptur at 29, 49, 69 days after transplanting (DAT). AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different regular, italic, bold letters denote significant differences according to Tukey's post hoc test ($P < 0.05$) among treatments at 29, 49, 69 DAT, respectively.

4.3.5 Discussion

Many reports well demonstrated beneficial effects of AMF, Tri, Pse on plant performance, yield as well as improving plant tolerance to abiotic and biotic stresses. Here the present study focused on plant growth, yield and defense enzymes induced by these microbes during the course of plant growth of three pepper genotypes under field conditions.

The positive effects of beneficial microbes on plant growth and yield have been described in many studies (Pereira et al., 2015; Yuan et al., 2016; Azarmi et al., 2016; Pascale et al., 2017; Yadav et al., 2015). Our analyzed results demonstrated generally that the application of AM, Tri, Pse and their combinations enhanced biomass production and fruit yield in pepper plants although the beneficial gains depended on specific combinations between varieties and microbes. Similarly, several reports showed that AM inoculation enhanced root and shoot dry

weight, fruit yield in pepper plants (Abdel Latef and Chaoxing, 2014; Abdel Latef, 2013; Boonlue et al., 2012; Tanwar et al., 2013), increased shoot dry weight in sunflower and maize plants (Aghababaei and Raiesi, 2015) whilst higher plant biomass in maize pretreated by *Trichoderma atroviride* under drought stress (Guler et al., 2016), in pistachio seedlings inoculated by Pse (Azarmi et al., 2016) were observed. In addition, Pascale et al. (2017) illustrated that *Trichoderma harzianum* and *Trichoderma atroviride* improved grape yield and quality. Using *Pseudomonas fluorescens* with organic fertilizer showed maximum enhancement in the growth, the yield of cucumber (Ahamd et al., 2015). Most noticeably, in our study, multi-use of three microbes (AM+Tri+Pse) had the best increment in root and shoot fresh as well as dry weight and fruit yield in all pepper cultivars, which may indicate that there were synergistically beneficial impacts from the microbes on plant production. Several examples of the synergistic effects on biomass were reported in bean plants with AM+Pse (Younesi and Moradi, 2014), in tobacco plants by using *Trichoderma harzianum* in bioorganic fertilizer and AM fungi (Yuan et al., 2016), greatest growth and yield in sunflower plants when combined inoculation of AM, *Trichoderma viride* and Pse were recorded (Yadav et al., 2015). The cumulative benefit of combining multi-bioinoculants may result from nutrient and water improvement, induced resistance or better tolerance to biotic and/or abiotic stresses, more effective protection from pest and plant diseases under field conditions.

It is believed that the antioxidant system with a diversity of constituents plays a crucial role in maintaining a dynamic equilibrium between ROS production and elimination in plants. POD, SOD, CAT are powerful ROS scavengers possessing different specificity of substrate and affinity for ROS (Polovnikova and Voskresenskaya, 2008; Wu et al., 2014, Jiang and Zhang, 2001; Harinasut et al., 2003). In addition, in spite of the fact that PPO is not considered as a constituent of ROS scavenging system, it participates in the control of oxidative processes (Polovnikova and Voskresenskaya, 2008; Mayer, 2006). Under environmental stresses, elevated PPO activity led to the formation of lignified barriers to limit ROS spreading (Mayer, 2006; Avdiushko et al., 1993; Polovnikova and Voskresenskaya, 2008). Our analysed results demonstrated that under field environment where unfavourable conditions frequently occurs including abiotic and biotic stresses, generally, application of the AM mixture with six different AM fungi species enhanced significantly PPO (increased by 140,4%), SOD (increased threefold), CAT activity (increased twofold) in leaves as compared to the control at 69 DAT (Table 7, 11, 12) whilst the leaf POD activity was always increased sharply and obtained highest values during the plant growth stages (Table 9). This finding is consistent with most previous mycorrhizal studies under stress conditions showing that AM plants stimulated antioxidative

enzymes in leaves of various plants (Pedranzani et al., 2016; Chu et al., 2016; Jiang et al., 2016; Hashem et al., 2016; Sarkar et al., 2016), and in pepper plants such as CAT when plants were exposed to 1 mM NaCl in the long term (Cekic et al., 2012), SOD and POD under salinity stress (Abdel Latef and Chaoxing, 2014). Nonetheless, in contrast to our results, the SOD, CAT levels in pepper leaves of AM plants were decreased or had no change in higher salt concentrations in comparison to its counterpart in the control (Cekic et al., 2012). Abdel Latef (2013) also reported that there were no considerable difference in SOD activity in pepper leaves of both AM (*Funneliformis mosseae*) and non-AM plants under Cadmium stress while the leaf POD activity was lowered in AM plants compared to the corresponsive in non-AM plants due to the fact that ascorbate peroxidase was enhanced as the main scavenger in AM plants. On the other hand, we found that AM plants experienced a substantial reduction in PPO (reduced by 76.1%), SOD (reduced by 50.5%), CAT activity (reduced by 33.7%) in leaves in comparison with non-inoculated plants at 29 DAT, even at 49 DAT with PPO activity (reduced by 19.3%) (Table 7, 11, 12), which may suggest that pepper plants gained benefits such as nutrient and water improvements from the symbiosis with the AM mixture, therefore, might produce less ROS, then lower the defense enzymes as in the observation of Kohler et al. (2008) that AM plants lessened SOD, CAT activity in leaves of *Lactuca sativa* under severe drought stress. Aghababaei and Raiesi (2015) also recorded that AM plants had considerably decreased POD, SOD, CAT while PPO level in these plants remained unchanged under Cadmium stress and earthworm attack. Our results of decreased PPO activity also is in accordance with earlier reports (Mollavali et al. 2016; Minton et al., 2016) while it is in contrast to result of Ismaiel et al. (2014) revealing that AM plants induced subtantial PPO activity in leaves of faba bean at both vegetative and flowering stage when the plants were irrigated with Nile water and wastewater, respectively. Moreover, different responses of AM inoculation to enzymatic activities in some plants has been shown (Cekic et al., 2012).

Trichoderma is a renowned beneficial fungus which can induce resistance in plants, strengthen the development of roots as well as plant growth. Similarly to *Trichoderma*, plant growth promoting bacteria such as *Pseudomonas fluorescens* also bring many advantages to their host plants owing to its capability of improving growth parameters and yield by various mechanisms (Walsh et al., 2001) and inducing metabolites in plants (Jaleel et al., 2007, 2009; Ghorbanpour et al., 2010), enhancing plant defenses against broad phytopathogens (Pieterse et al., 2001). According to our results, Tri application decreased or remained unchanged activities of defense enzymes in relation to its counterpart in control treatments during the plant growth (Table 7, 9, 11, 12). Instead of decreasing PPO, SOD activity in leaves as AM treatment did, the AM plants

supplemented with Tri did not change considerably PPO, SOD activity whereas, interestingly, these enzyme activities in the three-inoculant treatment were peaked at 29 DAT (Table 7, 11). Two combinations (AM+Tri), (AM+Tri+Pse) also exhibited a rise of all enzyme activities at 69 DAT in comparison with the control; nevertheless, there were weaker PPO, POD but stronger CAT activity than those of AM application merely. Several workers reported that application of *Trichoderma* spp. can improve defense enzymes in plants (Guler et al., 2016; Gajera et al., 2016) and co-inoculation of AM fungi and *Trichoderma* spp. have synergistic impacts on controlling phytopathogens (Saldajeno et al., 2008; Martínez-Medina et al., 2009; Srivastava et al., 2010). Yuan et al. (2016) demonstrated that co-inoculation of AM and Tri obtained the best result of inducing PPO and POD level in leaves of tobacco plants. Thus, our finding may indicate that AM combination with other microbes might better enhance the defense enzymes under field conditions but the only inoculation of mixed AM might be the most effective inducer in the long term.

In the case of Pse, this plant growth promoting bacterium lessened significantly PPO and CAT in leaves at 29 DAT, compared to the control (Table 7, 12). Nevertheless, POD and SOD activity were unchanged and increased by 27.8% in Pse inoculated plants, respectively, at the vegetative period (Table 9, 11). These results of PPO and CAT concur with those of Kumari et al., (2015) but are dissimilar to those of Azarmi et al. (2016) while the declines of SOD, POD activity are in agreement with the study of Azarmi et al. (2016) in pistachio seedlings but contrast to the work of Kumari et al., (2015) in soybean plants. Apparently, the combination of this bacterium with AM decreased significantly activities of all enzymes in relation with its counterpart in Pse inoculation alone, which is in accordance with the report of Kohler et al. (2008). In this report, combination of *Rhizophagus irregularis* or *Funneliformis mosseae* with plant growth promoting rhizobacterium (*Pseudomonas mendocina* Palleroni) dropped SOD, CAT level in leaves of *Lactuca saiva* L. as compared to non-microbe plants under severe drought stress. However, POD activity in our results is a contrast to that of the authors. This might suggest a complex and specific interaction between plants and microbial partners.

Plants respond to environmental adversities including biotic and abiotic factors by defense mechanisms in which ROS is the early response (Singh et al., 2011) acting as an essential signal for subsequent defenses (Király et al., 1993). However, due to its oxidizing capability, ROS can destroy proteins, lipids and DNA (Miller et al. 2010), leading to cell damage and death. Scavenging the overproduced ROS needs to occur synchronously to avoid oxidative damages *in planta*. PPO and POD are not only effective defense enzymes against abiotic and biotic stresses but also involved in ROS generation (Mayer, 2006) and lignification processes

(Avdiushko et al., 1993), while SOD and CAT are key ROS scavengers. Furthermore, PPO, SOD, CAT are metalloenzymes whose functions are affected partially by the availability of metals such as Cu, Fe, Mn, Zn in plants (Tang et al., 2015; Armada et al., 2016). These micronutrients can be enhanced in plants inoculated by the microbes. Therefore, the modulation of plant defense system by the beneficial microbes utilized could be an important gainful impact of plants cultivated under field conditions where many stresses can take place simultaneously and are associated with complicated interactions among biotic, abiotic and edaphic environments with frequent climate changes. AM, Tri, Pse, well known as plant performance enhancers can influence the pool of enzymatic scavengers and defense enzymes as our findings demonstrated, thus alleviating oxidative stress and reinforcing plant defense responses. In fact, the pattern of PPO, POD, SOD and CAT activity changes of the microbial treatments in our experiment were different from non-microbe plants over the entire time of pepper plant growth (Figure 20, 21, 22, 23 and Table 7, 9, 11, 12), overall enhancing the defense enzymes. Nonetheless, the effectiveness of the applied microorganisms was not always as defense stimulators but mainly in the later period of plant growth under field conditions. The differential capability of inducing defense enzymes among the inoculation with mixed AM, Tri, Pse as well as their combined uses in every period of plant growth was observed.

Our results also highlighted different responses among pepper genotypes to impacts of microbial inoculants on PPO, POD, CAT activities (Table 8, 10, 13). Furthermore, we found several combinations between microbial treatments and pepper cultivars showing the most effective enhancing in PPO, POD, CAT in the plant growth periods. For example, combined inoculation of three microbes, AM+Tri+Pse with highest SOD activity at 29, 49 DAT in Karpia and Kaptur; AM+Tri with high CAT level in Karpex and Kaptur; application of three inoculants with most elevated PPO level in all pepper genotypes. Thus, there were specific interactions between microbe as well as their combination and pepper genotype, which was described in many studies (Sensoy et al., 2007; Cekic et al., 2012).

4.4 Novel scientific results

1. Using seven AMF isolates with diverse species and origin to examine the ability to induce tomato plant resistance against Cmm, we found three levels of response on disease sensitivity of the host plant. Plants pretreated with *Rhizophagus irregularis* expressed highest induced resistance to Cmm whereas an intermediate resistance was induced by *Funneliformis mosseae, Claroideoglomus claroideum* and *Gigaspora margarita*.

2. Utilising ET-insensitive tomato mutant (Never ripe), we discovered that *Rhizophagus irregularis*-induced resistance against Cmm is dependent on ET signalling pathway.

3. Inoculation with *Septoglomus deserticola* or *Septoglomus constrictum* enhanced the tolerance of tomato plants under drought, heat and the combination of both stresses. Under heat stress, both mycorrhizal fungi simply alleviate oxidative stresses (MDA and H_2O_2) and enhance the effectiveness of enzymatic antioxidant systems such as SOD, POD and CAT in both roots and leaves. Under drought and the combined drought and heat stress, AM symbiosis are able to enhance water status and physiology as well as stress tolerance of host plants by regulating stomatal conductance, increased leaf water potential and relative content, modifying expression of aquaporin gene (*SlPIP2.7*) and ABA, JA biosynthetic gene (*SlLOXD*, *SlNCED*) in roots colonized by *Septoglomus constrictum*. SOD, POD and CAT enzyme activities in roots and leaves of colonized plants were also elevated whilst lowered leaf H_2O_2 and MDA content and higher F_v/F_m were recorded in AM plants.

4. Combined inoculation of AMF with two beneficial microbes (*Trichoderma* and *Pseudomonas fluorescens*) enhanced the highest plant biomass and yield in three pepper varieties (Karpia, Karpex, Kaptur) under field conditions. Not all pepper cultivars gained the same beneficial effects on the yield from the microbial inoculations. Karpia and Kaptur cultivars are dependent on microbial inoculations to increase their yield while Karpex is not.

5. Microbial inoculations modified the pattern of changes in defense enzymes, PPO, POD, SOD and CAT over the course of the experiment and enhanced activities of defense enzymes, especially in the later plant growth period. Efficacy of the applied microorganisms was not always as defense stimulators but mainly in the later period of plant growth under field conditions. The differential capability of inducing defense enzymes among the inoculation with mixed AM, Tri, Pse as well as their combined uses in every period of plant growth was observed. In addition, different responses among pepper genotypes to impacts of microbial inoculants on PPO, POD, CAT activities were recognized. We found several combinations between microbial treatments and pepper cultivars showing the most effective enhancing in PPO, POD, CAT activity in the plant growth periods under field conditions. Specific interaction between microbe as well as their combination and pepper genotype was highlighted.

5. CONCLUSION

Our studies proved that AMF inoculation influenced the host tolerance against some abiotic stresses and phytopathogens by the novel results obtained. One of our key findings revealed that AM colonization can induce systemic resistance to bacterial canker Cmm in tomato plants, however, not all of seven AMF isolates used in our experiment were able to enhance the resistance. Therefore, the efficiency of bio-protection by AM depends on isolates. In addition, ET signalling pathway is required for MIR against Cmm. Although mechanisms underlying Cmm resistance of AM plants have not been investigated yet, some mechanisms are proposed. Obviously, further studies are required to elucidate the mechanisms involved in AMF-induced resistance to Cmm with most effective AM species being *Rhizophagus irregularis*.

The results of our abiotic stress experiment highlighted that under optimum conditions (unstressed conditions) mycorrhizal colonization did not result in marked benefits to host tomato plants. Noticeably, AM inoculation can confer protection to plants against drought, heat and the combination of both stresses by alleviating oxidative stress and enhancing the enzymatic antioxidant system. Under water-related stresses, eg. drought and the integrated drought and high temperature stress, AM symbiosis were able to enhance water status and host physiology by sustaining more water balance status, tissue hydration for physiological performances *in planta* through mediating stomatal conductance, higher leaf water potential and relative water content. Mycorrhization also changed expression patterns of aquaporin and ABA, JA biosynthetic gene in roots associated with *Septoglomus constrictum.* These AM-induced modifications did not occur in plants subjected to heat stress. Nevertheless, the protective efficacy depends on specific AM isolates applied, in which *Septoglomus constrictum* triggered better plant tolerance to the abiotic stresses.

We also investigated beneficial effects of AMF and its combination with renowned microbes *Trichoderma* and *Pseudomonas fluorescens* on three pepper cultivars in the field where diverse abiotic and biotic stresses can occur in single and/or combined way throughout the season. AMF, Tri, Pse and their combinations had different positive impacts on plant growth, yield and a distinct potential to modulate defense enzymes over the time of plant growth under field conditions despite the fact that no combinations always enhance activities of the enzymes all over the periods of plant growth. Microbial inoculations altered the pattern of changes in defense enzymes over the course of the experiment and enhanced activities of defense enzymes, especially in the later plant growth period. Significant differences in modulating the enzymes

among genotypes in the periods of plant growth were observed. Some specific combinations between microbes and genotypes in each plant growth stage induced more effectively defense enzymes than others. Remarkably, the combination of AM with two other microbes Tri, Pse (triple inoculation) brought more benefits to host pepper plants when the plants obtained the highest yield and usually induced higher defense enzymes activities during the plant growth periods. Thus, AM application together with other compatible beneficial microbes could be more effective practice under field conditions. Importantly, the combination of microbes depended on genotypes to induce defense enzymes.

Our results altogether demonstrated that use of AM can enhance host plant tolerance or resistance against some abiotic stresses and phytopathgens. AM combination with other compatible microbes possibly provides a better enhancement in plant fitness, yield and stress tolerance under field conditions. There is an existence of specificity among AMF species/isolates and compatible interactions between beneficial microbe and cultivar in beneficial effects on host plants.

6. SUMMARY

A great number of early publications support that AMF are capable of enhancing plant fitness and resistance/tolerance to diverse stresses although not all AMF species or isolates are effective enhancers. Our study proved that AM colonization could strengthen the host plant tolerance against some abiotic stresses and phytopathogens with novel results through three different experiments in the controlled environment and in the field below.

Our first experiment was to find out whether AMF isolates can induce resistance to Cmm in tomato plants. Therefore, a pot experiment was conducted to assess the protective role of AMF against the bacterial canker of tomato Cmm. The eight treatments consisting of seven inoculations with different AMF isolates, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus intraradices*, *Rhizophagus* sp., *Septoglomus constrictum*, *Claroideoglomus claroideum*, *Gigaspora margarita* and non-inoculated control plants. Stems of seven-week tomato plants was infected with Cmm and disease severity index (DSI) was calculated during the next three weeks. Besides different responses to mycorrhizal inoculation on colonization processes, three levels of responses on disease sensitivity are recognized. Plants inoculated with *Rhizophagus irregularis* (Ri) showed both highest colonization and induced resistance to Cmm, while the effect of *Funneliformis mosseae*, *Gigaspora margarita* and *Claroideoglomus claroideum* were intermediate on colonization and high on induced resistance. Subsequently, Ri was chosen to inoculate ethylene-insensitive tomato mutant line Never ripe and its background (Pearson) to investigate the possible role of ethylene in the mycorrhiza-induced resistance (MIR). Our results showed that Ri can induce systemic resistance against Cmm in the Pearson background and ethylene-insensitivity in Nr plants impaired MIR, thus, ethylene is required for Ri-induced resistance against Cmm. To our knowledge, this is the first study to examine the effect of different AMF isolates on tomato plant response to Cmm and involvement of ethylene in MIR against Cmm.

The next experiment in the controlled environment was carried out to explore the impact of two AMF species, *Septoglomus deserticola* and *Septoglomus constrictum* on tomato plant tolerance to drought, heat stress, combined drought and heat stress. No substantial differences in physiological parameters were measured in non-AM and AM plants under non-stress conditions. Nonetheless, when plants were exposed to drought and drought+heat conditions AM plants exhibited distinctively higher levels of stomatal conductance, leaf water potential and relative water content, reduced stress status of plants with lessened MDA, H_2O_2, in line

with higher activities of POD, SOD and CAT in roots and leaves and eventually, higher plant biomass. In addition, lower expression levels of ABA-biosynthetic gene *SlNCED* and aquaporin gene *SlPIP2.7* were detected in droughted plants inoculated by S. constrictum and higher rates of transcription of JA-biosynthetic gene *SlLOXD* were found in the mycorrhizal plants exposed to drought and combined stress. Additive damages in plants under the combination of heat and drought stress were observed. In case of heat stress, lower MDA and H_2O_2 content along with enhanced antioxidative enzyme activities were recorded in AM plants while water relations and other physiological aspects of AM plants were not improved. Altogether, our results indicated that AM inoculation had a positive influence on the tomato plant tolerance to all stresses tested with different efficacy depending on the specific AM isolate.

Finally, we also implemented a field experiment at the experimental station of Szent István University, Gödöllő to investigate the impact of arbuscular mycorrhizal fungi only alone or together with *Trichoderma* and plant growth-promoting bacteria on plant growth, yield and defense enzymes of three pepper varieties, Karpia, Karpex, Kaptur. The seven inoculation treatments consisting of arbuscular mycorrhizal fungi (AM), *Trichoderma* (Tri), plant growth promoting bacteria (Pse) and their combinations (AM+Tri; AM+Tri+Pse; AM+Pse) together with three pepper hybrids and non-inoculation (control) plants were arranged in a randomized complete block design. Defense enzyme activities PPO), peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) of various treated plants were measured during the growth stages of plants. Our results showed that inoculation of AMF, Tri, Pse alone or in a combined way positively impacted the plant growth, yield and distinctly modulated defense enzymes during the growth stages of pepper plants under field conditions. The patterns of alterations in defense enzymes of microbial treatments were different from the ones of non-microbial treatments in three pepper cultivars (controls). Noticeably, the integrated use of AM, Tri, Pse led to the highest yield and usually induced higher defense enzymes activities in different plant growth stages. Therefore, AM combination with other beneficial microbes could be a promising practice in the open-field cultivation. Notably, the beneficial microbial combinations were dependent on genotypes in the induction of defense enzymes.

7. APPENDICES

A1. REFERENCES

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A2. SUPPLEMENTAL FIGURES

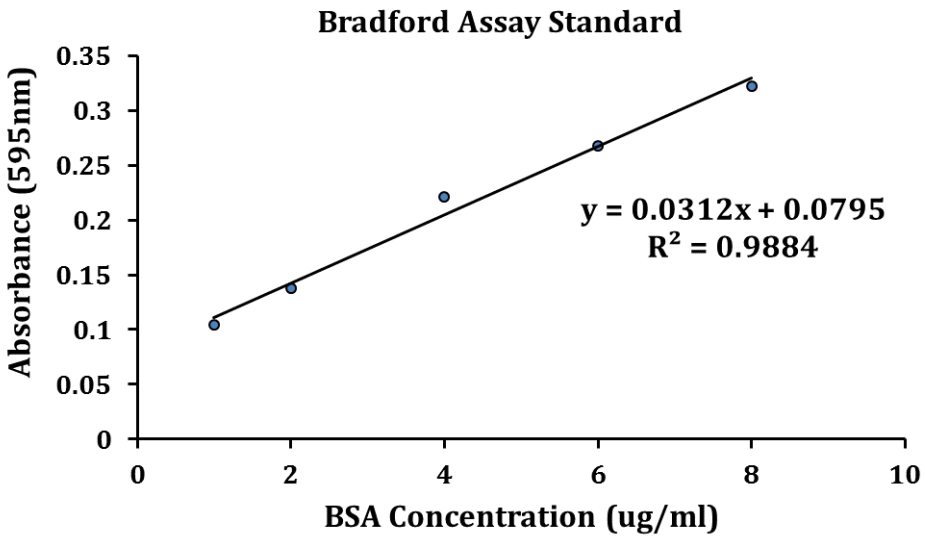

Figure 24. Bovine serum albumin standard curve for Bradford assay.

Figure 25. Three-week tomato plants with or without *Septoglomus constrictum* or *Septoglomus deserticola* were grown in a growth chamber.

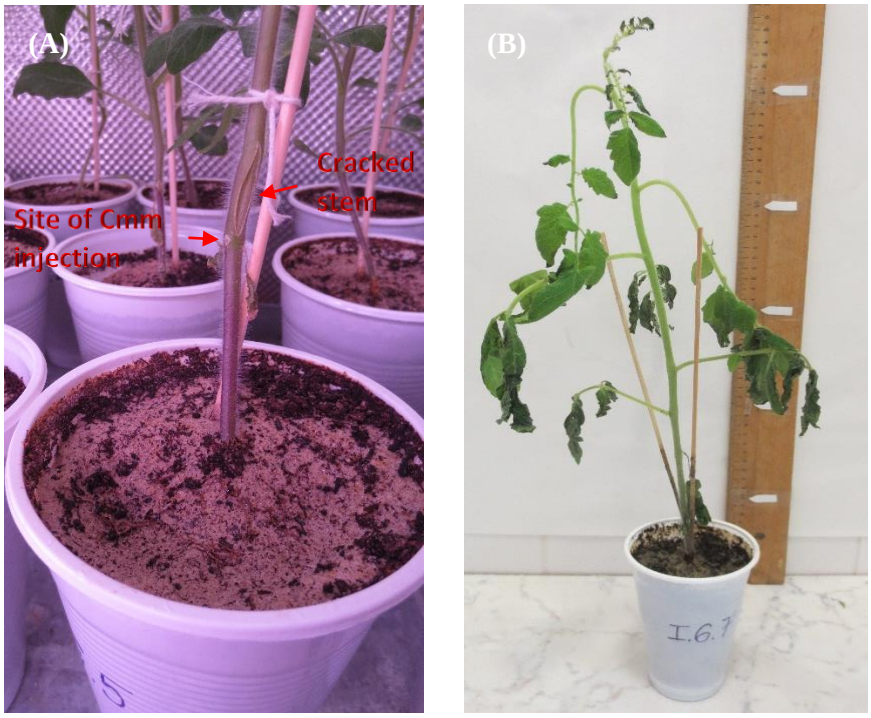

Figure 26. Cracked stem (A) and symptom of a tomato plant (B) after 17 days of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) infection

Figure 27. Tomato plants cultivar Pearson (A) and Never ripe (B) with or without *Rhizophagus irregularis* inoculation and both genotypes (C) after 18 days of *Clavibacter michiganensis* subsp. *michiganensis* infection.

Figure 28. Pepper plants cultivar Karpia, Karpex, Kaptur were grown in the experimental station of Szent István University.

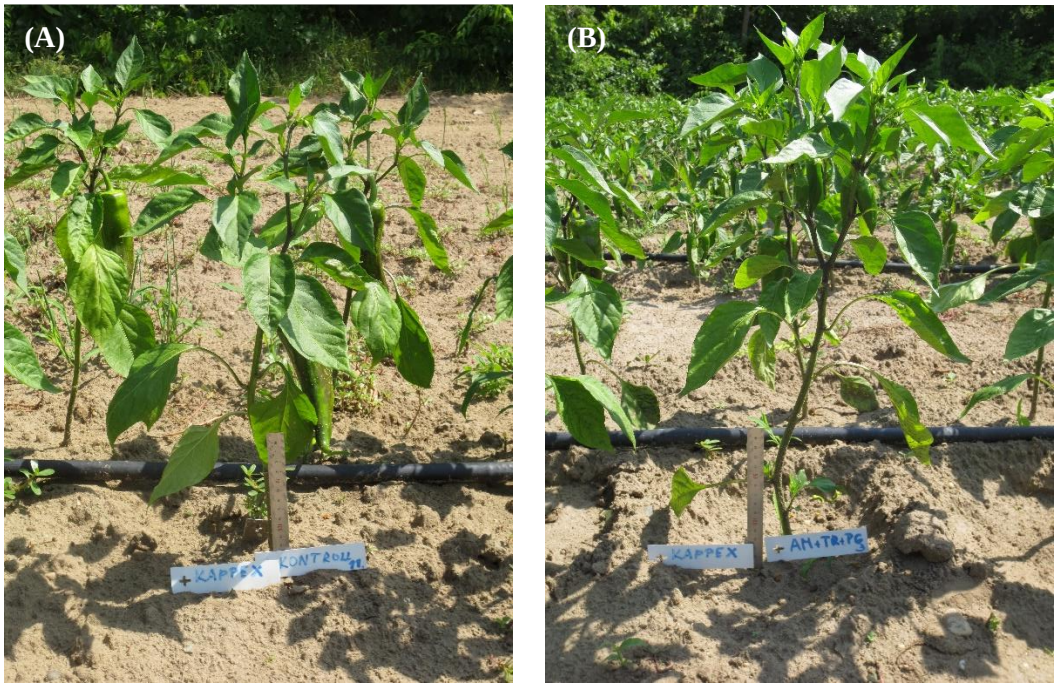

Figure 29. Pepper plants (Karpex cultivar) without microbial treatment (A) or with the triple application (*Trichoderma*, *Pseudomonas fluorescens*, arbuscular mycorrhizal fungi) (B) at 49 days after transplanting.

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