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Impact of arbuscular mycorrhizal fungi on polyphenol profiles of *Eclipta prostrata* L., and on defense system of tomato plants.

By

Imane Haddidi

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Title: Impact of arbuscular mycorrhizal fungi on polyphenol profiles of *Eclipta prostrata* L., and on defense system of tomato plants.

Discipline: Biological Sciences

Name of Doctoral School: Doctoral School of Biological Sciences

Head: Prof. Dr. Zoltán Nagy
Professor, DSc.
Hungarian University of Agriculture and Life Sciences,
Faculty of Agricultural and Environmental Sciences,
Institute of Biological Sciences

Supervisor: Prof. Dr. Posta Katalin
Professor, DSc.
Hungarian University of Agriculture and Life Sciences,
Faculty of Agricultural and Environmental Sciences
Institute of Biological Sciences
Department of Genetics, Microbiology and Biotechnology

....
Approval of Head of Doctoral School Approval of Supervisor

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ABBREVIATION

AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
APX	Ascorbate peroxidase
CAT	Catalase
D + H	Drought + heat stress
D + HS	Drought + heat shock
E. prostrata	*Eclipta prostrata*
E. p	*Eclipta prostrata*
FW	Fresh weight
GHS	Reduced glutathione
GR	Glutathione reductase
GST	Glutathione-S-transferase
MDA	Malondialdehyde
MIR	Mycorrhiza-induced resistance
NoS	Non-stress
POD	Peroxidase
PPO	Polyphenol Oxidase
PSI	Photosystem I
PSII	Photosystem II
RH	Relative humidity
ROS	Reactive oxygen species
SOD	Superoxide dismutase

1. INTRODUCTION

As sessile organisms, plants are not only confronted with single stress, but are exposed to a combination of various abiotic stresses that are interlinked and tend to occur at the same time in their natural or agronomic habitats (Wani et al., 2016; Zandalinas et al., 2018). Increased global mean temperature and changes in precipitation pattern, as a result of ongoing climate change, affect directly and indirectly the ecosystems, and represent the major limiting factors for agriculture production sustainability (Arunanondchai et al., 2018). The negative effects of global warming could be seen from great crop yield loss, and thus, poses an earnest problem regarding food security infrastructure around the world (Arunanondchai et al., 2018; Wani et al., 2016). Among stresses, drought, heat, and salt stresses are major abiotic stresses that potentially limit plant growth and development, which is often worse in the context of global warming (Lesk et al., 2016). Soil salinization has appeared as a major challenge worldwide, affecting natural resources and threatening food security. It is reported that around one billion hectares in more than 100 countries suffer salt problems, and salinization is rapidly increasing with an estimated annual addition of 0.3-1.5 million hectares of farmland (FAO, 2015).

In addition of salt stress, a very relevant abiotic stress given its agriculture implication is high temperature stress. Heat stress is becoming more prominent due to the global warming scenario, and their drastic effects on plant growth cause a severe menace to world food security (Hasegawa et al., 2018). For example, a study reported that 1 °C rise of temperature lead to the loss of more than 4.1% of global wheat yields (Liu et al., 2016). Another environmental issue which the world faces is drought stress. This phenomenon is a growing problem in agricultural ecosystems which jeopardizes plant growth and productivity (Duque and Setter, 2019; Rubin et al., 2017). According to the prediction of Intergovernmental Panel on Climate Change, global temperature will rise by 1.5 to 4.5°C until the end of the current century (Stocker et al., 2014), and this will enhance and amplify the severity, amplitude, and frequency of each stress spells mentioned above.

For that reason and under the current conditions, it is essential to develop a biotechnological method that focuses on enhancing plant tolerance and making agriculture more resilient, with the introduction and reuse of beneficial natural soil microbiota with economic and/or ecologic potential. Arbuscular mycorrhizal fungi (AMF), one of the most prevalent soil microbes, can colonize root of most terrestrial plant. These symbiotic fungi have shown to play considerable role in the fight

against various abiotic stresses such as drought, salinity, heavy metals, extreme temperature (low/high) , and play significant role in the plant growth, yields, nutrient uptake, and as well as known as bio-fertilizers (Begum et al., 2019b; Biró, 2005). Remarkably, arbuscular mycorrhizal (AM) fungi could enhance host plant tolerance to salt stress by an array of physiological and biochemical mechanisms, including higher water-use efficiency, photosynthetic capacity, ionic homeostasis maintenance, osmoprotection, cell ultrastructure preservation, and strengthened antioxidant metabolism (Evelin et al., 2019). In addition, AM symbiosis ameliorates detrimental effects of the drought stress in *Nicotiana tabacum* by upregulating antioxidant metabolism and osmolyte accumulation (Begum et al., 2020). Furthermore, AMF appear to have a positive effect on *Zea mays* under high temperature stress, through improved photosynthetic efficacy and protected PSII from damage (Mathur et al., 2018a).

Overall, the general objective of this PhD dissertation was to investigate the roles of arbuscular myccorhizal fungi in alleviation different abiotic stresses (drought, heat, and salinity) on two important models. The first one is a medicinal plant *E. p* which has been utilized as folk medicine in China, Japan, India, Vietnam, and other tropical regions for the cure of respiratory disorders, including cough and asthma, infectious hepatitis, cardiovascular ailments, and hemorrhagic diseases (Yu et al., 2020). The second one is tomato plant (*Solanum lycopersicum* L.) that considered as the second most important vegetable crop all over the world after potato (Wakil et al., 2017).

In particular, the following specific objectives have been accomplished:

1. Characterize the influence of AM inoculation and different proportion of sand/peat substrate on polyphenols content changes of *Eclipta prostrata.*
2. Investigate the interactive effects of two different salt levels (moderate, and high salt conditions) and AM inoculation on physio-biochemical parameters and polyphenol profiles of *Eclipta prostrata.*
3. Describe the defense enzymes in tomato plants (*Solanum lycopersicum* L.) under combined drought and heat, as well as drought and heat shock after mycorrhizal infection.

2. LITERATURE REVIEW

2.1. Arbuscular mycorrhizal fungi (AMF)

Arbuscular mycorrhizal fungi, are one of the widely distributed beneficial plant–microbe associations. This mutualistic relationship between plant root and fungi known since to the first appearance of land plants, which is older than 450 million years, and is considered a key step in the development of terrestrial plants (Redecker et al., 2000; Smith, 2009). Based on morphological characteristics of root tissues and host plant lineages, four major types of mycorrhizas: arbuscular mycorrhizas (AM), ectomycorrhizas (EcM), ericoid mycorrhiza (ErM), and orchid mycorrhizas (OrM) (Brundrett, 2017). Nowadays, AMF are found almost throughout the world in almost types of soil, more than 80 % of land plants including agricultural and horticultural crop species are able to form this type of interaction with fungi belongs to the phylum Glomeromycota (Andreo-Jimenez et al., 2015; Barea and Azcón-Aguilar, 1982; Smith, 2009; Song et al., 2019). The AMF proportion can be reach 36 % of the total soil biomass and 9-55 % of the soil microbe biomass (Olsson et al., 1999). However, the basis of this symbiotic union is on biotrophic nutrient exchange between the host plant and the fungal partner, while the plant provides up to 20 % of photosynthetic products (carbohydrate) to continue its life cycle, in turn the symbiont enables the plant to have access to the sparsely available nutrients (especially phosphorus and nitrogen) (Bao et al., 2019; Limpens and Geurts, 2018; Smith, 2009). Moreover, plants from the families *Amaranthaceae*, *Caryophylaceae*, *Chenopodiaceae*, *Cyperaceae*, *Juncaceae*, *Urticaceae*, *Poaceae*, *Polypodiales*, *Proteaceae* and *Brassicaceae* are rarely or never formed symbiotic relationship with fungus (Brundrett, 2017; M et al., 2018; Miransari, 2012).

2.1.1 The main structures of AMF and their functions

Extraradical, intraradical hyphae, vesicles, spores and arbuscules has been identified as the principal structures of arbuscular mycorrhiza fungi. Extraradical hyphae are responsible for the uptake of nutrients and water from the rhizosphere and also able for producing a new spores (Souza, 2015). The intraradical hyphae produced during the beginning of the symbiotic phase with limited growth and establish the infection units in the roots of the host plant and its responsible for the transfer of water, nutrients and metabolites from the extraradical hyphae to the root cortex of the host plant (Ramos et al., 2008a, 2008b, 2008c). Using the hyphae, AMF are able to bind soil particles and even interconnect different plants (van der Heijden and Horton, 2009). In the root cortex cell, a tree

shaped structures are formed from intraradical hyphae termed arbuscules which are known as the main interface for nutrient exchanges (Gutjahr and Parniske, 2013). Another important structure are the vesicles, originated from intraradical hyphae in their terminal or intercalary position in the root cortex. These are organs that storage large amounts of lipids during the development of mycorrhizae, and also accountable for the maintenance and growth of the fungus after stoppage of root metabolic function (Souza, 2015). Spores can be formed inside or outside the roots (Ramos et al., 2008a, 2008b, 2008c), and their process formation occurs within 3-4 weeks after the mycorrhiza colonization begin (Berbara et al., 2020). Moreover, spores are considered as structures of survival, resistance and responsible for dispersal and establishment (Souza, 2015).

2.1.2 Taxonomy of AMF

At present, AMF belonging to the Phylum Glomeromycota were removed from the Phylum Zygomycota based on physical information (morphological) like: spore morphology, spore formation, mycorrhizal and mycelial structures, and also by genetic features (β-tubulin and rRNA sequences) (Oehl et al., 2011). This reclassification contains three classes: Glomeromycetes, Archaeosporomycetes, and Paraglomeromycetes, with 5 orders, 14 families with 29 genera (Oehl et al., 2011). A few diverging classification was offered by (Redecker et al., 2013). Later, an important update in Glomeromycota taxonomy and classification, 15 families and 38 genera and approximately 338 AMF species are actually enumerated (Błaszkowski et al., 2015; Marinho et al., 2014; Sieverding et al., 2015). The update classification is shown in table (1)

Table 1. The update classification of the phylum Glomeromecota after Oehl et al. (2011) and updated by Aguilera et al. (2015)

Order	Class	Family	Genus
Glomerales	*Glomeromycota*	*Glomeraceae*	*Glomus*
			Dominikia
			Funneliformis
			Kamienskia
			Rhizoglomus
			Sclerocystis
			Septoglomus
			Simiglomus
		Enterophosporaceae	*Claroideoglomus*
			Albahypha
			Viscospora
			Entrophospora
Diversisporales	*diversisporales*	*Diversisporaceae*	*Diversispora*
			Corymbiglomus
			Otospora
			Redeckera
			Tricispora
		Sacculosporaceae	*Sacculospora*
		Pacisporaceae	*Pacispora*
		Acaulosporaceae	*Acaulosporu*

			Kuklospora
Gigasporales	*Gigasporales*	*Gigasporaceae*	*Gigaspora*
		Scutellosporaceae	*Scutellospora*
			Bulbospora
			Orbispora
		Dentiscutataceae	*Dentiscutata*
			Fuscutata
			Quatunica
		Intraornatosporaceae	*Intraornatospora*
			Paradentiscutata
		Racocetraceae	*Racocetra*
			Cetraspora
Archaeosporales	*Archaeosporomyctes*	*Ambisporaceae*	*Ambispora*
		Archaeosporaceae	*Archaeospora*
			Intraspora
			Palaeospora
		Geosiphonaceae	*Geosiphon*
Paraglomerales	*Paraglomeromycetes*	*Paraglomeraceae*	*Paraglomus*

2.1.3 Beneficial functions of AMF

Arbuscular mycorrhizal fungi (AMF) play a pivotal role in sustainable agriculture due to their positive effect on soil quality, plant growth, plant nutrition, nutrient cycling and nutrient losses (Begum et al., 2019b; Bender et al., 2015; Heijden et al., 2015; Parihar et al., 2019; Saia et al., 2020; Shao et al., 2018). They are essential for multiple functions in agroecosystem, like enhanced stress tolerance and resistance against biotic (pathogens, soil herbivory) and abiotic factors (salt, drought,

high or low soil pH, heavy metals, extreme temperature) (Diagne et al., 2020; Heijden et al., 2015; Smith, 2009).

Arbuscular mycorrhizal fungi contribute to aid in plant nutrients uptake in normal and stressed conditions, especially the immobile one, such as enhancing plant N acquisition, potassium (K), sulphur (S), phosphorus (P) and micronutrients from the soil through increasing the root surface area by extending their fine hyphae and exploring a larger soil volume and thus improves plant access to nutrients than is possible compared to the non arbuscular mycorrhizal plants (Jansa et al., 2019; Smith and Smith, 2011; Song et al., 2020). Furthermore, the fungal hyphae diameters are quite smaller than those of fine root hairs (3–7 μm versus 5-20 μm), therefore, are able to penetrate small pores (Allen and Allen, 2011; Dodd et al., 2000). Moreover, mycorrhizal colonization was found to contribute to increase total plant water uptake by approximately 20 %, and thus showing the role of this interaction in the water status of host plant (Ruth et al., 2011). Consequently, AMF play a significant role in P modifications and availability in soils. Wang et al. (2013) demonstrated that AMF-mediated decrease in pH can promote the phytate mineralization which considered as an organic P. It has been also reported that siderophore activity is associated with four AMF species, indicating that siderophores production that specifically chelates iron may be partly the reason for increased iron uptake by AMF (Goltapeh et al., 2008). Furthermore, the decomposition process of soil organic matter can accelerate fungal hyphae (Paterson et al., 2016). Although the higher spreading of hyphae system into the soil can offer and provide the development of many and different kinds of beneficial organisms, (Xavier and Germida, 2002) indicated that *Rhizobium* nodulation in legume hosts could be improved through co-inoculation with AMF. It is also important to highlight that the extent to which the partner plant benefits depend with the AMF species utilized (Kim et al., 2017).

Besides growth stimulation; AMF are able to provide many other ecosystem services. AM fungi can have a direct impact on the ecosystem, as they enhance the soil structure and aggregation (Leifheit et al., 2014, 2015; Rillig and Mummey, 2006). Due to the higher mycelial networks into the surrounding soil that can reach up to 30 m of fungal hyphae per gram soil, AMF symbionts are able to binding the soil particles in consequence improve soil structure (Cavagnaro et al., 2006; Wilson et al., 2009).

Moreover, glomalin released by hyphae and spores, contains 30–40 % C and its related compounds that play a key role in the increase of soil aggregate stability and soil quality, which contribute to increases the water holding capacity, and increase nutrient sequestration to the micro and macro aggregates in the soil, therefore, avoid nutrient leaching, decrease soil erosion, improve soil fertility, and avoid the pollution of ground and surface water (Bedini et al., 2009; Pal and Pandey, 2017; Prasad et al., 2018; Zou, 2016).

In terms of environment, AMF enhances interception of N by roots, which is likely to be associated with root length densities, may also reduce the risk of nutrient loss via leaching and/or as N_2O emissions, thereby reduce the risk of greenhouse gas (GHG) emissions from soils, thus suggesting that they could play a role in the mitigation of climate change (Cavagnaro et al., 2015; Storer et al., 2018). AM fungi could regulate N_2O emissions by improving plant N uptake and assimilation, therefore, a reduction of soluble N in the soil, and consequently, in a limitation of denitrification. AM fungi could change the physical conditions of soil, i.e., moisture, aggregation, and aeration, all of which influence indirectly the production and transport of GHG in soil (Bender et al., 2015).

2.1.4 Mycorrhiza-induced resistance (MIR)

Arbuscular mycorrhiza fungi (AMF) are one of the most beneficial soil microorganisms, that enhance plant growth and performance. AMF are able also to confer a faster and stronger systemic protection against below and above-ground attackers (such as soil-borne fungal and bacterial pathogens, nematodes, and pests) (Jung et al., 2012). This systemic protection are defined as mycorrhiza induced resistance (MIR), where priming of plant immunity and jasmonate signaling plays a plausible mechanism strategy operating in MIR (Martinez-Medina et al., 2016; Minton et al., 2016; Nair et al., 2015; Sanchez-Bel et al., 2016). The plant defense mechanisms are controlled through small molecules that act as signal transducers and regulate the expression of genes involved in defense (Jones and Dangl, 2006). Jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and ethylene (ET) has been considered as the prominent phytohormones in this regulatory mechanism (Pieterse et al., 2009).

Song et al. (2015), reported that tomato plants (*S. lycopersicum*) treated by *Funneliformis mosseae* against *Alternaria. solani* which causes tomato early blight disease by increases activities of β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) in tomato leaves. Three genes encoding pathogenesis-related proteins, PR1, PR2, and PR3, as well as

defense-related genes LOX, AOC, and PAL in tomato leaves where found. Moreover, JA signaling pathway, Oxylipin-related responses and induction of secondary metabolites including higher levels of the vitamins folic acid and riboflavin, indolic derivatives and phenolic compounds such as ferulic acid and chlorogenic acid have also been reported in *Rhizophagus irregularis* enhanced resistance against necrotrophic foliar pathogen *Botrytis cinerea* in tomato (Sanchez-Bel et al., 2016). Recently, Sanmartín et al. (2020), demonstrated that the protective ability of *Rhizoglomus irregularis* on tomato plant upon *Botrytis cinerea* infection seem to be associated with the primed accumulation of callose.

2.2. Abiotic stress and impact of AM fungi in stress alleviation

Abiotic stress is aggravated day by day due to the climate change abnormalities. The NASA report that the global surface temperature was 1.78 degrees Fahrenheit (0.99 °C) warmer in 2016 than in the middle of the 20th century ("SVS: Global Temperature Anomalies from 1880 to 2018" n.d.). Whereas IPCC revealed that in the late 21st century, the global mean surface temperature will rise by 1.5 to 4.5 ˚C (Change, 2014). Among abiotic stresses, drought, salinity, heavy metals, and heat directly or indirectly restrain plant growth and development or even decline the crops yield in many parts around the world (Mahalingam, 2015; Ramegowda and Senthil-Kumar, 2015). As stated by FAO World Soil Resources Reports,2000 that the total land area affected by drought, cold and salinity is 65, 57, 6 %, respectively. In addition, it was reported that abiotic stress has a drastic effect and considered as a principal reason to lose more than 50 % of crop yields over the world (Alcázar et al., 2006). Heat and drought represent the most important stressors and, their combination were recognized to have more devastating effects on crop production compared to each stress imposed individually, which interrupts the metabolic pathways of plants and affect its growth and development (Zandalinas et al., 2018). These stresses are more frequently in the semi-arid and hot growing regions in Mexico, Argentina, North Africa, South Africa, Australia, and the Mediterranean countries, and in high latitude, semi-arid growing regions of eastern and central Asia, Kazakhstan, the USA, and Canada (Tricker et al., 2018). Practically, a global-scale analysis reported that water scarcity responsible for 20–40 % of yield reductions on maize and wheat (Daryanto et al., 2016). Meanwhile, the destructive effect of temperature on crop yield (maize) has been estimated at a 3.8 %, over the past decades (1980-2008) (Lobell et al., 2011). Furthermore, drought and heat stress were accountable for wheat yield loss by more than 40 and 60 %, respectively (Zampieri et al., 2017). Moreover, it is reported that moderate soil salinity was responsible in yield losses of 55 %,

28%, and 15 % in corn, wheat and cotton, respectively. In contrast, a high soil salinity provoke a reduction of 55 % yield loss in cotton (Satir and Berberoglu, 2016). It was also found that among saline condition the drought can result a further stress-effects on the natural halophytes (Füzy et al., 2008a).

Besides the obvious negative effect on plant quality and final yields in the agriculture sector, abiotic stress poses an earnest problem regarding food security, and it is likely to exacerbate with anticipated change in the climate (Dubey et al., 2015; Lesk et al., 2016; R. S. Yadav et al., 2017; Zhao et al., 2017). In consequence, additional food production is required that should be reached at least 70 % by 2050 to feed and support the human population which grow rapidly and predicted to reach 9.1 billion, and thus considered as a very stiff task facing the world (Panta et al., 2014).

2.2.1 Drought stress on plant

Drought stress is one of important and prevalent stress factors affecting plant growth and productivity worldwide, especially in arid and semiarid area (Rao et al., 2006). Water scarcity cause morphological, physiological, biochemical, and molecular changes in plants. It is known that drought stress featured by higher transpiration rate, reduced leaf water potential, turgor pressure and disturbance of stomatal opening that reduce and limit CO_2 diffusion to chloroplasts (Farooq et al., 2009; Nakhforoosh et al., 2016; Panda et al., 2016). A limitation in the total nutrient availability in soil that lead to decrease in nutrients uptake by roots was occur under drought stress (Bista et al., 2018). Drought stress has been also demonstrated to induce a decrease in a fresh and dry weight of shoot and root on *Oryza sativa* L (Saha et al., 2019). Furthermore, reduction and/ or inhibition of photosynthesis that effect photosystems I and II (PSI, PSII), decrease the fluidity of thylakoid membrane and affect RUBISCO activity. Besides these effects, water deficiency was reported to be responsible for changes in proteins, amino acids, lipids, and hormonal balance (Liu et al., 2016; Meng et al., 2016). Water deficit also induces the overproduction of reactive oxygen species which leads to lipid peroxidation, damage in membrane functions and alteration of proteins synthesis (Pandey et al., 2015; Singh et al., 2015).

Plants evolved a plethora of mechanisms to withstand and adapt to unfavorable environmental conditions, that ensure through change at morpho-physiological levels (leaf rolling, reduced leaf water potential, decrease in stomata conductance) (Bortolheiro et al., 2017; Puglielli et al., 2019). Moreover, the biochemical levels are characterized by increase in antioxidative enzyme, decrease

efficiency of Rubisco, accumulation of proline, polyamine, and trehalose (Ammar et al., 2015; Conesa et al., 2016; Rady et al., 2020). Even more, synthesis of specific proteins like Late Embryogenesis Abundant (LEA) and dehydrins, and enhanced ABA has also proved to enhance plant tolerance to drought (Ding et al., 2016; Kamarudin et al., 2019). All of this acclimation responses are controlled by a complex signaling network and multigenic process, which starts from a signal perception to downstream functional that intervened by ABA, ion transport and transcription factors (TFs) summarized in Fig 1.

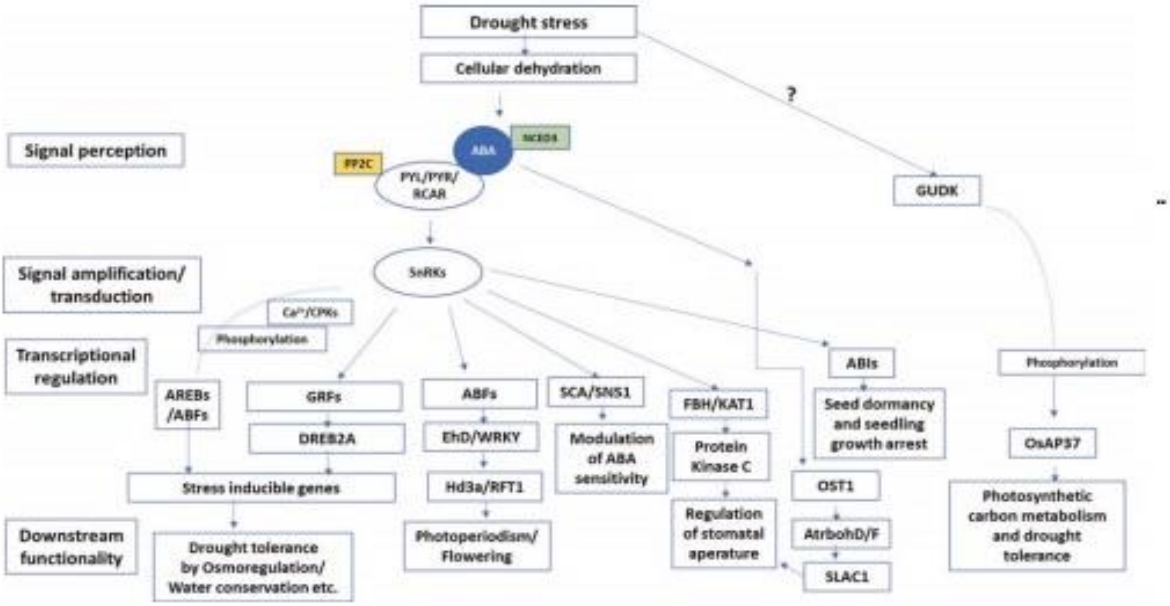

Figure 1. Schematic model of different response pathways under drought stress: ABA-dependent pathway operates through PP2C-PYR/PYL/RCAR complex which positively regulates AREB/ABF-SnRK2 pathway. SnRK2s further activate/regulate other TFs downstream by phosphorylat (reviewed by Ramegowda et al., 2014; Joshi et al., 2016; Hasanuzzaman, 2020)

2.2.2 Salinity stress on plants

Nowadays salt stress is one of the major abiotic factors that threaten sustainable agriculture and food safety (Ahanger et al., 2017). Salt stress is recognized to induce three kind of stresses: osmotic, ionic, and oxidative stress (Fig. 2). Reduced the water potential in plants and turgor loss at the cellular level, causes disturbance in the balance of essential nutrient such as a decrease in the uptake of nitrate, phosphorus, calcium, and magnesium, in contrast, increase salt levels uptake (Na^+ and Cl^-), thus leading to ionic toxicity and osmotic stress and, in consequence repress plant growth and

development under salt conditions (Forni et al., 2017; M. Hasanuzzaman et al., 2013; Rady et al., 2019; Vahdati and Lotfi, 2013; van Zelm et al., 2020). Salt stress also reported to reduce the transpiration rate, the relative water content and, increases the membrane permeability of plants (Chakraborty et al., 2018; Hniličková et al., 2017). Furthermore, salinity stress is often responsible for decrease in stomata aperture and the inhibition of photosynthesis, enzyme activity, protein synthesis (Hanin et al., 2016; Zhang et al., 2014). The excessive production of ROS was considered as important criteria that refer to salt stress, that cause membrane damage and these changes ultimately disturb whole physiological and biochemical processes in plants (Ahanger et al., 2018).

Therefore, plants perform diverse morphology and/or structure, and also in metabolic and physiological strategies in warding off these harsh environmental conditions. These mechanisms comprise ion homeostasis and compartmentalization of Na^+ and Cl^- ions in the vacuole (Abd El-Mageed et al., 2020; Kumar et al., 2013). Accumulation of compatible solutes such as proline, glycine betaine and trehalose, was also considered as another mechanism to mediate salt tolerance in plants. These compatible solutes insure osmotic balance at the cellular level (Kumar et al., 2013).For example, higher osmotic adjustment was associated with higher levels of proline and soluble sugars that has been proved using salt-tolerant genotype relative to sensitive one under salt stress (Maswada et al., 2018). While production of ROS scavengers is necessary to prevent destructive ROS, that has been reported to be important strategy for plant salt adaptation under saline conditions (Parihar et al., 2015)

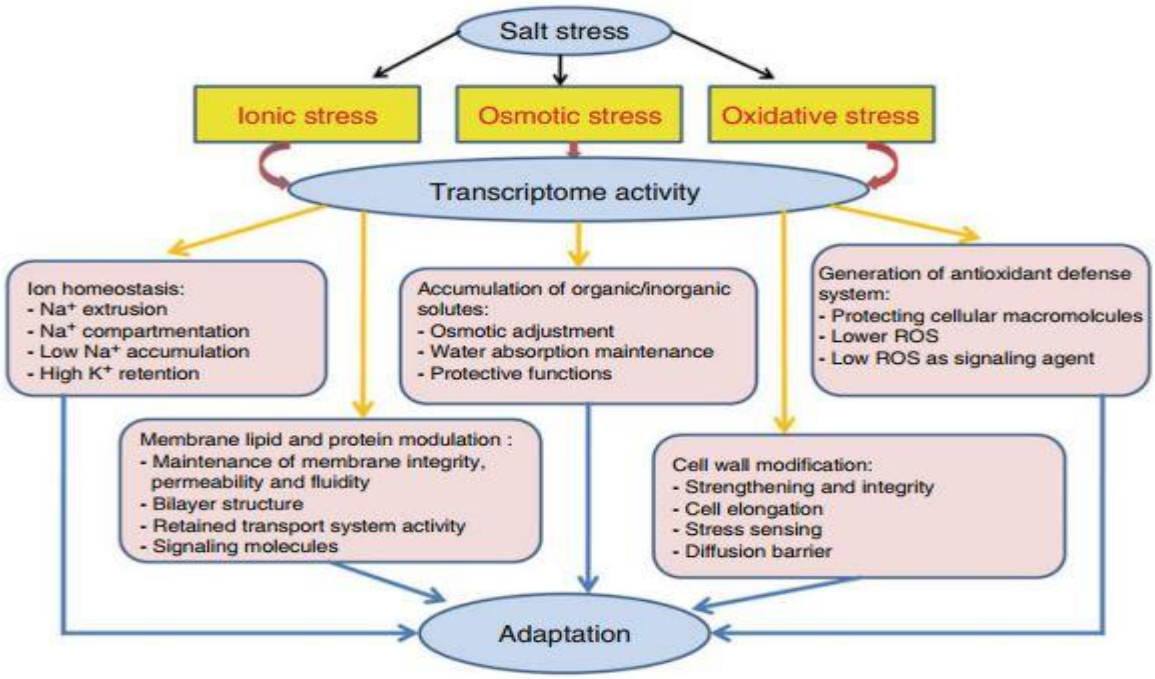

Figure 2. Effect and tolerance mechanism of plant under salt stress (Giri and Varma, 2019).

2.2.3 Extreme temperature stress on plants

High temperature is a serious phenomenon that is likely to aggravate with the anticipated change in the climate (Rogelj et al., 2018; Sorokin and Mondello, 2018). An increase in temperature above the thresholds affect all stages of plant growth and development, where the reproductive stage was more sensitive compare to the vegetative stage (Prasad et al., 2017). The effect of the increment of temperature cause adverse alterations that can occur on a molecular level up to a whole plant level. Heat stress is expected to cause reduction in plant growth and yields through prohibition of seed germination, increase of leaf senescence, and decrease of the overall dry weight of plants that were documented in various studies (Johkan et al., 2011; Kumar et al., 2011). Higher temperature has a greater effect on photosynthetic organelles of plants, and affects RuBisCo and RuBisCo activase enzymes of the Calvin cycle (Bindumadhava et al., 2018; HanumanthaRao et al., 2016; Hu et al., 2020). Heat stress exerts a negative influence on stomata density and nutrient uptake (Giri et al., 2017; Sharma et al., 2015; Zhao et al., 2017). Meanwhile, heat stress was often cause the overproduction and accumulation of harmful oxygen species (ROS) such as the superoxide anion, hydroxyl radical, hydrogen peroxide, and singlet oxygen (Pistelli et al., 2019; Telfer et al., 2018).

All these alterations provoke and owing to oxidative stress and determine plant growth and productivity.

Heat tolerant plant offer various adaptation features through avoidance (leaf rolling, transpiration cooling, closure of stomata and reduction of water loss), and tolerance mechanisms by production of compatible solutes (sugars, proline, glycine betaine), accumulation of secondary metabolites (carotenoids, flavonoids, isoprenoids), antioxidant defense enzymes, regulating heat shock proteins/chaprones (HSP60, HSP70, HSP90, HSP100, and the small HSP), RNA-binding proteins (RBPs), and phytohormones (such as abscisic acid, brassinosteroids, cytokinins, auxins) to endure heat stress and provides heat-stress tolerance, which controlled by signaling cascades and transcription (Fig. 3) (Ahammed and Yu, 2016; Alamri et al., 2019; Balfagón et al., 2018; Dawood and El-Awadi, 2018; Hasanuzzaman, 2020; Mirza Hasanuzzaman et al., 2013; Sharma et al., 2020).

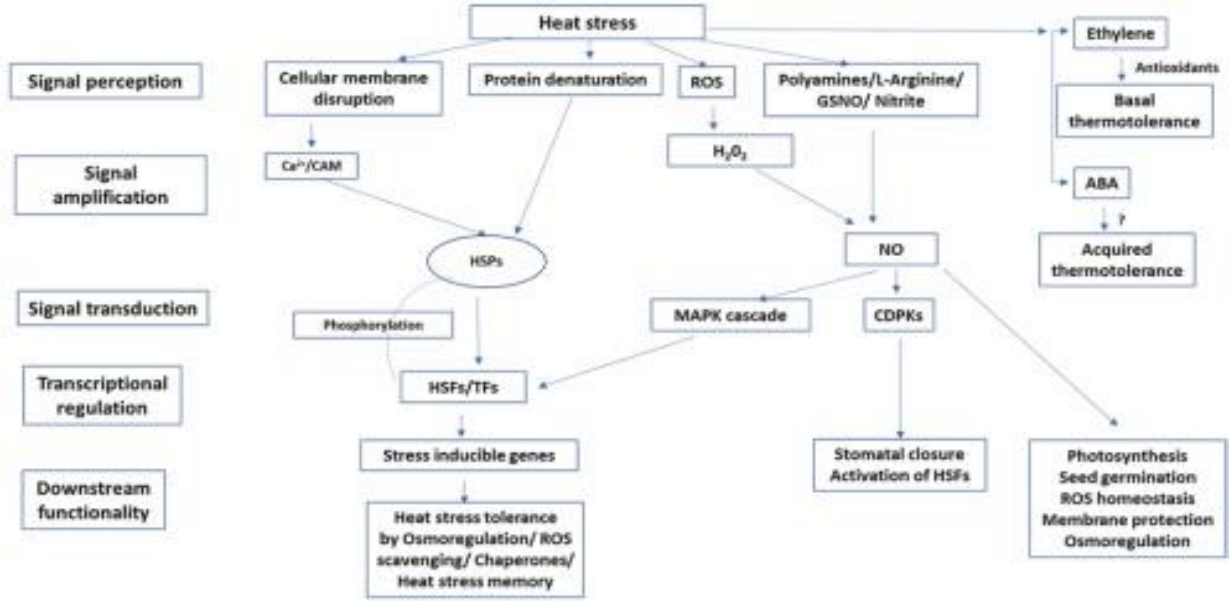

Figure 3. Schematic model of different response pathways under heat stress: Heat stress induces Ca2+ inflx, thereby triggering MAPK cascade leading to gene expression. Secondary signals like ROS, H2O2, NO, and ABA are other modes leading to heat stress tolerance. (reviewed by Awasthi et al., 2015; Guo et al., 2016; Hasanuzzaman, 2020; Parankusam et al., 2017)

- **Arbuscular mycorrhiza plant tolerance to abiotic stress**

As we discussed above, plants created a plethora of mechanisms to cope with and adapt to the harsh environmental stresses. However, these mechanisms are not always enough and can be ineffective to

endure these stressors. In this regard, arbuscular mycorrhizal fungi knowns to affect multiple functions in agroecosystems, and also proved to confer an array of advantages under divers abiotic stresses (salinity, drought, cold, heat, nutrient-deficiency, and toxic metals) and play significant role in the plant growth, yields and as well as nutrients acquisition (Heijden et al., 2015; Latef et al., 2016).

2.2.4 Mechanisms of AMF in alleviation drought stress in host plants

The extent of water shortage is becoming serious threat to plant growth and crop productivity, that exerts osmotic stress in the plant. Mycorrhization has been well documented to cope with the stress by recruiting two important strategies: drought mitigation and/or drought tolerance (Fig. 4), that ensured through morphological, physiological, biochemical, and molecular responses (Posta and Duc, 2019). AMF can enhance plant performance and growth of the host plant under water scarcity. As outlined by Pavithra and Yapa, (2018), AMF inoculation could increase plant growth, yield, and soil moisture and thus increase drought tolerance of soybean under drought stress. Indeed, increase in shoot, root, and total biomass, as well as increase in surface area of lateral roots that helped the partner plant to absorb water and nutrients was stimulated under drought stress in trifoliate orange inoculated with *Funneliformis mosseae* (Huang et al., 2017). *Zea mays* L pretreated with *Rhizophagus irregularis* increased N, Mg, P, K, and Ca under ample water and/or drought stress (Zhao et al., 2015). Improving soil structure, modification in the root architecture, in turn enhancing soil water holding, aggregate stabilization and mineral nutrition, in droughted mycorrhizal plants has been reported (Wu and Ying-Ning, 2017; Zou et al., 2017). Drought tolerance as a result of increased water potential, stomata conductance, osmotic root hydraulic conductivity, and water use efficiency has been demonstrated in several studies (Quiroga et al., 2017; Wu et al., 2017; Zhao et al., 2015). Enhancing plant photosynthetic efficiency and protect photosynthetic apparatus by AMF is another mechanism to mitigate drought stress (Mathur et al., 2018b). *Septoglomus constrictum* colonized tomato plants subjected to drought stress and drought + heat stress increased maximal photochemical efficiency of photosystem II (F_v/F_m) (Duc et al., 2018). *Rhizophagus irregularis* inoculation to *Ricinus communi*s exhibited enhanced content of chlorophyll a, b, carotenoid, and total chlorophyll in comparison to those without the AM fungi treatment (Zhang et al., 2018). The increases of these parameters maybe related to increase in nutrients uptake, especially N and Mg that are considered as important structural components of chlorophyll (Posta and Duc, 2019). They have also been reported to stimulate plant stress tolerance by altering and enhancing different

metabolites such as sugars, proline, starch, glycine betaine and polyamines. These osmolytes contribute to reduction of osmotic potential, and participate in the maintenance of leaf water potential, thereby a greater osmotic adjustment (Wu et al., 2017). Several studies reported that plants inoculated with AMF overcome the stress caused by water depletion through higher accumulation of proline (Mo et al., 2016; Zhang et al., 2018). By contrast, other studies have indicated AM-induced decline in proline accumulation under water scarcity (Zou et al., 2013). Higher accumulation of total phenols in plant inoculated with AMF ameliorated the deleterious effects of drought-induced oxidative damage (Begum et al., 2019a). Under water restriction, AMF known to increase the activities of different antioxidant enzymes, as well as the non- antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), peroxidase (POD), which considered as an important feature in plant defense to scavenge the harmful effects of ROS, and hence, contribute to the maintenance of redox homeostasis (Benhiba et al., 2015; Essahibi et al., 2018; Rani et al., 2018). A study conducted by Duc et al. (2018) reported that AM symbiosis increases plant protection against oxidative stress by decreasing the level of lipid peroxidation (MDA), hydrogen peroxide (H_2O_2), and accumulation of antioxidative enzymes such as SOD, POD, and CAT in roots and leaves under drought condition. Furthermore, AMF overcome the negative effect of drought stress by alternating hormonal balance and expression of genes involved in stress response. Abscisic acid is one of the key plant hormones, that play an important role under stress condition. Abscisic acid is not only responsible for stomatal closure to prevent water loss, but also have the ability to modulate transpiration rate, root hydraulic conductivity, and activate different responsive genes under water restrictions (Lim et al., 2015). In study conducted by Liu et al. (2016) showed that trifoliate orange seedlings colonized by *F. mosseae* increased the levels of abscisic acid (ABA), methyl jasmonate (MeJA) and zeatin riboside (ZR) under drought stress. Downregulation of ABA biosynthetic gene (SINCED) under water stress in tomato plant colonized by *Septoglomus constrictum* with greater water status and higher stomata conductance showed the beneficial effect of AMF on stress tolerance (Duc et al., 2018). Moreover, modulation of aquaporin genes under water shortage by AMF has been reported in several studies (Quiroga et al., 2017; Recchia et al., 2018).

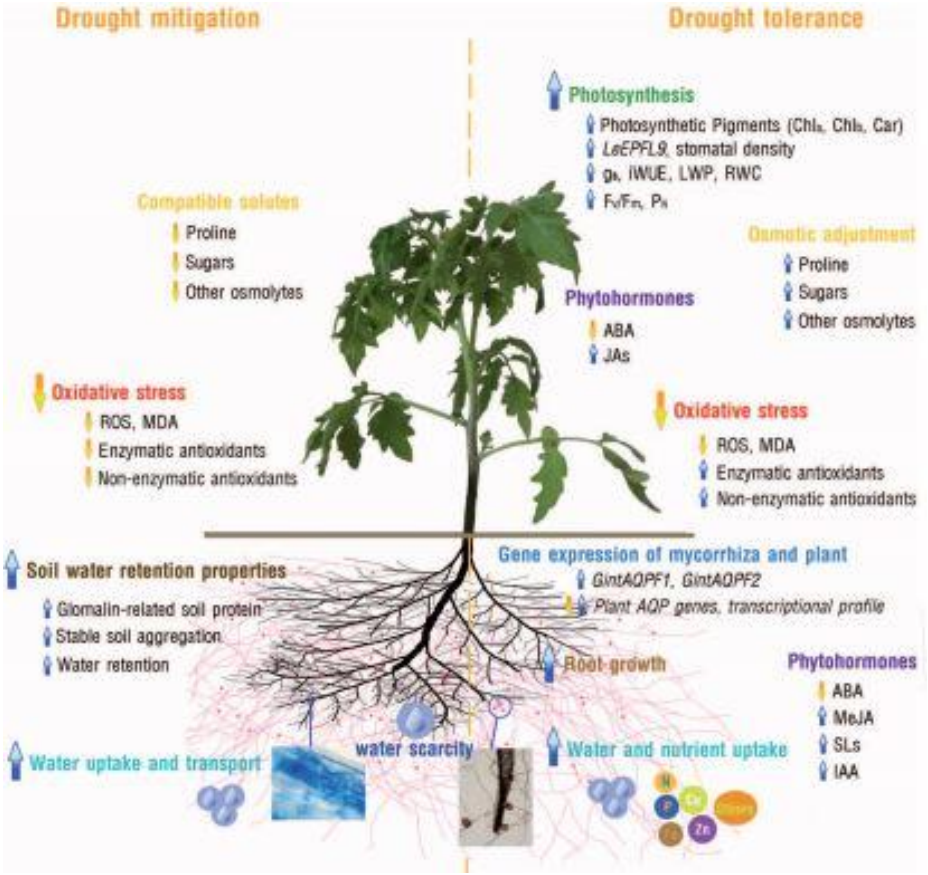

Figure 4. Strategies of mycorrhizal plants to cope with water scarcity,that is, drought mitigation and drought tolerance. Multiple benefits/mechanisms could be simultaneously induced by arbuscular mycorrhizal fungi in the host plant exposed to water deficit. The blue arrows show increase/up-regulation, whereas the orange arrows indicate decrease/down-regulation, relative to control non-mycorrhizal plants. Italic words indicate genes. ABA, abscisic acid; AQP, aquaporin; Car, carotenoids; Chla, chlorophyll a; Chlb, chlorophyll b; Fv/Fm, maximum quantum efficiency of PSII; gs, stomatal conductance; IAA, indole-3-acetic acid; iWUE, intrinsic water use efficiency; JAs, jasmonates; LWP, leaf water potential; MDA, malondialdehyde; MeJA, methyl jasmonate; PN, net photosynthesis rate; ROS, reactive oxygen species; RWC, relative water content; SLs, strigolactones (Posta and Duc, 2019).

2.2.5 Mechanisms of AMF in alleviation of salt stress in host plants

AMF are well known for their inherent capability to mitigate the osmotic, ionic, and oxidative stresses exerted by the salt stress, that is insured through an array of mechanisms (Fig. 5) (Füzy et al., 2007). In consequence, AMF can promote plant growth and development. AMF inoculation has been reported to alleviate salt stress in many plant species such as tomato, wheat, cucumber, and several native plants on the Hungarian steppe (Biró et al., 2002; Füzy et al., 2008b; Landwehr et al., 2002; Rivero et al., 2018; Sallaku et al., 2019; Zhu et al., 2018). Under salt stress, AMF has been

observed to increase available water content required for plant growth, through improving soil structure via extra radical hyphae and glomalin, thereby preventing the adverse effect of excess salt accumulation (Zhang et al., 2017; Zou et al., 2015). It has also been reported that plant inoculated with AMF under salinity stress enhanced nutrients uptake, and prevented toxic ions accumulation, resulting in improved ionic balance (Sallaku et al., 2019). In this regard, AMF was shown to diminish the mobilization of Na^+ to prevent their excess levels to plant tissues by their sequestration into vacuoles, vesicles, and intraradical mycelium (Mardukhi et al., 2011; R. S. Yadav et al., 2017). Under salt condition, lettuce plant colonized with AMF exhibited higher biomass production, enhanced synthesis of proline, augmented N uptake, and noticeable alterations in ionic relations, particularly lowering Na^+ accumulation, compared to the counterpart non-mycorrhizal plants (Santander et al., 2019). Mycorrhizal inoculation of *Citrus aurantium* increased shoot and root dry matter, root infections, spore production, and improved the concentration of N, P, K, Zn, Mn, and Cu in plant tissues (Satir et al., 2016). Another study conducted by Elhindi et al. (2017) showed an increase in chlorophyll content, gas exchange, water use efficiency, improvement in the uptake of K^+, P, and Ca^{+2}, and increase in K/Na, and Ca/Na ratio in sweet basil plants inoculated with *Glomus deserticola*, with lowering in Cl^- and Na^+ uptake under salt stress. In addition, under salinity stress, a meta-analysis revealed the positive effect and the efficiency of AMF on the performance of photosystem II in the partner plants, which was depending on photosynthetic types (C4 species had higher photosynthesis performance compare to C3 and CAM species), plant group (monocotyledon had better photosynthesis than dicotyledon), life cycle (annual species more than perennial), plant types (woody more than herbaceous) as well as, considered *Rhizophagus irregularis* as the most efficient AMF species on photosynthetic performance (Wang et al., 2019). Moreover, AMF counteract salt-induced oxidative and ionic stress by altering plant physiological level through enhanced stomatal conductance, photosynthetic rate, chlorophyll content, and leaf water potential (Ait-El-Mokhtar et al., 2019; Upreti et al., 2016). Furthermore, inoculation of AMF was also reported to induce the accumulation of various compounds such as proline, polyamine, glycine betaine, sugars, organic acids, amino acids, and trehalose, thus maintain osmotic adjustment, therefore, prevent dehydration of cells (Chen and Murata, 2011; Parihar et al., 2020). More recently, Wang et al. (2020) attributed the positive effect of AM colonization to mediate salt tolerance in maize plants by increased photosynthetic capacity and induction of enzymatic and non-enzymatic antioxidant system to overcome the deleterious effects caused by ROS. Plants associated with AMF presented heightened of hormonal concentrations under salt condition such as cytokinins and

abscisic acid concentrations (Khalloufi et al., 2017). Furthermore, several studies revealed mycorrhiza-mediated salt tolerance through the stimulation of expression of genes related to K^+ acquisition and Na^+ extrusion into the soil (Chen et al., 2017; Evelin et al., 2019; Porcel et al., 2016). In black locust, AMF maintain ion homeostasis by the induction of expression of genes encoding for membrane transport proteins and aquaporins (*SOS1*, *HKT1*, *NHX1*, and *SKOR*) (Chen et al., 2017).

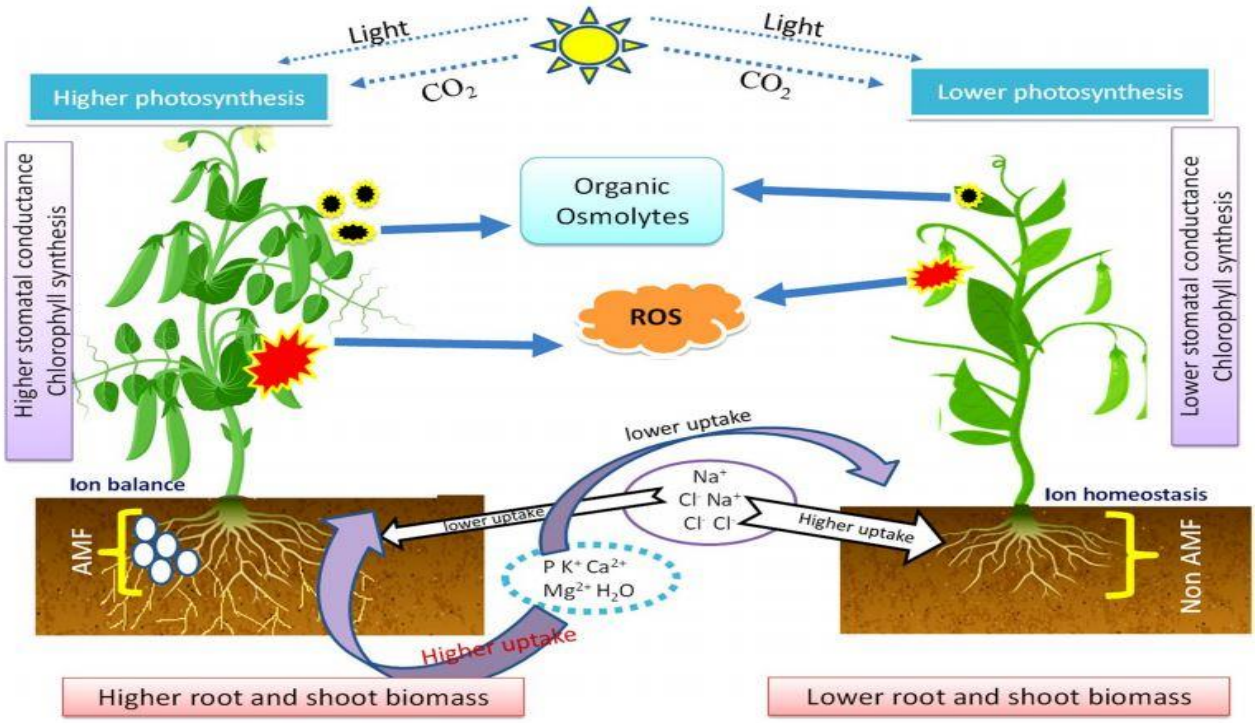

Figure 5. Mechanisms of AM fungi to mediateplant tolerance to salt stress (Diagram showing t comparative efect of salt stress on mycorrhizal and nonmycorrhizal plants. AM fungi (1) improve the uptake of selective nutrients, (2) reduce the ROS level by upregulatin antioxidant enzyme system, (3) facilitate greater accumulation of organic osmolytes, (4) enhance photosynthetic activity and (5) enhance water uptake due to stomatal conductance which results in higher root and shoot biomass production. Overall, AMF improves the plant performance and production under salt stress conditio (Parihar et al., 2020).

2.2.6 Mechanisms of AMF in alleviation of extreme temperature stress in host plants

Many studies revealed that AMF can be harnessed for improving plant growth under higher/and or lower temperature stress. AMF is a key biological agent in the agriculture sector. They have shown their efficiency and capacity to deal with extreme temperature conditions (Fig. 6). AMF has been described to avoid cell dehydration and improve water status, in result of increased stomata

conductance, relative water content, and improve water use efficiency (Liu et al., 2014). AMF was also reported to increase nutrients uptake by the way of increase plant performance to tolerate temperature stress (Zhu et al., 2017). Photosynthesis is one of the most sensitive process, that can be greatly influenced by temperature stress. However, it has been demonstrated that AMF mitigate the deleterious effects of higher temperature on plant growth is mainly related to increase photosynthetic capacity, and protect the damage of photosynthetic apparatus (Mathur and Jajoo, 2020). A study conducted by Chu et al. (2016), demonstrated that *Glomus mosseae* could help the growth of *Elymus nutans* Griseb with increased chlorophyll a and b and total chlorophyll content. Temperature stress was responsible for unbalance in the ROS production, that resulted in the oxidative damage of lipid, proteins, and nucleic acids (Pistelli et al., 2019). However, AMF has been reported to minimize these effects through the induction of antioxidant enzymes, as well as non-antioxidant systems such as PPO, POX, and GST (Duc et al., 2018; Mayer et al., 2017). Production of different compounds like: proline, sugar, and polyamine reported as another mechanism implemented by AMF to counteract the harsh extreme temperature, these compound recognized to reducing osmotic stress, thereby, maintain turgor pressure of the cell (Chen et al., 2014; Zhu et al., 2017). Other strategies are implicated in AM plant tolerance to temperature stress, among these strategies, the expression of plant aquaporin genes. Liu et al. (2014) reported an upregulation of *PIP1;1*, *PIP1;3*, *PIP2;1*, and *PIP2;5* gene expression in AM rice (*Oryza sativa*) plants under lower temperature.

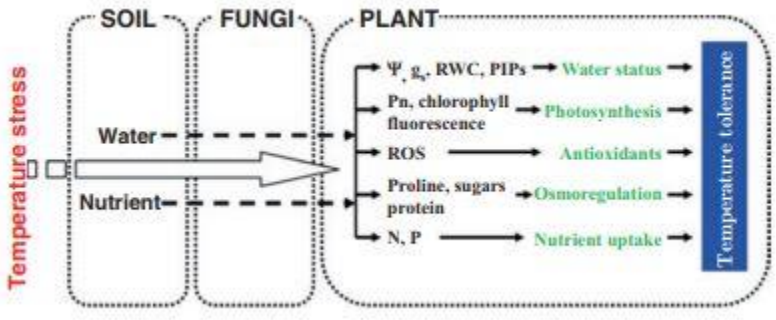

Figure 6. Mechanisms of AM fungi to mediate plant tolerance to temperature stress (Zhu et al., 2017)

2.3. Stress combination

In nature, plants are not only subjected to a single stress but are often encountered with multiple stressors that occur concurrently. Many reports revealed that plants have a distinctive response to

combined stresses, that differ and cannot be deduced from their responses when taken individually (Correia et al., 2018; Zandalinas et al., 2018). Positive, negative, unknown stress combinations, and lack of interactions are shown in (Fig. 7), such as drought and heat, salinity and heat, UV with heat, and high light and heat that leads to disproportionate damage in growth, physiology and crops yield (Alharby et al., 2020; Awasthi et al., 2017; Mittler and Blumwald, 2010; Suzuki et al., 2014). These responses are mostly imposed a specific set of complex and distinct regulatory pathways, controlled by the implication of diverse genes, metabolites, proteins, and physiological responses, that related to stress intensity and plants species as well (Hu et al., 2015; Perdomo et al., 2015; Zandalinas et al., 2016b, 2016a; Zinta et al., 2014). The combination of higher temperature with water deficit results in the rapid decline of growth and performance of plants which reported in different studies (Boeck et al., 2016; Handayani and Watanabe, 2020; Niinemets, 2016; Zandalinas et al., 2017; Zheng et al., 2019). Negative interactions in case of combine drought and salinity stress have also been reported in study conducted by Ahmed et al. (2013) on cultivated barley, that revealed decrease in plant growth, chlorophyll content, net photosynthetic rate (Pn), maximal photochemical efficiency pf PSII (Fv/Fm), water potential and osmotic potential. Recently, Al-Elwany et al. (2020), reported that combined drought and salt stress had negative effect on Chili pepper growth by affecting photosynthetic apparatus . In contrast, a positive effect on plants can be found between two different stress applied at the same time, where the combinations of ozone and drought, in which decrease ozone intake through stomata related to the reduction in stomatal conductance in response to drought stress (Iyer et al., 2013).

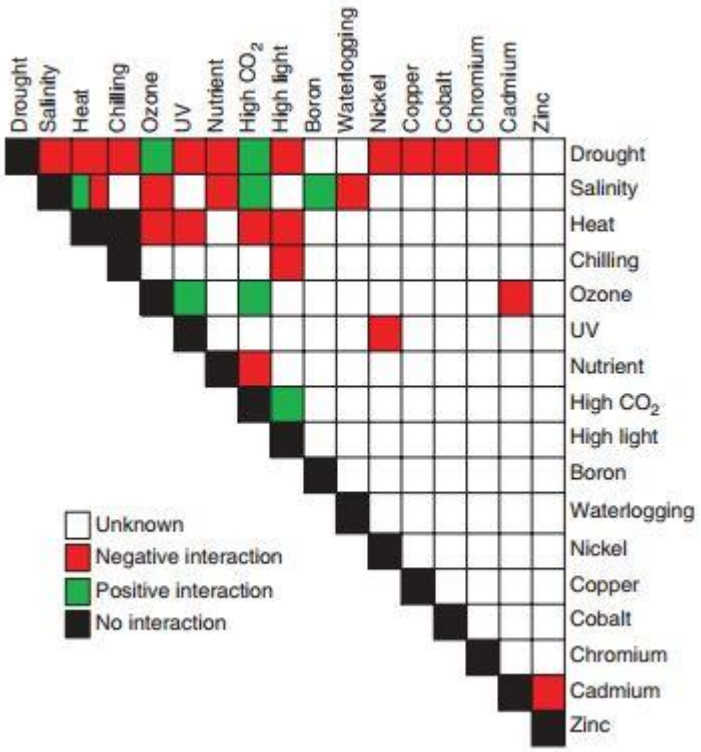

Figure 7. The stress matrix. Different combinations of potential environmental stresses that can affect crops in the field. Adapted from (Mittler, 2006) and modified in (Zandalinas et al., 2018).

In summary, environment factors linked to climate change such as drought, high salinity, and low/high temperature have innumerable effects on plant growth, development, and yield and are often interconnected and have almost a common denominator impact which cause cellular damage and secondary stresses, such as osmotic and oxidative stress. For this, the plant requires to evolve different strategies to cope with a harsh environment, responding upon activation of cascades of molecular networks, starting from the stress signal specific receptor-mediated signal perception followed by signal amplification by secondary messengers, and further transmission through either protein kinase/disruption of regulatory protein activity resulting in the activation of specific transcription factors (MYC/MYB. bZIP, CBF/DREB). These transcription factors can further activate or suppress functional genes, and regulate numerous metabolic network involved in stress tolerance or resistance (Fig. 8) (Hasanuzzaman, 2020; Lokhande and Suprasanna, 2012; Wang et al., 2003).

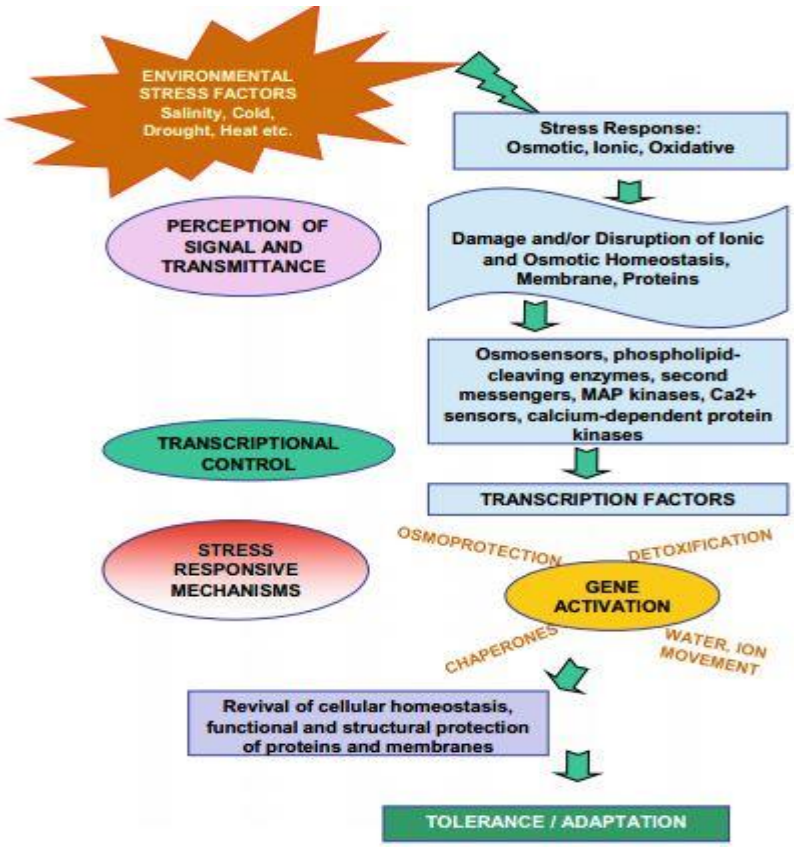

Figure 8. The complex response of plants to abiotic stresses (Lokhande and Suprasanna, 2012)

2.4. Eclipta prostrata

Eclipta prostrata Linn. (*E. p*), commonly known as "false daisy" in English, "Mo Han Lian" in Chinese, or "Bhringraj" in Ayurveda (Fang et al., 2015; Feng et al., 2019; Puri, 2003) (Fig. 9). *E. p* is an annual herb, belongs to the Asterales order, and family of Asteraceae/Compositae, It is a native plants of Asia, but it was also distributed in tropical, subtropical, and warm temperature areas around the world such as America, Africa, (Liu et al., 2012). The genus name *Eclipta* is derived from the Greek ekleipta, meaning "Deficient" with reference to the absence of the pappus on achenes (Chung et al., 2017). This herb is generally characterized by cylindrical stem, with longitudinal ridges, 2–5 mm in diameter; externally greenish-brown or dark green (Wagner et al., 2011). *E. p* was recognized to use as folk medicine such as respiratory troubles, hemorrhagic diseases, skin diseases, cardiovascular aliments and infectious hepatitis, and was also recognized to use as vegetables in many parts of the world (Fang et al., 2015). It is an important medicinal plant, which has been used in conventional systems of medicine and also by traditional healers,

particularly in China, Japan, India, Vietnam, and other regions in the cure for various diseases (Yu et al., 2020) (Fig. 9).

Figure 9. *Eclipta prostrata* (A); the flower of *E. p* (B); the fruits of *E. p* (C) (Feng et al., 2019).

2.4.1 Chemical compounds of *E. p*

Due to their excellent therapeutic and medicinal value, many studies have discussed the phytochemical and pharmacological aspects of *E. p* (Feng et al., 2019). Various phytochemical classes have identified and purified from the aerial, or whole plant including triterpenes, flavonoids, coumestans, thiopenes, steroids, from these compounds, coumestans was considered as the most typical bioactive constituents of *E. p*, that comprise wedelolactone and demethylwedelolactone. Furthermore, some researches showed that *E. p*, contains different natural compounds such as the highest content of tannin (11.86 %), followed by saponin (1.7 %), alkaloid (0.34 %), and flavonoid (0.87 %) (Dhandapani and Sabna, 2008). Importantly, environments, or other factors such as season, harvest time, storage time, and geographical sources may lead to variability in these bioactive components in *E. p* (Chung et al., 2017).

2.4.2 Pharmacological activities of compounds extracts from *E. p*

E. p is among the widely used plant for both nutritional and medical aspects due to the valuable contents of bioactive phytochemicals. Moreover, a different bioactive compounds isolated from *E. p*

have reported to display significant pharmacological features particularly for health-promoting, that curing and preventing many diseases such as:

- **Hepatoprotective effect**

Luo et al. (2018) carried out a study to demonstrate the hypatoprotective effect of E. p. the investigation revealed that wedelolactone, an important bioactive compound of *E. p,* exhibited hepatoprotective activity, that decrease liver inflammation and hypatocytes apoptosis, attenuated leukocyte infiltration and T-cell activation in concanavalinA-induced liver injury in mice. Wedelolactone, apigenin, and luteolin extracted from *E. p* displayed anti-HCV activity in vitro and in cell culture system (Manvar et al., 2012)

- **Anti-osteoporotic effect**

Ursolic acid, oleanolic acid, echinocystic acid and their derivatives extracts from *E. p* were evaluated for their anti-osteoporotic potential by Deng et al. (2015). Another recent study, reported that the extracts of *E. p* obtained with ethanol were evaluated for their anti-osteoporotic by regulating gut microbiota (GM) in mice (Zhao et al., 2019).

- **Anti-tumor and Antioxidant effects**

Arya et al. (2015) reported that the chloroform fraction of the methanol extract was able to selectively inhibit breast cancer cells over non-tumorigenic cells, which mainly related to the presence of luteolin. Moreover, Eclalbasaponin II promoted apoptosis and autophagy in human ovarian cancer cells by the regulation of JNK, p38, and mTOR signaling (Cho et al., 2016). However, recent study conducted by revealed that *E. p* extracts reduced oral cancer metastasis by inhibiting MMP2 enzymes (Liao et al., 2018). Moreover, the data provided by demonstrated that alcoholic extract of *Eclipta alba* provides protection against free radical and intracellular ROS and show anticancer activity (N. K. Yadav et al., 2017)

- **Anti-microbial effect**

Gurrapu and Estari, (2017) carried out a study to demonstrate the anti-microbial effect of *Eclipta*. The results of this investigation indicated that alkaloids isolated from leaves suppressed the growth of five human pathogenic bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella*

boydii, *Staphylococcus aureus* and *Streptococcus faecalis*. By broth microdilution assay and agar well diffusion.

On the other hand, it was reported that increased accumulation and production of such phytochemicals in medicinal plants was achieved through the introduction of AM fungi, and therefore enhance their quality for therapeutic purposes (Avio et al., 2017; dos Santos et al., 2017; Gashgari et al., 2020; Wu et al., 2021). Moreover, the enhancement of bioactive constituents such as polyphenols by AM fungi was linked to an increase in the biomass of plants due to better nutrition, expression of genes related to the defense system of plants, or related to changes in phytohormones due to mycorrhization that demonstrated by many studies (Adolfsson et al., 2017; Al Jaouni et al., 2018; Gashgari et al., 2020; Toussaint et al., 2008, 2007). Recently, Gashgari et al. (2020) asserted that AMF caused a considerable increments in the contents of the majority of the measured phenolic acids and flavonoids in *P. hortense* and *M. pulegium,* as compared to the untreated plants. Such improvement in the contents of phenolic acids and flavonoids was accompanied with enhancement in the total antioxidant capacity (TAC) of AMF-treated plants.

Finally, the biosynthesis and accumulation of secondary metabolites, especially the bioactive constituents in medicinal plants inoculated with AMF could increase for example the antioxidant capabilities of the treated plants (Gashgari et al., 2020), thus optimizing their pharmacological properties. AMF appears as a promising path to support and add value for the medicinal herb in the drug industry.

3. MATERIALS AND METHODS

3.1. Target plants

Seeds of *Eclipta prostrata* (L.) from Hong Dai Viet Ltd (Vietnam), and *Solanum lycopersicum* (L.) var. MoneyMaker seeds (Sieberz Ltd., Gödöllő, Hungary) were used in our experiments.

3.2. Arbuscular mycorrhizal fungi inocula

The mycorrhizal commercial inoculant Symbivit® containing six AMF species [a mixture of *Rhizophagus irregularis (G. intraradices), Funneliformis mosseae (G. mosseae), Claroideoglomus etunicatum (G. etunicatum), Claroideoglomus claroideum (G. claroideum), Rhizoglomus microaggregatum (G. microaggregatum),* and *Funneliformis geosporum (G. geosporum)*] (Symbiom Ltd., Lanskroun, Czech Republic; www.symbiom.cz) was utilized in the experiments part (3.3.1 and 3.3.2)

Three different mycorrhizal fungal inocula, *Funneliformis mosseae* (collection of Szent István University), *Rhizophagus irregularis* USK F1 (collection of the University of Silesia in Katowice), and *Funneliformis coronatum* (Giovann.), originated from Prof. Janusz Blaszkowski (Department of Plant Protection, West Pomeranian University of Technology, Szczecin, Poland), were used. All strains were cultured with *Zea mays* (L.) and *Plantago lanceolata* (L.) separately for five months in sterilized sand. These species were utilized in the experiments part (3.3.3).

A mixture of spores, mycelia, infected root fragments, and sand from cultures was harvested for mycorrhizal inoculation. For each treatment, the inoculums were applied 4 cm below the depth of seeding in each pot.

3.3. Plant growth and experiment design

3.3.1 Impact of arbuscular mycorrhizal fungi and different proportion of sand/peat media on polyphenols content in *E. p*

This experiment was implemented between February to June, 2016. The *E. p* seeds were treated with 1 % NaOCl, then seeds were washed with distilled water several time and put on the filter paper in Petri dishes at 26 ^{o}C for germination for three days. Germinated seeds were placed in 0.5 liter plastic pots filled with an autoclaved mixture of sand and peat. Sand and peat were prepared in different proportions (100:0; 80:20; 60:40; 40:60; 20:80 and 0:100). The sand:peat substrate properties are presented in table 5. The analysis of P and k elements were performed according to Hungarian standards (MSZ) (MSZ EN ISO 11885:2009 and MSZ 21470-50:2006 3.1.3., 3.2.3)

using ICP-OES instrument. The total nitrogen using CNS analyzer (Fisons NA 1500 CNS analyzer)(Schumacher, 2002). The calcium carbonate content was determined using Scheibler calcimeter (Gowing, 2021). Dry matter content was determined by drying the sample at 105 °C for 24 h (Jones, 2001). Experiments were carried out in ten biological replicates for each sand-peat proportion with two treatments: plants inoculated with 15 grams of commercial product of arbuscular mycorrhizal fungi Symbivit® [a mixture of *Rhizophagus irregularis (G. intraradices), Funneliformis mosseae (G. mosseae), Claroideoglomus etunicatum (G. etunicatum), Claroideoglomus claroideum (G. claroideum), Rhizoglomus microaggregatum (G. microaggregatum),* and *Funneliformis geosporum (G. geosporum)*]; while the control sample, where the plants were not inoculated with AMF (No AM) were received 15 grams of autoclaved Symbivit®, resulting in total of 120 pots. The pots were put in a climatic chamber EKOCHL 1500 (24/28 °C, 60 % relative humidity, 16/8 h photoperiod, the light intensity 600 μmol m^{-2} s^{-1}). During plant growth (seven weeks), plants were watered three times per week.

After 7 weeks of growth, mycorrhizal colonization, polyphenols, total phenolic content, and proline were determined. Fully expanded leaves (excluding petioles) and root samples were immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis.

3.3.2 Impact of AM inoculation and salinity stress on plant performance and polyphenol profiles of *E. p*

This experiment was carried out from September, 2016 to January, 2017. A factorial experiment was performed using a randomized complete block design with two factors: (1) salinity levels (0, 100, and 200 mM NaCl) (Chauhan and Johnson, 2008), and (2) mycorrhizal inoculation (inoculated with either the mixture of six AMF species (90 grams of Symbivit product) or the sterilized AM inoculant as control). After surface-disinfected seeds were germinated, they were sown in each plastic pot with 10 x 6 x 14 cm in size containing 3 kg of sterilized sand and peat (60:40 %) (v/v) substrate. Each treatment had ten biological replicates; therefore, six treatments (3 salinity levels x 2 mycorrhizal inoculations) with 10 replicates resulted in a total of 60 pots (one plant per pot). Pots were put in a climatic chamber EKOCHL 1500 (24/28 °C, 60 % relative humidity, 16/8 h photoperiod, the light intensity 600 μmol m^{-2} s^{-1}). During plant growth (eight weeks), non-stress plants were watered with 100 ml of tapping water per pot one time a week while salt stress treatments were applied by watering with 100 ml of 100 mM or 200 mM NaCl for each pot once a week. Shoot and root weight, plant height, leaf number, leaf area, stem diameter, chlorophyll

fluorescence, mycorrhizal colonization rate were examined at four and eight weeks of growth. Fully expanded leaves (excluding petioles) were immediately frozen in liquid nitrogen and stored at -80 °C until analysis of proline, superoxide dismutase, peroxidase, catalase, and polyphenol components.

3.3.3 Mycorrhizal tomato plant tolerance to combined drought and heat stress

The experiment was implemented between May to August, 2018. *Solanum lycopersicum* (L.) var. MoneyMaker seeds (Sieberz Ltd., Gödöllő, Hungary) were surface sterilized by immersion into 2.5 % sodium hypochlorite containing 0.02 % (v/v) Tween-20 for 30 min, then seeds were washed several times with sterile distilled water for 10 min. Seeds were germinated on moist filter paper in Petri dishes for 3 days at room temperature. The pre-germinated seeds were sown in plastic pots containing 0.5 kg of sterilized sand and peat mixture (4:1, v/v). Plants were randomly distributed and grown in a climatic chamber with 600 µmol $m^{-2}s^{-1}$ photon flux density, at 26/20 °C, with 16/8 hours' day/night temperature and 60 % relative humidity at Institute of Genetics, Microbiology, and Biotechnology, Szent István University, Gödöllő, Hungary.

Experiments were carried out in eight replicates for four tomato treatments: control sample, where the plants were not inoculated with AMF (No AM); plants inoculated with *R. irregularis*; plants inoculated with *F. mosseae*; and plants inoculated with *F. coronatum*. For all four tomato treatments, a total of 96 pots were used, where three stress effects were examined: control with 100 % field capacity, no stress (NoS); simultaneous heat and drought stress (D + H); and simultaneous drought and heat shock stress (D + HS). All plants were grown under the same non-stress conditions for six weeks, after which different stress applications were implemented (Fig. 34). First of all, 32 pots were kept in non-stress conditions (growing conditions described above), while drought stress was applied for 64 pots. Drought stress was achieved by watering the plants at 40 % field capacity for 14 days. Half of the drought pots (32 pots) were exposed to high temperature, that is, 38 °C for 16 h during the daytime and 30 °C for 8 h at night for the last 5 days of the experiment (from day 9, till day 14 of the drought period), while relative humidity and light intensity remained the same as in non-stress conditions. The rest of the drought pots (32 pots) were subjected to even higher temperature conditions (heat shock) at 45 °C. This took place only for 6 h before the plants were harvested.

Plants were harvested at the end of the combined stress applications (14 days after the start of drought stress). Fully expanded leaves and root samples were immediately frozen in liquid nitrogen and stored at –80 °C until further analysis.

3.4. Plant growth assessment and study on AMF colonization

3.4.1 Assessment of mycorrhizal colonization

From each treatment, a total of sixty (1 cm long) root pieces were randomly selected from both non-AM and AM plants, after which they were cleaned with 10 % KOH for 10 min, followed by the acidification of the segments with 2 % hydrochloric acid and 0.05 % trypan blue (1:1:1 proportion of water/glycerol/lactic acid), and left overnight (Trouvelot et al., 1986). The mycorrhizal colonization ratio was calculated by the gridline intersection method (Giovannetti and Mosse, 1980)

3.4.2 Growth rate and plant biomass

In salt experiment and impact of different mycorrhizal fungi species on plant growth, and polyphenols content of *E. p,* shoot weight, root weight, leaf number, stem diameters, leaf area were recorded at one week after transplanting the seedling, and at four and eight-weeks growth period. Afterwards, shoot and root were dried in a hot-air oven at 60 °C for three days to determine their dry weight.

In combine drought and heat experiment, four tomato plants for each treatment were used for the estimation of fresh plant biomass.

3.4.3 Measurement of leaf area

Leaf area was determined according to the method of (Glozer, 2008). The measurements were implemented on the fourth leaf from a single plant's shoot apex in each treatment with five biological replicates. Afterwards, the selected leaves were scanned with scan machine and the average leaf area was calculated through using Image J software.

3.4.4 Chlorophyll fluorescence determination

The maximum quantum efficiency of photosystem II photochemistry (Fv/Fm), a chlorophyll fluorescence parameter, was measured after 30 min of dark-adaption using a Walz-PAM 2500 (Germany) fluorometer according to the method of Oxborough and Baker, (1997).

3.5. In vitro and instrumental assessment of plant-physiological respond

3.5.1 Proline content determination

Proline content was quantified by the acid ninhydrin procedure of Bates et al. (1973). A half gram of leaf samples from each treatment was homogenized in a mortar with 10 ml of 3 % aqueous sulfosalicylic acid. Afterward, it was centrifuged at 12.000 g for 15 min. Two ml of the supernatant, 2 ml of glacial acetic acid, and 2 ml ninhydrin acid were blended, then incubated at 100 ºC for 1 hour. The reaction was terminated in an ice bath; subsequently, the chromophore was extracted with 4 ml toluene. Its absorbance at 520 nm was measured by U-2900 UV-VIS spectrophotometer (Hitachi). Proline concentration was estimated from the standard curve and calculated on fresh weight basis as follow:

*[(µg proline/ml * ml toluene) / 115.5 µg/ µmole] / [(g sample)/ 5] = µmoles proline/g of fresh weight material.*

3.5.2 Total phenolic content determination

Total phenolic concentration was determined by Folin–Ciocalteu assay (Lister and Wilson, 2001). 2 g of leaves were blended well in a mortar with 20 ml of 60 % ethanol, subsequently filtered. 1 ml filtrate and 0.5 ml Folin-Denis reagent were transferred to a tube, then mixed completely. Next, 1 ml of saturated Na_2CO_3 was added after 3 min at room temperature. The mixture was completed to 10 ml with distilled water and incubated for 30 min at room temperature. The absorbance at 760 nm was recorded using gallic acid as standard. Total phenolic content was presented as mg gallic acid per g fresh weight.

3.5.3 Determination of hydrogen peroxide accumulation

Here, 0.5 g frozen (leaf/root) sample was grounded in a mortar with liquid N_2 to obtain a homogeneous, fine powder. After that, 5 mL of cold 0.1 % (*w*/*v*) trichloroacetic acid (TCA) kept in an ice bath was added and the obtained solution was centrifuged at 12.000 g for 15 min at 4 °C. Next, 0.5 mL of the supernatant was added to 0.5 mL of 100 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). The reaction occurred for 1 h in darkness at room temperature. Hydrogen peroxide (H_2O_2) concentration in leaves and roots was measured by the U-2900 UV-VIS spectrophotometer (Hitachi) at 390 nm (Alexieva et al., 2001).

3.5.4 Determination of malondialdehyde (MDA) content

The lipid peroxidation level was determined by homogenizing 0.2 g of (leaf/root) sample in 5 mL 0.1 % TCA, then the mixture was centrifuged at 10.000 g for 5 min. Then, 1 mL of the previously centrifuged supernatant was mixed with 4 mL of 20 % TCA containing 0.5 % thiobarbituric acid (TBA). The solution was heated in a water bath (95 °C) for 15 min, then immediately cooled in an ice bath (Heath and Packer, 1968). MDA was calculated based on absorbances at 532 nm and 600 nm using a double beam spectrophotometer. Lipid peroxidation level was expressed in nmol MDA amount, where the extinction coefficient was 155 $mM^{-1}cm^{-1}$.

3.5.5 Determination of defense enzyme activities

Here, 0.5 g of frozen leaf and root samples from treatments was placed in a pre-cooled sterile mortar and homogenized in liquid nitrogen (N_2) together with 3 mL Tris-HCl buffer (50 mM, pH 7.8) containing 7.5 % (*w*/*v*) polyvinyl-pyrrolidone K25 and 1 mM Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA). The solution was centrifuged for 20 min at 4 °C at 10.000 g. The supernatant was further used to measure enzyme activities such as peroxidase, polyphenol oxidase, catalase, and glutathione S-transferases. Until further use, samples were kept in a freezer. A double beam spectrophotometer (Hitachi U-2900 UV-VIS) was used to determine enzyme activities. The protein concentration of all leaf extracts was determined according to the method of (Bradford, 1976).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically at 560 nm following the method of Beyer Jr and Fridovich, (1987). The reaction mixture (2 ml) consisted 50 mM phosphate buffer (pH 7.8), 2 mM EDTA, 0.025 % Triton X-100, 55 µM Nitroblue tetrazolium (NBT), 9.9 mM L-methionine, 20 µl of the crude extract, and 20 µl of 1 mM riboflavin. The absorbance was read at 560 nm. One unit of SOD activity (U) was defined as the required enzyme volume to lead to 50 % inhibition of the NBT decline under the assay conditions.

Catalase (CAT, EC 1.11.1.6) activity was measured by the method of Aebi, (1984). The reaction mixture consisted of 1 ml of 10 mM of hydrogen peroxide and 2 ml of 50 mM potassium phosphate buffer (pH 7.0) and 20 µl leaf extract. The absorbance decrease at 240 nm of the reaction was recorded as the deposition level of H_2O_2. The enzyme activity was presented as the changes in absorbance mg^{-1} protein min^{-1} (For the experiment 3.3.2). The catalase (CAT, EC 1.11.1.6) activity was measured using a Catalase assay kit purchased from Sigma-Aldrich, St. Louis, MO, USA

(product identification: CAT 100). The activity was measured for the solution containing 5 µL of plant extract mixed with 500 µL 1X assay buffer and 500 µL H_2O_2 (20 mM), and the degradation of hydrogen peroxide was monitored and recorded at 240 nm wavelength for 5 min (For the experiment 3.3.3).

Peroxidase (POD, EC 1.11.1.7) activity was assessed as the protocol of Rathmell and Sequeira, (1974). Solution preparation: 10 µL plant extract was mixed with the reaction mixture 2.2 mL (0.1 M sodium phosphate buffer (pH 6.0), 100 µL of 12 mM H_2O_2, and 100 µL of 50 mM guaiacol). Similar to the other enzyme amount measurements, the spectrophotometric determination was done at 436 nm for 5 min. The enzyme activity was calculated by the changes in absorbance. POD activity was expressed in mmol $mL^{-1}min^{-1}$, where the extinction coefficient was 25.5 $mM^{-1}cm^{-1}$.

Polyphenol oxidase (PPO, EC 1.10.3.1) activity was measured by the modified procedure of Fehrmann and Diamond, (1967). The assay mixture contained 2.2 mL reaction mixture (0.1 M sodium phosphate buffer (pH 6.0), 1 mM Na_2EDTA, and 20 mM catechol) with 100 µL of plant extract. The degree of quinone formation was recorded at 400 nm wavelength in 5 min. Furthermore, PPO activity was expressed in µmol $mL^{-1}min^{-1}$, where the extinction coefficient was 1150 $M^{-1}cm^{-1}$.

Glutathione S-transferase (GST, EC 2.5.1.18) activity was determined using an assay kit purchased from Sigma-Aldrich, Missouri, USA (product identification: CS0410). The reaction mixture contained substrate solution (980 µL Dulbecco's phosphate buffer saline, 10 µL of 200 mM L-Glutathione reduced, and 10 µL of 100 mM 1-Chloro-2,4-dinitrobenzene (CDNB) with 2 µL of plant extract. As described by Habig et al, (1974), the absorbance of the solution was measured for 5 min at 340 nm. GST activity was expressed in µmol $ml^{-1}min^{-1}$, where the extinction coefficient for CDNB conjugate at 340 nm was 9.6 $mM^{-1}cm^{-1}$.

3.6. HPLC determination of polyphenols

From each well-homogenized aerial part of fresh material of *E. p*, a 0.5 g sample was taken and crushed in a crucible mortar with quartz sand. Twenty ml of a mixture of 44 % EtOH, 44 % MeOH, 10 % water, and 2 % acetic acid were gradually added with crushing and then transferred to a 100 ml Erlenmeyer flask. The macerate was subjected to an ultrasonication force using an ultrasonic water bath device (Model USD-150, Raypa) for 4 min, followed by mechanical shaking (GLF3005) for 15 min. The mixture was kept overnight at 4 °C and filtered through Albet-DF400125 type

filter paper. Before injection on to the HPLC column, it was further cleaned up by passing through a 0.22 mm PTFE HPLC syringe filter. Nucleosil C18-100, 3µm, 240 x4.6 mm Protect-1 HPLC column (Macherey- Nagel, Duren, Germany was used to separate phenolic compounds using a gradient elution of 1 % formic acid in water (A) and acetonitrile (B) with a flow rate of 0.6 ml min-1. Gradient elution began with 2 % B, changed to 13, 25, and 40 % B in 10, 5, and 15 min, respectively, and finally turned to 2 % B in 5 min. The HPLC determination was performed using Hitachi Cromaster HPLC with a Model 5160 pump, a Model 5260 autosampler, a Model 5310 column oven, and a Model 5430 diode-array detector. The separation and data processing were operated by OpenLab CDS software. The peaks were identified by comparing their retention times and spectral characteristics with available standards such as quercetin-3-arabinoside, luteolin-glucoside, luteolin-7-O-glucoside, luteolin, wedelolactone, demethyl-wedelolactone, caffeic acid, 3,4-O-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4-O-caffeoylquinic acid, 4,5-dicaffeoylquinic acid, 5-O-caffeoylquinic acid, ferulic acid, feruloylquinic acid (Sigma-Aldrich Ltd., Hungary). For the quantification of phenolic compounds, each peak area was integrated at the maximum absorption wavelength, and the concentrations were calculated by relating the areas of peaks to those of the available external standards (Merken and Beecher, 2000). The standard materials were singly injected as external standards and chromatographed with the samples as well.

3.7. Statistical analysis

Statistical analysis was implemented using the SAS 9.1 (SAS Institute, Cary, NC) package for Windows. All data were evaluated by one-way analysis of variance in the first experiment and two-way factorial analysis of variance (ANOVA) with AM inoculation and salt stress in the second experiment. The last experiment, all data were evaluated by one and two-way analysis of variance (ANOVA). Means were compared by Tukey post-hoc test at $P < 0.05$ for the first experiment, while the other experiments were compared by Duncan post-hoc test ($P < 0.05$). A two-tailed test was applied to compare the same treatments between four weeks and eight weeks. Principal component analysis (PCA) was carried out by the XLSTAT program to determine the different interactions among variables and treatments, and patterns in polyphenolic data of *E. p* with and without AMF under non-stress and salinity conditions.

4. RESULTS AND DISCUSSIONS

4.1. Characterize the influence of AM inoculation and different proportion of sand/peat substrate on polyphenols content of *Eclipta prostrata*

4.1.1 Mycorrhizal inoculation rate

The microscopic observation of root colonization showed a good symbiotic establishment among symbivit and *E. p* roots. No infection was detected in non-inoculant plants. Moreover, root mycorrhizal colonization was varied from 20 to 80 % under different ratios of growth substrate, where plant grown in the presence of a substrate containing a sand/peat ratio of 60/40 % (v/v) had a highest percentage of root colonization (76.23 % ± 15.6) as compared with the others ratios followed by a higher sand proportion at the same rate with peat 80:20; 100:0; 40:60; 20:80; and 0:100 % (v/v) (Fig. 10). There were no significant differences in root colonization at higher than 40 % (v/v) peat ratios.

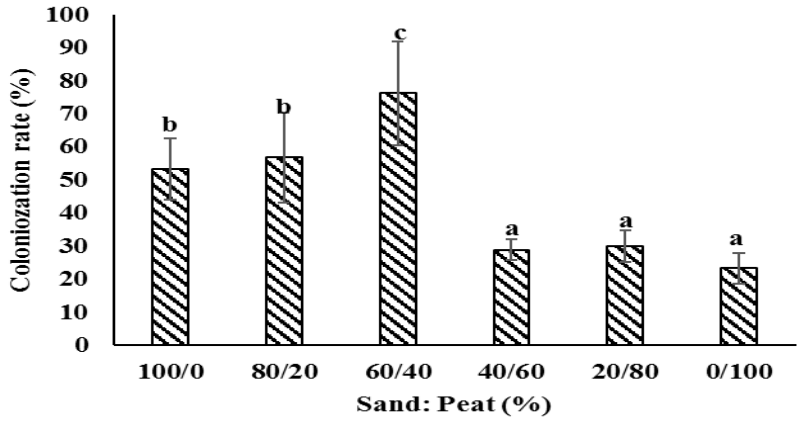

Figure 10. Mycorrhizal colonization rate (%) of *E. p* after 7 weeks of growth at different proportion of sand and peat as a growth substrate. Different letters indicate a significant difference according to the Tukey posthoc test ($P < 0.05$).

4.1.2 Proline concentration

In this experiment, proline concentration of the leaves revealed to be affected by both mycorrhizal fungi and growth substrate. At 100 % peat proportion, both inoculated and non-inoculated plants reaching the highest level of proline concentration (Fig. 11).

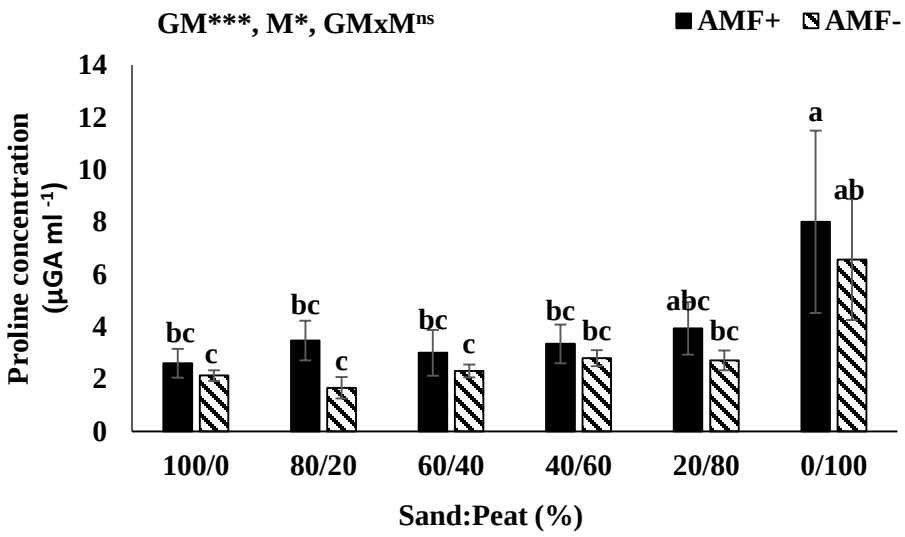

Figure 11. Proline concentration in the leaves of *E. p* after seven weeks of growth at different proportion of sand and peat as the growth substrate. Different letters indicate a significant difference, according to the Tukey posthoc test ($P < 0.05$). xNS = contrast is nonsignificant, *significant at $P < 0.05$, ***significant at $P < 0.001$. GM, growing media effect. M, mycorrhizal inoculation effect. GM×M, the interaction between mycorrhizal inoculation and growing media.

4.1.3 Total phenolic concentration

A significant difference effects of growth substrate ratios on the concentration of total phenolic content (TP) of aerial parts was found. In contrast no significant effects of mycorrhizal inoculation were detected (Fig. 12). the increase in peat concentration more than 40 % (v/v) decreased the phenolic content of plants. Moreover, higher phenolic content was found in inoculated and non-inoculated plants grown in a 60/40 % (v/v) sand and peat substrate.

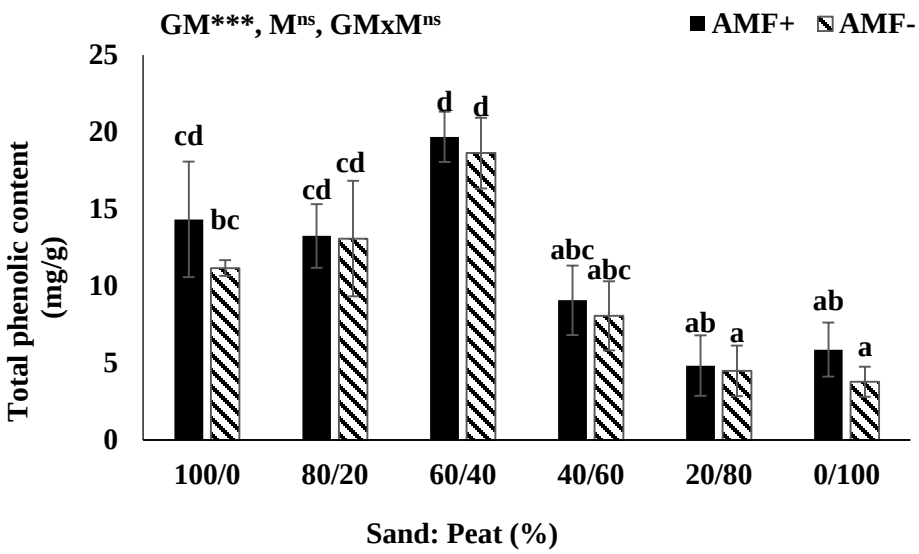

Figure 12. Total phenolic content (TP) in leaves of *E. p* after 7 weeks of growth at different proportion of sand and peat as the growth substrate. Different letters indicate a significant difference according to the Tukey post-hoc test ($P < 0.05$). xNS = contrast is nonsignificant, *significant at $P < 0.05$, ***significant at $P < 0.001$. GM, growing media effect. M, mycorrhizal inoculation effect. GM×M, the interaction between mycorrhizal inoculation and growing media.

4.1.4 HPLC analysis of polyphenols profile from the leaves of *E. p*

HPLC-DAD was utilized for detection and identification of different polyphenols from the aerial part of *E. p*. The gradient elution applied was able to efficiently separate nine phenolic compounds namely five hydroxycinnamates (protocatechuic acid; 5-O-caffeoylquinic acid; 4,5-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; 4-O-caffeoylquinic acid); two flavonoids (quercetin-3-arabinoside and luteolin), and two coumarins (dimethylwedelolactone; and wedelolactone) (Fig. 13, 14 and 15), with dimethylwedelolactone and wedelolactone being abundant in all of the samples examined.

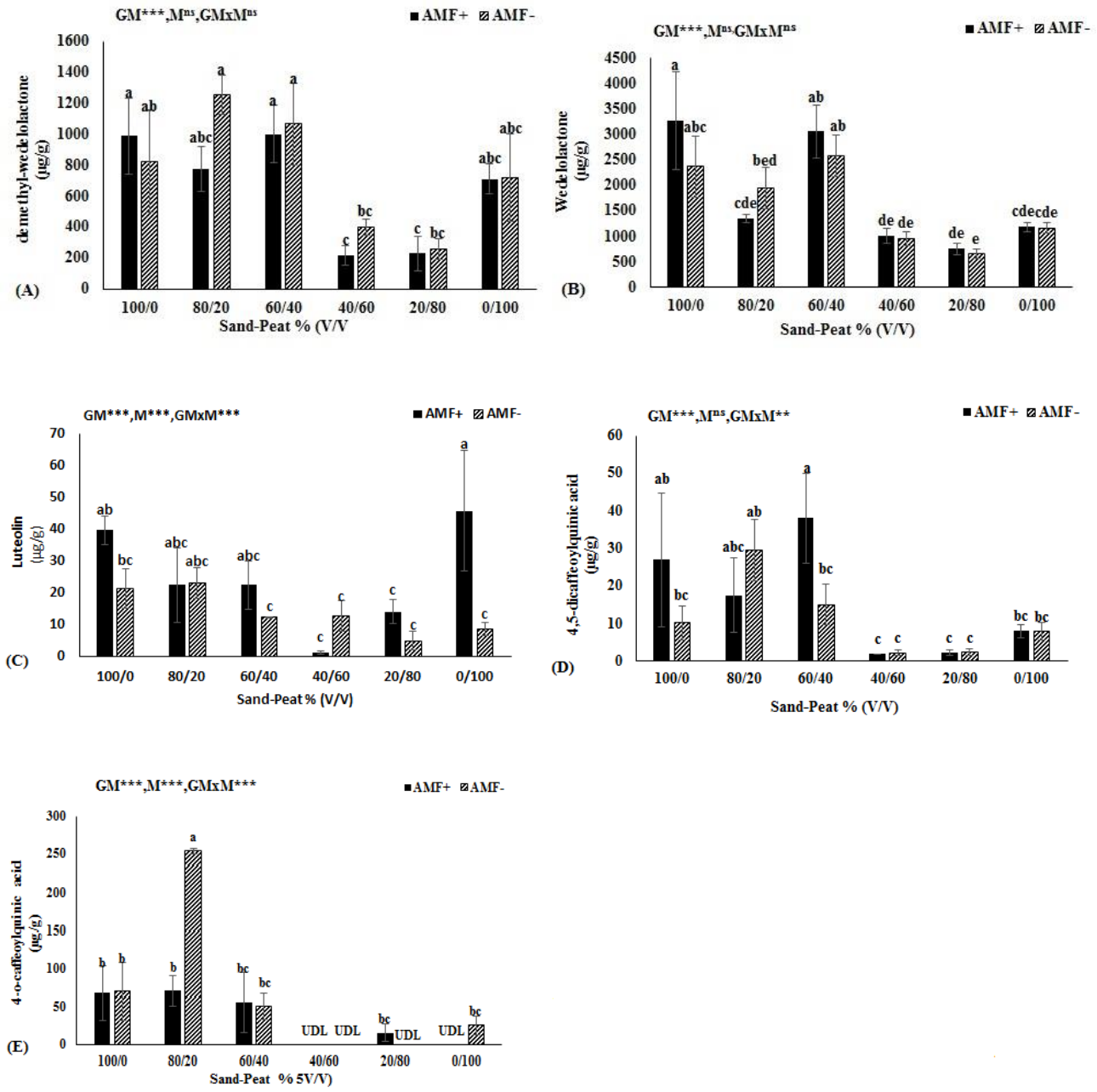

Figure 13. Change in the concentration of major polyphenols: demethyl wedelolactone (A), wedelolactone (B), luteolin (C), 4,5-dicaffeoylquinic acid (D), 4-O-caffeoylquinic acid (E) in leaves of *E. p* inoculated with arbuscular mycorrhiza or not inoculated at different proportion of sand and peat growing media. Each bar shows the mean ± standard deviation (n = 3). Different letters indicate significant difference according to the Tukey test ($P < 0.05$) among sand and peat ration. ^{x}NS = contrast is nonsignificant, *significant at $P < 0.05$, ***significant at $P < 0.001$. GM, growing media effect. M, mycorrhizal inoculation effect. GM×M, the interaction between mycorrhizal inoculation and growing media. UDL, under the detection limit.

The content of all individual polyphenols was affected, to a high extent, by the proportions of peat and sand in the growing media. Moreover, there was a considerable effect of mycorrhizal inoculation (M) on the contents of four hydroxycinnates (at least $P < 0.05$), and two flavonoids (at least $P < 0.05$). In addition, the interactions between two main effects (GM×M) were found (at least $P < 0.05$), except dimethyl-wedelolactone, wedelolactone, and 3,5-dicaffeoylquinic acid. In the inoculated plants, such a tendency held true only for protocatechuic acid, 5-O-caffeoylquinic acid, quercetin-3-arabinoside, and 3,5-dicaffeoylquinic acid (Fig. 14). In both inoculated and non-inoculated samples, peat proportions between 60 % and 80 % caused a drastic decrease in the content of all polyphenols detected in the extracts compared to others treatment except wedelolactone (Fig. 13 and 14). Notably, a drastic decrease in the polyphenol content did not occur with 100 % peat in both inoculated and control samples. With AMF inoculation, the concentration of luteolin was 45.74 mg/g at a 0/100 % (v/v) sand and peat mixture, which was significantly higher than that determined in the other treatments ($P < 0.05$) (Fig. 13 C). The average content of luteolin; 3,5-dicaffeoylquinic acid; wedelolactone; 4-O-caffeoylquinic acid; and protocatechuic acid was higher by 75 %, 37 %, 10 %, 41 %, and 67 %, respectively, in mycorrhizal inoculated plants compared to their levels in the control ones. Whereas the content of 5-O-caffeoylquinic acid; dimethyl-wedelolactone; 4,5-dicaffeoylquinic acid; and quercetin-3-arabinoside was lower by 25 %, 13 %, 47 %, and 31 %, respectively. The highest level of protocatechuic acid (41.87 mg/g) was recorded in a 60/40 % (v/v) sand and peat mixture by AMF+ (Fig. 14 F). In addition, the highest levels of wedelolactone, the major polyphenol, were found in plants grown in peat proportion between 0 % and 40 % in both inoculated and non-inoculated treatments.

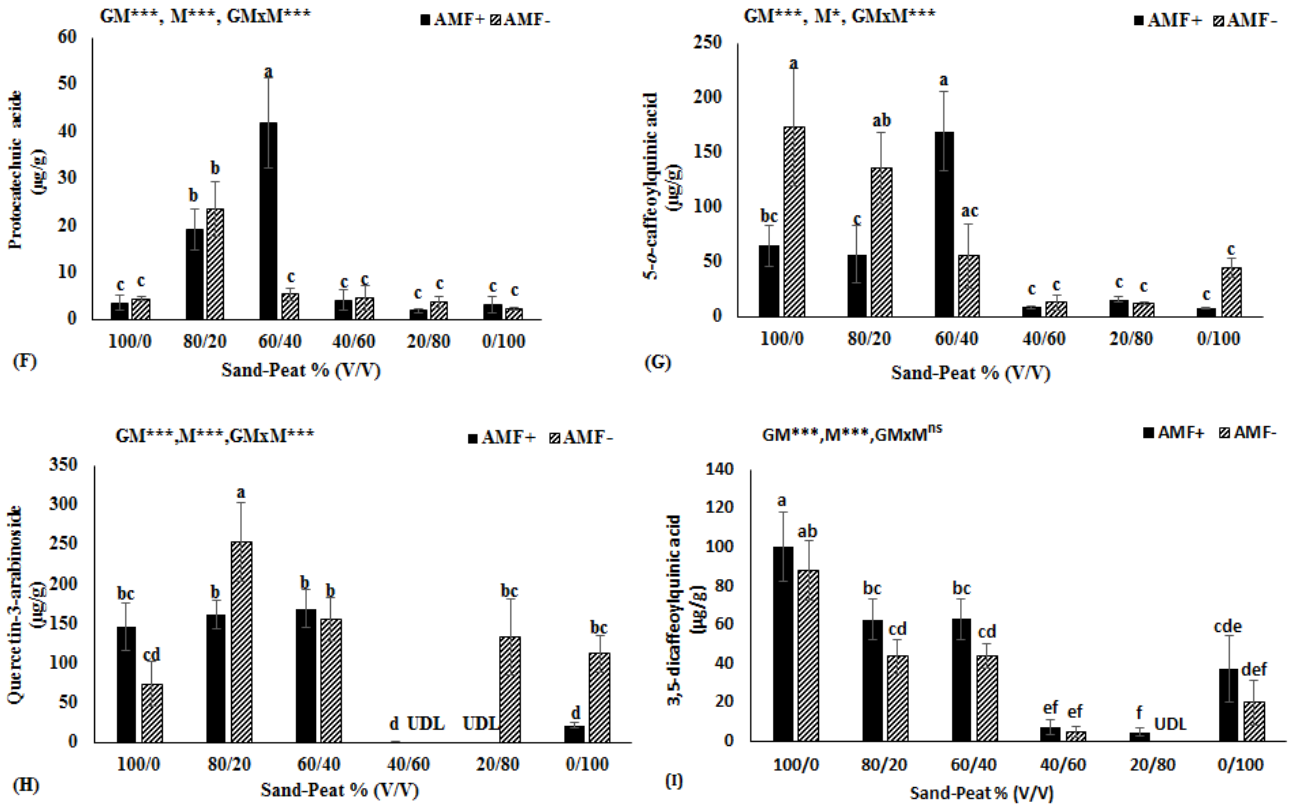

Figure 14. Change in the concentration of major polyphenols: Protocatchuic acid (F), 5-O-caffeoylquinic acid (G), Quercetin-3-arabinoside (H), 3,5-dicaffeoylquinic acid (I) in leaves of *E. p* inoculated with arbuscular mycorrhiza or not inoculated at different proportion of sand and peat growing media. Each bar shows the mean ± standard deviation (n = 3). Different letters indicate significant difference according to the Tukey test ($P < 0.05$) among sand and peat ration. ^{x}NS = contrast is nonsignificant, *significant at $P < 0.05$, ***significant at $P < 0.001$. UDL = under detection limit. GM, growing media effect. M, mycorrhizal inoculation effect. GM×M, the interaction between mycorrhizal inoculation and growing media. UDL, under the detection limit.

The principal component analysis (PCA) was applied to assess the data on phenolic content in *E. p* plants determined by HPLC. As demonstrated in Figure (17A) and Principal component 1 (Factor1) explains up to 55.58 % of the total variance and is characterized mainly by protocatechuic acid; 5-O-caffeoylquinic acid; demethyl-wedelolactone; 4,5-dicaffeoylquinic acid; quercetin-3-arabinoside; wedelolactone; and 3,5-dicaffeoylquinic acid. Principal component 2 (Factor 2), explaining 12.52 %, is contributed mainly by luteolin-glucoside. The PCA scatter plot showed 68.10 % of the total variability in the phenolic data set. Through PCA and AHC, it is important to note that the C treatment cluster group has significant differences in polyphenolic contents compared to other treatments (Fig. 16 and 17 A, B).

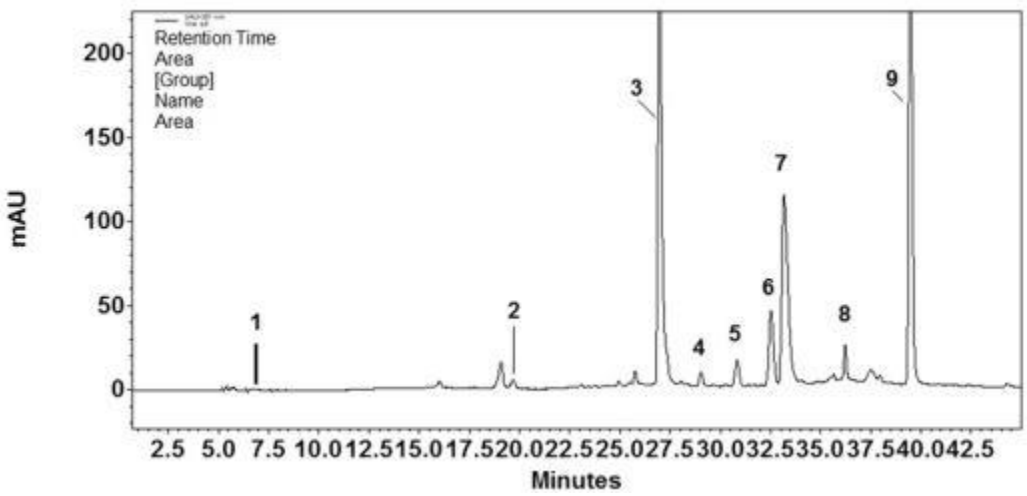

Figure 15. HPLC profile of polyphenols from leaves of *E. p* separated on C18 Protect-1, 250x4,6 mm eluated with gradient of Acetonitril in 1 % formic acide solution. Peak identifications: 1= Protocatechuic acid; 2= 5-O-caffeoylquinic acid; 3= Dimethylwedelolactone; 4= 4-O-caffeoylquinic acid; 5= 3,5-dicaffeoylquinic acid; 6= 4,5-dicaffeoylquinic acid; 7= Quercetin-3- arabinoside; 8= Luteolin; 9= Wedelolactone.

4.1.5 Discussion in the influence of AMF and growth substrate on polyphenol profiles of *E. p*

Many reports have shown the characteristics and feasibility of AMF introduction to sustainable plant production (Gupta et al., 2019; Oliveira et al., 2016). Moreover, AM symbiosis has been found to boost the biomass and secondary metabolite contents of several medicinal plants (Beltrame et al., 2019; Pandey et al., 2018); however, its impact on polyphenol profiles of *E. p* plants under different proportions of growth substrates has not been studied before.

Our results indicated that root colonization is significantly affected by growth substrates proportions, where the greatest extent of mycorrhizal colonization was recorded at 60/40 (v/v) sand peat ration, which might be due to the suitable physicochemical properties of this mixture at that proportion thus agreeing with the findings of Stevens et al. (2011) and Dhen et al. (2018). In contrast higher peat proportion more than 40 % decreased the root colonization by AMF consistent with finding of Patil et al. (2015) who reported that AM colonization and spore number decrease with the increment of super phosphate, as well as with the results of previous researchers (El Amerany et al., 2020; Ma et al., 2007; Marschner et al., 2006; Paradi et al., 2003). On the other hand, some workers demonstrated that the effect of AMF symbiosis does not correlate with colonization level (Toussaint et al., 2007) and was most effective when root colonization scored from 20 % to 30 % (Feldmann et al., 2009).

In addition to the positive effects of AM fungi on nutrient uptake, this mutualistic relationship frequently give a balance to different stress conditions (Estrada et al., 2013; Latef et al., 2016). However, in our study, higher proline content for both AMF and non-AMF treatments was recorded at 100 % peat substrate. Proline is considered as an important amino acid, that plays a key role in plants: it protects the plants from various stresses and also helps plants to recover from stress more rapidly (Hossain et al., 2014).

Medicinal plants recognized by the synthesis of different secondary metabolites such as alkaloids, terpenoids, and phenolics. These bioactive compounds not only play an important role in human health but also can uplifting in plant defense against biotic and abiotic stress factors (Bhattacharya et al., 2010; Lattanzio et al., 2006; Pandey et al., 2018). It is well documented that AM fungi improve both quality and the quantity of various important bioactive compounds on medicinal plants, and it was suggested to utilize as a path for crop biofortification (Dutta and Neog, 2016; Jugran et al., 2015; Yang et al., 2017; Zubek et al., 2015). The importance of flavonoids in ultraviolet protection has been proved using mutant ultraviolet-hypersensitive phenotypes of Arabidopsis (Ryan et al., 2001). Preformed antibiotic compounds such as phenolic and polyphenolic compounds are ubiquitous in plants and play an important role in non-host resistance to pathogens (Lattanzio et al., 2006). Furthermore, Stoms, (1982) showed that the polyphenolic compounds have a crucial role in regulating the growth and development of plants. The phenolic compounds have antioxidant properties also, which can reduce the peroxidation of membrane lipids by decreasing their fluidity in consequence, limiting the diffusion of free radicals that have been proved using *Allium sativum* L (Bozin et al., 2008). Moreover, there is another valuable property of polyphenolic compounds: having the ability and capacity to chelate heavy metal ions, as demonstrated in a study with *Nympheae* sp (Lavid et al., 2001). A study conducted by Bencherif et al. (2019), demonstrated that the endogenous AMF in Algerian steppic semi-arid areas stimulate the polyphenol compounds and antimicrobial activity of *T.gallica*. The measurement of leaves polyphenol profiles of *E. p* are consistent with the previous finding where wedelolactone, and dimethyl-wedelolactone considered as the main components of medicinal plant *E. p* (Fang et al., 2015; Murali et al., 2002). Furthermore, the polyphenols luteolin-glucoside and 4,5-dicaffeoylquinic acid were recorded in the *E. p* extract (Fang et al., 2015).

Dimethyl-wedelolactone (DWL) is an important polyphenolic component in the profile of *E. p* due to its antihepatoma toxic properties. Using 60/40 % sand/peat as a growth substrate, we

found about ten times higher concentration of DWL than reported by Murali et al. (2002). Some minor poly-phenols (such as 5-O-caffeoylquinic acid, quercetin-3-arabinoside, 4-O-caffeoylquinic acid, and protocatechuic acid) are detected for the first time in the extract of *E. p* samples. Protocatechuic acid plays an essential role in the tolerance of rice during anaerobic flooding germination, promotion of shoot elongation, and the increase in chlorophyll b (Khanh et al., 2018). Our results indicate that both growth substrate and mycorrhizal inoculation, leads to further changes in the production of secondary metabolites of *E. p*. These findings confirm some previous work, where qualitative changes due to AMF were recognized in alkaloid, terpene, flavonoid, and phenolic acids in some medicinal plants (Zeng et al., 2013), but not in *E. p*. The mechanism by which AMF changed the quality and quantity of production of the active ingredients of *E. p* can be multidirectional and not well understood (Toussaint et al., 2007). AMF symbiosis could significantly enhance the contents of some secondary metabolites of medicinal plants might be due to the upgraded nutrient availability to plants (Chandra et al., 2010). Enhanced the overall uptake of phosphorus and nitrogene by AMF resulting in an increment of nutritional status, and eventually increased synthesis of amino acids and specific metabolites in medicinal plants (Johansen et al., 1996; Jugran et al., 2015; Zubek et al., 2012). Our results showed higher phenolic content, and an increase of main polyphenols (such as wedelolactone; luteolin; 4,5-dicaffeoylquinic acid; and quercetin-3-arabinoside) of mycorrhizae inoculated plants compared with the controls (Fig. 13, 14). Avio et al. (2017) asserted that AMF responsible in further rise of polyphenols and antioxidants in red and green Oak leaf lettuce cultivars. These findings also agree with other studies where *Cynara cardunculus* and *Ocimum basilicum* plants increased the phenolic contents (Zeng et al., 2013). Furthermore, other reports conclude that the changes in the total phenols concentration can be related by the influence pathways producing fatty acids, amino acids, and apocarotenoids in the cycle of tricarboxylic acids, which results in sub-products that interfere in the integration of phenolic compounds (Lohse et al., 2005). Moreover, the sugar content that was regulated by AMF colonization was found to be responsible in the activation or inactivation of gene expression phenols biosynthesis pathways (Mota-Fernández et al., 2011). In addition, it was reported that changes in phytohormone production in plants inoculated with AMF could also affect the quantity and quality of secondary metabolites (Bencherif et al., 2019; Jeong et al., 2007; Jugran et al., 2015; Mandal et al., 2015, 2013). Jeong et al. (2007), reported that auxin's and cytokinin's concentrations affect the production of secondary metabolites. These phytohormones concentration in the host plant could be change as a result of mycorrhization. Mandal et al. (2013) showed that the induction of jasmonic

acid pathway in mycorrhizal *Stevia rebaudiana* plant enhanced the biosynthesis of stevioside and rebaudioside. Another reason could be attributed in the synthesis of secondary metabolite that AMF further induce defense related compounds including proteins and phenolic compounds in plants (Cameron et al., 2013; Fontana et al., 2009; Volpin et al., 1994). However, contrasting results have been reported that AMF decreased the phenolic content in *Ocimum basilicum* and *Salvia* (Lee and Scagel, 2009), and it was also influenced by host plants genotype.

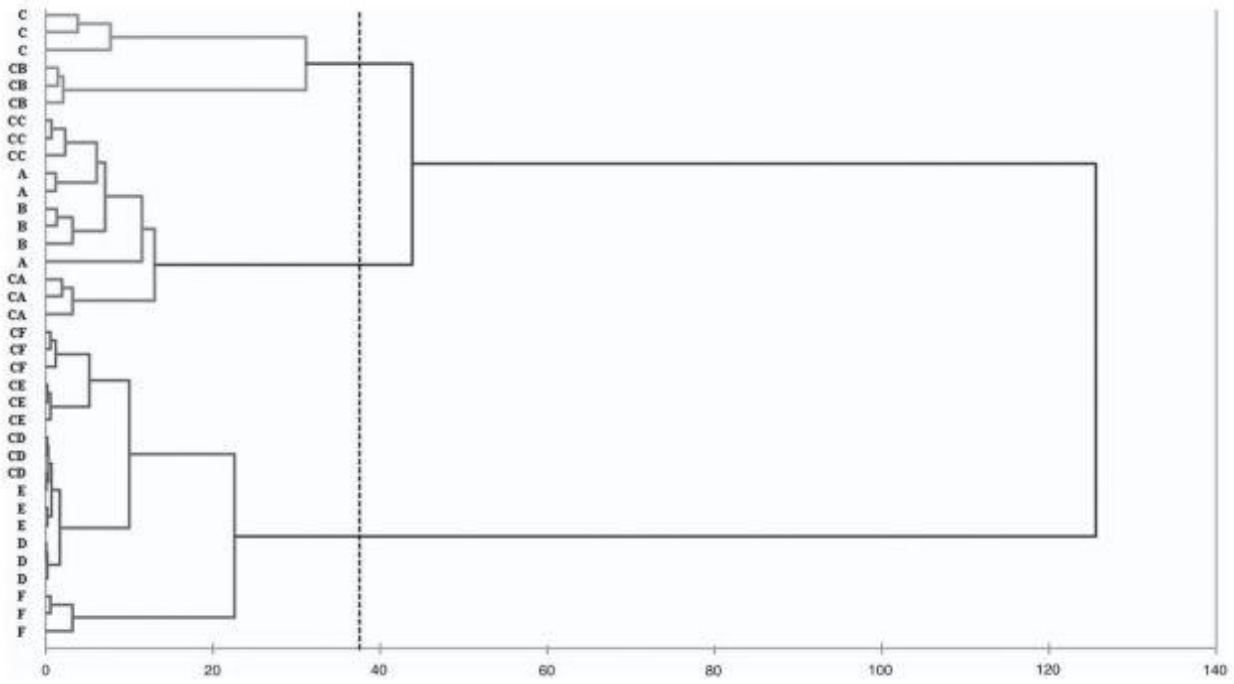

Figure 16. Dendrogram of the different treatments with and without AMF obtained from the hierarchical cluster analysis. AMF+: mycorrhizal plants, AMF–: nonmycorrhizal plants; A: AMF+ 100/0 sand/peat % (v/v); B: AMF+ 80/20 sand/peat % (v/v); C: AMF+ 60/40 sand/peat % (v/v); D: AMF+ 40/60 sand/peat % (v/v); E: AMF+20/80 sand/peat % (v/v); F: AMF+ 0/100 sand/peat % (v/v); CA: 100/0 sand/peat % (v/v); CB: 80/20 sand/peat % (v/v); CC: 60/40 sand/peat % (v/v); CD:40/60 sand/peat % (v/v); CE: 20/80 sand/peat % (v/v); CF: 0/100 sand/peat % (v/v).

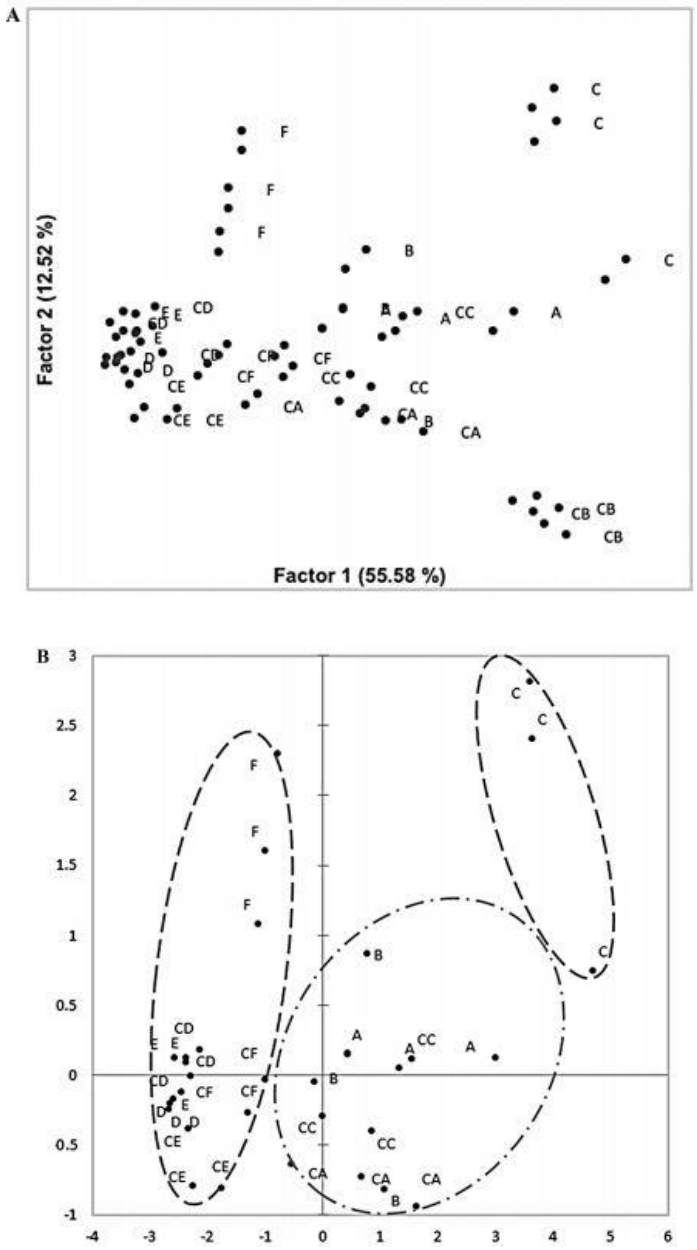

Figure 17. Principal component analysis of polyphenolic data of different treatments. A scatter plot (PC1 versus PC2). (A), A zoomed in scatterplot on the cluster containing treatments (B). A= AMF+ 1 00/0 sand/peat % (v/v); B= AMF+ 80/20 sand/peat % (v/v); C= AMF+ 60/40 sand/ peat % (v/v); D= AMF+ 40/60 sand/peat % (v/v); E= AMF+ 20/80 sand/peat % (v/v); F= AMF+ 0/1 00 sand/peat % (v/v); CA= 1 00/0 sand/peat % (v/v); CB= 80/20 sand/peat % (v/v); CC= 60/40 sand/peat % (v/v); CD= 40/60 sand/peat % (v/v); CE= 20/80 sand/peat % (v/v); CF= 0/1 00 sand/peat % (v/v).

4.2. The interactive effects of salinity stress and AM inoculation on physio-biochemical parameters and polyphenol profiles of *Eclipta prostrata*

4.2.1 Root colonization and growth parameters

Microscopic observation of the roots showed that no mycorrhizal colonization in non-AM plants during plant growth was detected. After four weeks of growth, the mycorrhizal colonization rate of AM plants obtained 54 % under non-stress conditions, while the rate was 58.4 % in those treated with 100 mM NaCl (Fig. 18). No significant differences could be found between mycorrhizal plants under non-stress conditions and salt stress at 100 mM NaCl. Nonetheless, high salinity (200 mM NaCl) considerably decreased the colonization percentage to 29.6 % at this plant growth stage. Interestingly, we did not find any substantial differences in mycorrhizal colonization rates among colonized plants under non-stress and saline conditions at eight weeks. Their rates were 51.9 %, 47.4 %, and 43 % in mycorrhizal plants under non-stress, moderate, and high salt stress. The percentage of AM colonization in AM plants under high saline conditions at the later stage was significantly elevated ($P < 0.05$) relative to those at the early stage.

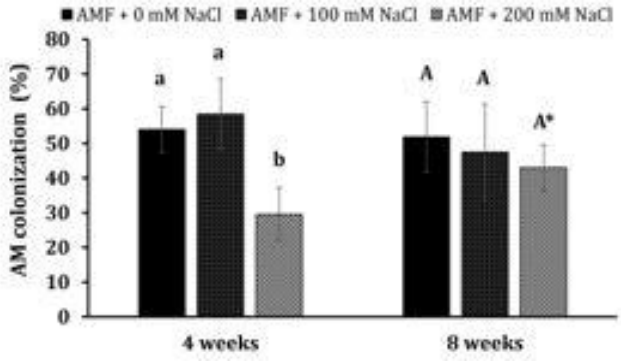

Figure 18. Arbuscular mycorrhizal colonization in roots of *E. p* in inoculated plants under non-stress, moderate (100 mM NaCl), and high saline (200 mM NaCl) conditions four and eight weeks after inoculation. * indicates a significant difference at $P<0.05$ according to the two-tailed test in the same treatments between four weeks and eight weeks after inoculation.

Exposure of *E. p* plants to salt stresses, particularly at high salt stress (200 mM NaCl), led to a considerable decrement in all growth parameters tested at both plant growth stages (Table 2). Under non-stress conditions, mycorrhizal inoculation substantially enhanced root weight (by 625 and 75.6 %), shoot weight (111 and 125 %), leaf number (89 and 107 %), stem diameter (70 and 47.1 %), and leaf area (81.5 and 99.6 %) at four and eight weeks as compared to those of non-AM plants, while

plant height remained unchanged in AM plants. In the presence of 100 mM NaCl, the increase in shoot weight (by 93 % at eight weeks), leaf number (68.1 and 96.3 %), leaf area (59.7 and 88.5 %) at both times measured in colonized plants were observed, compared to those of non-AM plants. AM colonization also markedly elevated leaf area at four weeks (by 101 %) in plants treated by 200 mM NaCl in comparison to those of corresponding uncolonized plants.

Table 2. Growth parameters of *E. p* inoculated with arbuscular mycorrhizal fungi or not inoculated under non-stress, moderate, and high saline conditions four and eight weeks after inoculation

Treatment		**Root weight (g/plant)**		**Shoot weight (g/plant)**		**Leaf number (leaf/plant)**		**Plant height (cm/plant)**		**Stem diameter (mm/plant)**		**Leaf area (cm^2/plant)**	
Stress conditions	**Arbuscular mycorrhizal**	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
Non-stress	AMF -	0.17 ± 0.05 ^{c}	1.68 ± 0.56 ^{b}	1.43 ± 0.42 ^{bc}	4.4 ± 0.7 ^{b}	10.0 ± 1.5 ^{d}	15.2 ± 1.5 ^{bc}	11.3 ± 6.0 ^{ab}	24.6 ± 6.6 ^{a}	2.0 ± 0.3 ^{b}	2.93 ± 0.2 ^{b}	8.73 ± 0.7 ^{b}	11.93± 2.8 ^{bc}
	AMF +	1.08 ± 0.4 ^{a}	2.95 ± 0.72 ^{a}	3.02 ± 0.4 ^{a}	9.9 ± 2.6 ^{a}	18.9 ± 2.9 ^{a}	31.5 ± 10.1 ^{a}	13.7 ± 6.0 ^{a}	25.5 ± 6.5 ^{a}	3.4 ± 0.7 ^{a}	4.31 ± 0.8 ^{a}	15.85 ±2.6 ^{a}	23.81 ±3.7 ^{a}
100 mM NaCl	AMF -	0.53 ± 0.3 ^{b}	0.15 ± 0.05 ^{c}	0.99 ± 0.33 ^{cd}	1.4 ± 0.1^{c}	9.4 ± 0.4 ^{d}	11.1 ± 1.5 ^{c}	6.9 ± 2.2 ^{bc}	10.9 ± 3.0 ^{b}	1.9 ± 0.1^{bc}	2.13 ± 0.1^{bc}	5.24 ± 0.8 ^{cd}	7.63 ± 1.0 ^{c}
	AMF +	0.78 ± 0.3 ^{ab}	0.41 ± 0.06 ^{c}	1.82 ± 0.6 ^{b}	2.7 ± 0.3 ^{b}	15.8 ± 3.2 ^{b}	21.8 ± 3.7 ^{b}	7.9 ± 2.1^{bc}	13.3 ± 2.2 ^{b}	2.4 ± 0.1 ^{b}	2.45 ± 0.1 ^{bc}	8.37 ± 0.6 ^{b}	14.38± 3.7 ^{b}
200 mM NaCl	AMF-	0.03 ± 0.00 ^{c}	0.13 ± 0.02 ^{c}	0.44 ± 0.1^{d}	0.9 ± 0.4 ^{c}	9.4 ± 0.5 ^{d}	9.0 ± 1.7 ^{c}	3.8 ± 0.5 ^{c}	5.0 ± 0.6^{c}	1.4 ± 0.2 ^{c}	2.06 ± 0.4 ^{c}	3.28 ± 0.6 ^{d}	7.28 ± 1.5 ^{c}
	AMF +	0.11 ± 0.02 ^{c}	0.19 ± 0.04 ^{c}	0.66 ± 0.5 ^{d}	1.6 ± 0.6 ^{c}	12.8 ± 1.1 ^{c}	11.7 ± 0.3 ^{c}	5.6 ± 0.7 ^{c}	10.1 ± 2.1 ^{b}	1.8 ± 0.3 ^{bc}	2.36 ± 0.4 ^{bc}	6.60 ± 0.5 ^{bc}	9.15 ± 1.8 ^{bc}
Source of variation (ns = not significant, *$P \leq$ 0.05, **$P \leq$ 0.01, ***$P \leq$ 0.001)													
Mycorrhizal inoculation (M)		*	*	***	***	***	***	ns	ns	**	**	***	***
Salt stress (S)		***	***	***	***	**	**	*	***	***	***	***	***
M x S		ns	*	ns	**	*	ns	ns	ns	ns	ns	*	*

The means ± standard deviation is shown (n = 5 for mycorrhizal colonization, n = 3 for other parameters). Different letters in each column indicates significant difference according to the Tukey test ($P < 0.05$) among treatments 4 and 8 weeks after inoculation. AMF, arbuscular mycorrhizal fungi; ns, non-significant difference.

4.2.2 Chlorophyll fluorescence

A slight increase in the maximal photochemical efficiency of photosystem II (*Fv/Fm*) was found in plants under salt stress conditions, no significant differences between mycorrhizal and non-mycorrhizal plants were found under the same conditions at both stage of plant growth (Fig. 19).

Nevertheless, non-stress mycorrhizal plants showed a considerable augmentation in *Fv/Fm* during plant growth. Mycorrhizal treatment was the main factor substantially influencing *Fv/Fm* at four and eight weeks ($P < 0.01$), whereas the effect of salt stress was statistically significant on this parameter at 4 weeks ($P < 0.05$).

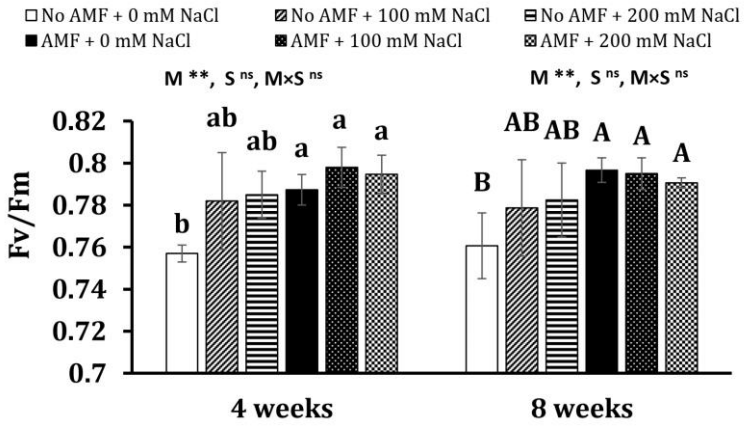

Figure 19. Maximal photochemical efficiency of photosystem II (Fv/Fm) in leaves of *E. p* inoculated with arbuscular mycorrhizal fungi or not inoculated under non-stress, moderate (100 mM NaCl), and high saline (200 mM NaCl) conditions four and eight weeks after inoculation. AMF, arbuscular mycorrhizal fungi. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments according to the Duncan test ($P<0.05$) four weeks and eight weeks, respectively, after inoculation. **, significant differences at $P<0.01$. ns, not significant. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M×S, the interaction between mycorrhizal inoculation and salt stress.

4.2.3 Proline concentration

Salinity heightened proline concentrations in mycorrhizal and non-mycorrhizal plants at four weeks (Fig. 20). In detail, 4.7 and 8.2 folds of proline content in non-AM plants exposed to 100 and 200 mM NaCl over the control (non-AM plants) were detected while 5.3 and 6.8 folds of proline level in AM plants under moderate and high saline conditions over non-stress mycorrhizal plants, respectively, were recorded. There are no significant differences between AM and non-AM plants under non-stress and high saline conditions. A nearly similar trend was observed at eight weeks of growth. Plants exposed to salt stresses substantially accumulated a higher proline content in comparison to non-exposed ones. Notably, under moderate salinity, the proline level in AM plants was 116 % higher than non-AM plants. The effect of mycorrhizal inoculation (M) and salt stress (S) were statistically significant on proline concentration measured at four and eight weeks (at least $P < 0.05$) with an existence of the interaction between two factors at eight weeks ($P < 0.05$).

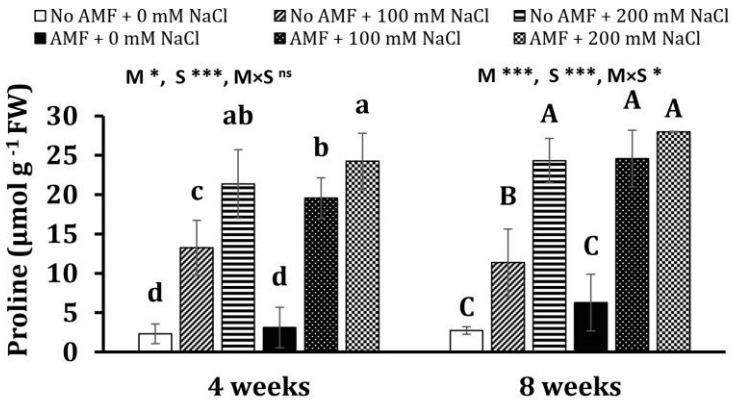

Figure 20. Proline concentration in leaves of *E. p* inoculated with arbuscular mycorrhizal fungi or not inoculated under non-stress, moderate (100 mM NaCl), and high saline (200 mM NaCl) conditions four and eight weeks after inoculation. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments according to the Duncan test (P<0.05) four weeks and eight weeks, respectively, after inoculation. *, ***, significant differences at P< 0.05, 0.001. ns, not significant. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M×S, the interaction between mycorrhizal inoculation and salt stress.

4.2.4 Antioxidant enzymatic activities

At the early stage of plant growth, mycorrhizal plants substantially increased POD activity under moderate salt stress. In contrast, the activity of this enzyme decreased in non-AM plants subjected to 100 mM NaCl (Figure 21A). No significant differences could be seen in other treatments. At the later stage, POD activity was considerably lowered (by 80.8 %, $P < 0.05$) in non-AM plants under non-stress conditions and (by 37.4 %, $P < 0.05$) in moderate-salted mycorrhizal plants, but it was substantially leaped (by 140 %, $P < 0.05$) in uncolonized plants exposed to 200 mM NaCl. Salt treatments remarkably induced almost three and seven folds higher POD activity in non-stress uncolonized plants subjected to 100 mM and 200 mM NaCl, respectively, at eight weeks. In contrast, both saline levels did not elevate POD activity in colonized plants. However, AM inoculation triggered an increase in POD activity by nearly six folds in non-stress plants. Under moderate salt and high salt stress, no significant differences in POD activity were found between non-AM and AM plants. Mycorrhizal treatment markedly impacted POD at four weeks ($P < 0.01$), while salinity remarkably affected POD at eight weeks ($P < 0.05$).

In terms of SOD activity, observed differences between mycorrhizal and non-mycorrhizal plants under non-stress and salt levels were not statistically significant at four and eight weeks (Figure 21B). There were substantial increments in this enzyme activity in non-AM plants under high salt

stress (by 257 %, P<0.05), non-stress AM plants (by 131 %, P < 0.05), and mycorrhizal plants exposed to moderate saline conditions (by 112 %, P < 0.05) at the later stage versus the early stage of plant growth. Salinity considerably affected SOD at four weeks (P < 0.05).

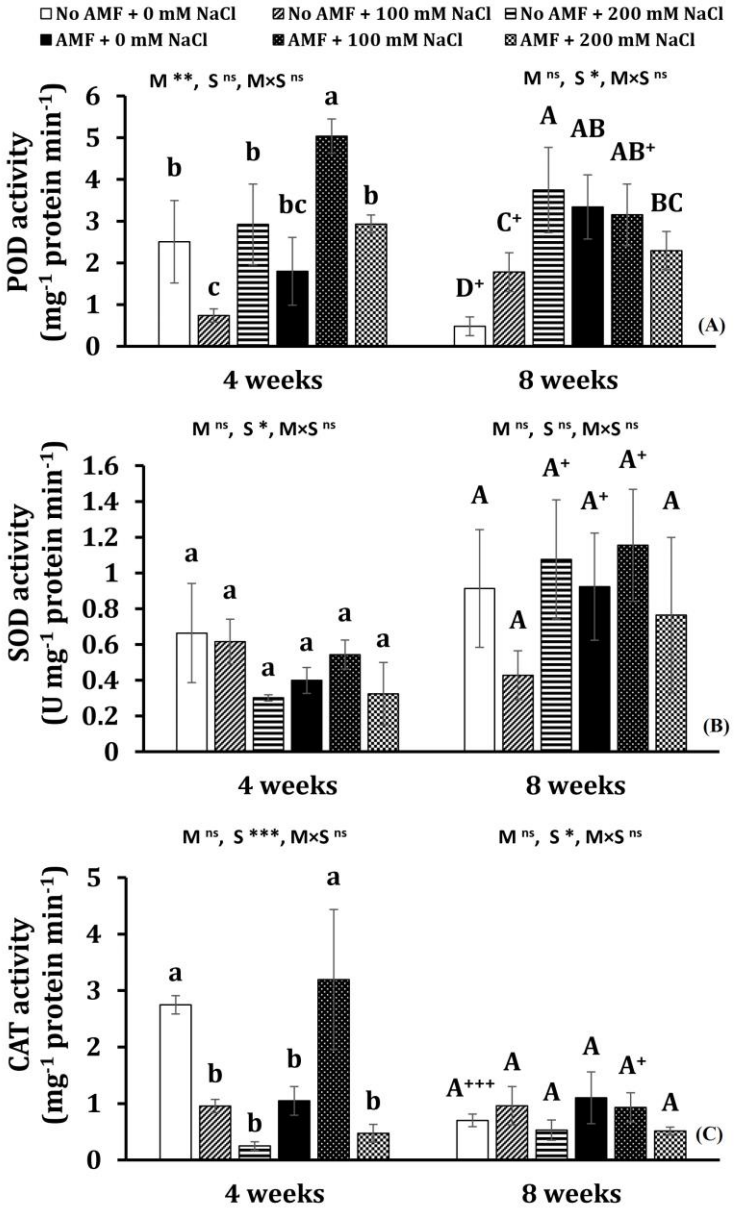

Figure 21. Peroxidase (POD) (A), superoxidase dismutase (SOD) (B), and catalase (CAT) (C) activity in leaves of *E. p* inoculated with arbuscular mycorrhiza or not inoculated under non-stress, moderate (100 mM NaCl), and high saline (200 mM NaCl) conditions four and eight weeks after inoculation. AMF, arbuscular mycorrhizal fungi. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments according to the Duncan test (*P*<0.05) four and eight weeks, respectively, after inoculation. +, ++, +++ indicate a significant difference between the same treatments four weeks and eight weeks after inoculation at *P*<0.05,

P<0.01, and *P*<0.001, respectively, according to the two-tailed test. ns, non-significant. *, **, ***, significant differences at *P*< 0.05, 0.01, 0.001. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M×S, the interaction between mycorrhizal inoculation and salt stress.

Under non-stress conditions, mycorrhizal application significantly dropped CAT activity in plants at four weeks (Figure 21C). Moderate salt stress triggered a substantially higher level (by 205 %) of this enzyme activity in colonized plants but remarkably lessened it (by 65.2 %) in non-AM plants as compared to corresponding ones. When plants were exposed to high salt concentration, no changes in CAT activity were recorded in mycorrhizal plants. Conversely, CAT activity was markedly reduced (by 90.9 %) in uncolonized plants in comparison with those under non-stress conditions. Under all conditions, no significant differences in CAT activity were found in both mycorrhizal and non-mycorrhizal plants at eight weeks of growth. Nevertheless, profound declines in CAT activity in non-AM plants under non-stress (by 292 %, $P < 0.001$) and AM plants exposed to moderate salt stress (by 70.8 %, $P < 0.05$) eight weeks versus four weeks after inoculation were observed. Salinity remarkably affected CAT at four ($P < 0.001$) and eight weeks ($P < 0.05$).

4.2.5 Arbuscular mycorrhizal fungi altered individual phenolic compounds of *E. prostrata* under non-saline and saline conditions

The quantitative and qualitative measurements of polyphenols in leaves of *E. p* were implemented by HPLC-DAD analysis. The gradient elution applied was able to efficiently separate fourteen phenolic constituents in plants four weeks after growth, namely eight hydroxycinnamates (caffeic acid; ferulic acid; 3,4-O-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; 4-O-caffeoylquinic acid; 4,5-dicaffeoylquinic acid; 5-O-caffeoylquinic acid; feruloylquinic acid), four flavonoids (luteolin-glucoside; luteolin; luteolin-7-O-glucoside; quercetin-3-arabinoside), and two coumarins (wedelolactone and demethyl wedelolactone) (Figure. 22A), but only thirteen components of polyphenols (feruloylquinic acid was under detection limit) were determined in eight-week plants (Figure 22B).

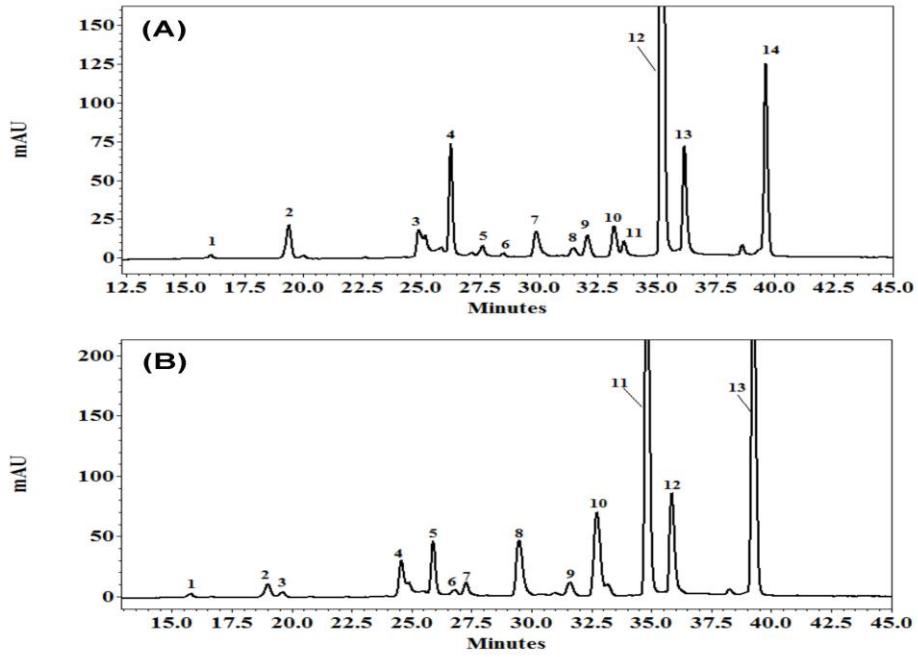

Figure 22. HPLC profile of polyphenols from leaves of *E. p* separated on C18 Protect-1,250x4,6mm eluated with gradient of Acetonitril in 1 % formic acide solution four weeks (A) and eight weeks (B) after inoculation. Peak identifications of (A): 1= 5-O-caffeoylquinic acid; 2= 4-O-caffeoylquinic acid; 3= caffeic acid; 4= 3,4-O-dicaffeoylquinic acid; 5= 3,5-dicaffeoylquinic acid; 6= uteolin-glucoside; 7= luteolin-7-O-glucoside; 8= ferulic acid; 9= quercetin-3-arabinoside; 10= demethyl wedelactone; 11= feruloylquinic acid; 12= 4,5-O-dicaffeoylquinic acid; 13= luteolin; 14= wedelolactone. Peak identifications of (B): 1= 5-O-caffeoylquinic acid; 2= 4-O-caffeoylquinic acid; 3= caffeic acid; 4= 3,4-O-dicaffeoylquinic acid; 5= 3,5-dicaffeoylquinic acid; 6= luteolin-glucoside; 7= luteolin-7-O-glucoside; 8= ferulic acid; 9= quercetin-3-arabinoside; 10= demethyl wedelactone; 11= 4,5-dicaffeoylquinic acid; 12= luteolin; 13= wedelolactone.

Among polyphenols, wedelolactone and/or 4,5-dicaffeoylquinic acid were abundant in all plants under different conditions. At the early stage of growth, the content of the total and individual polyphenols was mainly affected by salinity, whereas both mycorrhizal inoculation and salt stress influenced phenolic production at the later growth stage (Figure 23 and 24). In detail, after four weeks of growth, there was a considerable effect of mycorrhizal inoculation (M) on the contents of four flavonoids (at least $P<0.05$), five hydroxycinnamic acids (at least $P<0.01$), and demethyl wedelolactone ($P<0.001$). Salinity had a substantial impact on the level of all polyphenol compounds tested (at least $P<0.05$), except dimethyl wedelolactone and 5-*O*-caffeoylquinic acid. Interactions between two main effects on 3,5-dicaffeoylquinic acid ($P<0.001$), ferulic acid ($P<0.01$), feruloylquinic acid ($P<0.001$), 4,5-dicaffeoylquinic acid ($P<0.05$), and luteolin ($P<0.01$) were found.

When plants reached eight weeks of age, mycorrhizal colonization significantly influenced all polyphenol compounds (at least $P<0.05$), except dimethyl wedelolactone. Likewise, salinity elicited sharp changes in all polyphenols (with at least $P<0.01$). Interactions between two main effects on most polyphenols were recorded (at least $P<0.05$, except luteolin-glucoside, and demethyl wedelolactone).

After four weeks of growth, mycorrhizal colonization resulted in a significant increase in the total polyphenols (by 139 %) in non-stress plants. Such a tendency was observed in the content of wedelolactone (105 %), 3,5-dicaffeoylquinic acid (404 %), 4,5-dicaffeoylquinic acid (1281 %), feruloylquinic acid (2901 %). Moderate salinity significantly induced higher total phenolics (166 %), and seven individual polyphenols such as wedelolactone (134 %), ferulic acid (239 %), 3,5-dicaffeoylquinic acid (842 %), 4-5-dicaffeoylquinic acid (1436 %), 4-*O*-caffeoylquinic acid (336 %), caffeic acid (171 %), luteolin (287 %) in uncolonized plants at four weeks, while these increments were not found under high salt stress, except ferulic acid, 5-*O*-caffeolquinic acid, and luteolin. By contrast, under both salt stresses, the decrement trend was seen in the content of total polyphenols and wedelolactone, luteolin-7-glucoside, feruloylquinic acid in mycorrhizal plants, being more severe under high salt stress, whereas there were no significant changes in the concentration of ferulic acid, 4,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, quercetin-3-arabinoside, and 3,4-*O*-dicaffeoylquinic acid, in colonized plants as compared to the counterparts of non-stress mycorrhizal ones. Noticeably, under moderate salinity, the concentration of wedelolactone, ferulic acid, and 4-*O*-caffeoylquinic acid were substantially higher in non-AM plants than AM plants. Nevertheless, the fungal symbiont markedly enhanced the content of luteolin (62.3 %) in host plants in relation to those of non-AM plants. Besides, demethyl wedelolactone was under the detection limit in colonized plants under such stress, but their feruloylquinic acid was detectable. When exposed to high salinity (200 mM NaCl), fungal colonization positively influenced the level of 3,5-dicaffeoylquinic acid (37 folds more than that of the corresponding uncolonized plants), 4,5-dicaffeoylquinic acid (17 folds), feruloylquinic acid (detectable versus undetectable), and luteolin-glucoside (detectable) but negatively affected the content of ferulic acid (decreased by 268 % over the corresponding uncolonized plants) in colonized plants at 4 weeks.

After eight weeks of growth, salinity led to a significant reduction in the content of total polyphenols and 11 phenolic compounds in non-AM plants, being more severe under high saline conditions. In

eight-week mycorrhizal plants, moderate salinity also depressed the content of total polyphenols and eight phenolic substances, but the descending trend was alleviated in most bioactive compounds under high saline conditions. The level of few metabolites such as ferulic acid, 4-*O*-caffeoylquinic acid, and luteolin-glucoside was even profoundly enhanced by 93.7, 204, and 74 %, respectively, in AM plants exposed to high salinity relative to non-stress AM plants. Noticeably, after eight weeks of growth in the presence of 200 mM NaCl, the concentrations of all phenolic compounds were sharply inclined in mycorrhizal plants in relation to the counterparts of non-AM plants, except dimethyl wedelolactone. The highest and lowest increase induced by AMF were 4-*O*-caffeoylquinic acid (more than ten folds) and luteolin-glucoside (160 %), respectively.

Interestingly, significant changes in the content of phenolic compounds in non-AM and AM plants were observed over time. Under non-stress conditions, there were substantial increases in the content of most polyphenols in AM (eight phenolics) and non-AM plants (ten phenolics) at eight weeks versus their levels in the corresponding plants at four weeks. Considerable decreases in the content of seven individual phenolics were found in uncolonized plants treated with 100 mM NaCl eight weeks after growth versus those four weeks after growth. By contrast, substantial inclines in the concentration of three polyphenols, while a dramatic decrement in luteolin level (73 %) were detected in colonized plants exposed to moderate salinity at eight weeks relative to counterparts of those at four weeks. A significant augmentation in the level of two polyphenols whilst remarkable declines in four phenolics concentrations were found in uncolonized plants subjected to 200 mM NaCl at eight weeks versus four weeks. Contrariwise, pronounced increases in the concentration of all phenolic compounds were recorded in colonized plants exposed to high salinity at the early stage of plant growth relative to counterparts of those at the later stage.

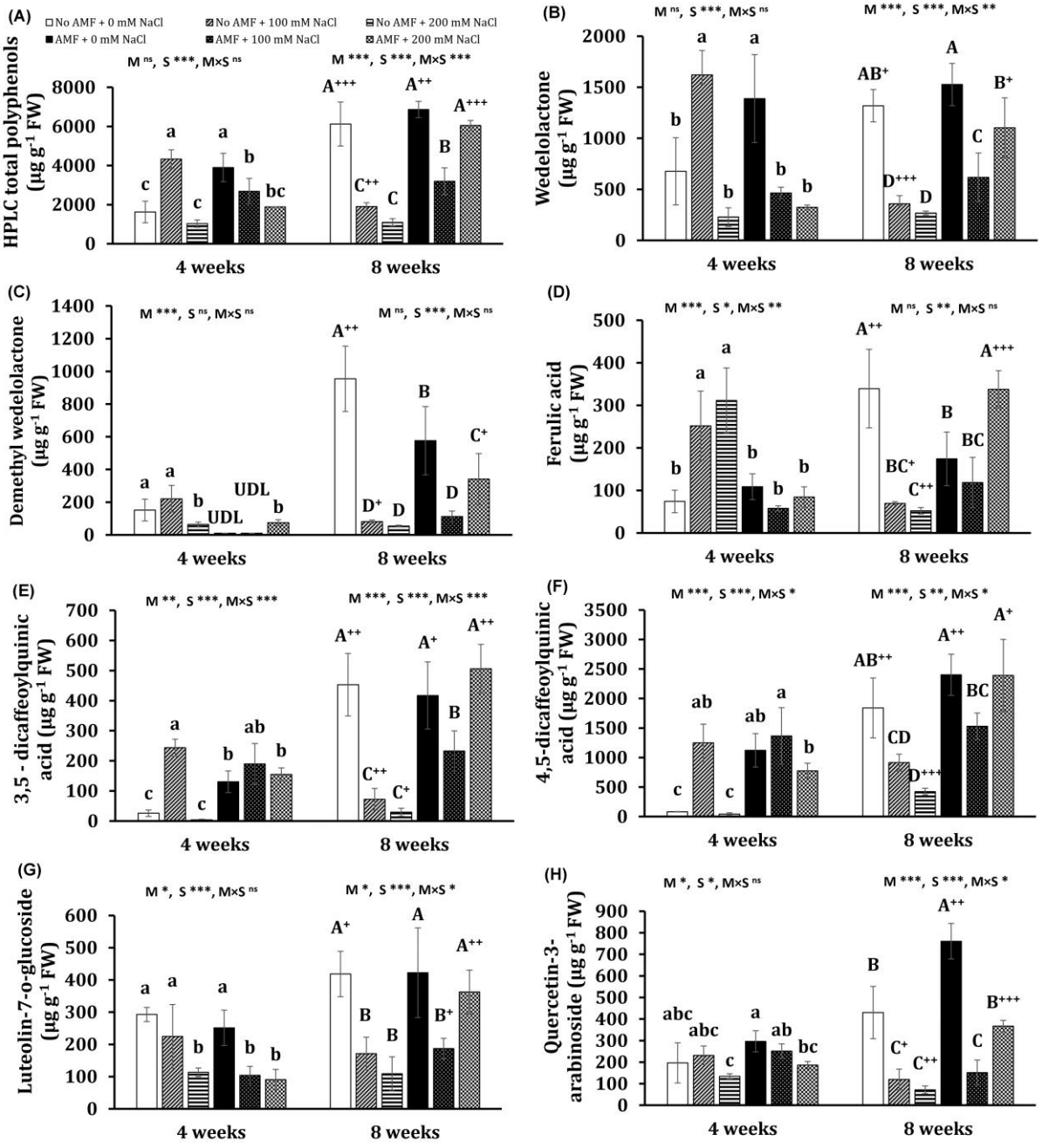

Figure 23. Contents of HPLC total polyphenols (A) and major polyphenols: wedelolactone (B), demethyl wedelolactone (C), ferulic acid (D), 3,5-dicaffeoylquinic acid (E), 4,5-dicaffeoylquinic acid (F), luteolin-7-O-glucoside (G), and quercetin-3-arabinoside (H) in leaves of *E. p* inoculated with arbuscular mycorrhiza or not inoculated under non-stress, moderate (100 mM NaCl), and high saline (200 mM NaCl) conditions four and eight weeks after inoculation. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments four and eight weeks after inoculation, respectively, according to the Duncan test (P<0.05). +, ++, +++ indicate significant differences between the same treatments four weeks and eight weeks after inoculation at P<0.05, P<0.01, and P<0.001, respectively, according to the two-tailed test. ns, non-significant. *, **, ***, significant

differences at *P*< 0.05, 0.01, 0.001. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M×S, the interaction between mycorrhizal inoculation and salt stress. UDL, under the detection limit.

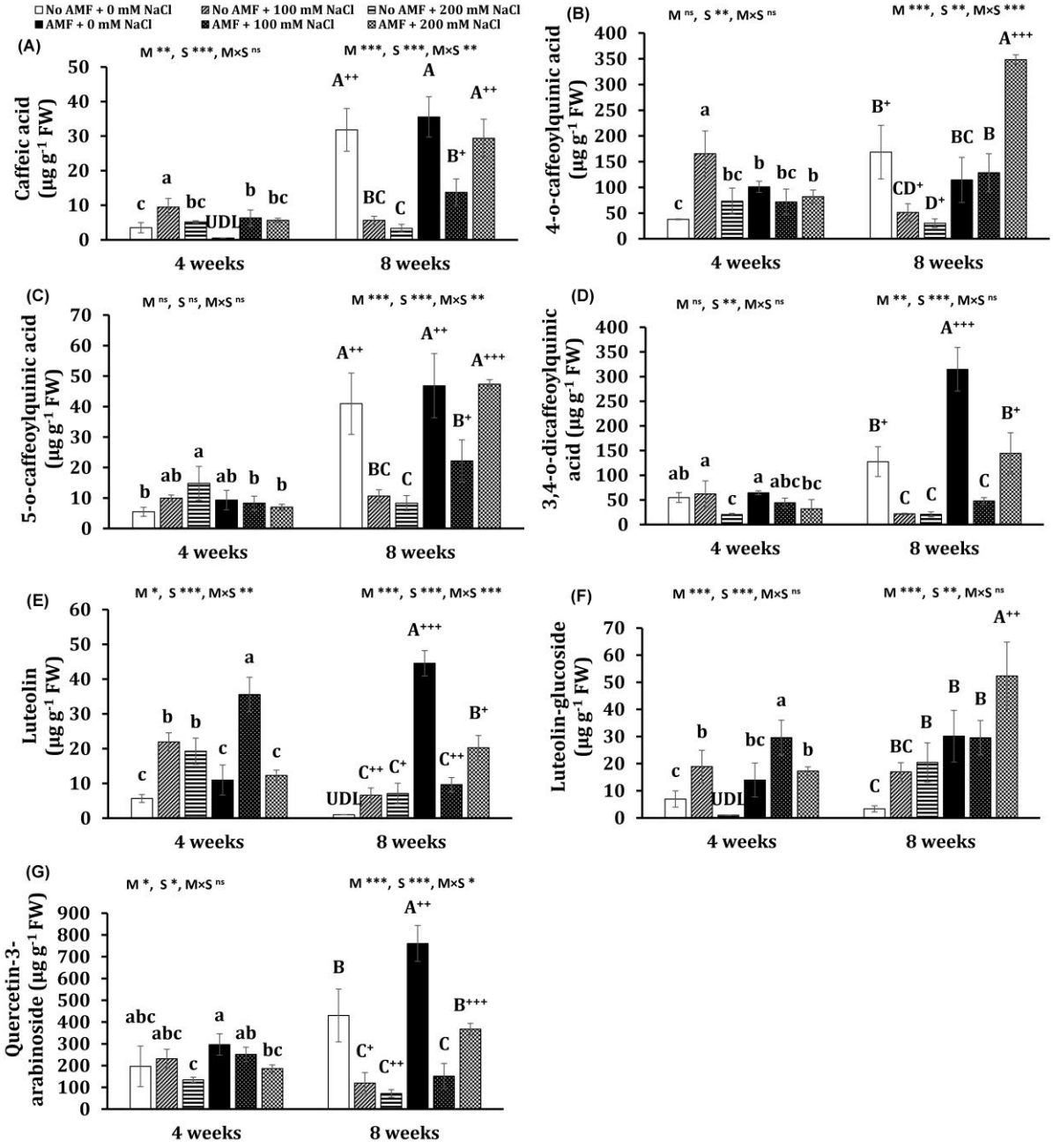

Figure 24. Contents of polyphenols: caffeic acid (A), 4-O-caffeoylquinic acid (B), 5-O-caffeoylquinic acid (C), 3,4-O-dicaffeoylquinic acid (D), feruloylquinic acid (E), luteolin (F), luteolin-glucoside (G) in leaves of *E. p* inoculated with arbuscular mycorrhiza or not inoculated under non-stress, moderate (100 mM NaCl), and high saline (200 mM NaCl) conditions four and eight weeks after inoculation. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments four and eight weeks after inoculation,

respectively, according to the Duncan test (P<0.05). +, ++, +++ indicate significant differences between the same treatments four and eight weeks after inoculation at P<0.05, P<0.01, and P<0.001, respectively, according to the two-tailed test. ns, non-significant. *, **, ***, significant differences at P< 0.05, 0.01, 0.001. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M×S, the interaction between mycorrhizal inoculation and salt stress. UDL, under the detection limit.

4.2.6 Principal component analysis (PCA) of individual polyphenols

Principal component analyses of individual polyphenols were performed, independently for each harvest time, to correlate variables determined under different conditions at four and eight weeks. The results demonstrated that 55.6 and 80.8 % of the total variation were explained by the first two principal components (PC1 and PC2) at four and eight weeks, respectively (Figure 25). After four weeks of growth, 33.6 % of the total variation was covered by the PC1, which had strong positive associations mainly with wedelolactone, 4,5-dicaffeoylquinic acid, quercetin-3-arabinoside, 4-*O*-caffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, and luteolin-glucoside. PC2, covering 21.9 %, was contributed primarily by caffeic acid (positive association) and feruloylquinic acid (negative association). In the next stage of plant growth (8 weeks), as much as 67.5 % of the total variation was covered by the PC1, which was positively influenced by all phenolic compounds (13 individual polyphenols with luteolin and luteolin-glucoside having fewer impacts). PC2 explaining 13.3 % of the total variance is positively influenced mainly by luteolin-glucoside and luteolin but negatively impacted principally by demethyl wedelolactone. At four weeks, high positive correlations between ferulic acid and 5-*O*-caffeoylquinic acid, demethyl wedelolactone and caffeic acid, 4-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and luteolin-glucoside, luteolin-glucoside and 4,5-*O*-dicaffeoylquinic acid, quercetin-3-arabinoside and 3,4-*O*-dicaffeoylquinic acid, and luteolin-7-*O*-glucoside and feruloylquinic acid could be seen, whereas there were negative associations between feruloylquinic acid/luteolin-7-*O*-glucoside and ferulic acid/5-*O*-caffeoylquinic acid (Figure 25A). At eight weeks, there were robust positive correlations between 4-*O*-caffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid, 5-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and quercetin-3-arabinoside, and wedelolactone and luteolin-7-*O*-glucoside (Figure 25B). The biplot also demonstrated a relatively clear discrimination among the groups of the control treatment (C_0) and mycorrhizal treatment (A_0) under non-saline conditions, the control treatment under 200 mM NaCl (C_{200}), and the other group (A_{100} + A_{200} + C_{100}) at four weeks. Differences among C_{200}, C_0, and

A_0 groups were distinguished by PC1, while PC2 discriminated between the salinity stresses and non-saline groups at 4 weeks. Four different clusters—C_0, A_{100} (AM inoculation under 100 mM NaCl), $A_0 + A_{200}$ (AM treatment under non-stress and high salt stress conditions), and $C_{100} + C_{200}$ (control treatment in the presence of 100 and 200 mM NaCl) were recognized at 8 weeks. Obviously, AM inoculation under non-saline and high saline conditions influencing individual polyphenols was different from the other groups at the later stage of plant growth.

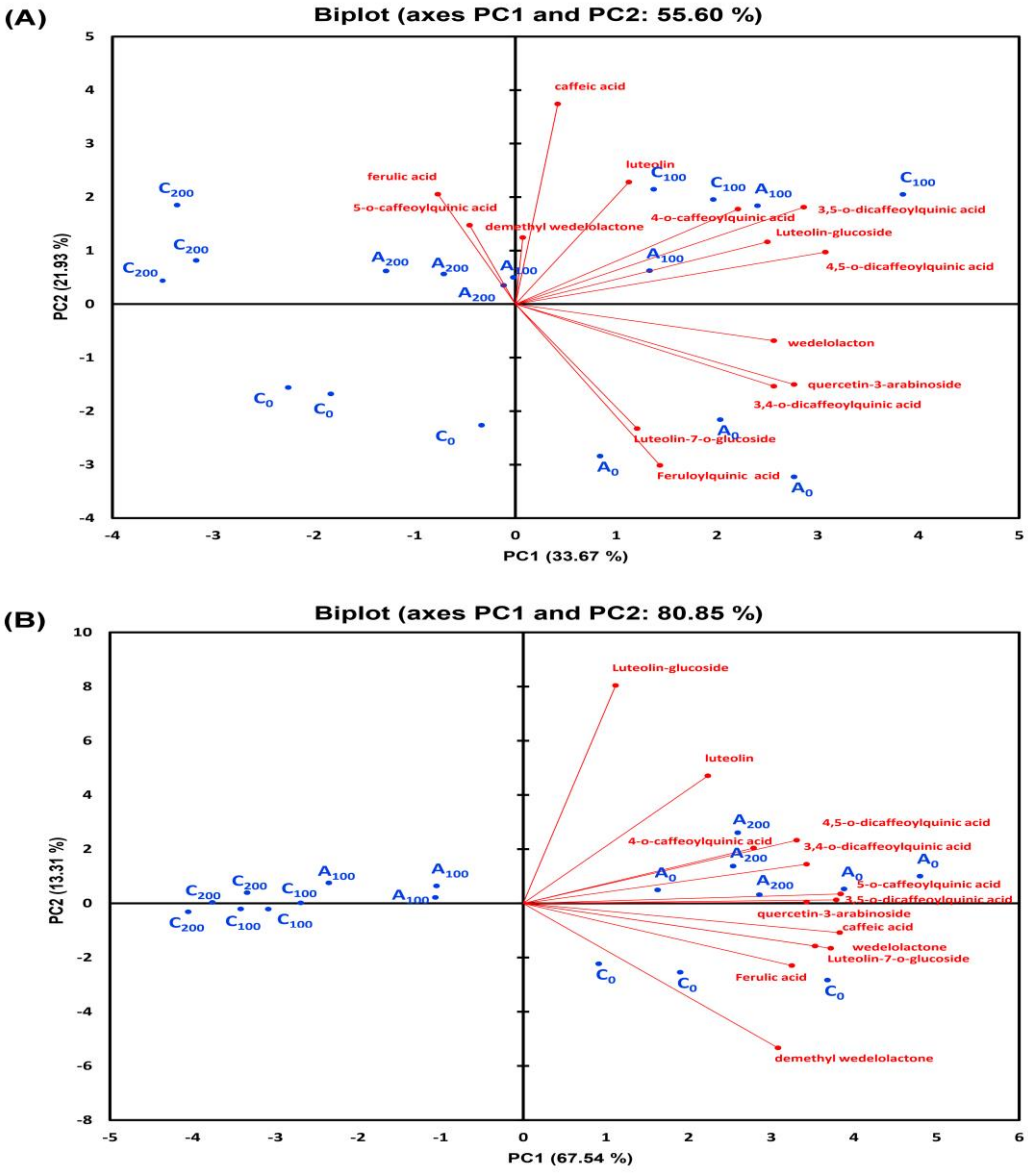

Figure 25. Principal component analysis of individual polyphoenols in leaves of *E. p* inoculated with arbuscular mycorrhiza or not inoculated under non-stress, saline conditions four weeks (A) and eight weeks (B) after inoculation. C0: control treatment (plants without mycorrhiza) under non-saline conditions, C_{100}: control treatment exposed to 100 mM NaCl, C_{200}: control treatment exposed to 200 mM NaCl, A0: mycorrhizal treatment under non-saline conditions, A_{100}: mycorrhizal treatment exposed to 100 mM NaCl, A_{200}: mycorrhizal treatment exposed to 200 mM NaCl.

4.2.7 Discussion in the effect of AMF on growth, physico-chemical parameters, and polyphenols alteration in *E. p* under salt stress

It is well documented that AMF affect directly or indirectly the growth and performance of various plant species under salinity stress (Ait-El-Mokhtar et al., 2020; Amanifar and Toghranegar, 2020; Santander et al., 2019), responses of *E. p* inoculated with arbuscular mycorrhizal fungi to salt stress, particularly in terms of phytochemical constituents, have not been investigated before. In the current work, under high saline level (200 mM NaCl), the percentage of mycorrhizal colonization was markedly declined after four weeks of plant growth, but the decrease was unnoticeable at the eight weeks of plant growth (at later stage). The negative impact of salt stress on AM colonization capacity at the beginning stage of plant growth could be explained by the direct inhibitory effect of NaCl on extraradical hyphal growth, sporulation and spore germination (Garg and Chandel, 2015), afterwards, AMF may tolerate and adapt to such level of salt at eight weeks of plant growth. Earlier investigations reported that salt stress reduced mycorrhizal colonization rate though it was based on AMF isolates and plant species (Santander et al., 2019; Wang et al., 2019).

As a result of salt stress, high osmotic potential and ionic imbalances affect negatively plant growth, due to the perturbation in normal metabolism, water, and nutrient uptake (Santander et al., 2017). Under salinity stress, the assessment of plant biomass represents the direct characteristic on symbiosis-mediated plant performance. AM inoculation has been proven to enhance the growth features of different plant species subjected to saline conditions, such as lettuce (Santander et al., 2019), date palm (*Phoenix dactylifera* L.) (Ait-El-Mokhtar et al., 2020), Alfalfa (Shi-chu et al., 2019) and medicinal plant *Valeriana officinalis* (L.) (Amanifar and Toghranegar, 2020). These results support that the beneficial effects of AM application on the growth parameters of host plants under salt stress are in accordance with our findings under moderate salinity (increased fresh shoot weight, leaf number, and leaf area). An array of strategies have been recruited to explain the higher

plant growth and development under salinity, such as improved nutrient uptake, net photosynthetic rate, stomatal conductance, relative water content, and osmoprotection; enhanced antioxidative enzymes; and maintenance of ionic homeostasis (Evelin et al., 2019). Furthermore, fungal symbiosis was shown to diminish the mobilization of NA^+ to prevent their excess levels to plant tissues by their retain probably into intraradical hyphae (Giri et al., 2007; Rivero et al., 2018). A substantial decrement in Na^+ translocation from roots to shoots was observed in colonized plants under salinity (Moreira et al., 2020), which may participate to the higher plant growth. However, the AM advantageous not obvious under high salt conditions (200 mM NaCl), this failure of plant growth might be in consequence to nutritional imbalance, excessive uptake of sodium (Na^+) and chloride (Cl^-) ions, and the over production of ROS (Isayenkov and Maathuis, 2019), the AM benefits have been also found linked to the intensity of salt stress. Moreover, our results proposed that *E. p* inoculated by AM fungi could be cultivated in agricultural areas with saline irrigation below 100 mM NaCl (EC = 10 dS m^{-1}) and/or slightly and moderately saline soils (EC of the saturation extract from 4 to 8 dS m^{-1}).

The osmolytes accumulation such as proline have a great role to mediate salt stress tolerance in plants, stabilizing cellular structures and membranes (Maswada et al., 2018; Meena et al., 2019). Our current study revealed that under moderate salt stress, proline production was stimulated by the fungal symbiosis during plant growth, in accordance with the report of Santander et al.(2019), highlighting its contribution to the reduction of oxidative damage. Moreover, there are a conflicting results, which Amanifar and Toghranegar, (2020) illustrated that under salt stresses, a lower proline level was found in leaves of medicinal plants *Valeriana officinalis* inoculated by either *R. irregularis* or *F. mosseae* while its higher concentration was recorded in roots treated by *F. mosseae*. It might be refer to the various functions of this free amino acid in belowground and aboveground parts of the plant (Kang et al., 2019). also may indicate mitigation of the stress (e.g., maintaining the ratio of K^+/ Na^+) upstream of proline synthesis (Evelin et al., 2019). The increase of proline level in our results may be linked with low oxidative damage in moderate-salted mycorrhizal plants. In fact, proline serves as an osmoprotectant and effective ROS scavenger, thus decreasing ROS damage as shown by the findings in this study.

As a fundamental structures of the photosynthetic apparatus, photosystem (PS) I and II are liable to saline conditions. Wang et al. (2019) reported that salt stress can affect negatively the process of photosynthesis through the demolish of the reaction center of PS II and disturbing the electron

transport from PS II to PS I. Our results demonstrate that salt stress was not affected the maximal photochemical efficiency of PS II (Fv/Fm), indicating that salinity did not impair the photosynthetic system under the experimental conditions. Besides, AMF did augment Fv/Fm under non-stress conditions but not under both salt stresses. Contradictory to the findings of Wang et al. (2019). The reasons may be accountable to differences in growth conditions, stress treatments, stress duration, as well as specific interaction between fungal and host plant, as found in earlier investigations (Amanifar and Toghranegar, 2020; Duc et al., 2018).

In order to avoid the oxidative stress caused by the generation of reactive oxygen species (ROS), plants upregulate the activities of antioxidant systems where SOD, POD, and CAT are important enzymes responsible for rapid scavenging of the harmful ROS. In the present study, under moderate salt stress, AMF highly stimulated the activity of POD at four weeks and CAT at four and eight weeks in *E. p* plants, while mycorrhizal application did not change the activity of other enzymes at both times of measurement. The reason could be explained by that POD and CAT were two fundamental antioxidative enzymes in mycorrhizal *E. p* plants to mitigate the oxidative stress caused by moderate saline conditions. By contrast, the fungal symbiosis even dropped POD activity at eight weeks, whereas did not change SOD, CAT activity in response to high salt stress. SOD functions as the first defense line to deal with ROS, catalyzing the dismutation of superoxide radical (O_2^-) or singlet oxygen ($^1O_2$) to H_2O_2 and O_2 (Mittler, 2002). H_2O_2 is a potentially destructive subproduct of oxygen metabolism and is scavenged from cell compartments via CAT and peroxidases (Mittler, 2002). Interestingly, Mayer et al. (2019) demonstrated that the defense enzymes induced by AMF varied with plant age, which is an agreement with our observation. Similarly, strengthened POD and CAT activity were reported in other plants inoculated with AMF under salinity conditions (Ait-El-Mokhtar et al., 2019; Santander et al., 2019).

Phenolic substances, the most pronounced secondary metabolites present in plants, play a crucial role in the formation of various biomolecules protecting plants against stresses (Saxena et al., 2015). Enhancing the phenolic content may contribute to osmoregulation, ROS protection, or the general defense systems of salt-stressed plants (Alqarawi et al., 2014). In the present study, and under moderate salt stress, The HPLC analysis demonstrated that total polyphenol content was increased at four weeks but declined at eight weeks in non-AM plants. Conversely, in mycorrhizal plants, the total phenolics did not changed at the first stage of plant growth under 100 mM NaCl. But was diminished at the later stage as compared with that of non-stress colonized plants. Moreover, the

decrement was alleviated in AM plants relative to the counterpart non-AM plants in moderate salt stress at 8 weeks. This might be linked to different mechanisms between non-AM and AM plants subjected to moderate salinity during the growth stage in the activation of phenolic production to diminish oxidative damage caused by ROS. Under sever salt stress (200 mM NaCl), total phenolic content in AM plants was significantly higher than that of non-AM plants, especially 8 weeks after inoculation; however, this response was not effective in detoxifying ROS in colonized plants as no higher biomass in mycorrhizal plants subjected to high salinity were found. Increased total phenolic level has been found in salt-stressed *Valeriana officinalis* plants and *Ephedra aphylla* plants due to mycorrhization (Amanifar and Toghranegar, 2020). Phenolics are produced in the shikimic acid pathway in plants using carbohydrate precursors (Lin et al., 2016), thus improved carbohydrate metabolism in colonized plants could uplift the biosynthesis of phenolic substances and/or supply energy source for the fungal symbiosis (Pedone-Bonfim et al., 2018). The interactive effects of AMF and salt stress on bioactive compounds of *E. p* plants have not been investigated before. Polyphenols have been identified to have antioxidant properties that incorporates into plant defenses against oxidative stress, as well as providing human health benefits such as antioxidants, antimicrobial activities, antihypertensive, anti-inflammatory, cardioprotective, anti-allergic, anti-arthritic, and anti-carcinogenic (Lin et al., 2016). In the last few years, many studies have been focusing on antioxidant polyphenols because of restrictions on the use of synthetic antioxidants and enhanced public awareness of health-related issues (Bhuyan and Basu, 2017). In our previous study, nine major polyphenols were identified and measured in *E. p* plants with wedelolactone and dimethyl wedelolactone were abundant in all treatments (Vo et al., 2019). Noticeably, in the present study, we extended identifications to 14 individual phenolics. The findings showed that wedelolactone, an important phenolic compound to prevent inflammatory diseases and cancer in human (Sarveswaran et al., 2012), was one of the two most main components of phenolic compounds in plants during growth stages under different conditions, which is in line with the earlier results (Vo et al., 2019). Also, wedelolactone, recognized to have an hepatoprotective activity, that decrease liver inflammation and hypatocytes apoptosis, attenuated leukocyte infiltration and T-cell activation in concanavalinA-induced liver injury in mice (Luo et al., 2018). The difference in the second main constituent of polyphenols (4,5-dicaffeoylquinic acid in the present experiment versus demethyl wedelolactone in the previous study) may be explained by to the different substrate volume we applied. Our previous findings showing that phenolics were altered by AMF (Vo et al., 2019), which is in line with our present results that under non-stress

conditions, AM colonization considerably influenced the content of six polyphenols in plants during different growth stages. The reasons may be linked to the mechanisms underlying AMF plant interaction during mycorrhization. Adolfsson et al. (2017), reported that AM colonization may induce a secondary metabolism response in the leaves and enhance abscisic acid biosynthesis and flavonoid and terpenoid biosynthesis regulated by jasmonate in the leaves.

Moreover, the modifications in the global metabolic such as the majority of sugars, organic acids, amino acids, fatty acids, and phenolic acids in mycorrhizal shoots leads to the alteration in the accumulation of phenolic compound (Saleh et al., 2020). Therefore, changes in carbohydrate metabolism in colonized plants may affect the biosynthesis of phenolic substances (Pedone-Bonfim et al., 2018). Interestingly, the AMF-induced changes in polyphenol profiles at both stages of plant growth in this work, contradictory to our previous one, in consequence to differences in plant age (4, 8, and 7 weeks) and substrate volume. The content of total and individual phenolic substances in AM and non-AM plants (8 versus 4 weeks) was also affected by plant age. In fact, many biological factors, including developmental ones, contribute to the accumulation of secondary metabolites in plants (Broun et al., 2006). Furthermore, the biosynthesis of secondary metabolites and their storage were affected by the development factors, in which alter the initiation and consequent differentiation of cellular structures related to their (Broun et al., 2006). Notably, developmental stages of the plant impact the expression pattern of biosynthetic genes of secondary metabolites (Sharma, 2018), which could explain the changes in the content of phenolic constituents during plant growth in this study. Salt stress stimulates phenolic compound accumulation in plants as a defense mechanism to stress (Parvaiz and Satyawati, 2008). Therefore, this abiotic stress led to a positive outcome in term of enhancing the production of secondary metabolite of many herbs (Behdad et al., 2020; Bistgani et al., 2019; Boughalleb et al., 2020).

In this study, salinity had the trend toward increasing and remaining phenolic compounds unchanged in non-AM plants under moderate (100 mM NaCl) and sever salt stress (200 mM NaCl), respectively, at the first stage of growth but substantial decreased them at the later stage. In contrast, the decline in polyphenols caused by salt treatments was observed in mycorrhizal plants during the growth stage, with the mitigation at the later growth stage. Different behaviors in individual phenolics accumulation between non-AM and AM plants under saline conditions may result from the difference in the biochemical and physiological status in the host due to mycorrhization and AM benefits. On top of that, mycorrhizal inoculation caused changes in the content of many tested

secondary metabolites of *E. p* plants under both salinity levels at the early stage of plant growth. Noticeably, at the later growth stage, AMF enhanced all phenolic components in the host plants under high salt stress (200 mM NaCl). Furthermore, as noted by Rivero et al. (2018), in response to salt stress, various compounds with antistress properties differentially accumulated in mycorrhizal roots. The fungal symbiont also influenced the age-related changes in the leaf metabolome and partially halted senescence in the leaves, as a consequence a better metabolite accumulation (Shtark et al., 2019). Taken altogether, the metabolic alterations associated with AMF tend to be the reason for the direct impact on polyphenol profiles of *E. p* plant under saline conditions during growth stages. A conflicting observations regarding the influence of AMF on the accumulation of phenolic compound under salt stress. A noticeable lower phenolics was found in leaves of two lettuce cultivars colonized by AMF under salt stresses (Santander et al., 2019). In contrast to other studies, that demonstrated a significant incline in phenolic substances in AM plants (Amanifar and Toghranegar, 2020; Hashem et al., 2018). However, most of the earlier studies only investigated polyphenol profiles at one harvest time. In this work, both plant age and stress intensity have a different trend (increase and decrease) in phenolic compounds under salt stress. It may be owing to the fact that the secondary metabolic pathways and their regulation are incredibly susceptible to environmental factors and growth stages; in which the expression of genes involved in their pathways or their encoded protein activities are susceptible and changed at different plant ages and/or in the presence of various stresses (Li et al., 2020; Sharma, 2018).

4.3. Mycorrhizal tomato plant tolerance to combined drought and heat stress

Plant Growth and Mycorrhizal Colonization

4.3.1 Root colonization and plant growth

Under no stress conditions, a similar trend was observed on fresh plant weight for both AM and non-AM plants. Moreover, both mycorrhiza and stress application significantly affected fresh plant biomass, and the interaction between them was also significant (Table 3).

Table 3. Effects of arbuscular mycorrhizal (AM) inoculation (M), stress application (S), and the interaction between (M) and (S) on the measured paramaters. Fresh shoot biomass; H_2O_2, hydrogen peroxide; MDA, malondialdehyde, POD, peroxidase; CAT, catalase; PPO, polyphenol oxidase; GST, glutathione-S-transferase

Parameters	Variables	Mean Squares	F-Value	D_f	*p*-Value
Fresh shoot biomass	AM inoculation (M)	4.548	7.42	3	** 0.0006
	Stress application (S)	131.045	213.85	2	*** <0.0001
	M*S	3.819	6.23	6	** 0.0002
Leaf H_2O_2 accumulation	AM inoculation (M)	4.991	18.09	3	*** <0.0001
	Stress application (S)	114.779	415.85	2	*** <0.0001
	M*S	2.26	8.19	6	*** <0.0001
Root H_2O_2 accumulation	AM inoculation (M)	0.7	13.07	3	*** <0.0001
	Stress application (S)	3.894	72.67	2	*** <0.0001
	M*S	0.551	10.28	6	*** <0.0001
Leaf MDA	AM inoculation (M)	0.152	19.89	3	*** <0.0001
	Stress application (S)	1.081	140.73	2	*** <0.0001
	M*S	0.074	9.74	6	*** <0.0001
Root MDA	AM inoculation (M)	0.002	5.3413	3	** 0.0037

	Stress application (S)	0.012	30.1518	2	*** <0.0001
	M*S	0.002	5.7469	6	*** <0.0001
Leaf CAT	AM inoculation (M)	3752.957	12.44	3	*** <0.0001
	Stress application (S)	20,880.46	69.23	2	*** <0.0001
	M*S	1688.4	5.6	6	*** <0.0001
Root CAT	AM inoculation (M)	1951.394	12.46	3	*** <0.0001
	Stress application (S)	16,928.51	108.05	2	*** <0.0001
	M*S	1110.936	7.09	6	*** <0.0001
Leaf POD	AM inoculation (M)	346.791	15.76	3	*** <0.0001
	Stress application (S)	6526.13	296.5	2	*** <0.0001
	M*S	202.406	9.2	6	*** <0.0001
Root POD	AM inoculation (M)	2609.255	23.94	3	*** <0.0001
	Stress application (S)	19,807.52	181.7	2	*** <0.0001
	M*S	1465.06	13.44	6	*** <0.0001
Leaf PPO	AM inoculation (M)	0.003	6.96	3	*** <0.0001
	Stress application (S)	0.08	155.71	2	*** <0.0001
	M*S	0.001	2.21	6	* 0.0155
Root PPO	AM inoculation (M)	0.002	9.76	3	*** <0.0001
	Stress application (S)	0.083	297.48	2	*** <0.0001
	M*S	0.002	8.39	6	*** <0.0001
Leaf GST	AM inoculation (M)	0.054	3.59	3	** 0.0023

	Stress application (S)	0.244	16.02	2	*** <0.0001
	M*S	0.092	6.05	6	*** <0.0001
Root GST	AM inoculation (M)	0.043	2.62	3	* 0.0185
	Stress application (S)	0.52	31.37	2	*** <0.0001
	M*S	0.045	2.77	6	** 0.0026

*, **, and *** indicate significant differences at $P < 0.05$, 0.01, and 0.001, respectively.

The fresh shoot biomass was reduced under imposed stresses compared with the NoS condition (Table 4), with almost 32 % of all plants subjected to D + H, while it decreased by 29 % under D + HS for root colonized by *R. irregularis* and *F. coronatum*. In contrast, plants inoculated by *F. mosseae* did not change the biomass as compared with the corresponding non-stress condition.

The assessment of root colonization under microscopic examinations showed a good symbiotic association between different arbuscular mycorrhizal species and tomato plant roots. No mycorrhizal colonization could be detected in the roots of non-mycorrhizal plants. Moreover, no significant differences in the colonization rates of plant inoculated with three AMF inoculums were detected following various stress treatments. The highest percentage of mycorrhization reached 63.82 % in plants treated with *R. irregularis* under D + H and 55.84 % in plants inoculated with *F. mosseae* under D + HS.

Table 4. Fresh plant biomass and AM colonization rate in plants not inoculated or inoculated by *R. irregularis*, *F. mosseae*, or *F. coronatum* under no stress, drought+ heat stress, and drought+ heat shock after eight weeks of growth

Stress Condition	**AM Inoculation**	**Fresh Plant Biomass (g plant^{-1})**	**AM Colonization (%)**
No stress	No AM	17.21 ± 0.33 ^{Aa}	0
	R. irregularis	17.86 ± 0.84 ^{Aa}	58.87 ± 6.90
	F. mosseae	17.17 ± 1.41 ^{Aa}	47.27 ± 7.35
	F. coronatum	17.23 ± 1.37 ^{Aa}	48.01 ± 7.11

Drought + heat stress	No AM	11.38 ± 0.43 ^{Bb}	0
	R. irregularis	12.28 ± 0.66 ^{Ba}	63.82 ± 11.47
	F. mosseae	12.02 ± 0.89 ^{Ba}	53.16 ± 6.44
	F. coronatum	11.79 ± 0.59 ^{Bba}	48.36 ± 9.07
Drought + heat shock	No AM	12.31 ± 0.34 ^{Cb}	0
	R. irregularis	12.75 ± 1.12 ^{Bb}	52.09 ± 9.45
	F. mosseae	15.72 ± 0.46 ^{Aa}	55.84 ± 8.14
	F. coronatum	12.18 ± 0.47 ^{Bb}	51.03 ± 7.93

AM, arbuscular mycorrhizal. No AM, without arbuscular mycorrhiza. For each parameter, the means ± standard deviations are presented (n = 4). Different minuscule within a column indicates significant differences among treatments under the same conditions (no stress, drought + heat stress, or drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$. Different capital letters within a column indicate significant differences of the same treatments (No AM, *R. irregularis, F. mosseae,* or *F. coronatum*) under different conditions (no stress, drought + heat stress, and drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$.

4.3.2 Accumulation of Hydrogen Peroxide and Lipid Peroxidation

D + H and D + HS significantly increased the generation of H_2O_2 in tomato plants, leading to considerable oxidative damage, which can be measured as malondialdehyde (MDA) content. Under NoS condition, no significant difference was observed in the H_2O_2 amount for tomato leaves: No AM = 3.06 ± 0.46 nmol g^{-1} FW, *R. irregularis* = 2.38 ± 0.48 nmol g^{-1} FW, *F. mosseae* = 2.39 ± 0.40 nmol g^{-1} FW, and *F. coronatum* = 2.96 ± 0.70 nmol g^{-1} FW (Figure. 26A). However, significant increases in leaf and root H_2O_2 content were detected in plants without AM inoculation only under stress conditions (D + H, D + HS). AM treatment significantly ($P < 0.0001$) reduced H_2O_2 concentration compared with that of non-AM plants (Table 2, Figure 27). In detail, plants inoculated with *F. mosseae* showed a considerably lower H_2O_2 accumulation in leaves under D + H stress relative to non-mycorrhizal ones (reduced by 35 %), for plants inoculated by both *R. irregularis* and *F. coronatum*, where the H_2O_2 decreased by 20 %. Under D + HS, no significant differences in H_2O_2 levels were observed among the three AMF species (*R. irregularis, F. mosseae,*

and *F. coronatum*), where AMF species reduced H_2O_2 levels in leaves by almost 23 % compared with no AMF under D + HS.

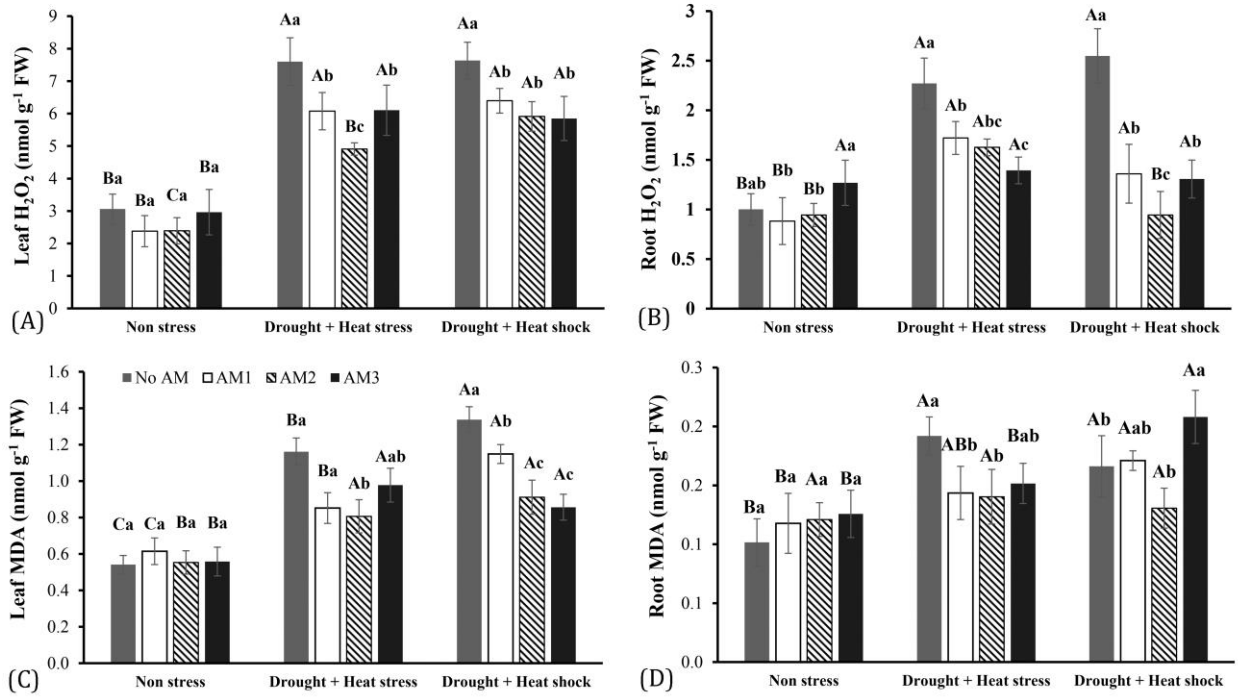

Figure 26. Hydrogen peroxide (H_2O_2) and malonaldehyde (MDA) accumulation in leaves (A,C) and roots (B,D) of non-inoculated plants (No arbuscular mycorrhizal (AM) and plants inoculated by R. irregularis (AM1), F. mosseae (AM2), or F. coronatum (AM3) subjected to non-stress (NoS), drought + heat stress, and drought + heat shock. Each bar represents the mean and standard deviation (n = 4). Different minuscules indicate significant difference among treatments under the same conditions (no stress, drought + heat stress, or drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$. Different capital letters indicate significant differences of the same treatments (No AM, *R. irregularis, F. mosseae,* or *F. coronatum*) under different conditions (no stress, drought + heat stress, and drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$.

In roots, there were significant ($P < 0.0001$) main effects of AM inoculation, stress application, and their interaction on root H_2O_2 accumulation (Table 3). The level of hydrogen peroxide was significantly higher in non-AM plants than in AM plants (increased by almost 61 %) under imposed stresses (D + H, D + HS) in comparison with no stress conditions and was significantly decreased in inoculated plants. In D + H, H_2O_2 levels were decreased by 24 %, 29 %, and 39 % in plants inoculated by *R. irregularis, F. mosseae, and F. coronatum*, respectively. Remarkably, under D + HS, plants inoculated with *F. mosseae* exhibited substantially reduced H_2O_2 accumulation by 63 %

compared with the non-inoculated plants and by 47 % and 49 % in plants treated by *R. irregularis* and *F. coronatum*, respectively.

Although under NoS, leaf MDA content did not change significantly, in both non-AM plants and AM plants, MDA increased as stress treatments were applied. AMF treatment significantly decreased MDA content compared with non-AM plants. Under the D + H stress, MDA content decreased by 27 %, 31 %, and 16 % in leaves, while the decreases were 14 %, 32 %, and 36 % under D + HS in *R. irregularis, F. mosseae,* and *F. coronatum*, respectively. In roots, AM plants showed a significant decrease in MDA levels for roots colonized *by R. irregularis, F. mosseae,* and *F. coronatum* by 25 %, 27 %, and 21 %, respectively, under D + H compared with the corresponding uninoculated plants. For D + HS plants, an increase in MDA level was observed in roots inoculated by *F. coronatum* (by 22 % compared with that of non-AM roots), while no significant differences among other treatments were found.

A significant positive correlation between leaf H_2O_2 and leaf MDA was found under D + H (r = 0.64 **), as well as between leaf H_2O_2 and root MDA (r = 0.61 *) (Table 6), while under D + HS, a substantial positive correlation between leaf H_2O_2 and leaf MDA (r = 0.89 ***) and leaf MDA and root H_2O_2 (r = 0.70 **) was also observed (Table 7).

Under different stress applications, the accumulation of H_2O_2 and MDA was higher in the leaves than in roots. However, H_2O_2 and MDA concentrations in the leaves and roots of AMF plants were lower than those of non-AM plants under combined stresses.

4.3.3 Defense Enzyme Activities

In this experiment, CAT, PPO, POD, and GST were selected, because these enzymes are known to be involved in ROS scavenging. Their activity was determined in shoots and roots of all plants for both inoculated and non-inoculated ones. Defense enzyme activity (CAT, PPO, POD, and GST) was stimulated and increased in both leaves and roots when they were exposed to the studied combined stresses. However, there was a slight difference between the inoculated and non-inoculated plants because the activity was significantly higher in mycorrhiza treated tomato plants.

No significant difference was observed in leaf POD activity among AM and non-AM plants under no stress conditions (Figure 27). POD activity significantly increased by 26 %, 38 %, and 60 % in plants treated with *R. irregularis, F. mosseae,* and *F. coronatum*, respectively, under D + H stress

compared with the corresponding non-AM plants. Furthermore, plants inoculated with *F. mosseae* and *F. coronatum* subjected to D + HS exhibited an additional boost in the activity of POD, where it increased by 86 % and 102 %, respectively, as compared with non-AM ones. In contrast, leaf POD drastically decreased (by 43 %) in plants colonized by *R. irregularis*, approaching the level found in non-stressed plants.

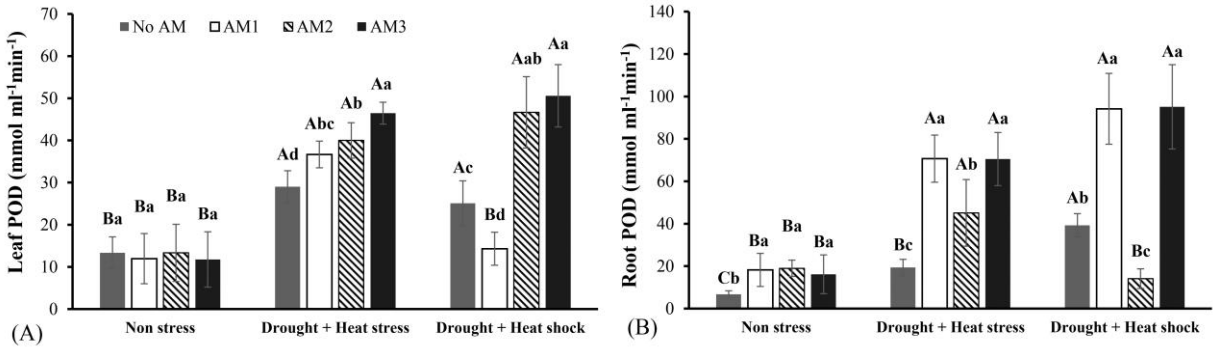

Figure 27. The activity of peroxidase (POD) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought + heat shock (D + HS). Each bar represents the mean and standard deviation (n = 4). Different minuscules indicate significant differences among treatments under the same conditions (no stress, drought + heat stress, or drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$. Different capital letters indicate significant differences of the same treatments (No AM, *R. irregularis*, *F. mosseae*, or *F. coronatum*) under different conditions (no stress, drought + heat stress, and drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$

In the case of roots, there were significant ($P < 0.0001$) main effects of AM inoculation and stress applications on POD activity, and an interaction between the two main factors (Table 3). Root POD activity appeared to be consistently increased in plants under both combined stress conditions compared with that of non-stress plants (Figure 27B). POD activity was strongly enhanced in roots colonized with *R. irregularis* and *F. coronatum* under D + H (increased by 268 %) and D + HS (increased by 141 % and 143 %, respectively) as compared with non-AM plants, while plants inoculated with *F. mosseae* showed a decrease in root POD activity by 64 % under D + HS (analysis of variance (ANOVA) results are shown in Table 3).

There were significant main effects of AM inoculation and stress imposed ($p < 0.0001$) and their interaction. The imposed stresses substantially induced leaf PPO activity in plants compared with non-stress conditions (Figure 28A). The data showed no significant differences among control, *R. irregularis, F. mosseae*, and *F. coronatum* under both D + H and D + HS (Figure 28A). In roots,

under D + H, PPO activity was increased by 43 % and 64 % in plants treated by *R. irregularis* and *F. coronatum*, respectively, compared with that in non-AM plants (Figure 28B), whereas it decreased by 24 % in plants inoculated with *F. mosseae*. Moreover, under D + HS, the highest root PPO activity (increased by 30 %) was in roots colonized by *R. irregularis*, while it decreased by 40 and 24 % in plants inoculated with *F. mosseae* and *F. coronatum*, respectively, compared with the corresponding non-AM plants.

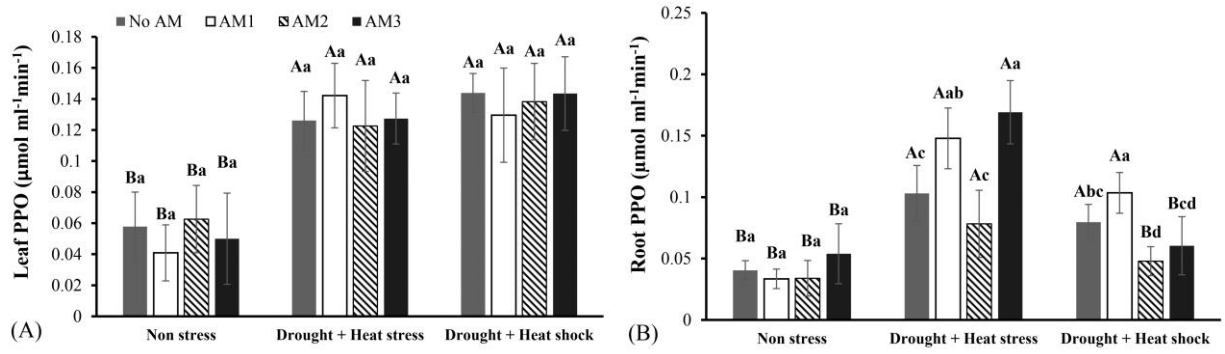

Figure 28. The activity of polyphenol oxidase (PPO) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought + heat shock (D + HS). Each bar represents the mean and standard deviation (n = 4). Different minuscules indicate significant differences among treatments under the same conditions (no stress, drought + heat stress, or drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$. Different capital letters indicate significant differences of the same treatments (No AM, *R. irregularis, F. mosseae,* or *F. coronatum*) under different conditions (no stress, drought + heat stress, and drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$.

No significant difference among treatments was observed under both no stress condition and D + H, as compared with the corresponding non-AM plants. Moreover, under D + HS, *R. irregularis* and *F. coronatum* enhanced leaf CAT activity by 42 % and 57 %, respectively, in plants as compared with that of uncolonized plants (Figure 29A). In roots, significantly higher activity of CAT was observed in plants exposed to both stresses, compared with that of plants under no stress conditions. Under D + H stress, no significant differences in CAT activity were observed among treatments (Figure 29B), while a higher CAT activity (increased by 30 %) was observed in plants inoculated with *F. mosseae* under D + HS, while plants inoculated with *R. irregularis* and *F. coronatum* increased root CAT activity by 4 and 11 %, respectively, as compared with non-AM plants. There were significant impacts of AMF inoculation and stress imposed ($P < 0.0001$) and their interaction (Table 3) on leaf CAT activity as well as on root CAT activity.

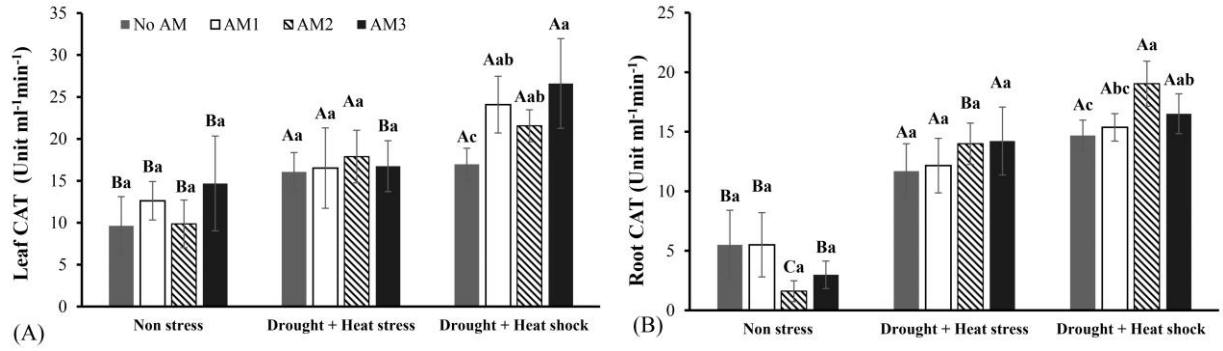

Figure 29. The activity of catalase (CAT) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought+ heat shock (D + HS). Each bar represents the mean and standard deviation (n = 4). Different minuscules indicate significant differences among treatments under the same conditions (no stress, drought + heat stress, or drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$. Different capital letters indicate significant differences of the same treatments (No AM, *R. irregularis, F. mosseae,* or *F. coronatum*) under different conditions (no stress, drought + heat stress, and drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$.

Leaf glutathione-S-transferase activity was significantly increased in plants inoculated with *F. mosseae* compared with that of non-AM plants under NoS conditions. Moreover, the same inoculant enhanced the GST activity (increased by 46 %) in colonized plants in comparison with uninoculated plants in D + H, while the same tendency was observed in roots colonized by *R. irregularis* and *F. coronatum* (Figure 30A). Under D + HS, no significant difference could be observed among non-AM plants and various AMF strains (*R. irregularis, F. mosseae,* and *F. coronatum*).

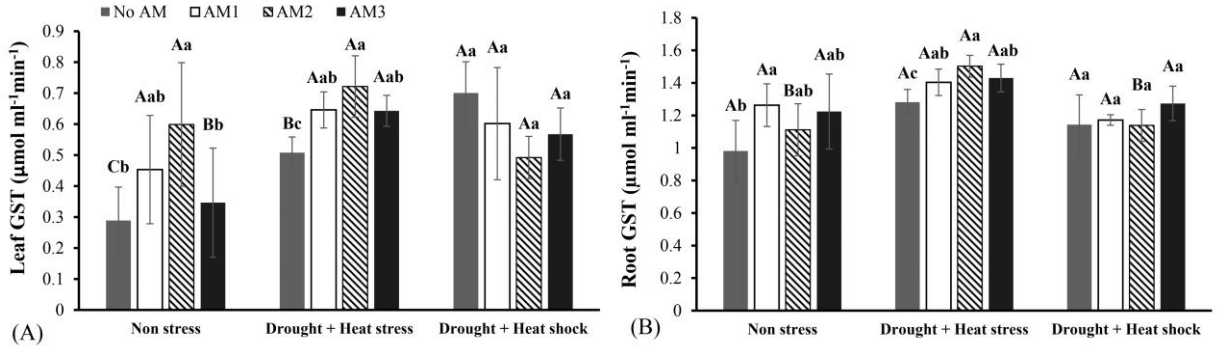

Figure 30. The activity of glutathione S transferase (GST) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought+ heat shock (D + HS). Each bar represents the mean and standard deviation (n = 4). Different minuscules indicate significant differences among treatments under the same conditions (no

stress, drought + heat stress, or drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$. Different capital letters indicate significant differences of the same treatments (No AM, *R. irregularis*, *F. mosseae*, or *F. coronatum*) under different conditions (no stress, drought + heat stress, and drought + heat shock) by Duncan's post hoc test at $P \leq 0.05$.

In roots, the two-way ANOVA test indicated a significant difference in AM inoculation, stress application, and their interaction (Table 3). A slight increase in root GST activity was determined in plants inoculated with *F. mosseae*, which increased by 17 % compared with non-inoculated ones, while a similar trend occurred in root GST activity for both plants inoculated by *R. irregularis* and *F. coronatum* (increased by 8 % and 22 %, respectively) under D + H. Moreover, no significant impact on root GST activity was observed among non-AM plants and AM treatments under D + HS.

Under D + H stress, arbuscular mycorrhizal colonization had a significant positive correlation with leaf GST (r = 0.65 **), leaf POD (r = 0.57 *), root GST (r = 0.53 *), and root POD (r = 0.72 **), but had a negative correlation with leaf H_2O_2 (r = −0.67 **), leaf MDA (r = −0.76 ***), root H_2O_2 (r = −0.69 **), and root MDA (r = −0.66 **) (Table 6), while under D + HS, a substantial positive correlation was found among AM colonization leaf CAT (r = 0.65 **) and root CAT (r = 0.51 *), but a negative correlation was found with leaf H_2O_2 (r = −0.74 **), leaf MDA (r = −0.69 **), and root H_2O_2 (r = −0.89 ***) (Table 7).

4.3.4 Discussion in the response of different AMF to enhancing the defense enzymes of tomato plant under combine drought and heat stresses

Combinations of drought and heat frequently occur in field conditions and under climate change, particularly in semi-arid and hot growing regions in Mexico, Argentina, North Africa, South Africa, Australia, and the Mediterranean countries, and in high latitude, semi-arid growing regions of eastern and central Asia, Kazakhstan, the USA, and Canada (Tricker et al., 2018). In the present study, we attempted to mimic the field condition of a relatively long-lasting drought period together with a short period of heat stress (heat shock), which usually takes place at mid-day (combined drought and heat shock), and the combination of a drought period with more prolonged heat stress, which is a regular occurrence in semi-arid and hot growing regions. Arbuscular mycorrhizal fungi establish mutualistic interactions with more than 80 % of all plant species, providing a direct physical link between the soil and plant roots (Coleman-Derr and Tringe, 2014; Lenoir et al., 2016; Mello and Balestrini, 2018). AMF is one of the most used biological agents in boosting plant growth, helping in photosynthesis, and acting as protection against biotic and abiotic stresses

(Cavagnaro et al., 2015; S. Liu et al., 2016). Nevertheless, there are still gaps in our knowledge regarding the regulatory mechanisms underlying AMF mediated tolerance under combined drought and heat stresses. As the aim of this study, we examined the effects of three AMF strains on tomato plant growth and their redox status when plants are exposed to combined heat and drought (D + H) and drought and heat shock (D + HS) stress. Our results indicated that inoculation with different AMF species (*R. irregularis, F. mosseae,* and *F. coronatum*) could enhance the tolerance of combined drought and heat stress of tomato plant by improving the antioxidant enzymes system, which in turn lowered cellular H_2O_2 and decreased lipid peroxidation (MDA).

The increase of plant biomass is the most obvious and direct feature reflecting symbiosis-mediated plant growth and performance under different abiotic stresses. Several investigation have been reported the clear role of AMF to stimulate plant growth under abiotic stress (Mo et al., 2016; Ye et al., 2019). Our results demonstrated that stress applications (D + H and D + HS) are negatively affected plant growth, where fresh plant biomass is significantly reduced compared with NoS (Table 4), this decrement might have been due to the effect on plants photosynthetic capacity, and thereby reduced stomatal conductance or the unavailability of nutrients. These results confirmed the finding of Quiroga et al. (2017), who demonstrated that water deficit negatively affected the growth of drought-sensitive maize pretreated with *R. irregularis*, strain EEZ 58 (Ri), and especially in the case of drought-tolerant maize pretreated with the same strain. Moreover, similar results were demonstrated earlier that combine drought and heat stress imped plant biomass (Boeck et al., 2016; Niinemets, 2016; Sehgal et al., 2017; Zandalinas et al., 2017, 2016b). In addition, Choudhury et al. (2017), reported that limited irrigation significantly reduced the root dry weight of *Medicago sativa* inoculated with the mixture of *R. intraradices* and *F. mosseae*. Contrariwise, *Echinacea angustifolia* seedlings inoculated with *R. irregularis* had higher dry weights than those of non-mycorrhizal seedlings under salt stress conditions (Chang et al., 2018).

On the other hand, the present investigation demonstrated that root colonization of tomato was not affected by different stress conditions (D + H and D + HS) as compared with the non-stressed control group, in which coincided with Pedranzani et al. (2016). This phenomenon can be attributed to the stress duration (limited period of stress applications), or perhaps the different species of AMF (*R. irregularis, F. mosseae,* and *F. coronatum*) used in our experiment have high efficiency with tomato roots, even when exposed to abiotic stress conditions. A study conducted by Boutasknit et al. (2020) asserted that drought does not affect root colonization because it is linked to the unchanged

carbon availability of the host plant. However, there are contradictory studies, in which mentioned the negative effect of water stress on root colonization in different plant species such as soybean (Porcel and Ruiz-Lozano, 2004), rice (Ruíz-Sánchez et al., 2011), *Ocimum gratissimum* (Hazzoumi et al., 2015), *Trifolium repens* (Xiao-Qing et al., 2017), and *Triticum aestivum* (Mathur et al., 2018b). Interestingly, Pearson correlation analysis in our study illustrated that mycorrhizal colonization had a close correlation with some defense enzyme activities, and a negative association with H_2O_2 and MDA levels under both combined stresses (Tables 6 and 7), highlighting the role of AMF in ROS metabolism in the host plants exposed to such simultaneous abiotic stresses.

The peroxidation of membrane lipids considered as typical criteria reflecting plant injury caused by various biotic and abiotic factors, that correlated with H_2O_2 and malondialdehyde (MDA) accumulation, while antioxidant enzymes are one of the important features in plant defense that scavenge the harmful effects of ROS, therefore maintain the redox homeostasis. In our experiment, H_2O_2 and MDA levels were measured to investigate the effect of AMF symbiosis on stress tolerance. MDA content (which is considered an end product of lipid peroxidation) decreased in plants treated with different AMF compared with non-AM plants under stress treatments (Figure 26). The MDA content was found higher in leaves than in roots in all treatments. Besides MDA content, H_2O_2 increased sharply in the leaves of the tomato compared with the roots when subjected to different stresses, but the accumulation was significantly decreased in the plants treated with different AMF species. Moreover, the higher H_2O_2 and MDA content in leaves compared with roots reflected a lower oxidative damage in tomato roots. However, the increment may be attributed to the direct exposure of leaves to light, which affects directly the photosynthesis machinery, photorespiration, and accordingly affects the mitochondria, chloroplast, and peroxisome, which are considered to be the major sites of ROS production (Apel and Hirt, 2004; Foyer and Noctor, 2005), in contrast to the roots that lack photosynthetic activity.

Besides, our results proved the ability of AMF in lowering oxidative damages. Moreover, this is consistent with previous reports, where the reduction in H_2O_2 and MDA accumulation under different stress treatments could be explained by a significant increase in defense enzyme activities like CAT, POD, and PPO of AM plants. As compared with non-inoculated plants, concerning all treatments, the reduction of H_2O_2 and MDA can be explained by better water availability and the accumulation of osmolytes, that proved in several studies (Chitarra et al., 2016; Pedranzani et al., 2016; Porcel and Ruiz-Lozano, 2004). AM symbiosis decreased H_2O_2 and MDA accumulation

under stress combination, identical to tomato plants under drought, heat, combined drought, and heat stress (Duc et al., 2018). Drought-stressed trifoliate orange seedlings colonized by *F. mosseae* showed similar results Huang et al. (2017). Alleviated H_2O_2 and MDA accumulation is also correlated with stress tolerance (Mirshad and Puthur, 2016). Furthermore, we also noted higher MDA content under different stress treatments in the leaves and roots; this is coherent with the higher generation rates of H_2O_2. Previous studies have also documented this observation in plants under different stress (Ara et al., 2013).

Antioxidant enzymes are known to have prominent beneficial roles in fighting against stress conditions. In the present study, combined drought and heat stress significantly stimulated the activities of antioxidant enzymes. Our results showed that different mycorrhizal species upon different stress exposures elicited a substantial change (increase, decrease, unchanged) in the activities of GST, CAT, PPO, and POD relative to non-AM plants in leaves as well as in roots (Figure 27, Figure 28, Figure 29 and Figure 30). A significant increase in leaf POD activity was found for all AMF species, except for plants treated with *R. intraradices* under combined drought and heat shock, where it has been reduced. *R. intraradices* and *F. coronatum* showed a much higher ability to promote POD activity in roots under both stresses. Remarkably, root PPO activity was noticeably increased by *R. irregularis* and *F. coronatum* upon D + HS. The change in different antioxidant enzyme activities in tomato plants in response to different stresses is related to the various forms of antioxidant enzyme metabolism; this has been proved by Sheikh-Mohamadi et al. (2017) using wheatgrass genotypes under drought and salinity, and is also consistent with the finding of Tommasino et al. (2018) using *Cenchrus ciliaris* (L.) under drought, heat, and their combination. Moreover, our results show a positive correlation between AM colonization and leaf CAT (r = 0.65 **) and root CAT (r = 0.51 *) (Table 7), where higher root CAT activity was found in plants inoculated with *F. mosseae* under D + HS (Figure 29). Being a tetrameric and heme-containing enzyme, CAT catalyzes the dismutation of hydrogen-peroxide into H_2O and O_2 (Sofo et al., 2015; Zhao et al., 2013). The change in different antioxidant enzymes shows enhanced resistance to oxidative stress-induced by drought, where the performance of AM plants differs depending on the AMF species (Chitarra et al., 2016; Tyagi et al., 2017). The use of AMF as a biological agent is the most efficient approach to cope with the single or combined effect of drought and heat stress through the neutralization of ROS and enhancement of the antioxidant enzymes to avoid oxidative stress (Duc et al., 2018). Furthermore, other studies reported that the changes in

antioxidant enzymes activities influenced by plant species, organ analyzed (like root or leaf), plant age, intensity and stress relation, and the difference among AMF genera and even between AMF strains within the same species (Mayer et al., 2017; Rodríguez et al., 2012). Another point should be shed light on it, to explain the patterns of antioxidant defense enzymes, that the generation of ROS stimulates the production of abscisic acid, which plays a pivotal role in signaling networks of plant response to different abiotic stresses, which leads to the up-regulation of genes controlling the production of different antioxidants enzymes (Guajardo et al., 2016; Sattar et al., 2020; Xu et al., 2013; Zhang et al., 2019). Our results are in accordance with other findings using different AMF and plant species such as wheat inoculated by *Glomus mosseae* (Rani et al., 2018); trifoliate orange treated by *F. mosseae* (Huang et al., 2014); date palm inoculated by *F. mosseae* (Benhiba et al., 2015); finger millet inoculated with *R. intraradices* (Sofo et al., 2015); and carob inoculated with *F. mosseae, Rhizophagus fasciculatus*, or *R. intraradices* (Essahibi et al., 2018). Recently, Al-Arjani et al. (2020) demonstrated that AMF inoculated plants were significantly more resilient to drought stress by the upregulation of the host antioxidant defense system, especially SOD in Ephedra foliate boiss inoculated by *Claroideoglomus etunicatum*, *R. intraradices*, and *F. mosseae*. Another study illustrated that GSTs could protect the plants from different abiotic stresses (Ding et al., 2017), which have the ability to catalyze the conjugation of tripeptide glutathione (GSH) to unfamiliar electrophilic and hydrophobic substrates to form less toxic or non-toxic peptide derivatives (Cetinkaya et al., 2014). Therefore, increased total GST enzyme and isoenzyme activity in heat stress with pre-salinity treatments according to treatment alone (salt, heat) leads to decreased hydrogen peroxide levels in maize leaves (Cetinkaya et al., 2014); many reports illustrated that ABA induces the GST activities in different plant species under individual or combined drought and heat stress (Halušková et al., 2009; Jiang et al., 2010; Kellős et al., 2008; Liu et al., 2013). Hashem et al. (2016) have also reported a strong positive effect of beneficial microbes in reducing oxidative stress through the upregulation of the antioxidant system in *Acacia gerrardii*. Cabral and coworkers found that inoculation with an AMF mixture including *R. irregularis* BEG140, *R. irregularis, F. mosseae* BEG95, *F. geosporum*, and *Claroideoglomus claroideum* in wheat (*Triticum aestivum*) plants mitigated the adverse effects of temperature stress at 35 °C in the daytime and 25 °C in the night for seven days (Cabral et al., 2016). Otherwise, Essahibi et al. (2018) clarified the greater capacity of carob inoculated by different AMF (*F. mosseae, R. fasciculatus,* or *R. intraradices*) to tolerate water stress through increased water and nutrients uptake, stomatal conductance, improved osmotic adjustment, and enhanced antioxidant system (SOD, APX, G-POD, CAT); furthermore, he

recommended the use of *F. mosseae* as a biological tool to improve carob tolerance to water deficit (Essahibi et al., 2018).

4.4. Novel scientific results

1. Using different ratios of sand and peat together with arbuscular mycorrhizal inoculations (Symbivit) of important medicinal plant *E. p,* we discovered that 60/40 % (v/v) sand and peat ratio considered as the suitable ration for the cultivation of this plant resulting in development of AMF and therefore enhance the contents of polyphenols.

2. Nine phenolic compounds were recognized and quantified using High-Performance Liquid Chromatography (HPLC), namely five hydroxycinnamates (protocatechuic acid; 5-O-caffeoylquinic acid; 4,5-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; 4-O-caffeoylquinic acid); two flavonoids (quercetin-3-arabinoside and luteolin), and two coumarins (dimethylwedelolactone; and wedelolactone) in aerial part of *E. p,* with dimethyl wedelolactone; and wedelolacone being plentiful in all treatments and we confirmed that AMF, growing media, and their interaction alter the secondary metabolites.

3. We were the first to describe data regarding the interactive impacts of AM inoculation and salt stress on physio-biochemical parameters and polyphenol profiles of *E. p.* Our work confirmed the efficiency of AM inoculation in the improvement of the growth and salt tolerance of *E. p* due to enhancing osmotic adjustment like proline, as well as increasing in antioxidant enzymes defense such as CAT (at 4 weeks), and POD (at 4 and 8 weeks). Moreover, these parameters showed significant differences depending on the age of the plant and severity of salt stress (moderate, or highly saline condition).

4. We proved that AM inoculation induced significant alteration in polyphenolic profiles concentration under moderate and severe salt stress. 4,5-dicaffeoyl-quinic acid and wedelolactone found to be the abundant polyphenols detected in all of the different samples of *E. p* under both levels of salt stress at the early stage of plant growth (after four weeks). Nearly all identified phenolic compounds through HPLC analysis were promoted in AM plants under higher salt stress.

5. We proved that *Funneliformis mosseae, Rhizophagus irregularis, and Funneliformis coronatum* have high efficiency with tomato roots under both combine drought and heat, and combine drought

and heat shock. All AM strains and especially *Funneliformis mosseae* enhanced the tolerance of tomato plant under both stresses applied through a considerable change in defense enzymes tested (PPO, POD, CAT, ad GST) between leaves and roots and depending AM species. The variations observed in the antioxidant enzymes in different organs (leaves, roots) proved the abilities of different strains in alleviating cellular oxidative damage, and therefore protect plants.

5. CONCLUSIONS AND PERSPECTIVES

The first part of this thesis revealed that the use of arbuscular mycorrhizal fungi and different sand and peat ratios influence the polyphenol profile of an important medicinal plant *E. p* which considered as a novel scientific results obtained. The key finding in this part that 60/40 % (v/v) sand and peat proportion appeared to be the preferable ratio and this results should be taken into account in the cultivation of *E. p*. The AMF inoculation successfully influence the polyphenol components of *E. p*. Furthermore, nine individual phenolic components were identified in the aerial part of *E. p* using HPLC-DAD analytical method. Further studies are required under various kinds of biotic and abiotic stress conditions, using single or mix arbuscular mycorrhizal fungi in an open field experiment.

In the next part, we examined the positive effects of AMF on *E. p* plant under two different levels of salt stress. The results highlighted that mycorrhizal inoculation (with mixed AMF) enhanced growth and salt tolerance of *E. p* against moderate salinity through improving osmotic adjustment (proline), enzymatic antioxidants such as CAT (at four weeks) and POD (at four and eight weeks), total phenolic content at eight weeks. Under high salinity, such benefits were not apparently observed, except remarkably higher total phenolic level in AM plants eight weeks after inoculation. Both Salt stress and mycorrhizal colonization elicited significant changes in the accumulation of phenolic constituents of *E. p*. Under moderate salinity, mycorrhizal inoculation showed a higher plant tolerance during plant growth, but under sever salt stress the higher phenolic compounds accumulated was found at the later plant growth stage. Mycorrhizal inoculation significantly augmented polyphenol concentration and yield depending on plant growth stages and severity of stress. Moreover, mycorrhizal application individually or in combination with salinity and harvest time would be a practical approach for optimizing individual polyphenol production in this medicinal plant.

This study shows how important the selection of the right date of harvest is for to uplift the bioactive compounds production for pharmaceutical, nutraceutical, functional food, and cosmetic industries. Moreover, AMF and moderate salt stress can be used to manipulate the pattern of individual polyphenol production. Further studies required to investigate other secondary metabolites in this medicinal plant colonized by AMF and/or exposure to different abiotic stresses to optimize their production. Other studies also needed to investigate other beneficial soil microorganisms such as the

use of PGPR alone or mixed with AMF will be interesting to take their effects on polyphenol profiles of *E. p* into account.

The last part of this thesis provides new evidence concerning the beneficial role of AMF symbiosis in the alleviation of ROS accumulation caused by combined drought and heat, and combined drought and heat shock stress in tomato plants. The change in H_2O_2, lipid peroxidation (MDA), defense enzymes (POD, PPO, CAT, and GST) in the leaves and roots were investigated in pot culture under both stresses applied. Our data revealed that both mycorrhiza and stress application significantly affected fresh plant biomass. Moreover, no significant differences in the colonization rates of plant inoculated with three AMF inoculums were detected following various stress treatments. The accumulation of H_2O_2 and lipid peroxidation (MDA) was much higher in leaves than in roots. However, inoculation with different AMF strains, and especially, *F. moseae,* could enhance tomato plants' tolerance by lowering H_2O_2 and MDA content, and changed the activities of antioxidant enzymes investigated. For example, higher POD and GST activities were found specifically in roots than in leaves of mycorrhizal plants. In contrast, higher CAT activities were found in leaves of mycorrhizal plants. However, PPO activities were not too prominent in leaves, while they increased in roots of plants inoculated by *R. irregularis* and *F. coronatum* under combined drought and heat stress. The efficiency of different AMF strains used in our experiment to endure combined drought and heat is of great importance for improving agriculture production in a vast area over the world. This experiment-drive to a novel path for further investigation that requiring intensive work regarding the functions of arbuscular mycorrhizal fungi (AMF) under different combined stresses.

Gathering all the outcomes of this dissertation together, AM fungi are able to promote plant growth, and increase the polyphenol profiles of *E. p,* as well as enhance their tolerance against salt stress. Furthermore, different strains of AM symbiosis provide a new insight on their efficiency to endure the harsh effect caused by combined drought and heat, and drought and heat shock in tomato plants.

6. SUMMARY

Given the current and growing impacts of climate change, which amplify the amplitude and severity of various abiotic factors that affect directly or indirectly the agriculture sectors worldwide. For that, the problem raised in the last few decades, is how it is possible to enhance growth and mediate plants more resistance/ tolerance against various stressors. Despite the current circumstance, many studies have reported on the beneficial effects of AMF to endure the negative effects of abiotic stresses, and considered as a promising solution to solve these issues. Based on our results, it can be concluded that AMF colonization are able to improve the growth and tolerance versus some major abiotic stresses of two important models a medicinal plant *E. p* and tomato plant (*Solanum lycopersicum*) with novel results that achieved through three different experiments in controlled condition.

Our first experiment was performed to examine the effect of arbuscular mycorrhizal inoculation and different proportions of growth substrate on root colonization, proline, and content of polyphenols in *E. p*. Mycorrhizal inoculation changes the content of some bioactive compounds of *E. p*, demonstrating considerable differences in polyphenol contents among various treatments assayed. Furthermore, the difference in the proportions of sand and peat, lead to the variation of nutrients supplies within the growth substrate. These variations had considerable influence on both mycorrhizal colonization and polyphenol profiles of *E. p*. Our results revealed that 60/40 % (v/v) sand and peat proportion is appeared to be the preferable ratio for *E. p* cultivation, in consequence increase the total phenolic content. The analyzes carried out made it possible to identify nine important bioactive phenolic compounds using high-performance liquid chromatography (HPLC) analysis, namely five hydroxycinnamates (protocatechuic acid; 5-*O*-caffeoylquinic acid; 4,5-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; 4-*O*-caffeoylquinic acid); two flavonoids (quercetin-3-arabinoside and luteolin), and two coumarins (dimethylwedelolactone; and wedelolactone), where the last two compounds (coumarins) were abundant in the all treatments. Moreover, some of them have not been recoreded before in the aerial part of *E. p* cultivars like 5-*O*-caffeoylquinic acid, quercetin-3-arabinoside, 4-*O*-caffeoylquinic acid, and protocatechuic acid.

However, the focus in the first part of the effect of AMF and different proportion of sand peat growing media on the polyphenol profiles, moreover the purpose of the second part was to investigate the interactive effects of salinity stress and AM inoculation on physio-biochemical features and secondary metabolites of *E. p*. As a follow-up the first experiment, we extended this

second experiment with the most suitable ration for *E. p* growth 60/40 % (v/v) sand and peat and with two different levels of NaCl concentration, one moderate (100 mM NaCl) and the other severe stress (200 mM NaCl) as salt stress. The finding indicated that AM mixture application improved growth and salt tolerance of *E. p* through increasing proline level at both plant stages (four and eight weeks) and total phenolic content at eight weeks, the activity of catalase at four weeks, and peroxidase at both plant stages. Under high salinity, such benefits were not clearly observed, except a higher total phenolic concentration in mycorrhizal plants at eight weeks. Using high-performance liquid chromatography, fourteen individual phenolic compounds were analyzed, with wedelolactone and/or 4,5-dicaffeoylquinic acid abundant in all treatments. Salinity and mycorrhizal inoculation sharply altered polyphenol profiles of *E. p*. Moderate salinity boosted phenolic compounds production in non- AM plants at four weeks, while at eight weeks, the decline in the content of phenolic compounds occurred in uncolonized plants subjected to both saline conditions. Mycorrhization augmented polyphenol concentration and yield under non-saline and saline conditions, depending on growth stages and severity of salt stress. Plant age influenced polyphenol profiles with usually a higher content of phenolic compounds in older plants and changed the production of individual polyphenols of both non-AM and AM plants under non-stress and salt stress conditions. A better understanding of factors affecting phenolic compounds of *E. p* facilitates the identification of new industrial applications of this medicinal plant.

Finally, AMF furnish various ecosystem utilities and it would be helpful to know how different AMF strains (*Rhizophagus irregularis*, *Funneliformis mosseae*, and *Funneliformis coronatum)* differ in their functionality to alleviate the negative effects caused by the combination of two major abiotic stresses: drought and heat as well as drought and heat shock on tomato plants (*Solanum lycopersicum*). A pot experiment was performed under controlled conditions at Institute of Genetics, Microbiology, and Biotechnology, Szent István University, Gödöllő, Hungary. The obtained results showed that combined drought and heat stresses had no significant impact on root colonization while fresh biomass was significantly affected by mycorrhiza and stress application. Higher accumulation of H_2O_2 and MDA was found in leaves that in roots under both stress applications. Furthermore, stressed AMF plants exhibited a decrease in hydrogen peroxide and malondialdehyde content in the cells, and especially, *F. moseae*, could enhance tomato plants' tolerance by lowering H_2O_2 and MDA content. All AM strains showed changes in defense enzyme activities (peroxidase

(POD), catalase (CAT), polyphenol oxidase (PPO), and glutathione S-transferase (GST) in leaves as well as in roots compared with their relative non-mycorrhizal plants.

6. ÖSSZEFOGLALÓ

A éghajlatváltozás felerősíti a különböző abiotikus stressz tényezők súlyosságát, amelyek közvetlenül vagy közvetetten érintik a mezőgazdasági ágazatokat. Ezért igen fontos annak megválaszolása, hogy miként lehet fokozni a növények toleranciáját a különféle stressz faktorokkal szemben. Számos tanulmány számol be az AMF jótékony hatásairól és az abiotikus stresszek negatív hatásainak enyhítésében betöltött szerepéről, de több információra van szükség a nagyobb mértékű alkalmazásukhoz. Munkámban cél volt annak tanulmányozása, hogy az AMF kolonizáció hogyan befolyásolja két target növény (*E. p, Solanum lycopersicum*) növekedését és toleranciáját.

Első kísérletünket azzal a céllal végeztük, hogy megvizsgáljuk az AMF oltás és szubsztrát különböző arányú hatását az *E. p* gyökérkolonizációjára, prolin-tartalmára és polifenol tartalmára. A mikorrhiza oltása megváltoztatja az *E. p* egyes bioaktív vegyületeinek tartalmát, ami jelentős különbségeket okoz a polifenol tartalomban a különböző vizsgált kezelések között. Mindemellett homok és tőzeg arányának változtatásával vizsgáltuk a gyógynövény polifenol profilját. Eredményeink alapján a 60/40 % (v / v) homok és tőzeg arány tűnik a legkedvezőbb aránynak az *E. p* termesztéshez, és annak fenoltartalmának változásához. Az elemzések kilenc fontos bioaktív fenolvegyület azonosítását tették lehetővé, nagy teljesítményű folyadékkromatográfiás (HPLC) analízissel. Nevezetesen öt hidroxicinnamátot (protokatechuinsav; 5-O-koffein-kininsav; 4,5-dikaffeoil-kininsav; 3,5- dikaffeoil-kininsav; 4-O-koffein-kininsav); két flavonoid (kvercetin-3-arabinosid és luteolin) és két kumarin (dimetil-wedelolakton; és wedelolakton). Kiemelendő, hogy az 5-oksafoil-kininsav, a kvercetin-3-arabinozid, a 4-O-koffein-kininsav és a protokatechuinsav kimutatását a gyógynövényből elsőként mi tettük meg.

Mindemellett megvizsgáltuk a só-stressz és az AM-oltás interaktív hatásait is az *E. p* másodlagos metabolitjaira. 60/40 % (v/v) arányú homok és tőzeg szubsztrátban vizsgáltuk meg közepes (100 mM NaCl) és magas só koncentráció (200 mM NaCl) mellett és AM oltás hatására.

Az AM oltás javította az *E. p* növekedését és sótoleranciáját azáltal, hogy mindkét vizsgált növényi stádiumban (négy és nyolc hét) megemelte a prolinszintet, és az összfenol-tartalmat, valamint a negyedik héten a kataláz és peroxidáz aktivitásokat. HPLC méréssel tizennégy fenol-vegyületet elemeztünk, melyben wedelolakton és 4,5-dikaffeoil-gazdagon fordult elő. A mikorrhizáció növelte a polifenol koncentrációját só jelenlétekor a növekedési szakaszoktól és a sós stressz súlyosságától

függően. A növény kora befolyásolta a polifenol profilokat, az idősebb növényekben általában magasabb volt a fenolos vegyületek tartalma, és megváltoztatta mind a nem AM, mind az AM növények egyedi polifenoljainak termelését stressz és só igénybevétele mellett. Az *E. p* polifenol vegyületeinek képződését befolyásoló tényezők jobb megismerése kulcs lehet e gyógynövény új ipari alkalmazásában illetve termesztésében.

Végül különböző AMF-törzsek (*Rhizophagus regularis, Funneliformis mosseae* és *Funneliformis coronatum*) hatását vizsgáltuk meg két fő abiotikus stressz (szárazság /hő-sokk, hő-stressz) kombinációjakor, paradicsom növényeken. A kapott eredmények azt mutatták, hogy a szárazság és hő együttes alkalmazása nem gyakorolt jelentős hatást a gyökér kolonizációjára, míg a friss biomasszát jelentősen befolyásolta. Továbbá a stressz jelenlétekor az AMF növények csökkent hidrogén-peroxid- és malondialdehid-tartalmat mutattak, mely különösen a *F. mosseae* oltáskor jelentkezett. Mindhárom AM-törzs hatására eltérő mértékű védelmi enzimaktivitásokat (peroxidáz, kataláz, polifenol-oxidáz és glutation-S-transzferáz) mértünk, ezáltal előnyhöz juttatva a mikorrhizált növényt stressz helyzetben.

7. APPENDICES

7.1. References

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7.2. SUPPLEMENTAL FIGURES AND TABLES

Table 5. The sand: peat substrate properties

	pH	Phosphorus (mg/kg)	Potassium (mg/kg)	Carbonate (%)	Total Nitrogen (%)	Dry matter content (m/m%)
Peat	6.45±0.06	1610 ± 14	3320 ± 24	0.94±0.018	42.93±0.347	15.7 ± 0.1
Sand	7.23±0.03	62.16± 0.78	2485 ± 11	0.01±0.0015	0.38±0.081	79.4 ± 0.3

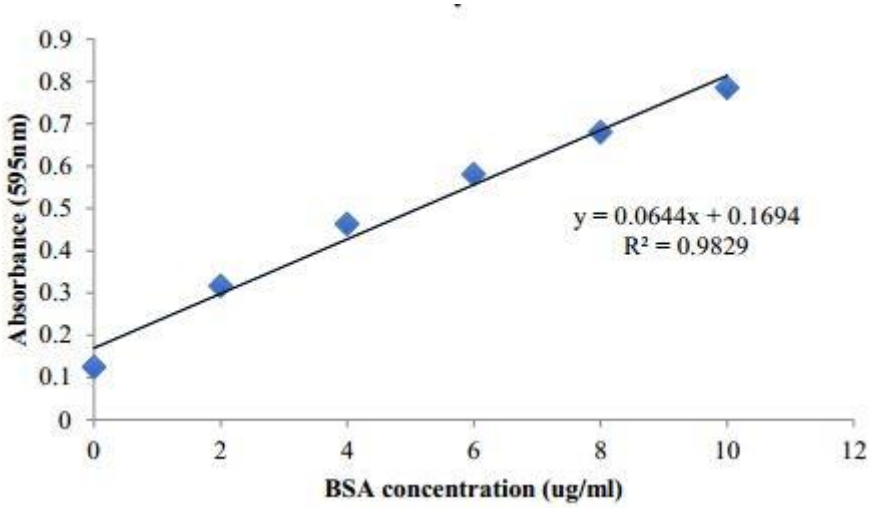

Figure 31. Bovine serum albumin standard curve for bradfird essay

Figure 32. *E. p* plants with and without arbuscular mycorrhizal fungi (Symbivit) after eight-weeks of growth in a growth chamber. Abbreviations: AM1 (Amf + 100/0 sand-peat % (v/v)), AM2 (Amf

+ 80/20 % (v/v) sand-peat), AM3 (Amf + 60/40 sand-peat % (v/v)), AM4 (Amf + 40/60 sand-peat % (v/v)), AM5 (Amf + 20/80 sand-peat % (v/v)), AM6 (Amf + 0/100 sand-peat % (v/v)). NONAM1 (100/0 sand-peat % (v/v)), NONAM2 (80/20 sand-peat % (v/v)), NONAM3 (60/40 sand-peat % (v/v)), NONAM4 (40/60 sand-peat % (v/v), NONAM5 (20/80 sand-peat % (v/v), NONAM6 (0/100 sand-peat % (v/v). (Photo by Vo, 2016)

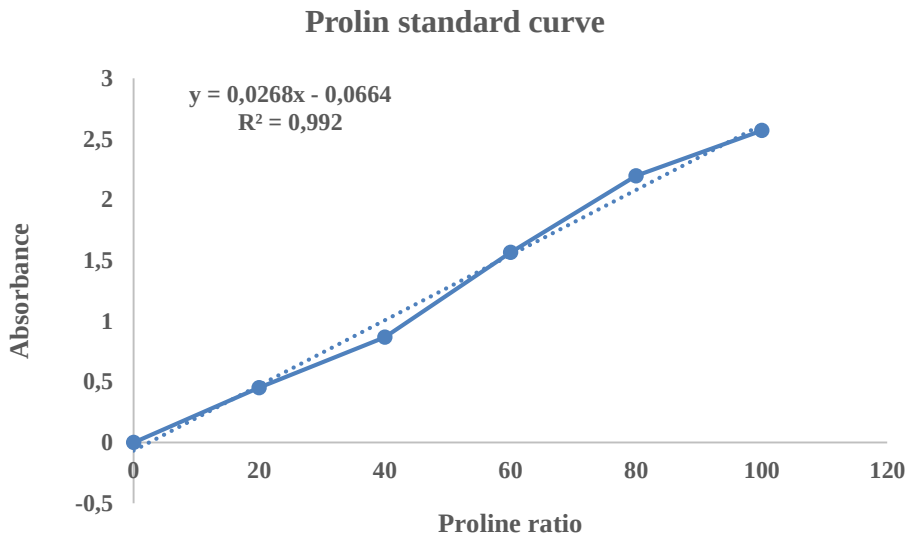

Figure 33. Proline standard curve

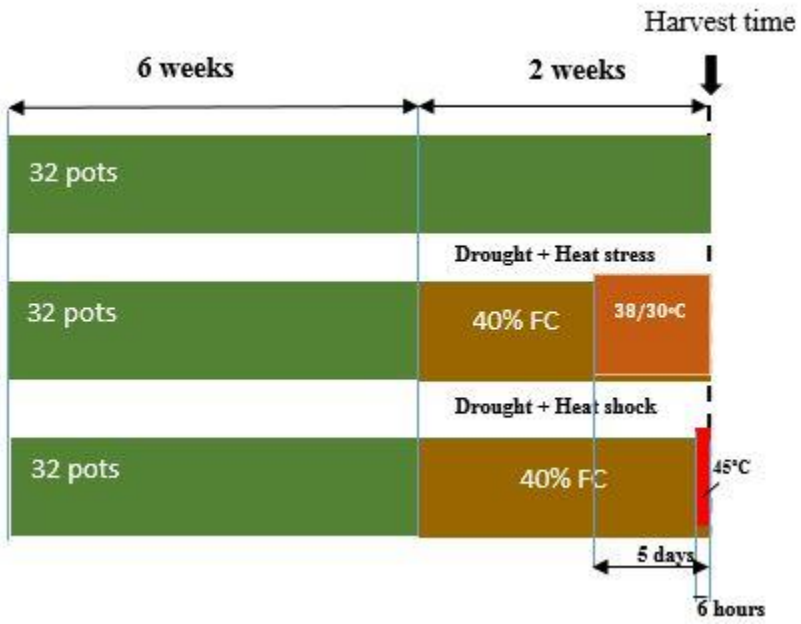

Figure 34. The experimental design for combined drought and heat stress, and combined drought and heat shock to tomato plants. Each combined stress or no stress, there were 32 pots (plants): 8 non-AM plants, 8 plants inoculated with *Rhizophagus irregularis*, 8 plants inoculated with *Funneliformis mosseae*, and 8 plants inoculated with *Funneliformis coronatum*.

- No stress: water 100 % field capacity, 600 µmol/m^2/s photon flux density, 26/20 °C with 16/8 hours' day/night temperature and 60 % relative humidity,
- Drought: 40 % field capacity, other conditions were the same as no stress
- Heat stress: 38/30 °C with 16/8 hours' day/night, other conditions were the same as no stress
- Heat shock: 45 °C for 6 hours, other conditions were the same as no stress

Table 6. Pearson correlation coefficients (r) among plant physiological parameters measured under drought and heat stress (D+H)

	AMF col.	FSB	Leaf CAT	Leaf GST	Leaf H_2O_2	Leaf MDA	Leaf POD	Leaf PPO	Root CAT	Root GST	Root H_2O_2	Root MDA	Root POD
AMF col.													
FSB	0.43^{ns}												
Leaf CAT	0.07^{ns}	-0.08^{ns}											
Leaf GST	0.65**	-0.01^{ns}	0.06^{ns}										
Leaf H_2O_2	-0.67**	-0.38^{ns}	-0.08^{ns}	-0.67**									
Leaf MDA	-0.76***	-0.19^{ns}	-0.32^{ns}	-0.57*	0.64**								
Leaf POD	0.57*	0.01^{ns}	0.21^{ns}	0.62*	-0.51*	-0.42^{ns}							
Leaf PPO	0.18^{ns}	-0.02^{ns}	0.11^{ns}	0.15^{ns}	0.33^{ns}	0.02^{ns}	0.15^{ns}						
Root CAT	0.33^{ns}	0.27^{ns}	0.03^{ns}	0.06^{ns}	-0.22^{ns}	-0.10^{ns}	0.12^{ns}	-0.02^{ns}					
Root GST	0.53*	0.24^{ns}	0.25^{ns}	0.68**	-0.86***	-0.59*	0.66**	-0.25^{ns}	-0.13^{ns}				
Root H_2O_2	-0.69**	-0.40^{ns}	-0.12^{ns}	-0.57*	0.63**	0.48^{ns}	-0.78***	-0.09^{ns}	-0.37^{ns}	-0.57*			
Root MDA	-0.66**	-0.20^{ns}	-0.42^{ns}	-0.62*	0.61*	0.75***	-0.72**	-0.20^{ns}	0.19^{ns}	-0.77***	0.64**		
Root POD	0.72**	0.25^{ns}	0.10^{ns}	0.58*	-0.41^{ns}	-0.45^{ns}	0.73**	0.24^{ns}	-0.05^{ns}	0.60*	-0.66**	-0.64**	
Root PPO	0.29^{ns}	-0.17^{ns}	-0.10^{ns}	0.15^{ns}	0.10^{ns}	-0.01^{ns}	0.53*	0.20^{ns}	-0.30^{ns}	0.12^{ns}	-0.29^{ns}	-0.28^{ns}	0.72**

(*), (**), (***): correlation is significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; ns: correlation is not significant. AMF col., arbuscular mycorrhizal colonization; FSB, fresh shoot biomass; CAT, catalase; GST, glutathione-s-transferase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase; PPO, polyphenol oxidase.

Table 7. Pearson correlation coefficients between all plant physiological parameters measured under drought + heat shock (D+HS) conditions

	AMF col.	**FSB**	**Leaf CAT**	**Leaf GST**	**Leaf H_2O_2**	**Leaf MDA**	**Leaf POD**	**Leaf PPO**	**Root CAT**	**Root GST**	**Root H_2O_2**	**Root MDA**	**Root POD**
AMF col.													
FSB	0.37^{ns}												
Leaf CAT	0.65**	-0.13^{ns}											
Leaf GST	-0.42^{ns}	-0.49^{ns}	-0.18^{ns}										
Leaf H_2O_2	-0.74**	-0.30^{ns}	-0.74**	0.37^{ns}									
Leaf MDA	-0.69**	-0.33^{ns}	-0.61*	0.44^{ns}	0.89***								
Leaf POD	0.36^{ns}	0.32^{ns}	0.35^{ns}	-0.44^{ns}	-0.48^{ns}	-0.66**							
Leaf PPO	-0.23^{ns}	-0.13^{ns}	-0.02^{ns}	0.27ns	-0.05^{ns}	-0.18^{ns}	0.01^{ns}						
Root CAT	0.51*	0.66**	0.25^{ns}	-0.56*	-0.41^{ns}	-0.54*	0.56*	-0.24^{ns}					
Root GST	0.14^{ns}	-0.35^{ns}	0.47^{ns}	-0.03^{ns}	-0.24^{ns}	-0.38^{ns}	0.17^{ns}	0.12^{ns}	0.11ns				
Root H_2O_2	-0.89***	-0.57*	-0.52*	0.66**	0.68**	0.70**	-0.47^{ns}	0.10^{ns}	-0.59*	-0.07^{ns}			
Root MDA	0.03^{ns}	-0.67**	0.42^{ns}	0.33^{ns}	-0.15^{ns}	-0.09^{ns}	0.10^{ns}	-0.15^{ns}	-0.43^{ns}	0.033^{ns}	0.20^{ns}		
Root POD	0.19^{ns}	-0.60*	0.49^{ns}	0.02^{ns}	-0.26^{ns}	-0.15^{ns}	-0.21^{ns}	-0.01^{ns}	-0.38^{ns}	0.31^{ns}	-0.09^{ns}	0.68**	
Root PPO	-0.21^{ns}	-0.32^{ns}	0.08^{ns}	0.27^{ns}	0.20^{ns}	0.45^{ns}	-0.68**	-0.18^{ns}	-0.47^{ns}	-0.18^{ns}	0.27^{ns}	0.14^{ns}	0.47^{ns}

(*), (**), (***): correlation is significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; ns: correlation is not significant. AMF col., arbuscular mycorrhizal colonization; FSB, fresh shoot biomass; CAT, catalase; GST, glutathione-s-transferase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase; PPO, polyphenol oxidase.

8. LIST OF PUBLICATIONS

Peer -reviewed scientific articles

Imane Haddidi, Nguyen Hong Duc, Szende Tonk, Eszter Rápó and Katalin Posta (2020). Defense Enzymes in Mycorrhizal Tomato Plants Exposed to Combined Drought and Heat Stresses, Agronomy 10, 1657. https://doi.org/10.3390/agronomy10111657 IF 2.6 Q1.

Au Trung VO**, Imane HADDID**, Hussein DAOOD, Zoltan MAYER and Katalin POSTA (2019). Impact of Arbuscular Mycorrhizal Inoculation and Growth Substrate on Biomass and Content of Polyphenols in *Eclipta prostrata*, HortScience 54(11), 1976-1983. DOI: https://doi.org/10.21273/HORTSCI14227-19 IF 0.906 Q2.

Nguyen Hong Duc, Au Trung Vo, **Imane Haddidi,** Hussein Daood and Katalin Posta (2021). Arbuscular mycorrhizal fungi improve tolerance of medicinal plant *Eclipta prostrata* (L.) and induce major changes in polyphenol profiles under salt stresses. Front. Plant Sci., 15 January 2021 https://doi.org/10.3389/fpls.2020.612299 IF 4;402Q1

Conferences

Imane HADDIDI, Au Trung VO, Katalin POSTA. (2018). Effect of arbuscular mycorrhizal fungi and different growing media on the growth of *Eclipta prostrata.* 18th congress of the African Assiciation of biological Nitrogen fixation (AABNF2018) April, 22nd-24th 2018.

Imane HADDIDI, Au Trung VO, Katalin POSTA. (2019). Changing phenolic and proline content of *Eclipta prostrata* under impact ofarbuscular mycorrhizal fungi and different sand/peat media. Abstract Book –18th Alps-Adria Scientific Workshop. Cattolica, Italy 1 st - 6th Aprildoi:10.34116/NTI. 2019.AA.23.

Imane Haddidi, Bence Gajdics, Nguyen Hong Duc, Katalin Posta (2021). Combined Heat and Drought Tolerance of Tomato Plants when Treated with different Arbuscular Mycorrhizal Fungi. 5th World Congress on Civil, Structural, and Environmental Engineering (CSEE'21) Lisbon, Portugal – June, 2021

Other scientific articles with another topic published during PhD program

Fadila ZEGHDOUDI, Larbi M. TANJIR, Naouel OUALI, **Iman HADDIDI** and Mounira RACHEDI (2019). Concentrations of trace-metal elements in the superficial sediment and the marine magnophyte, *Posidonia oceanica* (L) Delile, 1813 from the Gulf of Skikda (Mediterranean coast, East of Algeria). *CAHIERS DE BIOLOGIE MARINE*, *60*(3), 223-233. IF 0.6

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