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Ph.D. thesis

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

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1. BACKGROUND AND OBJECTIVES

Today, climate change abnormalities considered as a serious environmental issue. Indeed, abiotic stress such as drought, extreme temperature, and salinity, represent the most important stressors (Mahalingam, 2015; Ramegowda and Senthil-Kumar, 2015), and their combination were recognized to have more devastating effects on plant quality and final yields in the agriculture sector and considered as a principal reason to lose more than 50 % of crop yields (Alcázar et al., 2006). Therefore, pose an earnest problem regarding food security infrastructure (Dubey et al., 2015; Lesk et al., 2016; 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 as a very stiff task facing the world (Panta et al., 2014).

Given the current and growing impacts of climate change, that amplifies the amplitude and frequency of different abiotic stresses, 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., 2019).

The general objective was to investigate the beneficial effect of arbuscular myccorhizal fungi (AMF) in the alleviation and reduction of some abiotic stress (salt stress, and the combination of drought and heat and drought and heat shock) on two important host plants. The first one is a medicinal plant *E. p*rostrata 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 frequently consumed food over the world after potato (Wakil et al., 2017).

In particular, our studies were targeted to:

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 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. MATERIALS AND METHODS

2.1. 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.

2.2. 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 (2.3.1 and 2.3.2)

Three different mycorrhizal fungal inocula, *Funneliformis mosseae* (collection of Hungarian University of Agriculture and Life Sciences,), *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 (2.3.3).

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.

2.3. Plant growth and experiment design

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

Six different proportion of sand and peat were prepared (100:0; 80:20; 60:40; 40:60; 20:80 and 0:100). 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®; 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. After 7 weeks of growth, mycorrhizal colonization, polyphenols, total phenolic content, and proline were determined.

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

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). The 60:40 % (v/v) sand: peat proportion was chosen for cultivating *E. p* plants. Each treatment had ten biological replicates, resulting in a total of 60 pots. Shoot and root weight, plant height, leaf number, leaf area, stem diameter, chlorophyll fluorescence, mycorrhizal colonization rate, proline, superoxide dismutase, peroxidase, catalase, and polyphenol components.were examined at four and eight weeks of growth.

2.3.3 Mycorrhizal tomato plant tolerance to combined drought and heat stress

The (4:1, v/v) and: peat proportion was used for cultivating tomato plants. The 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) [40 % field capacity for 14 days 38/30 ^{o}C with 16/8 h (for the last 5 days)]; and simultaneous drought and heat shock stress (D + HS) [40 % field capacity for 14 days 42 ^{o}C in 6 h (just before harvesting)].

2.4. Measurement of parameters

The assessment of root colonization were determined following Giovannetti and Mosse, (1980) and Trouvelot et al. (1986)

Plant growth rate and biomass, the **s**hoot height, branch length, branch number, leaf number were recorded at the time as mentioned above in each experiment. shoot and root were dried in a hot-air oven at 60 °C for three days to determine their dry weight.

Leaf area was measured according to Glozer (2008)

Proline determination was measured following to Bates et al. (1973)

Determination of total phenolic content was measured by Folin–Ciocalteu assay according to Lister and Wilson, (2001).

Determination of polyphenols by using high performance liquid chromatographic analysis (HPLC) as described in Vo et al. (2019)

Hydrogen peroxide (H_2O_2) was determined by the method of Alexieva et al. (2001)

Malondialdehyde was determined according to Heath and Packer, (1968).

Enzyme assays

Measurement of superoxide dismutase (SOD, EC 1.15.1.1)enzyme activity was determined the method of Beyer Jr and Fridovich, (1987). The enzyme activity of catalase (EC 1.11.1.6) was determined by the method of Aebi, (1984), or using a Catalase assay kit purchased from Sigma-Aldrich, St. Louis, MO, USA (product identification: CAT 100). Measurement of the activity of the peroxidase (POD, EC 1.11.1.7) was determined according to Rathmell and Sequeira, (1974). Measurement of polyphenol oxidase (PPO, EC 1.10.3.1) activity was measured by the modified procedure of Fehrmann and Diamond, (1967). 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). Total protein was determined by the method of Bradford (1976).

2.5. 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.

3. RESULTS

3.1. Characterize the influence of AM inoculation and different proportion of sand/peat substrate on polyphenols content of *E. p*

3.1.1 Mycorrhizal inoculation rate and measured plant parameters

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). There were no significant differences in root colonization at higher than 40 % (v/v) peat ratios.

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

3.1.2 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), with dimethylwedelolactone and wedelolactone being abundant in all of the samples examined.

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. 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. 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 ($P < 0.05$). 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+. 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.

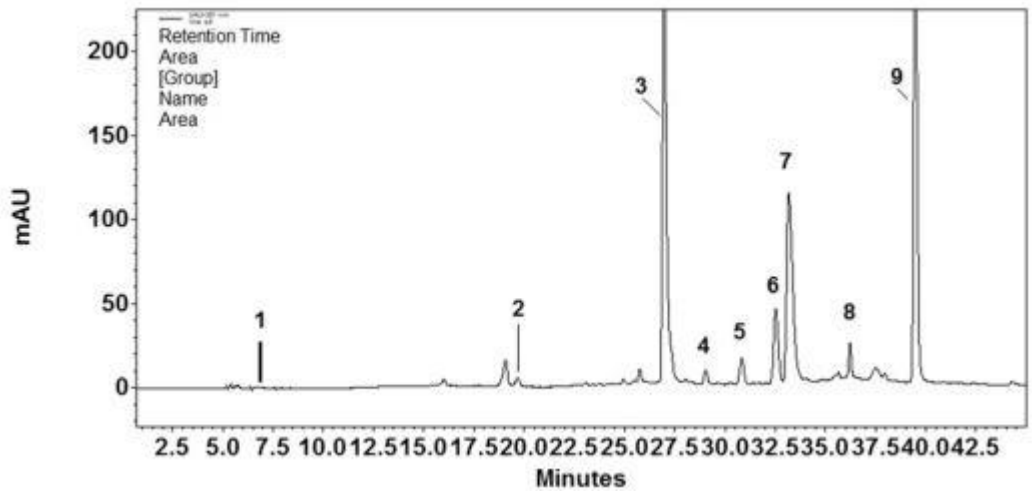

Figure 1. HPLC profile of polyphenols from leaves of *E. p* separated on C18 Protect-1, 250x4,6 mm eluated with gradient of Acetonitril in 1 % formic acide solution. Peak identifications: 1= Protocatechuic acid; 2= 5-O-caffeoylquinic acid; 3= Dimethylwedelolactone; 4= 4-O-caffeoylquinic acid; 5= 3,5-dicaffeoylquinic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

3.2. The interactive effects of salinity stress and AM inoculation on physio-biochemical parameters and polyphenol profiles of *E. p*

3.2.1 Root colonization

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. 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.

3.2.2 Proline concentration

Salinity heightened proline concentrations in mycorrhizal and non-mycorrhizal plants at four weeks. 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 ($P < 0.05$).

3.2.3 Polyphenol content

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), but only thirteen components of polyphenols (feruloylquinic acid was under detection limit) were determined in eight-week plants.

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 2 and 3). 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.

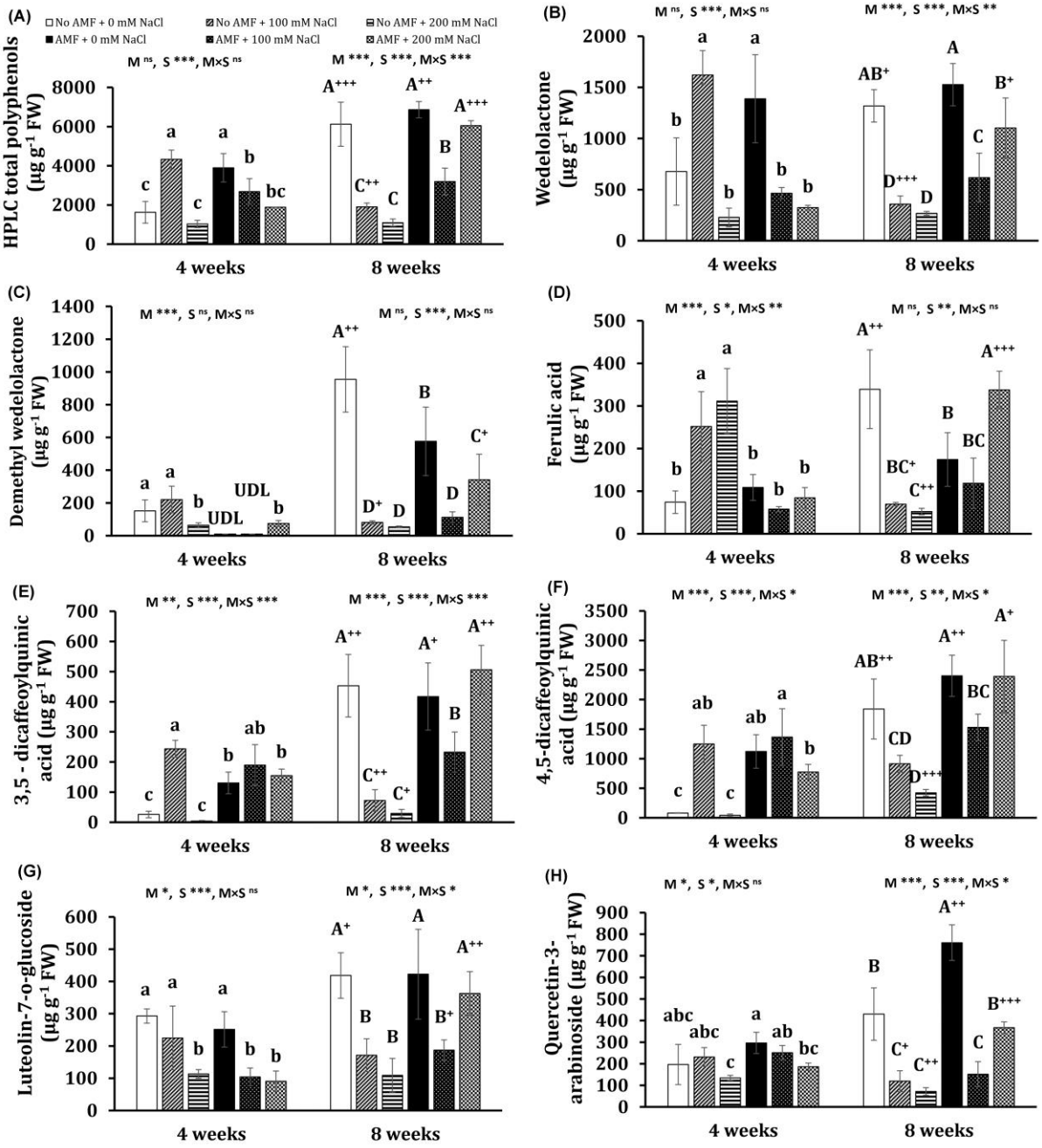

Figure 2. Contents of HPLC total polyphenols (A) and major polyphenols: wedelolactone (B), demethyl wedelolactone (C), ferulic acid (D), 3,5-dicaffeoylquinic acid (E), 4,5-dicaffeoylquinic acid (F), luteolin-7-O-glucoside (G), and quercetin-3-arabinoside (H) in leaves of *E. p* inoculated with arbuscular 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

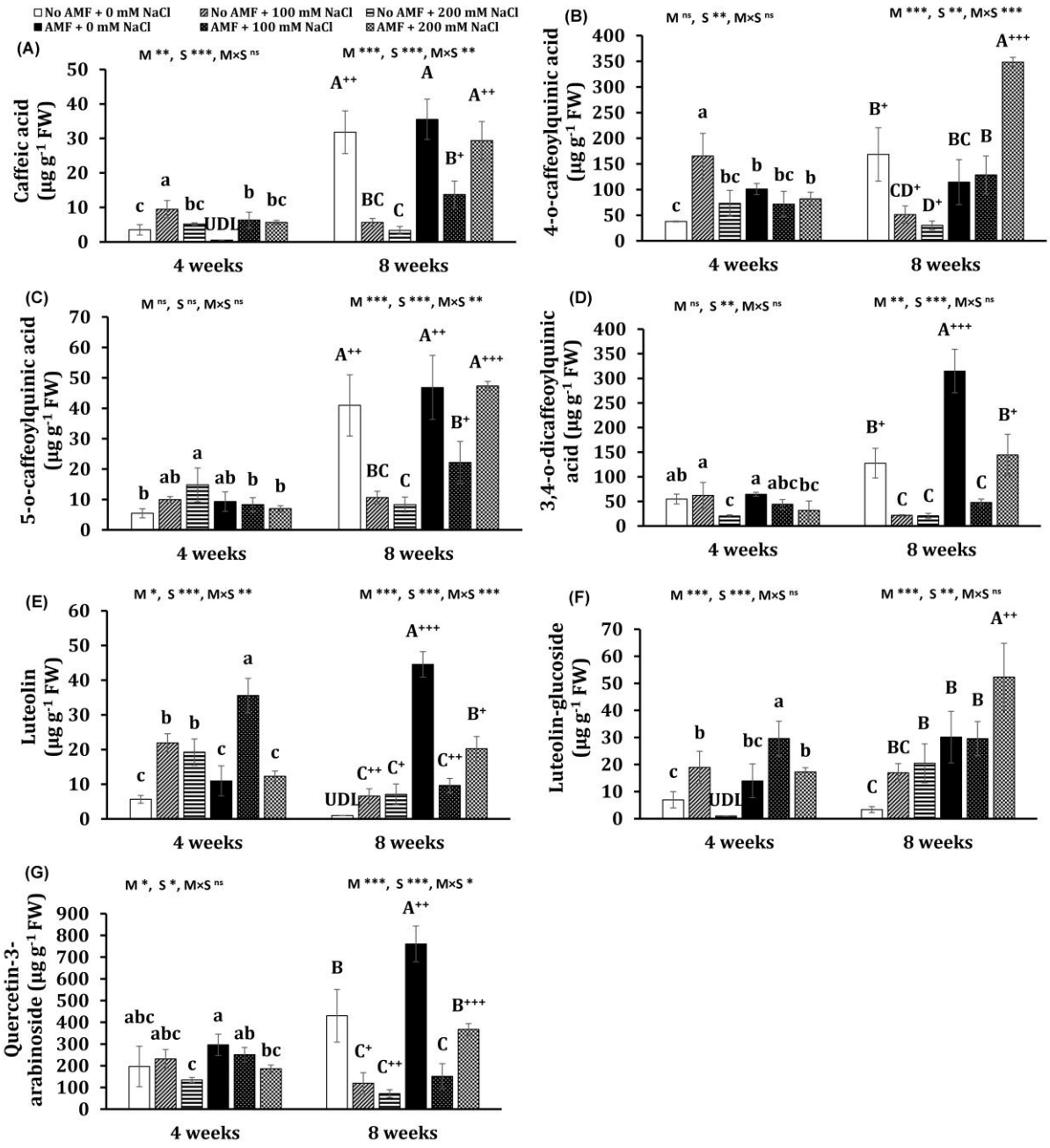

Figure 3. Contents of polyphenols: caffeic acid (A), 4-O-caffeoylquinic acid (B), 5-O-caffeoylquinic acid (C), 3,4-O-dicaffeoylquinic acid (D), feruloylquinic acid (E), luteolin (F), luteolin-glucoside (G) in leaves of *E. p* inoculated with arbuscular mycorrhiza or not inoculated under non-stress, moderate (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.

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

3.3.1 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. 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. 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.

In roots, 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.

3.3.2 Defense Enzyme Activities

No significant difference was observed in leaf POD activity among AM and non-AM plants under no stress conditions (Figure 4 A). 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.

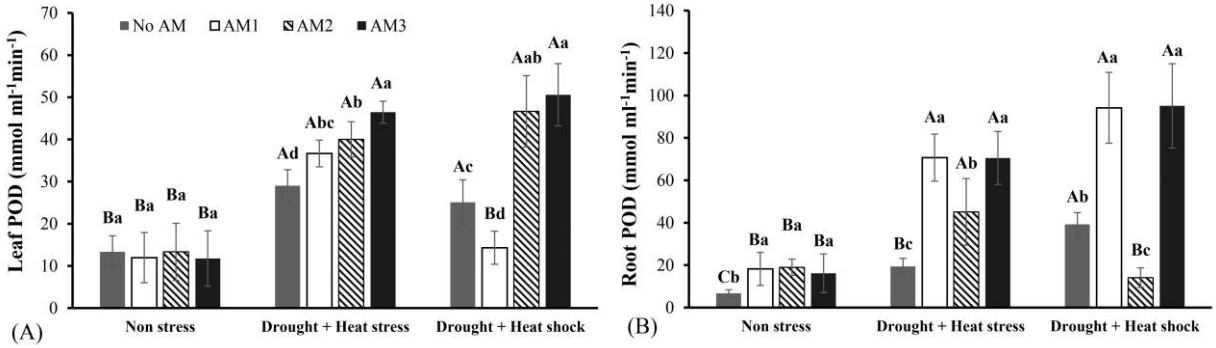

Figure 4. The activity of peroxidase (POD) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought + heat shock (D + HS). Each bar 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. coronat*um) 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, POD activity appeared to be consistently increased in plants under both combined stress conditions compared with that of non-stress plants (Figure 4 B). 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.

The imposed stresses substantially induced leaf PPO activity in plants compared with non-stress conditions (Figure 5A). The data showed no significant differences among control, *R. irregularis, F. mosseae*, and *F. coronatum* under both D + H and D + HS (Figure 5A). 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 5B), 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.

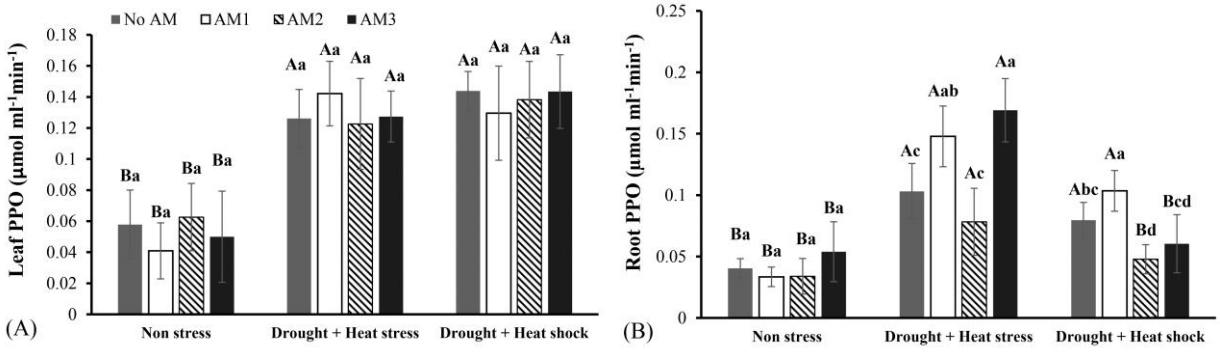

Figure 5. The activity of polyphenol oxidase (PPO) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought + heat shock (D + HS). 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 6A). 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 6B), 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.

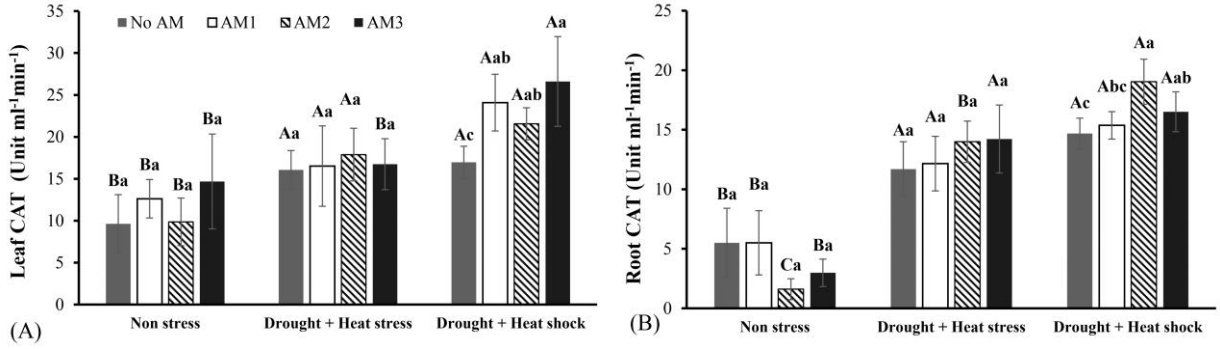

Figure 6. The activity of catalase (CAT) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought+ heat shock (D + HS). Each bar represents 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 7A). Under D + HS, no significant difference could be observed among non-AM plants and various AMF strains (*R. irregularis, F. mosseae,* and *F. coronatum*).

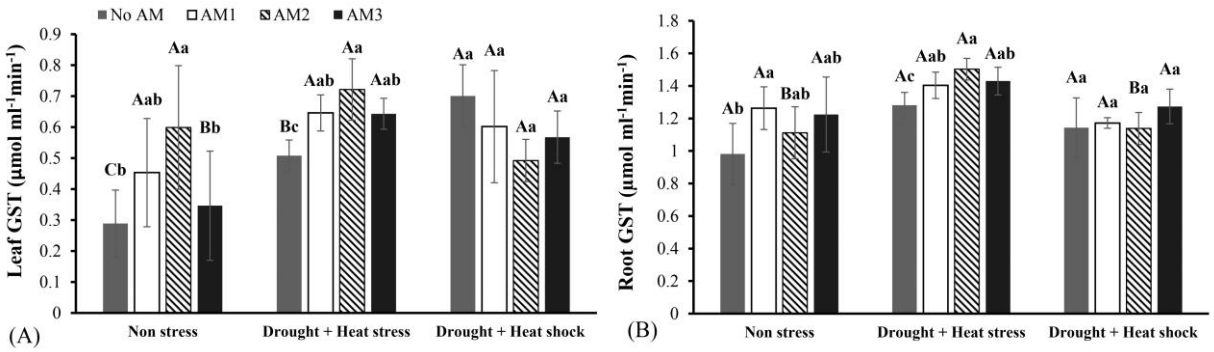

Figure 7. The activity of glutathione S transferase (GST) in leaves (A) and roots (B) of non-inoculated plants (No AM) and plants inoculated by *R. irregularis* (AM1), *F. mosseae* (AM2), or *F. coronatum* (AM3) subjected to non-stress (NoS), drought + heat stress (D + H), and drought+ heat shock (D + 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, 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.

3.4. 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 proportion 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 inoculation 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.

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.

4. 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.

5. 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

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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.

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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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