SZENT ISTVÁN UNIVERSITY

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

PhD Thesis

Nguyen Hong Duc

Godollo

2017

PhD school name: **Plant Science**

Discipline: **Plant Science**

Head: Prof. Lajos Helyes

Professor, DSc.

SZIE Faculty of Agricultural and Environmental Sciences,

Institute of Horticulture Technology Research

Supervisor: Prof. Posta Katalin

Professor, DSc.

SZIE Faculty of Agricultural and Environmental Sciences,

Institute of Genetics, Microbiology and Biotechnology

………………………..	………………………..
Prof. Lajor Helyes	Prof. Posta Katalin
Head of the PhD school	Supervisor

1. INTRODUCTION

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

Noticeably, under natural conditions plants are frequently associated with microbes, which directly modulates plant responses to stresses. Some plant-microbe interactions result in alleviating stress-related damages, enhancement of plant tolerance to environmental stresses (Turner et al., 2013; Ngumbi and Kloepper, 2014). As an important element of soils, microorganisms are an integral component of the agricultural system. Arbuscular mycorrhizal (AM) fungi, a ubiquitous soil microbe, can associate with the roots of most terrestrial plant species. These beneficial fungi have been reported to significantly contribute multiple benefits to its host plants (Bonfante and Genre, 2010). Enhancement of mineral nutrients, water supply, improved seedling survival, increased growth and yield, uniformity of horticultural crops, and earlier and increased flowering (Azcón-Aguilar and Barea, 1997; Vosátka and Albrechtová, 2008; Gaur et al., 1998; Kaya et al., 2009; Russo and Perkins-Veazie, 2010) were observed in AM colonized plants. The exploitation of AM symbiosis is one of the most effective practices to improve plant tolerance to abiotic stress (Birhane et al., 2012). Additionally, root colonization by AMF (arbuscular mycorrhizal fungi) enhances the plant's resistance to biotic or abiotic stresses (Birhane et al., 2012, Jung et al., 2012) through the remarkable reprogramming of plant functions, significant alterations in the hormonal balance and transcriptional profile, primary and secondary metabolism inside plants during AMF colonization of their host (Pozo et al., 2009).

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

Objectives

Our aims were to

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

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

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

2. MATERIALS AND METHODS

2.1 Target plants

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

2.2 Arbuscular mycorrhizal fungi inocula and other beneficial microbes

AMF species including *Funneliformis mosseae* BEG 12 (Fm), *Funneliformis geosporum* BEG 11 (Fg), *Rhizophagus irregularis* MUCL43194 (DAOM197198) (Ri), *Rhizophagus* sp. MUCL43204 (Rs), *Septoglomus constrictum* (formerly *Glomus constrictum* Trappe.) (Sc), *Septoglomus deserticola* BEG 73, *Claroideoglomus claroideum* BEG 23 (Cc), *Gigaspora margarita* BEG 34 (Gm), and Symbivit®, a commercial mycorrhizal product. (Symbiom Ltd., Lanskroun, Czech Republic; www.symbiom.cz) were utilized in our experiments. *Trichoderma harzianum* isolate (SzIE35), *Pseudomonas fluorescens* isolate (PK17) in the collection of Szent István University were used.

2.3 Mycorrhizal tomato plant tolerance to *Clavibacter michiganensis* subsp. *michiganensis*

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

This experiment was carried out from July to October 2015. There were eight treatments including plants inoculated separately with one of seven different AMF isolates altogether with non-AM plants. Thirty replicates of each treatment settled in a growth chamber. After 7 weeks of growth, bacterial pathogen *Clavibacter michiganensis* subsp. *michiganensis* (B.01778, National Collection of Agricultural and Industrial Microorganisms, Hungary) (Cmm) injection was performed. When plants reached 10 weeks of growth, plant biomass and mycorrhizal colonization, disease severity index were determined.

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

This experiment was implemented between April and July 2016. Tomato seeds (*Solanum lycopersicum* L.) of Never ripe (Nr), ethylene-insensitive mutant and its background Pearson were used. Before planting the seeds, inoculation with *Rhizophagus irregularis* (MUCL43194) and non-inoculation was implemented in each genotype. Cmm injection performed after 7 weeks of plant growth. Shoot fresh and dry weight, mycorrhizal colonization and disease severity index were examined at 10 weeks of plant growth.

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

This experiment was set up from November 2015 to January 2016. It consisted of three groups: non-AM plants, plants inoculated with AM fungi, *Septoglomus deserticola* BEG 73 or *Septoglomus constrictum* (formerly *Glomus constrictum* Trappe). Plants were distributed randomly and grown in a growth chamber (EKOCHIL 1500) at 26/20°C with 16/8 hour photoperiod, light intensity of 800 μmol m^{-2} s^{-1} and 60% humidity, When plants reached 6 weeks of age, the stress treatments were carried out.

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

2.5 Field experiment

The field experiment was conducted from May to September 2014 and described in detail in a publication of Duc et al. (2017). Briefly, three sweet pepper (*Capsicum annuum* L.) hybrids, Karpia, Karpex and Kaptur were used. Seedlings of pepper varieties were propagated at the beginning of April in a greenhouse. Then the seedlings were transplanted on 16th May, arranged in double rows with a distance of 0.8 m between beds, 0.3 m between the rows and 0.3 m between the plants. All treatments including seven microbial inoculations and three cultivars were arranged in a randomized complete block design. The seven microbial inoculations were arbuscular mycorrhizal fungi (AM), *Trichoderma* (Tri), plant growth promoting bacteria (Pse) and their combinations (AM+Tri; AM+Tri+Pse; AM+Pse) and non-inoculation (control) plants with 30 replications per treatment each cultivar. Leaves at the same level from five different plants per treatment were collected at 29, 49, 69 days after transplanting (DAT) and kept in the -80°C until enzyme assays.

2.6 Measurement of paramenters

Assessment of mycorrhizal colonization according to Vierheilig et al. (1998) and Giovanetti and Mosse (1980). Disease severity index (DSI) measurement at 7, 14, 17, 21 days post Cmm infection (dpi) using the formula described by Raupach et al. (1996). Measurement of tomato, pepper plant biomass and yield were conducted.

Leaf water potential was examined following the description of Boyer (1995). Relative water content (RWC) according to Cvikrová et al. (2013). Measurement of stomatal conductance was implemented using a porometer system (Delta-T AP4, UK). Chlorophyll fluorescence parameter, the maximum efficiency of PSII photochemistry after 30 minutes of dark-adaption (F_v/F_m) was determined using Walz – PAM 2500.

The concentration of H_2O_2 and lipid peroxidation level were measured according to the description of Alexieva et al. (2001) and Heath and Packer (1969), respectively. Protein concentration (Bradford, 1976), polyphenol oxidase (PPO, EC 1.10.3.1) (Fehrmann and Dimond, 1967), peroxidase (POD, EC 1.11.1.7) (Rathmell and Sequeira, 1974), superoxide dismutase (SOD, EC 1.15.1.1) (Beyer and Fridovich, 1987) and catalase (CAT, EC 1.11.1.6) (Aebi and Lester, 1984) activity were examined.

RNA from samples were isolated and used for cDNA synthesis. Aquaporin gene (*SlPIP2.7*) and the biosynthetic gene of Jasmonate (*SlLOXD*), abscisic acid (*SlNCED*) were examined by qPCR. The relative expression levels were normalized with the expression data of tomato Actin gene by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

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

3. RESULTS

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

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

Ri-induced resistance was also observed in the background plants inoculated by Ri at 7, 14, 17, 21 dpi (Figure 1). In addition, ethylene (ET) insensitivity limited disease development of Cmm due to the fact that DSI of Nr plants was considerably lower than that of the Pearson background during three weeks of Cmm infection. Remarkably, insensitivity of ET in Nr plants colonized with Ri eliminated the mycorrhiza-induced resistance (MIR) against Cmm when its DSI was similar to that of Pearson plants without Ri inoculation over the course of Cmm infection, suggesting that ET plays a key role in Ri-induced resistance against Cmm.

Noticeably, AM colonization failed to increase shoot fresh and dry weight in plants in our experimental conditions, where no remarkable differences in shoot fresh and dry weight between Pearson and Pearson+Ri, Nr and Nr+Ri were detected (Data not shown). Cmm significantly decreased shoot fresh by 34% and dry weight by 24% in Nr mutant and its background but the more pronounced reduction in shoot dry weight (52%) was in the treatment Nr+Ri+Cmm. Interestingly, AM colonization rate in Nr+Ri was increased by 17%, as compared to Pearson+Ri whilst this value was most severely reduced (28.7%) in Nr+Ri+Cmm.

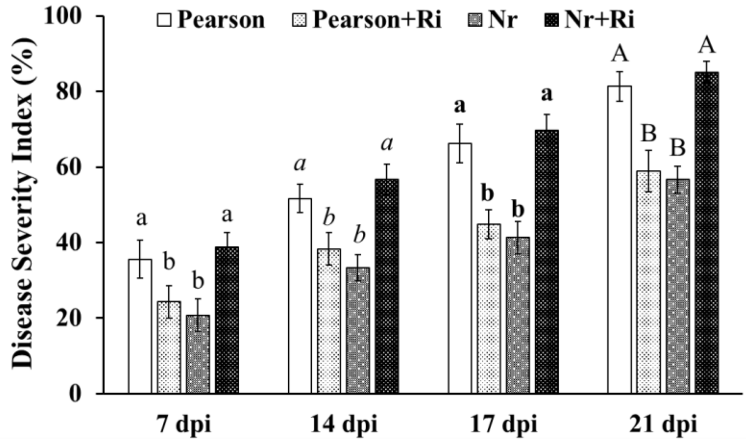


Figure 1. Disease severity index (DSI) of AM and non-AM plants at 7, 14, 17, 21 days post inoculation (dpi) of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in ethylene insensitive mutant (Nr) and its wild-type (Pearson). Ri, *Rhizophagus irregularis* MUCL 43194. Bars present means ± Standard Error. Different regular, italic, bold and capital letters denote significant differences in DSI among treatments at 7, 14, 17 and 21 dpi, respectively.

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

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

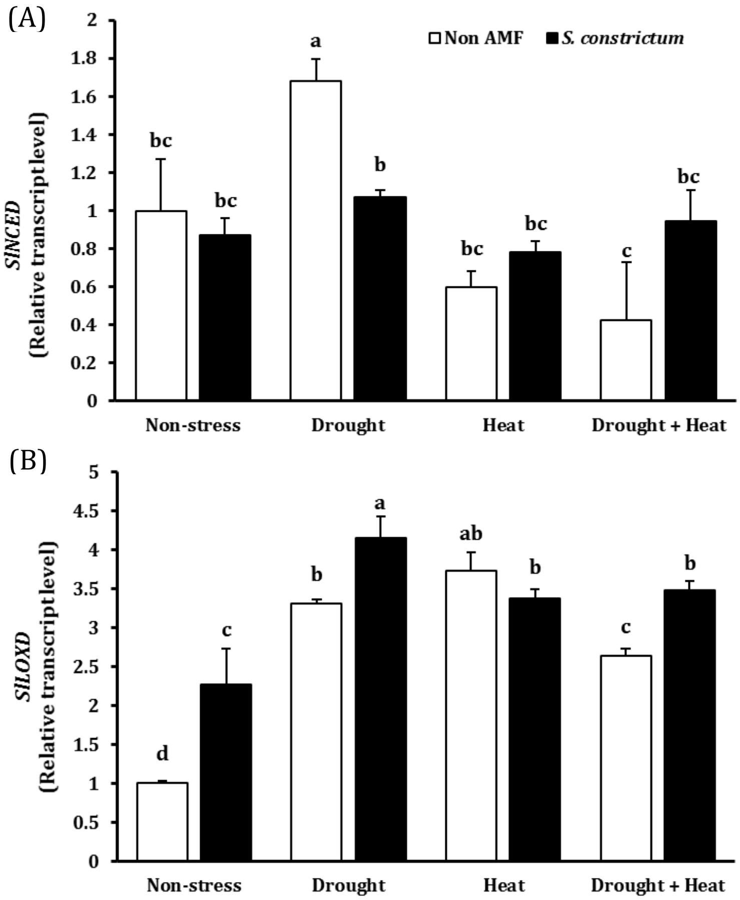
No significant variations in stomatal conductance (g_s), relative water content and leaf water potential between AM and non-AM plants in non-stress conditions were recorded (Data not shown). However, these physiological parameters were reduced sharply as a consequence of stresses, the reductions being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

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

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

Activities of antioxidant enzymes like POD, SOD, CAT in the leaves and roots of uninoculated and inoculated plants were not significantly different in normal growing conditions, but their levels increased in colonized plants under stress conditions. Non-AM plants exhibited considerably lower levels of POD activity than AM plants in stress treatments, although, no significant differences between the two AM species were detected, except for the better enhancement in plants colonized with *S. constrictum* in drought + heat stress. Similarly, the inoculation with *S. constrictum* considerably improved SOD activity under drought and drought + heat stress while AM colonization did not change enzyme activities under heat-stress conditions. CAT activity in both AM-plants increased in the similar fashion as plants were subjected to all stresses.

Based on the physiological performances of AM plants under stress conditions tested, only samples of *S. constrictum* pretreated plants were chosen for the analysis of the expression of ABA, JA biosynthetic gene and an important aquaporin gene. Drought treatment significantly upregulated *SlNCED* gene in roots of non-AM plants in relation to non-stress plants (Figure 2A). Remarkably, the gene expression was lowered in roots colonized by *S. constrictum* as compared with the non-inoculated ones under drought stress while no significant differences in the expression of root *SlNCED* gene between AM and non-AM plants were found under normal growing conditions and other stresses. Root *SlLOXD* gene in both AM and non-AM plants was upregulated by stresses (Figure 2B). Application of *S. constrictum* significantly increased *SlLOXD* gene expression under all conditions except heat stress in comparison with their counterparts in the non-AM plants. Although inoculation of *S. constrictum* enhanced the expression levels of root *SlPIP2.7* in normal growing conditions, drought and heat stress lessened it, while its transcript levels decreased under the combined stress (Figure 2C). Nonetheless, no significant differences in root *SlPIP2.7* expression between AM and non-AM plants were found under heat and drought+heat stress.



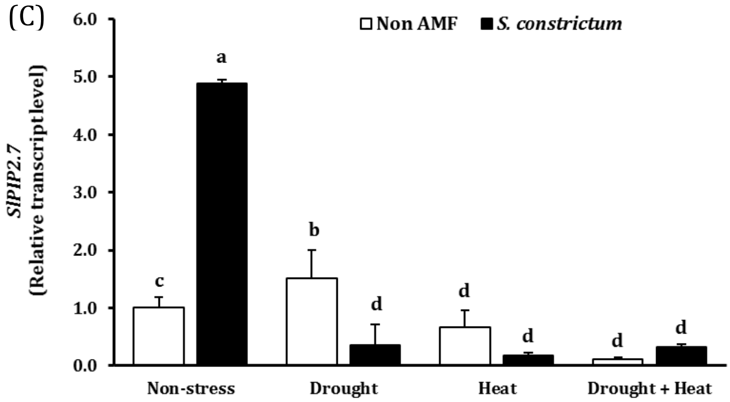


Figure 2. Expression of ABA-biosynthetic gene *SlNCED* (A), JA-biosynthetic gene *SlLOXD* (B), aquaporin genes *SlPIP2.7* (C) in roots of non-AM and *S. constrictum* inoculated plants under non-stress, drought, heat and combined stress conditions. Each bar represents mean ± standard deviation. Different letters indicate significant difference among treatments by Tukey's post hoc test at P ≤0.05.

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

Our analyzed results demonstrated generally that the application of AM, Tri, Pse and their combinations enhanced biomass production in pepper plants although the beneficial gains depended on specific combinations between varieties and microbes.

Inoculation with different microbes alone or together with others altered fruit yield of pepper plants in all pepper cultivars although significant differences depended on specific microbe-cultivar combinations (Table 1). The highest yield was recorded in AM+Tri+Pse combination as the best inoculation in Karpia and Karpex cv., while in Kaptur, the value was highest in plants pretreated by AM+Pse as the most enhancing application. Obviously, application of three inoculants gained highest fruit yield when the main effect of microbial inoculation was compared statistically, however, microbial applications had the greater effect on yield in Karpia and Kaptur (on average, increased 46% and 51%, respectively, in comparison to their non-inoculation treatment) (Table 1). No interaction between microbial treatment and cultivar in fruit yield was recognized.

Table 1. Fruit Yield (g) of microbial inoculations of three pepper cultivars (Karpia, Karpex, Kaptur).

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

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

There were significant differences in the PPO activity among three pepper varieties over time. Karpia and Karpex cultivars were more sensitive to the PPO activity improvement in leaves from microbial applications than Kaptur one. Using only AM enhanced increasingly PPO activity in three varieties whereas its combination with Tri or Tri + Pse decreased it at 49 DAT, then recovered but not completely at 69 DAT. By contrast, the pattern of PPO changes in Tri treatment and the control were increased at the middle phase (49 DAT), then declined at the end (69 DAT).

All inoculations had an increasing trend of POD level during the pepper plant growth in Karpia and Karpex variety; nevertheless, this trend only occurred in AM treatment in Kaptur whereas the pattern of POD activity changes in the control plants of all cultivars peaked at the middle stage, dropped at the final stage. Our results also indicated a substantial difference among three pepper genotypes in the duration of plant growth and Karpia variety had the highest POD activity at the later fruiting phase.

During the plant growth, the overall pattern of SOD activity for all treatment peaked at 49 DAT and finally declined. Most noticeably, AM and its combinations alleviated this drop at the final stage, especially AM and AM+Tri which had four-fold and five-fold of SOD level compared to the control, respectively. In terms of varieties, there were no significant differences of SOD level among the three.

CAT activity was on the downward trend during the pepper plant growth, however, few microbial combinations, triple application in Karpia or dual inoculation of AM and Tri in Karpex and Kaptur produced the upward trend of CAT activity. Apparently, application of beneficial microbes alleviated the decreased trend in the pepper plants. Kaptur cultivar showed the highest CAT level compared to others.

Novel scientific results

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

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

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

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

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

the most effective enhancing in PPO, POD, CAT activity in the plant growth periods under field conditions. Specific interaction between microbe as well as their combination and pepper genotype was highlighted.

5. CONCLUSION

AM colonization can induce systemic resistance to bacterial canker caused by Cmm in tomato plants, however, not all of seven AMF isolates used in our experiment were able to enhance the resistance. Therefore, the efficiency of bio-protection by AM depends on isolates. In addition, ethylene signalling pathway is required for MIR against Cmm. Although mechanisms underlying Cmm resistance of AM plants have not been investigated yet, some mechanisms are proposed. Obviously, further studies are required to elucidate the mechanisms involved in AMF-induced resistance to Cmm with most effective AM species being *Rhizophagus irregularis*.

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

We also investigated beneficial effects of AMF and its combination with renowned microbes *Trichoderma* and *Pseudomonas fluorescens* on three pepper cultivars in the field where diverse abiotic and biotic stresses can occur in single and/or combined way throughout the season. AMF, Tri, Pse and their combinations had different positive impacts on plant growth, yield and a distinct potential to modulate defense enzymes over the time of plant growth under field conditions despite the fact that no combinations always enhance activities of the enzymes all over the periods of plant growth. Microbial inoculations altered the pattern of changes in defense enzymes over the course of the experiment and enhanced activities of defense enzymes, especially in the later plant growth period. Significant differences in modulating the enzymes among genotypes in the periods of plant growth were observed. Some specific combinations between microbes and genotypes in each plant growth stage induced more effectively defense enzymes than others. Remarkably, the combination of AM with two other microbes Tri, Pse (triple inoculation) brought more benefits to host pepper plants when the plants obtained the highest yield and usually induced higher defense enzymes activities during the plant growth periods. Thus, AM application together with other compatible beneficial microbes could be more effective practice under field conditions. Importantly, the combination of microbes depended on genotypes to induce defense enzymes.

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

6. REFERENCES

Abdel Latef, A. A. H., Chaoxing, H. (2014) Does the inoculation with *Glomus mosseae* improve salt tolerance in pepper plants? *J Plant Growth Regul* **33**(3) :644−653.

Abdel Latef, A. A. H., Miransari, M. (2014) The role of arbuscular mycorrhizal fungi in alleviation of salt stress. In: Miransari, M. (Ed.), Use of microbes for the alleviation of soil stresses. *Springer Science+Business Media*, New York, USA, pp. 23−38.

Abdel Latef, A.A. H. (2013) Growth and some physiological activities of pepper (*Capsicum annuum* L.) in response to cadmium stress and mycorrhizal symbiosis. *Journal of Agricultural Science and Technology*, **15**: 1437–1448.

Abdel Latef, A. A. H., Chaoxing, H. (2011a) Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition, antioxidant enzymes activity and fruit yield of tomato grown under salinity stress. *Sci Hortic*, **127**(3): 228−233.

Aebi, H., Lester, P. (1984) Catalase in vitro. *Meth Enzymol*, **105**: 121–126.

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E. (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell & Environment*, **24**(12): 1337-134.

Azcón-Aguilar, C., Barea, J. M. (1997) Applying mycorrhiza biotechnology to horticulture: significance and potentials. *Scientia Horticulturae*, **68**(1-4): 1-24.

Beyer, W. F., Fridovich, I. (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal Biochem*, **161**(2): 559–566.

Birhane, E., Sterck, F. J., Fetene, M., Bongers, F., Kuyper, T. W. (2012). Arbuscular mycorrhizal fungi enhance photosynthesis, water use efficiency, and growth of frankincense seedlings under pulsed water availability conditions. *Oecologia*, **169**(4): 895–904.

Bonfante, P., Genre, A. (2010) Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. *Nature Communications* **1**: 48.

Boyer, J. S. (1995) Measuring the water status of plants and soils. *Academic Press*, San Diego. 178 p.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**(1-2): 248-254.

Cvikrová, M., Gemperlová, L., Martincová, O., Vanková, R. (2013). Effect of drought and combined drought and heat stress on polyamine metabolism in proline-over-producing tobacco plants. Plant physiology and biochemistry **73**: 7-15.

Duc, N. H., Mayer, Z., Pék, Z., Helyes, L., Posta, K. (2017). Combined inoculation of arbuscular mycorrhizal fungi, Pseudomonas fluorescens and Trichoderma spp. for enhancing defense enzymes and yield of three pepper cultivars. *Applied ecology and environmental research* **15**(3): 1815-1829.

Fehrmann, H., Dimond, A. E. (1967) Peroxidase activity and phytophthora resistance in different organs of the potato plant. *Phytopathology*, **57**: 69-72.

Gaur, A., Adholeya, A., Mukerji, K. G. (1998) A comparison of AM fungi inoculants using *Capsicum* and *Polianthes* in marginal soil amended with organic matter. *Mycorrhiza*, **7**(6): 307–312.

Giovanetti, M., Mosse, B. (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytology*, **84**(3): 489-500.

Hajiboland, R. (2013) Role of arbuscular mycorrhiza in amelioration of salinity. In: Ahmad P, Azooz MM, Prasad MNV (eds), Salt stress in plants: signalling, omics and adaptations. *Springer*, New York, pp. 301−354.

Hatfield, J. L., Walthall, C. L. (2015) Meeting global food needs: Realizing the potential via Genetics × Environment × Management interactions. Agronomy Journal, 107(4): 1215-1226.

Heath, R. L., Packer, L. (1969) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys*, **125**(1): 189-198.

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., Pozo, M. J. (2012) Mycorrhiza-induced resistance and priming of plant defenses. J Chem Ecol **38**(6): 651–664.

Kaya C, Ashraf M, Sonmez O, Aydemir S, Tuna AL, Cullu MA (2009) The influence of arbuscular mycorrhizal colonisation on key growth parameters and fruit yield of pepper plants grown at high salinity. *Sci Hort*, **121**(1): 1−6.

Livak, K. J., Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, **25**(4): 402–408.

Ngumbi, E., Kloepper, J. (2014) Bacterial-mediated drought tolerance: current and future prospects. *Appl Soil Ecol*, **105**: 109–125.

Pozo, M. J., Verhage, A., García-Andrade, J., García, J. M., Azcón-Aguilar, C. (2009) Priming plant defence against pathogens by arbuscular mycorrhizal fungi. In: Azcón-Aguilar, C., Barea, J. M., Gianinazzi, S., Gianinazzi-Pearson, V. (eds.), Mycorrhizas - Functional Processes and Ecological Impact. *Springer Berlin Heidelberg*.

Rathmell, W. G., Sequeira, L. (1974) Soluble peroxidase in fluid from the intercellular spaces of tobacco leaves. *Plant Physiology*, **53**(2): 317-318.

Raupach, G. S., Liu, L., Murphy, J. F., Tuzun, S. T., Kloepper, J. W. (1996) Induced systemic resistance in cucumber and tomato against *cucumber mosaic cucumovirus* using plant growth-promoting rhizobacteria (PGPR). *Plant Dis*, **80**: 891-894.

Russo, V. M., Perkins-Veazie, P. (2010) Yield and nutrient content of bell pepper pods from plants developed from seedlings inoculated, or not, with microorganisms. *Hort Science*, **45**(3): 352–358.

Turner, T. R., James, E. K., Poole, P. S. (2013) The plant microbiome. *Genome Biol*, **14**(6): 209.

Vierheilig, H., Coughlan, A. P., Wyss, U., Piché, Y., (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology*, **64**(12): 5004-5007.

Vosátka, M., Albrechtová, J. (2008): Theoretical aspects and practical uses of mycorrhizal technology in floriculture and horticulture, pp 466–479. In: Teixeira da Silva, J. A. (ed). Floriculture, ornamental and plant biotechnology. Advances and topical issues. *Glob. Sci. Books Ltd.*, Takamatsu, Japan.

Zhou, J., Wang, J., Li, X., Xia, X-J., Zhou, Y-H., Shi, K., Chen, Z., Yu, J-Q. (2014) H_2O_2 mediates the crosstalk of brassinosteroid and abscisic acid in tomato responses to heat and oxidative stresses. *Journal of Experimental Botany*, **65**(15): 4371–4383.

RELATED PUBLICATIONS

Peer-reviewed scientific articles

Duc, N. H. – Mayer, Z. – Pék, Z. – Helyes, L. – Posta, K., 2017. *Combined inoculation of arbuscular mycorrhizal fungi, Pseudomonas fluorescens and Trichoderma spp. for enhancing defense enzymes and yield of three pepper cultivars*. Applied Ecology and Environmental Research. 15(3):1815-1829. DOI: 10.15666/aeer/1503_18151829 (**IF: 0.792**).

Nguyen Hong Duc, Posta Katalin. *Mycorrhiza-induced alleviation of plant disease caused by Clavibacter michiganensis subsp. michiganensis in tomato* and role of ethylene in mycorrhiza-induced resistance (submitted for Acta Biologica Hungarica)

Nguyen Hong Duc, Posta Katalin. *Arbuscular mycorrhizal fungi alleviates negative effects of drought, heat stress, combined drought and heat stress on tomato plants* (submitting for a journal with impact factor)

Hong Duc Nguyen, Au Trung Vo, Katalin Posta, 2017. *Impacts of arbuscular mycorrhizal fungi on plant growth and yield of three pepper genotypes.* Columella: Journal of Agricultural and Environmental Sciences. 4(1):49-51. DOI: 10.18380/SZIE.COLUM.2017.4.1.suppl.

Conferences

Nguyen Hong Duc, Vo Trung Au, Posta Katalin, 2017. *Arbuscular mycorrhizal fungi improve tolerance to heat stress in tomato plants*. Poster. Asia Mycological Congress, Ho Chi Minh City, Vietnam, 10-13th Oct 2017.

Vo Trung Au, **Nguyen Hong Duc**, Posta Katalin, 2017. *How do arbuscular mycorrhizal fungi affect the growth of Eclipta prostrata under different nutrient supplies?* Poster. Asia Mycological Congress, Ho Chi Minh City, Vietnam, 10-13th Oct 2017.

Nguyen Hong Duc, Vo Trung Au, Posta Katalin, 2017. *Impacts of arbuscular mycorrhizal fungi on plant growth and yield of three pepper genotypes*. Presentation. 16th Alps – Adria Workshop - Synergism in science. Opatija, Croatia, 3 – 8th Apr 2017.

Vo Trung Au, **Nguyen Hong Duc,** Posta Katalin, 2017. *Impact of arbuscular mycorrhizal inoculation and nutrient supply on the growth of medicinal plant, Eclipta prostrata* L. Presentation. 16th Alps – Adria Workshop - Synergism in science. Opatija, Croatia, 3 – 8th Apr 2017.

Mayer Zoltán, Posta Katalin, **Nguyen Hong Duc,** 2017. *Gene expression of glutathione-S-transferase in sunflower (Helianthus annuus L.) plants inoculated with arbuscular mycorrhizal fungi under temperature stress*. Poster. 16th Alps – Adria Workshop - Synergism in science. Opatija, Croatia, 3 – 8th Apr 2017.

Nguyen Hong Duc, Mayer Zoltan, Posta, 2016. *Effect of arbuscular mycorhizal fungi and its combinations with Tricoderma and plant growth promoting rhizobacteria on antioxidant superoxide dismutase activity in three pepper cultivars*. Poster. Nature conservation investigations in NATURA 2000 sites in sustainable development in the Carpathian basin III. Szent István University, Godollo, Hungary, 17-18 Mar 2016.

Mayer Z., **N. H. Duc**, Posta K., 2015. *Arbuscular mycorrhizal (AM) fungi as an useful biotechnological tool for increasing plant defence mechanisms to alleviate different stresses*. Presentation. 6th CASEE conference – Latest Trends in Bioeconomy in Danube Region. Slovak University of Agriculture in Nitra, Slovak Republic, 24-26 May 2015.

Other scientific articles with other topic published during PhD program

Zoltán Mayer, **Nguyen Hong Duc,** Zita Sasvári and Katalin Posta. 2017. *How influence arbuscular mycorrhizal fungi the defense system of sunflower during different abiotic stresses.* Acta Biologica Hungarica 68(4): 376–387. (**IF: 0.506**)

Zoltán Mayer, **Nguyen Hong Duc,** Katalin Posta, 2017. *Gene expression of glutathione-S-transferase in sunflower (Helianthus annuus* L.*) inoculated with arbuscular mycorrhizal fungi under temperature stresses*. Columella: Journal of Agricultural and Environmental Sciences. 4(1):69-72. DOI: 10.18380/SZIE.COLUM.2017.4.1.suppl.