COMBINED INOCULATION OF ARBUSCULAR MYCORRHIZAL FUNGI, *PSEUDOMONAS FLUORESCENS* AND *TRICHODERMA* SPP. FOR ENHANCING DEFENSE ENZYMES AND YIELD OF THREE PEPPER CULTIVARS

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Abstract. Field experiment was carried out at the experimental station of Szent István University, Gödöllő, Hungary to explore the impact of arbuscular mycorrhizal fungi only alone or together with *Trichoderma* and plant growth-promoting bacteria on defense enzymes and yield of three pepper varieties. The seven inoculation treatments consisting of arbuscular mycorrhizal fungi (AM), *Trichoderma* (Tri), plant growth promoting bacteria (Pse) and their combinations (AM+Tri; AM+Tri+Pse; AM+Pse) together with three pepper hybrids and non-inoculation (control) plants were arranged in a randomized complete block design. Defense enzyme activities polyphenol oxidase (PPO), peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) of various treated plants were measured just before flowering, representing the most sensitive stage of plants. The results showed that AM+Tri+Pse treatment enhanced the yield most among microbial inoculations. Highest yield was recorded in the triple treatment in Karpex cultivar, however, Karpia and Kaptur variety obtained more improved yield by microbial inoculations. Defense enzymes activities generally were most induced in the combination of three inoculants in cultivars whereas different responses in induction of defense enzymes were found in other microbial treatments, depending on specific interactions between microbe and pepper genotype. These results suggested that the triple application brought more benefits to the host plant.
Keywords: *biofertilizers, Capsicum annuum L., antioxidative enzymes, pepper productivity, pepper varieties*

Abbreviations:

AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorhizal fungi
Tri	*Trichoderma*
Pse	*Pseudomonas fluorescens*
DAT	Days after transplanting
ROS	Reactive oxygen species
PPO	Polyphenol oxidase
POD	Peroxidase
SOD	Superoxide dismutase
CAT	Catalase

Introduction

Pepper (*Capsicum annuum* L.) is one of the main vegetables, cultivated worldwide and has important nutritional and economic values. It represents high pharmaceutical values due to its abundance of vitamins A, B6, C, E, K and minerals (manganese,

potassium) moreover a very good source of dietary fiber (Malik et al., 2011; Yang et al., 2010; López et al., 2012).

Besides plant varieties, pepper production and quality are fluctuated due to various stress conditions (high temperature, drought, pathogens) which often cause up to 70% losses in yield giving a barrier in pepper production (Gajanayake et al., 2011).

There is a growing awareness of sustainable agriculture, how to minimize the damage, reduced chemical inputs and ensure protection of environmental stresses. The application of beneficial microbes could be an important technique giving environment friendly way to enhance yield of pepper and plant resistances to abiotic and biotic stresses.

Among effective microorganisms arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria together with *Trichoderma* are the most frequently used inoculants (Singh et al., 2011).

AMF can colonize 80% of terrestrial plant species including many important agricultural and horticultural plants (Smith and Read, 2008). There are important aspects from mycorrhizal colonization including 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). Furthermore, root colonization by AMF can improve the plant resistance to biotic or abiotic stresses (Pereira et al., 2015) through significant alterations in the hormonal balance and transcriptional profile, primary and secondary metabolisms (Jung et al., 2012; López-Ráez et al., 2010).

Similarly, plant growth promoting bacteria also contribute positively to plant fitness through direct and indirect mechanisms, induction of resistance by production of phytohormones, solubilization of inorganic phosphates, increased iron nutrition through iron-chelating siderophores and volatile compounds that affect the plant signaling pathways (Ahemad and Kibret, 2014; Lugtenberg and Kamilova, 2009).

Trichoderma spp. are free-living filamentous fungi and some of them are the most potent agents for the biocontrol of soil borne plant pathogens (Elad, 1996). It has been known for many years that they produce a wide range of antibiotic substances and that they parasitize other fungi (Sivasithamparam and Ghisalberti, 1998). In addition, they inhibit or degrade pectinases and other enzymes that are essential for plant-pathogenic fungi (Zimand et al., 1996).

The study of interactions between beneficial microorganisms associated with plant roots is important, because such interactions might either enhance or inhibit the beneficial effects of individual species. It has also been indicated that some *Trichoderma* strains may influence AM fungi activity (Martinez et al., 2004; Martínez-Medina et al., 2009).

Several studies have demonstrated a positive effect of the dual or combined inoculation on plant performance in the presence as well as in the absence of plant pathogens (Datnoff et al., 1995; Siddiqui and Mahmood, 1996; Chandanie et al., 2009), while others reported a reduction in plant shoot and root dry weights (McAllister et al., 1994). Nonetheless, the studies on the interactions between *Trichoderma* spp. and AMF had contrast results such as antagonistic, neutral, and synergistic impacts on plants (Green et al., 1999; Martínez-Medina et al., 2009).

Although use of the beneficial microbes is widely investigated in many plants, little attention has been paid to defense enzyme activities induced by multiple application of the microbial inoculants and offered by different pepper hybrids under field conditions.

Therefore, the aim of this study was to examine the potential of AM mixture, *Trichoderma*, *Pseudomonas fluorescens* and their combined applications for improvement of fruit yield and inducing defense enzymes (PPO, POD, SOD, CAT) in different pepper genotypes under field conditions following the practice.

Materials and methods

Plant growth and experiment design

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

Table 1. Chemical properties of the soil in the experiment.

pH	EC (mS cm^{-1})	Organic matter (%)	NO$_3$ (N)	P$_2$O$_5$	K$_2$O	Ca	Mg	Fe	Cu	B	SO$_4$ (S)	Cl	HCO$_3$	Na
			mg kg^{-1}											
7.1	0.254	1.61	15.6	36.0	60	140.3	24.3	208	7.6	0.5	4.0	0	534	10

The average temperatures and precipitations were 12.8^{o}C and 8.95 mm in May; 16.8^{o}C, 7.2 mm in June; 18.7^{o}C, 8.84 mm in July; 15.7^{o}C, 11 mm in August; 12.6^{o}C, 8.21 mm in September 2014, respectively.

Seedlings of pepper varieties were propagated at the beginning of April in a greenhouse using special horticulture substrate [Klasmann TS3: 80% white sphagnum peat and 20% frozen black sphagnum-peat, slow-release 14N–16P–18K (w/w/w) fertilizer, pH 6.00] for 7 weeks. Then the seedlings were transplanted on 16th May, arranged in double rows with a distance of 0.8 m between beds, 0.3 m between the rows and 0.3 m between the plants. 72 g of NPK and 36 g of Ca(NO$_3$)$_2$ per square meter were applied for the whole season and drip irrigation was used to maintain substrate moisture content close to field capacity values (20% w/w) during the growth period.

All treatments including seven microbial inoculations and three cultivars were arranged in 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 days after transplanting (DAT) and kept in the -80°C until enzyme assays. Roots from five plants per treatment were taken just before harvesting for estimation of mycorrhizal colonization rate. The pepper harvesting was performed randomly by hand at the biological maturity stage in August and evaluated for plants in each treatment.

Microbial inoculations

Before transplanting mycorrhizal fungi in a commercial product Symbivit® (mixture of *Glomus intraradices, G. mosseae, G. etunicatum, G. claroideum, G. microaggregatum, G. geosporum*) produced by Symbiom Ltd. (Lanskroun, Czech Republic; www.symbiom.cz) was applied at 25 g of inoculum per pepper seedling into the planting hole and seedlings were planted immediately (AM treatment).

Trichoderma harzianum isolate (SzIE35) has previously been isolated and preserved in the collection of Szent István University. The isolate was cultured on Potato Dextrose Agar (PDA) at $25^{o}C$ for 4 days and the inocula was produced in potato-dextrose broth shake culture at (150rpm) for 1 week at $25^{o}C \pm 2^{o}C$. The cultures were filtered through a double layer of sterilized gauze and clean conidial suspension was prepared at the concentration of 10^{7} ml^{-1}. 10 ml fresh prepared conidia with the volume of 0.2 liter sterile water were implemented to the seedlings 27 days after transplanting (Tri treatment).

Pseudomonas fluorescens isolate (PK17) originated from the collection of the Szent István University was prepared for plant growth-promoting bacteria inoculation. The PK17 inoculum was prepared by growing in liquid R2A medium (Difco) at $25°C$ for 36 h, suspended in 0.1 M MgSO$_4$ buffer, washed twice and re-suspended in distilled water at 10^{8} cfu per ml. 10 ml fresh bacterium suspension was applied to the respective treatments (10^{8} cfu ml^{-1}) 27 days after transplanting (Pse treatment).

Enzyme extraction and measurement of enzymes activities

0.5 g of frozen ($-80^{o}C$) leaf material of each sample from treatments was homogenized in liquid N$_2$ with 3 ml of 50 mM Tris-HCl buffer (pH 7.8) containing 7.5% (w/v) polyvinyl-pyrrolidone K25 and 1 mM Na$_2$EDTA, and centrifuged at 10,000 x g for 20 minutes at $4^{o}C$. The supernatants were used for measuring peroxidase, polyphenol oxidase, superoxide dismutase and catalase activities, the protein concentration of all leaf extracts was estimated according to the method of Bradford (1976).

Polyphenol oxidase (PPO, EC 1.10.3.1) activity was measured by modified Fehrmann and Dimond (1967) method. The 2.2 ml of reaction mixture made up of 0.1 M sodium phosphate buffer (pH 6.0), 1 mM Na$_2$EDTA, 20 mM catechol with 200 µl of the crude leaf extract was used to assay the enzyme activity at 400 nm in 10 minutes. Changes of absorbance per protein concentration per unit time was estimated.

Peroxidase (POD, EC 1.11.1.7) activity was determined by Rathmell and Sequeira (1974) method. Briefly, 10 µl plant extract was added to 2.2 ml of reaction mixture consisting of 0.1 M sodium phosphate buffer (pH 6.0), 100 µl of 50 mM Guaiacol, 100 µl of 12 mM H$_2$O$_2$. The absorbance was recorded at 436 nm in 5 minutes. The enzyme activity was calculated by the changes of absorbance per mg protein per minute.

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

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

Assessment of mycorrhizal colonization of AMF

Samples for estimating root colonization were collected before harvesting. Five randomly chosen pepper plants from the same treatment were dug out with a soil core of $25 \times 25 \times 25$ cm. The roots and the soil were stored in separate plastic bags at 4°C until processing within 24 h. Approximately, 500 mg of fine roots from each plant were transferred to separate tubes and were subjected to the staining technique of Vierheilig et al. (1998). Internal fungal structures (hypae, arbuscules, vesicules) were examined under a stereomicroscope at × 100 magnification and the percentage of root length colonized calculated using the gridline intersect method (Giovannetti and Mosse, 1980).

Statistical analysis

SAS 9.1 (SAS Institute, Cary, North Carolina) package for Windows was used for statistical analysis. All data was evaluated by two-way factorial analysis of variance (ANOVA) and Tukey's Post hoc test at P < 0.05.

Results and discussion

Mycorrhizal colonization

AM colonization in mycorrhizae-inoculated plants was remarkably higher than in others without AM inoculation although no significant differences could be found among mycorrhizal treatments (*Fig. 1*). Interestingly, the combination of three inoculants reached maximum colonization percentage in roots, up to 59% in Karpia cv. whereas the rates still gained approximately 30% in no microbial treatment under field conditions. No substantial differences in mycorrhizal colonization rates among pepper cultivars and no interaction between microbial inoculations and cultivars were recorded (*Table 2*).
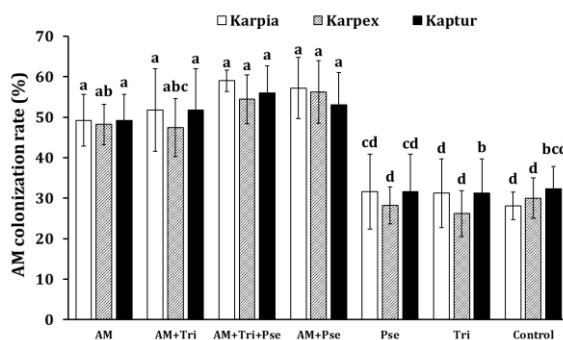

Figure 1. Mycorrhizal colonization of microbial inoculations of three pepper cultivars, Karpia, Karpex, Kaptur. AM, Arbuscular mycorrhizal fungi; Tri, Trichoderma; Pse, Pseudomonas fluorescens. Each bar presents mean ± standard deviation. Different letters denote significant differences among treatments according to Tukey's post hoc test (P < 0.05).

Table 2. *Significance of two main effects (microbial inoculation, M and cultivar, C) and their interaction between M and C on different parameters.*

Parameters	Microbial inoculation (M)	Cultivar (C)	M x C
AM colonization rate	***	ns	ns
Fruit yield	***	ns	ns
Enzyme activities:			
PPO	***	***	***
POD	***	***	***
SOD	***	ns	ns
CAT	***	***	***

ns, non significant. ***, significant differences at P<0.001 according to Tukey's post hoc test.

Fruit yield

Inoculation with different microbes alone or together with others altered fruit yield of pepper plants in all pepper cultivars although significant differences depended on specific microbe-cultivar combinations (*Table 3*). 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 main effect of microbial inoculation was compared statistically, however, microbial applications had greater effect on yield in Karpia and Kaptur (on average, increased 46% and 51%, respectively, in comparison to their non-inoculation treatment) (*Table 3*). No interaction between microbial treatment and cultivar in fruit yield was recognized (*Table 2*).

Table 3. *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%	

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 Tukey's post hoc test (P < 0.05).

The positive effects of beneficial microbes on yield have been described in many studies (Pereira et al., 2015; Yuan et al., 2016; Azarmi et al., 2016; Pascale et al., 2017;

Yadav et al., 2015). Various reports showed that AM inoculation enhanced fruit yield in pepper plants (Abdel Latef and Chaoxing, 2014; Abdel Latef, 2013; Boonlue et al., 2012; Hernádi et al., 2012; Tanwar et al., 2013) and in tomato (Bakr et al., 2017), which is dissimilar to our result. Pascale et al. (2017) illustrated that *Trichoderma harzianum* and *Trichoderma atroviride* improved grape yield and quality while using *Pseudomonas fluorescens* with organic fertilizer showed maximum enhancement in yield of cucumber (Ahamd et al., 2015). Our results demonstrated that combined three microbes (AM+Tri+Pse) had best increment in fruit yield in Karpia and Karpex cv., which may indicate that there were synergistically beneficial impacts from the microbes on the yield. Several examples of the synergistic effects on biomass were reported in bean plants with AM+Pse (Younesi and Moradi, 2014), in tobacco plants with using *Trichoderma harzianum* in bioorganic fertilizer and AM fungi (Yuan et al., 2016), greatest growth and yield in sunflower plants when combined using AM, *Trichoderma viride* and Pse (Yadav et al., 2015). The cumulative benefit of combining multi-bioinoculants may collectively result from nutrient and water improvement, induced resistance or enhanced tolerance to biotic and/or abiotic stresses, more effective protection from pest and plant diseases under field conditions. Nevertheless, beneficial effects of microbial inoculations on pepper production (Russo, 2006; Russo and Perkins-Veazie, 2010), yield and fruit quality in tomato plants (Bal and Altintas, 2006) were not observed.

Antioxidative and defense enzymes

The leaf PPO activities were lowered due to Pse inoculation in Karpia and Kaptur, application of AM alone, Tri alone and combination of AM and Pse in three pepper cultivars as compared to their control plants (*Fig. 2A*). The highest values of PPO were found in the triple inoculated treatments (AM, Tri, Pse), particularly in Karpia the activity of PPO was most pronounced. Dual inoculation of AM and Tri made no changes in the PPO activity of each cultivar (P<0.05). Using mycorrhizal inoculation alone induced significantly the highest POD activity in leaves of Karpex and Kaptur variety as compared to their control (*Fig. 2B*). It is worth mentioning that other microbial treatments in Karpia and Kaptur substantially declined or made no change in POD levels while in contrast, AM+Tri and Pse treatment triggered higher POD activities in Karpex. Remarkably, the integrated inoculation of AM, Tri, Pse in Kaptur increased this enzyme level in relation to the control.

AM treatment in Karpia and Kaptur, Tri application in Kaptur variety decreased substantially the leaf SOD activity while the inoculation of Pse in Karpia and Karpex, combination of AM+Tri+Pse in Karpex and Kaptur cultivar increased considerably this enzyme (*Fig. 2C*). Besides that, other treatments did not change significantly the SOD level compared to their control in each variety. On the other hand, all microbial treatments lowered significantly the CAT activity in leaves except the case of Pse inoculation in Karpex, Tri treatment in Kaptur (*Fig. 2D*).
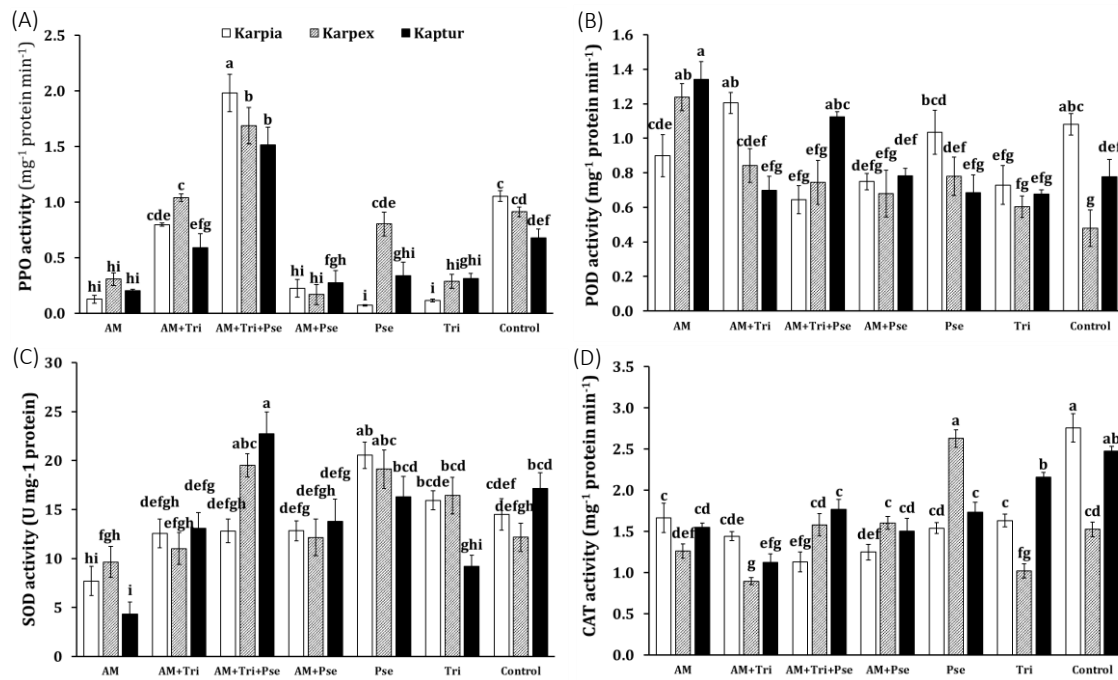
Figure 2. *Polyphenol oxidase (PPO) (A), peroxidase (POD) (B), superoxide dismutase (SOD) (C), catalase (CAT) (D) activity of microbial treatments in leaves of three pepper cultivars, Karpia, Karpex, Kaptur at 29 days after transplanting. AM, Arbuscular mycorrhizal fungi; Tri, Trichoderma; Pse, Pseudomonas fluorescens. Each bar presents mean ± standard deviation (n = 5). Different letters denote significant differences among treatments according to Tukey's post hoc test (P < 0.05).*

Significant differences in the PPO, POD, CAT activities among three pepper varieties were found after 29 days of transplanting (*Table 4*). Karpex cultivar showed the highest PPO activity whereas strongest POD activity was recorded in Karpia and more dominant CAT activity was in Kaptur. No significant differences in SOD activity among cultivars were observed.

Table 4. *Main effects of cultivar on PPO activity in leaves of various microbial inoculations.*

Enzyme activities	Karpia	Karpex	Kaptur
PPO (mg^{-1} protein min^{-1})	0.62 b	0.74 a	0.57 b
POD (mg^{-1} protein min^{-1})	0.90 a	0.76 b	0.87 a
SOD (U mg^{-1} protein)	13.84 ns	14.29 ns	13.80 ns
CAT (mg^{-1} protein min^{-1})	1.63 b	1.50 c	1.75 a

ns, non significant. Different letters in each row denote significant differences according to Tukey's post hoc test (P < 0.05) among pepper cultivars.

Under field conditions, plants are frequently confronted with environmental adversities including biotic and abiotic factors during their life. To deal with environmental stresses, plants possess elaborate defense mechanisms that are activated depending on specific stressors, where defense enzymes are essential components in stress-defended responses in plants. Among these enzymes, PPO and POD are crucial ones involved in ligninfication processes and phenols formation, resulting in defense

barriers for strengthening the plant cell structure (Avdiushko et al., 1993). PPO is usually produced during wounding, pathogen invasion or herbivore attack, considered as plant defense against pathogens, herbivores, also related to ROS generation (Mayer, 2006) whereas POD belongs to Pathogenesis-Related Proteins 9 (PR-9) family, responsive to pathogen invasions (Ray et al., 1998) but can produce as well as scavenge H_2O_2 in the first and next phase, respectively (Siegel 2003). ROS mainly consisted of singlet oxygen ($^1O_2^-$), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$). ROS is also inevitable by-products generated in many normal biochemical pathways in plants. *In planta*, ROS production and detoxification are balanced under normal metabolic processes, regulated by cellular redox homeostasis (Pospísil, 2012). When plants are subjected to abiotic and biotic stresses ROS is overproduced, breaking the balance, leading to toxification and degradation of lipids, proteins, DNA by their oxidation capacity, eventually causing cell death (Wu et al. 2014; Foyer and Noctor, 2005). To cope with oxidative destruction, plants have developed both ROS non-enzymatic and enzymatic scavengers consisted of SOD and CAT. SOD is the first defense line against ROS (Alscher et al., 2002) owing to its capability of catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 (Wu et al. 2014), then CAT and other antioxidative enzymes detoxify H_2O_2 to H_2O and O_2 (Apel and Hirt, 2004). Thus effective activation of these enyzmes can enhance stressed plant tolerance, prevent damages caused by abiotic and biotic stresses.

Our analysed results demonstrated that under field environment application of the AM alone mostly decreased significantly PPO, SOD, CAT activity in leaves of all varieties as compared to their controls (*Table 5*, *Fig. 2A, 2C, 2D*), which is in line with observations of Kohler et al. (2009), Mollavali et al. (2016), Minton et al. (2016). The finding may suggest that pepper plants gained benefits such as nutrient and water improvements from the symbiosis with the AM, therefore, might produce less ROS, then lower the defense enzymes. On the other hand, inoculation of AM also triggered highest POD levels (*Table 5*), particularly in Karpex and Kaptur cultivar (*Fig. 2B*), which is consistent with a result in pepper plants under salinity stress (Abdel Latef and Chaoxing, 2014). In contrast with our results, other studies show mycorrhizal treatments stimulated antioxidative enzymes in leaves of various plants (Pedranzani et al., 2015; Chu et al., 2016; Jiang et al., 2016; Hashem et al., 2016; Sarkar et al., 2016) in pepper plants such as CAT when plants exposed to 1 mM NaCl in the long term (Cekic et al., 2012), SOD under salinity stress (Abdel Latef and Chaoxing, 2014).

Table 5. Main effects of microbial inculation on defense enzymes activity in leaves of three pepper varieties.

Defense enzyme activity	AM	AM+Tri	AM+Tri+Pse	AM+Pse	Pse	Tri	Control
PPO (mg^{-1} protein min^{-1})	0.21 d	0.81 b	1.72 a	0.26 d	0.40 c	0.23 d	0.88 b
POD (mg^{-1} protein min^{-1})	1.16 a	0.91 b	0.84 bc	0.73 cd	0.83 bc	0.67 d	0.78 cd
SOD (U mg^{-1} protein)	7.22 c	12.22 b	18.35 a	12.93 b	18.67 a	13.85 b	14.60 b
CAT (mg^{-1} protein min^{-1})	1.49 c	1.15 d	1.49 c	1.45 c	1.96 b	1.60 c	2.25 a

AM, Arbuscular mycorrhizal fungi; Tri, *Trichoderma*; Pse, *Pseudomonas fluorescens*. Different letters in each row denote significant differences among microbial treatments according to Tukey's post hoc test ($P < 0.05$).

The plant growth promoting bacteria Pse lessened significantly PPO and CAT in leaves, compared to the control, however, SOD activity were greatly increased (*Table 5*), particularly in Karpia and Karpex variety (*Fig. 2C*) whilst POD remained unchanged in Pse pretreated plants with exception of the higher increase in Karpex (*Table 5, Fig. 2B*). Azarmi et al. (2016) showed that pistachio seedlings inoculated by *Pseudomonas fluorescens* triggered significant increases in PPO, POD, SOD, CAT ativities in leaves under Zn and NaCl stresses. By contrast, soybean plants treated by *Pseudomonas* sp. reduced considerably CAT, PPO, SOD, POD activity in leaves in comparison with the non-inoculated plants (Kumari et al., 2015). Apparently, the combination of these bacteria with AM decreased significantly the activities of all enzymes in relation with its counterpart in Pse inoculation alone (*Table 5*). This observation is in accordance with the report of Kohler et al. (2008) that combination of *Rhizophagus irregularis* or *Funneliformis mosseae* with *Pseudomonas mendocina* Palleroni dropped SOD, CAT level in leaves of *Lactuca saiva* L. under severe drought stress. However, POD activity in our results is in contrast to that of the authors. This might suggest a complex and specific interaction between plants and microbial partners under particular stresses.

Application of Tri reduced PPO, CAT activities but remained unchanged POD, SOD levels (*Table 5*), which is inconsistent with a previous study in maze plants under drought stress (Guler et al., 2016). Instead of decreasing PPO, SOD activity in leaves as AM plants did, AM+Tri did not change considerably PPO, SOD levels whereas, intriguingly, these enzyme activities in the three-inoculant treatment (AM+Tri+Pse) leaped (*Table 5*). In detail, integrated AM+Tri+Pse induced highest PPO in all cultivars (*Fig. 2A*), relatively high POD level in Kaptur (*Fig. 2B*), highest SOD in Karpex and Kaptur (*Fig. 2C*). The reasons for these increases may be that multiple inoculation with three different microbes activated the host plant defense system greater than single or dual inoculation did before plants recognized the applied microbes as non-pathogenic ones, then host plants might take an advantage of the induced/primed defense system as an important mechanism to protect themselves against unfavoured conditions in the field. Several workers reported that application of *Trichoderma* spp. can improve defense enzymes in plants (Guler et al., 2016; Gajera et al., 2016) and co-inoculation of AM fungi and *Trichoderma* spp. have synergistic impacts on controlling phytopathogens (Saldajeno et al., 2008; Martínez-Medina et al., 2009; Srivastava et al., 2010). Yuan et al. (2016) illustrated that plants inoculated by *Glomus mosseae* or *Trichoderma harzianum* amended bioorganic fertilizer elevated significantly PPO and POD activity; however, the co-inoculation gained the highest PPO and POD level as compared to the non-inoculated control.

Overall, our findings highlighted different responses among pepper genotypes to impacts of microbial inoculants on yield and defense enzymes activities. AM, Tri, Pse, well known as plant performance enhancers can influence the pool of enzymatic scavengers, defense enzymes and positively impact on fruit yield in all pepper varieties. In fact, PPO, POD, SOD and CAT activity of the microbial treatments in our experiment were different from non-microbe plants. The differences in microbe-induced enzymes activities even took place among cultivars, which may be due to host genetic variation among pepper varieties, compatibility of interaction between microbe and pepper genotype. Noticeably, effectiveness of the applied microorganisms and their combinations were not always defense stimulators under field conditions. Inconsistent results in defense enzymes from previous studies may be originated from different experiment conditions that most work were implemented in controlled environments,

different species and/or strains of microbes applied, specific microbe-cultivar associations. Indeed, in field environment, many stresses can occur simultaneously and are associated to complicated interactions among biotic, abiotic and edaphic environments, thus plants often tolerate combinations of different stresses. Modulation of plant defense system by the beneficial microbes utilized could be an important gainful impact of plants cultivated under field conditions, which might lead to better plant protection from various stresses, eventually increased fruit yield in pepper plants. In this study, specific interactions between microbe as well as their combination and pepper genotype were also observed, which was described in many studies (Sensoy et al., 2007; Cekic et al., 2012).

In conclusion, each beneficial microbe and their combined inoculations have a different potential to modulate defense enzymes and positively influence on fruit yield under field conditions. Karpia and Kaptur variety gained more beneficial effects from microbial inoculations on yield than Karpex. Remarkably, using combination of three different microbes (AM+Tri+Pse) generally gained synergic results in induction of defense enzymes as well as enhanced yield, which may be due to collective mechanisms of host protection induced by three diverse inoculants.

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