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Effect of *Trichoderma* spp. on Growth Promotion and Antioxidative Activity of Pepper Seedlings

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HIGHLIGHTS

- Two different *Trichoderma* strain-dependent modes of actions.
- Perspective *Trichoderma* isolates induce formation and accumulation of ROS.
- Positive correlation between PPx, SOD, CAT activity and germination.
- Nine *Trichoderma* isolates significantly increase root weight of pepper seedlings

Abstract: One of the main challenges in pepper production is to enhance seed germination energy and germination, and to grow healthy nursery plants with strong root system. *Trichoderma* species colonize roots as they grow and provide season-long benefits to plants, which is why *Trichoderma* species are widely used as plant growth promoter agents and promoters of plant defence mechanisms. This study evaluated the effectiveness of seed biopriming with *Trichoderma* isolates for growth promotion of pepper plants in early stage and their effects on seedling physiology. Nine out of ten *Trichoderma* isolates positively affected root weight of pepper seedlings, while three out of ten positively affected shoot weight. Root and shoot lengths were mainly unaffected. Germination energy was positively affected by five isolates with up to 40% increase compared to the control, while germination was significantly enhanced by two isolates with up to 22% increase. Considering seedling physiology, two different strain-dependent modes of actions were expressed. Promising *Trichoderma* isolates induced formation and accumulation of reactive oxygen species (ROS) which acted as signal molecules that increased germination energy and germination. Positive correlation was found between pyrogallol peroxidase, superoxide dismutase, catalase activity and germination in plants treated with these isolates.

Keywords: biopriming; germination; growth enhancement; seeds; ROS.

INTRODUCTION

Pepper (*Capsicum* spp.) is one of the world's major vegetable and spice crops. The most cultivated species is *Capsicum annuum* L., originating from Central America. The total cultivation area under pepper in the world was almost 2 million hectares in 2018 [1]. The main challenge in pepper production, besides dealing with diseases, is to enhance germination energy and seed germination, and to grow healthy nursery plants with strong root system. Seed deterioration is also an important issue in crop production and can cause seed to be discarded [2]. Therefore, the increase of germination and restoration of seed quality is often required. Seed priming has successfully demonstrated to improve seed germination and seedling establishment for many field crops, such as wheat, sugar beet, maize, soybean, and sunflower [3].

Living organisms are constantly exposed to biotic and abiotic stress and are forced to efficiently balance growth, reproduction and defence under adverse conditions. Plants contain numerous enzymatic (superoxide dismutase, peroxidase and catalase) and non-enzymatic (glutathione, ascorbic acid, α -tocopherol, and phenolic compounds) mechanisms, which prevent oxidative damage by scavenging free radicals before they attack membranes or other seed components [4]. During the germination process, strong increase in the respiratory activity and enhanced production of reactive oxygen species (ROS) was recorded [5]. Reactive oxygen species are usually considered as toxic molecules in seed physiology, but nowadays there is evidence that ROS also function as signalling molecules that activate in a wide range of defence responses to various stimuli [5]. Therefore, cellular levels of ROS have to be tightly controlled at both production and consumption levels [6]. Cellular antioxidant machinery, involving detoxifying enzymes and antioxidant compounds, eliminate potentially toxic ROS produced under stressful conditions or control ROS concentrations due to regulation of various signalling pathways. Non-toxic levels of ROS must be maintained in order to utilize them as signalling molecules. Evolutionally, it is assumed that - when cells once learn to deal with ROS toxicity, they are able to utilize ROS as signalling molecules [7].

Considering the growing demand for organic food production, the use of biofertilizers and biopesticides is an alternative for sustaining high eco-friendly production. The fact that *Trichoderma* species are resistant to the most chemical pesticides, classifies those species as good biocontrol and integrated control agents [8,9]. The main biocontrol mechanisms of *Trichoderma* species are mycoparasitism, antibiosis and competition for soil nutrients and space [10]. Those mechanisms make *Trichoderma* strongly aggressive to a broad range of phytopathogenic fungi [11], but besides biocontrol, *Trichoderma* species have been known as plant growth promoter agents and promoters of plant defence mechanisms [8,12]. *Trichoderma harzianum* fully colonizes roots as they grow and provides at least season-long benefits to plants [13]. A series of morphological and biochemical changes, initiated by *Trichoderma* spp. colonization and penetration of plant root tissues, are considered to be a part of the plant defence response [14]. Specific strains of *Trichoderma* spp. can stimulate defence responses in its host plants and are known as one of the best induced systemic resistance (ISR) agents [12]. Additionally, *Trichoderma* spp. root colonization also frequently enhances root growth and development, crop productivity, resistance to abiotic stress and nutrients uptake and utilization [14]. It is important to underline that within a single *Trichoderma* species, not all isolates are capable of promoting plant growth. For that reason, plant - *Trichoderma* sp. interaction depends on the plant species and the genotype used as well [15]. So far, it has been reported that *T. harzianum*, *T. viride* and *T. pseudokoningi* improve growth of sweet and/or hot pepper, but the physiology of plant - *Trichoderma* interaction remains unclear [16,17]. Therefore, the aim of this study was to test the ability of *Trichoderma* strains to promote growth of pepper plants in early stage and their effects on the seedling physiology.

MATERIAL AND METHODS

Isolation of biocontrol agents

Trichoderma spp. isolates were obtained from soil samples originating from various soil types and localities in Serbia, mainly from the Vojvodina Province in 2012. Selective water agar (WA) media amended with streptomycin was used to isolate *Trichoderma* from soil samples by particle-plating method. A total of 20 soil particles (of weight 0.0001 g per particle) per sample were analyzed seven days after incubation at room temperature. The emerging fungal colonies were observed under microscope, and transferred on Potato Dextrose Agar (PDA) and Synthetic low-Nutrient Agar (SNA) media for further analyses of

morphological characters. All *Trichoderma* isolates were refined to single-spore isolates for further research and preserved at +4 °C.

Preparation of conidia suspension

A conidia suspension of ten tested *Trichoderma* isolates was prepared from seven days old culture of the *Trichoderma* isolate flooded with 10 mL of sterilized distilled water and scraped by sterile glass rod. Such suspensions were filtered through cheesecloth, and conidial concentrations were adjusted to 10^6 conidia per mL by Neubauer's haemocytometer.

Plant material and seed treatment with *Trichoderma* spp.

Trichoderma spp. growth promoting activity was tested on 'California Wonder' sweet pepper seeds at Laboratories of Institute of Field and Vegetable Crops, Novi Sad during 2014. Pepper seeds were sterilized in 1% sodium hypochlorite solution for 5 min and rinsed in sterile distilled water. Sterilized seeds were treated with each *Trichoderma* isolate suspensions separately, according to the modified Mukhtar and coauthors [18] method. Seeds were dipped in seed-coating suspensions for 30 min and air dried on filter paper for 24 h at room temperature. Seeds treated with sterile distilled water were used as a control.

Growth promoting activity test

One hundred of sweet pepper seeds treated with *Trichoderma* suspensions were germinated in four replicates by Blotter method in a growth chamber with 12 h photoperiod at 25 ± 1 °C [19]. Germination energy (GE) and germination (G) were calculated on day seven and day 14, respectively, as a percentage of germinated seeds. Shoot and root maximal length, and weight of all pepper seedlings were measured on day 14. Vigour Index (V.I.) was calculated according to Asaduzzaman and coauthors [16]. Immediately after length and weight measurements, root and shoot samples were frozen at -80 °C for further enzyme analyses.

Enzyme extraction

Biochemical analyses were performed at the Laboratory for Biochemistry, University of Novi Sad Faculty of Agriculture, during 2014. Frozen shoots and roots (2 g each) were homogenized in 10 mL of phosphate buffer (0.1 M, pH 7.0). Homogenates were centrifuged for 20 min at $10.000 \times g$ and filtered. The supernatants were used to test enzyme activity and to determine intensity of lipid peroxidation.

Membrane lipid peroxidation and protein content

Lipid peroxidation (LP) was measured at 532 nm using the thiobarbituric acid test (TBA) test [20]. The enzyme extract (0.5 mL) was incubated with 2 mL of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid for 40 min at 95 °C. The reaction was stopped by cooling on ice bath for 10 min and the product was centrifuged at $10.000 \times g$ for 15 min. The total amount of TBA-reactive substances is given as nmol malondialdehyde equivalents (MDAE) mg protein^{-1} . Protein content in homogenates was determined using bovine serum albumin as a protein standard [21]. The Bradford assay is based on the direct binding of Coomassie brilliant blue G-250 dye (CBBG) to proteins at arginine, tryptophan, tyrosine, histidine, and phenylalanine residues.

Assay of catalase activity

Catalase (CAT) (EC 1.11.1.6) activity was determined according to Sathya and Bjorn [22]. The decomposition of H_2O_2 was followed as a decrease in absorbance at 240 nm. The enzyme extract (0.02 mL) was added (0.02 mL extract of shoots or 0.05 mL extract of roots, separately) was added to the assay mixture containing 1 mL of 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2 . The activity of the enzyme was expressed as IU per 1 mg of protein (IU mg^{-1} protein).

Assay of superoxide dismutase activity

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed according to the method of Mandal and coauthors [20] slightly modified by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 75 μM NBT, 0.1 mM EDTA, 13 μM L-metionine, 2 μM riboflavin and 0.015 mL of the enzyme extract. It was kept under a fluorescent lamp for 20 min, and then the absorbance was read at 560 nm. One unit of the

SOD activity was defined as the amount of enzymes required to inhibit reduction of NBT by 50%. The activity of the enzyme was expressed as IU mg⁻¹ protein.

Assay of peroxidase activity

Peroxidase (EC 1.11.1.7) activity was measured using guaiacol (guaiacol peroxidase; GPx) and pyrogallol (pyrogallol peroxidase; PPx) as substrates [23]. Peroxidase activity (GPx and PPx) was expressed as IU mg⁻¹ protein. Pyrogallol peroxidase activity: includes the measurement of the content of purpurogallin - a product of pyrogallol oxidation. The enzyme extract (0.02 mL) was added to the assay mixture containing 3 mL of 180 mM pyrogallol and 0.02 mL of 2 mM H₂O₂. The absorbance was recorded at 430 nm during 5 minutes. Guaiacol peroxidase activity consists of the assay of tetraguaiacol - a coloured product of guaiacol oxidation in the investigated sample. The enzyme extract (0.03 mL) was added to the assay mixture containing 3 mL of 20 μM guaiacol and 0.02 mL of 3 mM H₂O₂. The absorbance was recorded at 436 nm during 5 minutes.

Glutathione peroxidase (GSH-Px) activity

Glutathione peroxidase (GSH-Px) activity was assessed in the brain spectrophotometrically [24]. Applying this technique, GSH formation was assayed by measuring the conversion of nicotinamide adenine dinucleotide phosphate (NADPH) to NADP. The oxidation of NADPH was measured spectrometrically at 340 nm. The assay mixture contained 0.05 M phosphate buffer at a pH of 7.0, 5 mM EDTA, 2 mM NaN₃, 1 mM GSH, 0.2 mM NADPH, and 4 μg of GSH reductase. The sample (0.02 mL) was added to 2.88 mL of the assay mixture. The reaction was initiated by the addition of 0.1 mL of 2.5 mM H₂O₂. A blank assay with buffer instead of a sample was used to correct for any non-enzymatic oxidation of GSH and NADPH by peroxide.

Reduced glutathione

Reduced glutathione (GSH) was determined using Ellman reagent [25] and expressed as mg GSH mg⁻¹ protein. In this method, 0.5 g of plants tissues were homogenized in 5 mL 20% TCA solution. The samples were centrifuged at 10.000 × g for 15 min. 2.5 mL of 0.1 M potassium phosphate buffer (pH 7.5) with 1 mM EDTA and 0.5 mL of 3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added in 0.5 mL supernatant and after 20 min the absorbance was measured at 412 nm. The glutathione concentration was determined by a standard curve prepared with GSH standard.

Total antioxidant activity (TAA)

The reduction of molybdenum (VI) to molybdenum (V) by the root and shoot extracts was used to assess the total antioxidant activity following the method described by Berk and coauthors [26]. The standard curve for total antioxidant was plotted using ascorbic acid solution. The standard curve was constructed using different concentrations of ascorbic acid, and the results were expressed as mg ascorbic acid equivalents (AAE) per milligram of protein (mg AAE mg⁻¹ protein). An aliquot of 0.01 mL of the sample solution containing extract of peppers was combined in a glass tube with 5.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95 °C for 90 min, after the samples had cooled to ambient temperature, the absorbance of the aqueous solution was measured at 695 nm against a blank. A typical blank solution contained 5.0 mL of the reagent solution, and it was incubated under the same conditions as the rest of the samples.

2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging activity

The scavenging activity of the plant extracts on ABTS^{•+} radical was measured according to the method of Re and coauthors [27] with some modifications. The standard curve for total antioxidant capacity (activity) was plotted using ascorbic acid solution. ABTS^{•+} cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 24 h before use. ABTS^{•+} solution was then diluted with water to obtain an absorbance of 0.900 at 734 nm. After the addition of 0.02 mL of plant extract to 3.0 mL of diluted ABTS^{•+} solution, the absorbance was measured at 2 hours after the initial mixing. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times.

Statistical analyses

The data were processed in Statistica 12.0 (StatSoft, Dell Inc., USA) using two-factorial analysis of variance (isolate and plant organ being the first and second factor) with Duncan's multiple test intervals at a significance level of 0.05. Data in percentages (GE and G) were transformed by ArcSin transformation. Pearson correlation coefficients were determined separately by plant organs at the significance level of probability 0.05 and 0.01. Average values from each isolate were transformed and used for Principal Component Analysis (PCA).

RESULTS

Growth Promoting Activity

In this study we were able to detect growth responses of pepper seedlings as early as 14 days post-inoculation with *Trichoderma* isolates. In addition, the pathogen-free environment enabled us to study the direct influence of *Trichoderma* isolates on the growth response of pepper seedlings without any disease suppression.

Table 1. Duncan test of growth parameters of pepper seedlings treated with *Trichoderma* spp. isolates

Isolate	RL (cm)	SL (cm)	RW (mg)	SW (mg)	GE (%)	G (%)	V.I.
TR-1	31.67 ^{cde} (5.15)	21.34 ^{cd} (0.66)	2.19 ^e (0.38)	21.30^a (0.44)	51^c (4.03)	55 ^b (4.17)	294.96 ^{bc}
TR-2	38.26 ^{ab} (2.41)	25.86^{abc} (1.46)	3.77^d (0.18)	22.50^a (0.73)	67^a (5.38)	70 ^{cd} (6.99)	469.07^a
TR-3	41.60^a (1.45)	11.13 ^{ef} (0.08)	6.61^{ab} (0.70)	17.92^b (0.32)	58^{ac} (1.55)	89^a (7.59)	473.80^a
TR-4	33.28 ^{bcd} (0.79)	8.64 ^f (0.53)	6.75^a (0.58)	12.95 ^d (0.37)	18 ^b (2.07)	79 ^{acd} (0.46)	329.20 ^c
TR-5	27.22 ^{defg} (1.13)	9.68 ^f (0.39)	5.37^{bc} (0.26)	11.72 ^e (0.34)	4 ^d (4.31)	68 ^{bcd} (4.86)	253.59 ^{bc}
TR-6	23.36 ^{fg} (3.18)	8.46 ^f (0.28)	4.55^{cd} (0.28)	10.30 ^f (0.59)	6 ^d (5.99)	70 ^{bcd} (2.18)	221.03 ^b
TR-7	25.74 ^{efg} (1.09)	8.18 ^f (0.77)	5.49^{abc} (0.11)	11.54 ^e (0.34)	4 ^d (3.63)	74 ^{bcd} (0.76)	250.93 ^{bc}
TR-8	31.19 ^{cde} (0.72)	9.45 ^f (0.29)	5.82^{abc} (0.50)	13.20 ^d (0.41)	12 ^{bd} (3.55)	81 ^{acd} (2.63)	335.99 ^c
TR-9	28.87 ^{cdef} (1.75)	9.48 ^f (0.13)	4.79^{cd} (0.28)	13.73 ^{cd} (0.35)	49^c (3.35)	84^{ac} (3.20)	328.57 ^c
TR-10	28.99 ^{cdef} (3.64)	8.89 ^f (0.53)	5.91^{abc} (0.23)	13.53 ^d (0.41)	55^{ac} (1.15)	78 ^{acd} (0.79)	294.67 ^{bc}
H ₂ O	34.47 ^{bc} (4.94)	16.82 ^{de} (0.53)	1.96 ^e (0.33)	14.80 ^c (0.52)	27 ^b (4.02)	67 ^{bd} (0.81)	339.94 ^c

*Legend: RL – Root Length; SL – Shoot Length; RW – Root Weight; SW – Shoot Weight; GE – Germination energy; G - Germination; V.I. – Vigour Index; Values with different letters in the columns differ significantly at a significance level of $p < 0.05$; SE values are given in parentheses.

All *Trichoderma* tested isolates, with the exception of TR-1, had significant positive effects on root weight of pepper seedlings. Besides root weight, some isolates positively affected up to five out of six measured growth parameters (Table 1). Germination energy was positively influenced by five isolates, while germination was significantly enhanced by two isolates (Table 1). Considering all significant positive influences and Vigour Index, isolates TR-2 and TR-3 can be selected as the most promising. Shoot length and weight were mostly negatively affected, which can be explained by higher plants activity in roots enhancement.

Seedling Physiology Analyses

According to PCA the first five components which were explained in the analysis were higher than eigenvalue 1. These five components explained 91.74% of the total variance, while the first component accounts for approximately 41.82% variation. Considering the impact of tested traits on the major components (Table 2), the most positive effect in the first factor was noted for GSH, ABTS and GSH-Px all in root. In the second component, the most significant effect was noted for average length (AL) and

average weight (AW) of shoots, and germination energy. Since these traits mostly affected the first two components, these the traits also mostly affected the distribution of strains in Figure 1.

Correlations of these properties with GE, G, AW, and AL were more or less negative, which means that GSH, GSH-Px, and ABTS (all in the root) should be as low as possible. If one looks at the individual charts of these three variables (Figs 2A, 2B and 2C) in root, it can be noticed that pepper seeds treated with the isolates TR-2, TR-3, TR-4, TR-9, and TR-10 had the lowest values for GSH, GSH-Px, and ABTS, and the highest germination values (Fig. 2). Therefore, the strains TR-1, TR-5, TR-6, TR-7 and control (H₂O), as shown in Figure 1, were grouped above the rest of previously mentioned isolates, because they had overall higher values for these three biochemical parameters and low germination.

According to Figure 1, all tested *Trichoderma* isolates grouped according to their ability to enhance germination and growth parameters into three groups. Group I consists of three isolates which did not enhance the GE (TR-5, TR-6, and TR-7). Group II consists of two isolates (TR-4 and TR-8) that poorly affected GE, but final G was satisfying which would classify them as isolates with lower effect. Group III consists of isolates with positive effect on both root weight and G (TR-3, TR-9, and TR-10). Isolates TR-2 and TR-1 affected high shoot length and weight with low difference between GE and G.

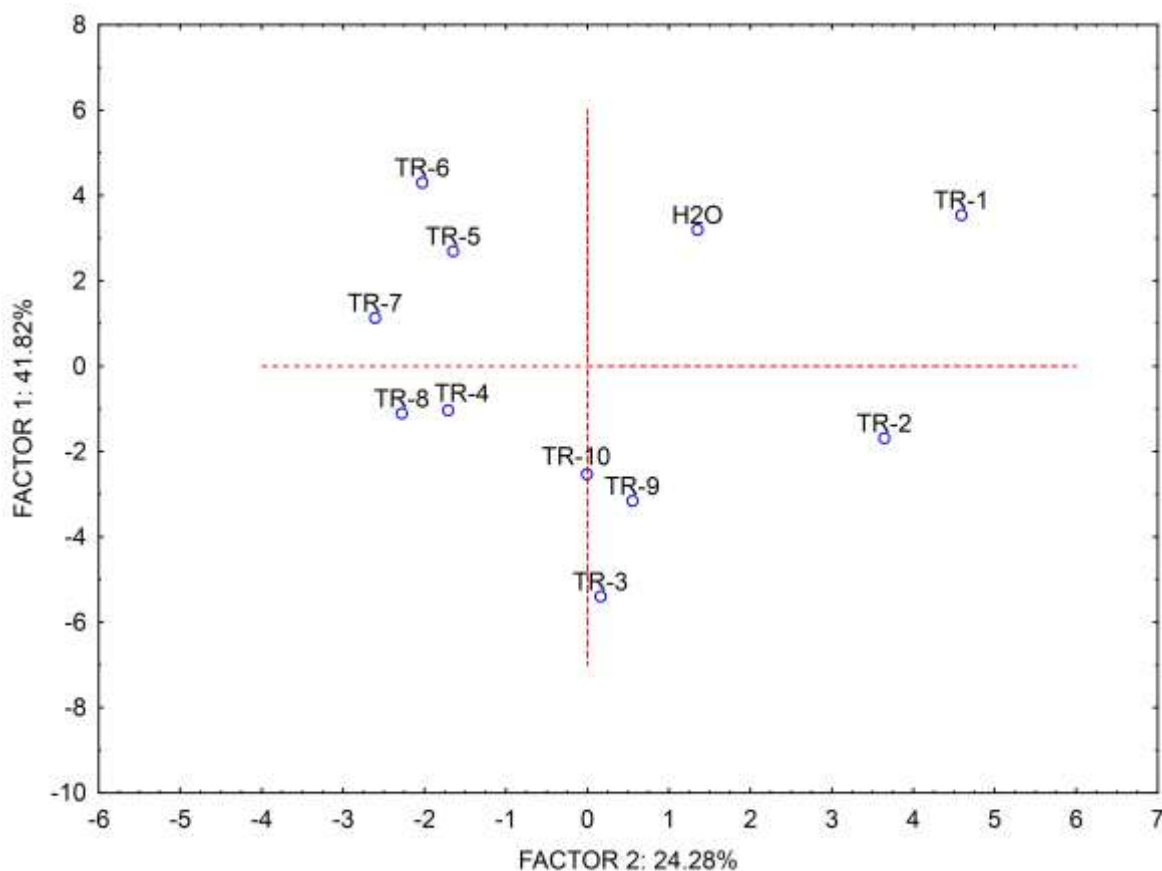


Figure 1. PCA biplot of evaluated *Trichoderma* isolates according to all tested parameters

Table 2. PC scores for all analysed traits in pepper seedlings

Traits	PC 1	PC 2	PC 3	PC 4	PC 5
Shoot					
GSH (mg GSH mg ⁻¹ protein)	-0.288	-0.549	-0.272	-0.412	0.153
TAA (mg AEE mg ⁻¹ protein)	-0.547	-0.316	-0.695	-0.247	0.120
ABTS (mg AEE mg ⁻¹ protein)	-0.349	-0.401	-0.736	-0.230	0.034
SOD (IU mg ⁻¹ protein)	-0.745	-0.406	-0.046	-0.037	-0.039
GPx (IU mg ⁻¹ protein)	-0.788	0.313	0.155	-0.379	-0.153
PPx (IU mg ⁻¹ protein)	-0.833	0.398	0.158	-0.235	-0.143
LP (nmol MDA mg ⁻¹ protein)	-0.624	0.494	-0.417	-0.095	-0.298
GSH-Px (IU mg ⁻¹ protein)	0.397	-0.307	0.602	-0.430	-0.291
CAT (IU mg ⁻¹ protein)	-0.789	0.441	-0.153	-0.219	-0.127
AL (cm)	0.136	0.896	-0.346	-0.009	-0.009
AW (g)	-0.219	0.903	-0.262	0.079	0.117
Root					
GSH (mg GSH mg ⁻¹ protein)	0.926	0.074	-0.222	-0.060	-0.242
TAA (mg AEE mg ⁻¹ protein)	0.061	-0.610	-0.447	0.593	-0.034
ABTS (mg AEE mg ⁻¹ protein)	0.808	0.153	-0.298	0.428	-0.128
SOD (IU mg ⁻¹ protein)	-0.803	-0.493	0.129	0.169	0.088
GPx (IU mg ⁻¹ protein)	-0.738	-0.002	0.464	0.385	-0.122
PPx (IU mg ⁻¹ protein)	-0.887	0.015	0.295	0.291	-0.079
LP (nmol MDA mg ⁻¹ protein)	0.069	-0.687	-0.349	-0.039	-0.575
GSH-Px (IU mg ⁻¹ protein)	0.838	-0.009	-0.151	-0.137	-0.455
CAT (IU mg ⁻¹ protein)	-0.688	-0.178	0.113	0.372	-0.529
AL (cm)	-0.560	0.506	-0.528	0.299	0.028
AW (g)	-0.606	-0.697	0.048	0.089	0.291
GE (%)	-0.566	0.792	0.123627	0.051	-0.092
G (%)	-0.869	-0.417	-0.099842	-0.089	-0.168

*Legend: GSH - Reduced glutathione; TAA - Total antioxidant activity; ABTS - Radical Cation Scavenging Activity; SOD - Superoxide dismutase; GPx - Guaiacol peroxidase; PPx - Pyrogallol peroxidase; LP - Lipid peroxidation; GSH-Px - Glutathione peroxidase; CAT - Catalase; AL - Average length; AW - Average weight; GE - Germination energy; G - Germination.

In pepper shoots, significant positive correlation with germination was noticed with SOD, TAA, PPx, GPx, LP, CAT, and GSH. Additionally, significant positive correlations were also found between GE and AW with GPx, PPx, LP, and CAT (Table 3).

In pepper roots, significant positive correlations were found between germination and root weight with SOD, GPx, PPx, and CAT. Significant positive correlation was found between germination energy and GPx and PPx. On the contrary, significant negative correlations were found for GSH and GSH-Px with all root growth parameters - GE, G, root length and weight (Table 3).

Table 3. Correlations of enzymes, antioxidant activity and growth parameters in pepper shoot (above 1) and root (below 1)

	L	W	G	GE	CAT	SOD	GSH-Px	GPx	PPx	LP	GSH	TAA	ABTS
L	1.000	0.883**	-0.349*	0.562**	0.315	-0.395*	-0.317	0.164	0.193	0.454**	-0.444**	-0.084	-0.094
W	0.010	1.000	-0.114	0.745**	0.550**	-0.158	-0.472**	0.353*	0.467**	0.620**	-0.364*	0.006	-0.113
G	0.208	0.418*	1.000	0.266	0.395*	0.666**	-0.156	0.455**	0.489**	0.435*	0.356*	0.513**	0.421*
GE	0.517**	-0.208	0.267	1.000	0.656**	0.076	-0.351*	0.663**	0.751**	0.704**	-0.299	-0.067	-0.205
CAT	0.249	0.405*	0.512**	0.342	1.000	0.406*	-0.305	0.763**	0.820**	0.737**	0.121	0.409*	0.167
SOD	0.119	0.798**	0.553**	0.005	0.610**	1.000	-0.066	0.562**	0.474**	0.209	0.212	0.571**	0.409*
GSH-Px	-0.331	-0.562**	-0.518**	-0.428*	-0.342	-0.711**	1.000	-0.060	-0.230	-0.437*	0.022	-0.393*	-0.301
GPx	0.207	0.466**	0.432*	0.505**	0.781**	0.639**	-0.638**	1.000	0.891**	0.577**	0.002	0.320	0.146
PPx	0.335	0.543**	0.572**	0.558**	0.783**	0.723**	-0.744**	0.955**	1.000	0.643**	0.018	0.275	0.082
LP	-0.132	0.179	0.141	-0.495**	0.167	0.077	0.286	-0.155	-0.137	1.000	0.117	0.374*	0.284
GSH	-0.268	-0.517**	-0.653**	-0.437*	-0.514**	-0.714**	0.885**	-0.676**	-0.767**	0.181	1.000	0.541**	0.595**
TAA	0.072	0.361*	0.134	-0.515**	0.194	0.311	-0.011	-0.059	-0.059	0.538**	0.029	1.000	0.907**
ABTS	-0.069	-0.542**	-0.571**	-0.307	-0.397*	-0.645**	0.694**	-0.534**	-0.647**	0.098	0.786**	0.344*	1.000

*Legend: Correlations marked with single asterisk are significant at p < 0.05, and with double asterisk are significant at p < 0.01;

These results suggest that *Trichoderma* spp. could express two different strain-dependent modes of actions. One mode of action can be noticed in seedlings treated with TR-2, TR-3, TR-9, and TR-10, where positive correlation between PPx, SOD, and CAT activity and germination was registered, and ROS species acted as signal molecules which increased energy and germination. Another mode of action can be noticed in seedlings treated with *Trichoderma* isolates TR-5, TR-6, and TR-7 where ROS decreased and they did not act as signal molecules, which lowered germination energy and germination confirmed as well.

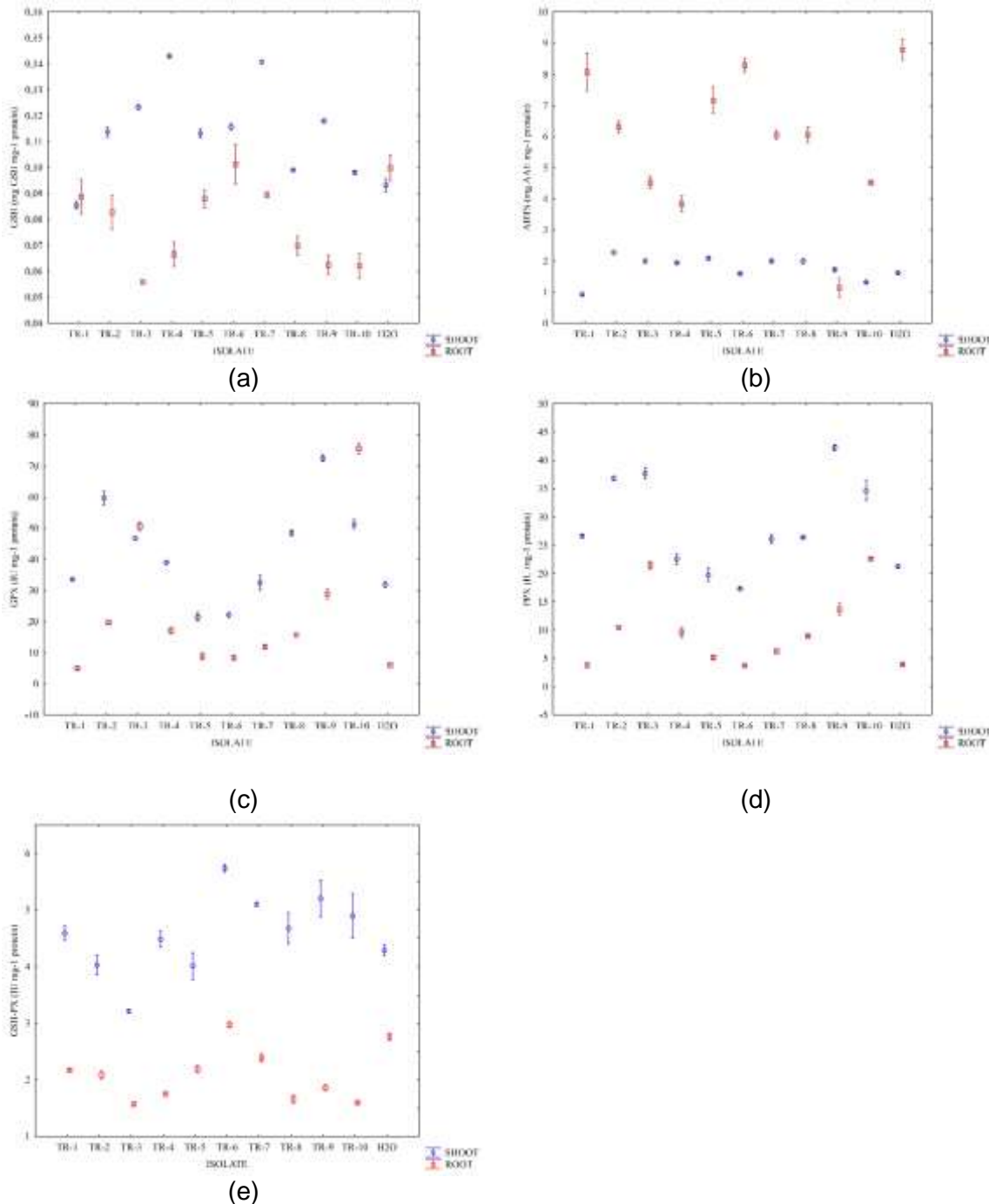


Figure 2. *Trichoderma* isolates effects on: A) reduced glutathione (GSH), B) Radical Cation Scavenging Activity (ABTS), C) guaiacol peroxidase (GPx), D) pyrogallol peroxidase (PPx), E) glutathione peroxidase (GSH-Px) in roots and shoots of pepper seedlings. Means of three independent experiments with three replicates; Bars represent standard errors.

DISCUSSION

According to growth promoting analyses in this study, the most noticeable is the significant positive effect of *Trichoderma* isolates on the root weight of pepper seedlings. Besides root weight, some isolates positively affected the shoot weight as well. Root enhancement caused by *T. harzianum* treatment has been also reported for sweet pepper [28], and cucumber plants with 24% of roots dry weight and 45% of root length increase [29]. Significant increase of shoot length of pepper seedlings treated with *T. harzianum* was reported by Inbar and coauthors [30], and by Joshi and coauthors [31] who registered both root and shoot length increase of chilli plants. In our study, root and shoot lengths were mainly unaffected by *Trichoderma* isolates – only one isolate out of ten tested had significant positive effect on these variables. This is in agreement with Tucci and coauthors [15], who noticed that *T. atroviride* and *T. harzianum* modified the root architecture of tomato in a differential way, and in most cases root length was unaffected, whereas lateral development was stimulated. Stimulated lateral development of pepper seedlings roots was the cause of the significant increase of root weight, although root length was unaffected in our study.

In current study, GE was positively affected by five isolates up to 40% increase in comparison with the control, while germination was significantly enhanced at the end only by two isolates, up to 22% of increase. Increase in seedling emergence up to 30% was observed by Yedia and coauthors [29] on cucumber seeds sown in *Trichoderma*-treated soil. Celar and Valic [32] studied the effects of different *Trichoderma* species on germination of onion, radish, spinach, carrot, tomato, cabbage, pea, red beet, chicory, lettuce, and maize seeds. In most cases, it only influenced the germination speed, but not the final number of germinated seeds, which was also the case in our study. Uniformity of pepper plants is very important in seedling production because those plants that germinate later will be significantly shorter and less competitive.

At the seedling physiology level, this study showed that *Trichoderma* could express two different strain-dependent modes of actions. Even though some authors [33,34] demonstrated that *Trichoderma* species alleviate the effects of stress caused by any other biotic or abiotic factor, by deactivation of stress- and defence-related systems, our results indicate that some strains of *Trichoderma* could induce formation and accumulation of ROS. The increase of antioxidative enzymes activity (CAT, SOD, oxalate oxidase, peroxidase, etc.) suggests the involvement of ROS production in plants colonized with *Trichoderma* spp. [35,36].

Recent information, suggests that ROS are not only stress signal molecules, but also have an important role in plant growth and development [37]. It could be supposed that during the colonization of pepper seed and seedling with some isolates, ROS species acted as triggering molecules which increased energy and germination. That is why a positive correlation between PPx, SOD, and CAT activity and germination was registered in plants treated with these isolates.

On the other hand, some of *Trichoderma* tested isolates had a completely different mode of action. Increased ROS formation led to the increase of glutathione content in plant tissue and consequently higher GSH-Px activity. As a potent scavenger of free radicals, glutathione enhanced the antioxidant effect of the system itself which is proved by ABTS test. Shores and Harman [36] reported that other stress-related proteins that were up-regulated included glutathione-dependent enzymes. Plants detoxify some contaminants by conjugating them, or their metabolites, to glutathione. By increasing antioxidant activity in the system, the level of ROS decreased and they did not act as signal molecules. Because of that, lower germination and germination energy were observed in this study in plants treated with these isolates.

CONCLUSIONS

Perspective *Trichoderma* isolates induced formation and accumulation of ROS, which acted as signal molecules that increased germination energy and germination. Positive correlation was found between pyrogallol peroxidase, superoxide dismutase, catalase activity and germination in plants treated with these isolates. Nine out of ten *Trichoderma* isolates significantly increased root weight of pepper seedlings, while three out of ten positively affected shoot weight. Root and shoot lengths mainly remained unaffected.

After all, not all *Trichoderma* strains were effective for pepper growth promotion, but *Trichoderma* strains which can induce formation and accumulation of ROS, as an important factor in plant growth and development, are desirable for germination and growth improvement of pepper seeds.

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REFERENCES

1. FAOSTAT, 2018 [internet]. Food and Agriculture Organisation of the United Nations. [cited 2020 February 10]. Available from: <http://www.fao.org/faostat/en/#data/QC>
2. Siri B, Vichitphan K, Kaewnaree P, Vichitphan S, Klanrit P. Improvement of quality, membrane integrity and antioxidant systems in sweet pepper (*Capsicum annuum* Linn.) seeds affected by osmopriming. *Aust J Crop Sci.* 2013; 7(13):2068-73.
3. Sadeghian SY, Yavari N. Effect of water-deficit stress on germination and early seedling growth in sugar beet. *J Agron Crop Sci.* 2004; 190(2):138-44.
4. Waskiewicz A, Beszterda M, Golinski P. Nonenzymatic Antioxidants in Plants. In: Parvaiz A, editor. *Oxidative Damage to Plants*. Elsevier; 2014. p. 201-34.
5. Bailly C. Active oxygen species and antioxidants in seed biology. *Seed Sci Res.* 2004; 14: 93–107.
6. Breusegem FV, Vranová E, Dat JF, Inzé D. The role of active oxygen species in plant signal transduction. *Plant Sci.* 2001; 161(3):405-14.
7. Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F. ROS signalling: the new wave? *Trends Plant Sci.* 2011; 16 (6): 300-9.
8. Harman GE. *Trichoderma* – not just for biocontrol anymore. *Phytoparasitica.* 2011; 39:103-8.
9. FRAC, 2016 [internet]. Fungicide Resistance Action Committee. Resistance tables Benz. [cited 2017 Aug 17]. Available from: http://www.frac.info/docs/default-source/expert-fora/2004-06-11resistance_tables_benz.pdf.
10. Howel CR. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* 2003; 87:4-10.
11. Vinale F, Sivasithamparam K, Ghisalberti E, Marra R, Woo SL, Lorito M. *Trichoderma* – plant – pathogen interactions. *Soil Biol Bioch.* 2008; 40:1-10.
12. Shores M, Harman GE, Mastouri F. Induced systemic resistance and plant responses to fungal biocontrol agents. *Ann Rev Phytopath.* 2010; 48:21-43.
13. Harman GE. Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Dis.* 2000; 84: 377-93.
14. Saba H, Vibhash D, Manisha M, Prashant KS, Farhan H, Tauseef A. *Trichoderma* – a promising plant growth stimulator and biocontrol agent. *Mycosphere.* 2012; 3(4):524-31.
15. Tucci M, Ruocco M, De Masi L, De Palma M, Lorito M. The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol Plant Pathol.* 2011; 12:341-54.
16. Asaduzzaman M, Alam MJ, Islam MM. Effect of *Trichoderma* on Seed Germination and Seedling Parameters of *Chili*. *J Sci Found.* 2010; 8(1-2):141-50.
17. Bal U, Altintas S. Application of the Antagonistic Fungus *Trichoderma harzianum* (TrichoFlow WP™) to Root Zone Increases Yield of Bell Peppers Grown in Soil. *Biol Agric Hortic.* 2012; 24(2):149-63.
18. Mukhtar I, Hannan A, Atiq M, Nawaz A. Impact of *Trichoderma* species on Seed Germination in Soybean. *Pak J Phytopath.* 2012; 24(2):159-62.
19. Maslienko LV. Substantiation and development of a microbiological method for controlling sunflower diseases [dissertation in Russian]: Krasnodar University, Russia; 2005. 71 p.
20. Mandal S, Mitra A, Mallick N. Biochemical characterization of oxidative burst during interaction between *Solanum lycopersicum* and *Fusarium oxysporum* f. sp. *lycopersici*. *Physiol Mol Plant Pathol.* 2008; 72:56-61.
21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248-54.
22. Sathya E., Bjorn M. Spectrophotometric Assays for Antioxidant Enzymes in Plants. In: Sunkar R, editor. *Plant Stress Tolerance*. Oklahoma USA: Humana Press; 2010. p. 273-81.
23. Morkunas I, Gmerek J. The possible involvement of peroxidase in defense of yellow lupine embryo axes against *Fusarium oxysporum*. *J Plant Phys.* 2007; 164:185-94.
24. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med.* 1967; 70:158–69.
25. Sedlak J, Lindsay H. Estimation of total protein bound and non-protein sulphhydryl groups in tissue with Ellman's reagent. *Anal Biochem.* 1968; 25:192-205.
26. Berk S, Tepe B, Arslan S, Sarikurkcü C. Screening of the antioxidant, antimicrobial and DNA damage protection potentials of the aqueous extract of *Asplenium ceterach* DC. *Afr J Biotechnol.* 2011; 10 (44): 8902–08.
27. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad Biol Med.* 1999; 26(9-10):1231–37.
28. Idowu OO, Oni AC, Salami AO. The interactive effects of three *Trichoderma* species and damping-off causative pathogen *Oythium aphanidermatum* on emergence indices, infection incidence and growth performance of sweet pepper. *Int J Recent Sci Res.* 2016; 7(4):10339-347.
29. Yedidia I, Srivastva AK, Kapulnik Y, Chet I. Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant Soil.* 2001; 235(2):235–42.
30. Inbar J, Abramsky M, Cohen D, Chet I. Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. *Eur J Plant Pathol.* 1994;100 (5):337-46.
31. Joshi BB, Bhatt RP, Bahukhandi D. Antagonistic and plant growth activity of *Trichoderma* isolates of Western Himalayas. *J Environ Biol.* 2010; 31(6):921-28.

32. Celar F, Valic N, Effects of *Trichoderma* spp. and *Gliocladium roseum* culture filtrates on seed germination of vegetables and maize. *J Plant Dis Protect.* 2005; 112(4):343–50.
33. De Oliveira AB, Gomes-Filho E, Eneas-Filho J, Tarquinio Prisco J, Mendes Alencar LN. Seed priming effects on growth, lipid peroxidation, and activity of ROS scavenging enzymes in NaCl-stressed sorghum seedlings from aged seeds. *J Plant Interact.* 2012; 2 (7): 151-59.
34. Chepsergon J, Mwamburi L, Kipkemboi KM. Mechanism of Drought Tolerance in Plants Using *Trichoderma* spp. *Intl J Sci Res.* 2014; 3(11):1592-95.
35. Brotman Y, Landau U, Cuadros-Inostroza A, Takayuki T., Fernie A., Chet I, et al. *Trichoderma*-Plant Root Colonization: Escaping Early Plant Defense Responses and Activation of the Antioxidant Machinery for Saline Stress Tolerance. *PLoS Pathogens* [internet]. 2013 [cited 2017 Avg. 19]; 9(3):e1003221. Available from: <https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1003221>
36. Shoshitaishvili M, Harman G. The Molecular Basis of Shoot Responses of Maize Seedlings to *Trichoderma harzianum* T22 Inoculation of the Root: A Proteomic Approach. *Plant Phys.* 2008; 147(4): 2147–63.
37. Karuppanapandian T, Moon JC, Kim C, Manoharan K, Kim W. Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. *Aust J Crop Sci.* 2011; 5(6):709-25.



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