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RESEARCH ARTICLE

Increased resistance of drought by *Trichoderma harzianum* fungal treatment correlates with increased secondary metabolites and proline content

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Abstract

Plant secondary metabolites play vital role in plant stress response. In this study we investigated whether root colonization of tomato (*Solanum lycopersicum*) infected by *Trichoderma harzianum* leads to alterations in the biosynthesis of secondary plant metabolites including phytohormones and osmolyte proline under drought stress. Exposure of tomato to drought caused a drastic decline in plant growth and physiological parameters. Tomato inoculated with *T. harzianum* showed increased root and shoot growth and chlorophyll pigments as compared to uninoculated controls as well as drought stressed plants. Proline and total soluble protein content was increased in plants inoculated with *T. harzianum* under both normal as well as drought conditions. An obvious increase in phenol and flavonoid content was observed due to *T. harzianum*. In addition, *T. harzianum* inoculated plants maintained higher levels of growth regulators indole acetic acid, indole butyric acid, and gibberellic acid under drought stress. Improved secondary metabolites which play an important role in plant stress tolerance by *T. harzianum* may have coordinately worked for bringing the growth regulation by protecting membranes from reactive oxygen species (ROS) and enhance plant growth through accessing more nutrients by root system.

Keywords: antioxidants, proline, polyphenols, tomato, drought, *Trichoderma harzianum*

1. Introduction

Drought is an abiotic stress resulting in devastation of agricultural crops and causing considerable losses in yield (de Vries *et al*. 2012). Though the general effects of drought stress on plant growth are known, the different tolerance mechanisms involved vary from one plant species to another (Farooq *et al*. 2009). Plants show a range of variations in drought tolerance potential and the mechanisms involved may include up- and down-regulation of several physiological and biochemical traits (Ahmad *et al*. 2010; Hameed *et al*. 2014). Plants have potential mechanisms to withstand stress, the induced oxidative damage through the antioxidant defense systems (Ahanger *et al*. 2014a; Hashem *et al*. 2016). In addition to this, accumulation of osmotic

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constituents like proline, and sugars during stress enable plants to maintain the osmoregulation and recover from the oxidative stress quickly (Jatav *et al*. 2012; Ahmad *et al*. 2015). Changes in the endogenous levels of phytohormones are another typical response of plants counteracted by stress (Hashem *et al*. 2016). The ability of plants to tolerate abiotic stress also depends on their association with microbes, such as mycorrhizal fungi, rhizobial and plant-growth-promoting rhizobacteria, endophytic fungi, which play a vital role in modulating their physiological processes (Ali *et al*. 2014; Egamberdieva *et al*. 2016; Hashem *et al*. 2016). Among microbes, *Trichoderma* species have the potential to induce host plant tolerance to several biotic and abiotic stresses including salinity and drought, by its involvement in root growth promotion, maintenance of nutritional uptake and in addition triggers protective mechanisms to avert the oxidative damage (Ahmad *et al*. 2015). Gusain *et al*. (2014) have observed enhanced drought tolerance in rice due to *Trichoderma harzianum* T35 colonisation and they evidenced that *T*. *harzianum* promoted activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and thereby preventing oxidative damage to rice through quick elimination of reactive oxygen species (ROS). Mastouri *et al*. (2012) observed that the enhanced tolerance of tomato to water stress by *T*. *harzianum* T22 was due to the ability of plants to remove damaging ROS, which was accompanied by an increase in the activity of antioxidant enzymes. Since plant secondary metabolites play vital role in plant stress response, we studied whether root colonization by *T. harzianum* leads to alterations in the biosynthesis of secondary plant metabolites under drought stress.

2. Materials and methods

2.1. Fungi, seeds and pot experiments

The endophytic mold, *T. harzianum* Rifai (TH) was isolated previously from the rhizosphere of tomato plants grown in Ismailia, Egypt, using *Trichoderma* selective medium (Williams *et al*. 2003). The identification was carried out up to species level according to Domch *et al*. (1993). Inoculum of *T*. *harzianum* was produced on potato dextrose agar (PDA) medium (Booth 1977) in Petri plates, at continuous light at 24°C for 10 d to produce abundant conidia (Resende *et al*. 2014). The conidia were collected in 0.05% (w/v) carboxymethylcellulose (CMC) as sticker, and then adjusted to 5.0×10^9 conidia mL^{-1}.

Seeds of tomato (*Solanum lycopersicum* L., *var*. Rio Grande) (Heirloom & Perennial Ltd., Turkey) were used for pot experiments. The seeds of tomato were sown in nursery plastic plates containing sand, perlite and peat (1:1:1, v/v/v) under control growth chamber conditions. The mean temperature was 28.0°C and photoperiod 16 and 8 h, for day and night (200 μE m^{-2} s^{-1} light intensity), respectively, with average relative humidity (RH) (65.0±2)%. In the 2nd true leaf stage, the root of seedlings were immersed in conidial suspension (5.0×10^9 conidia mL^{-1}) of TH for 1 h and transplanted to plastic pots (2 kg volume) containing sand, perlite and peat (1:1:1, v/v/v). Plants were grown under greenhouse climate conditions described above for more than 10 wk after transplantation. The experimental designed as completely randomized design with five replicates per each treatment. The drought stress was achieved by dissolving polyethylene glycol (PEG-8000; Sigma Chemical Co., St. Louis, MO, USA) in Hoagland Solution (Hoagland and Arnon 1950) to get water potential of –1.2 MPa according to Plaut and Federman (1985). Plants were bottom irrigated (100 mL per pot) every 3 d. Control pots were used as reference for each treatment. At end of the experiment (after 10 wk), the plant was removed very carefully from the pots and the length and depth of shoot and root system were measured, respectively. Leaf samples were collected by excising the leaf at the petiole from five replicates for biological analysis. The shoot and root systems were dried at 110°C for 24 h to measure plant biomass.

2.2. Photosynthetic pigments

For estimation of photosynthetic pigments, the uppermost fresh leaf samples (100 mg) were extracted in acetone and the absorbance of supernatant was recorded at 622, 664, and 440 nm using spectrophotometer (Lichtenthaler and Wellburn 1983).

2.3. Estimation of total soluble phenolic and flavonoid compounds

Total phenolics were estimated in the fresh uppermost leaf samples following Slinkard and Singleton (1977). After extraction with ethanol (80%, v/v), the supernatant was reacted with Folin and Ciocalteau's reagent (Julkunen-Tiitto 1985) and optical density of the mixture was read at 750 nm. Standard of pyrogallol was used for calculation. Content of flavonoid was estimated according to Zhishen *et al*. (1999) using catechin as standard. Samples were extracted in methanol and the absorbance was recorded at 510 nm and content of flavonoid was expressed as mg g^{-1} fresh weight (FW).

2.4. Determination of proline

Fresh uppermost leaf sample (0.5 g) was extracted in 3% sulphosalicylic acid and centrifuged for 10 min at 10 000×g. 2 mL of supernatant was incubated with equal

volume of acid ninhydrin and glacial acetic acid at 100°C for 1 h. Reaction was terminated by keeping samples on ice bath and proline was separated using toluene and the absorbance was read at 520 nm (Bates *et al*. 1973). Proline concentration was determined using calibration curve and expressed as μg g^{-1} FW.

2.5. Extraction and quantification of plant growth regulators

For determining the endogenous levels of plant growth regulators, apical bud leaves were extracted in 80% aqueous acetone (4:1, v/v) containing butylated hydroxytoluene (10 mg L^{-1}) and were purified using EtOAc and $NaHCO_3$ (Kusaba *et al*. 1998). Quantification of gibberellins (GA_1 and GA_4) was done using gas chromatograph-mass spectrometer (GC-MS) with selected ion monitoring (SIM) mode, and standards of GA_1 and GA_4 were used as references (Lee *et al*. 1998). Abscisic acid (ABA) was extracted as per the method of Qi *et al*. (1998) and computations were done using standard ABA as reference. For quantification of indole acetic acid (IAA) and indole butyric acid (IBA), method of Kelen *et al*. (2004) was adopted. Purified extract residue was subjected to HPLC on a column of PEGASIL ODS (6 mm×150 mm, Senshu Kagaku, Tokyo, Japan) and quantifications were done from the standard curves of IBA ranging from 10 to 200 ng mL^{-1}. For salicylic acid, estimation extracts were vacuum-dried at room temperature and the concentration of salicylic acid (SA) were estimated adopting Siegrist *et al*. (2000) using HPLC equipped with fluorescence detector (LC-2010 AHT; SHIMADZU, Japan).

2.6. Statistical analysis

The experimental design was a completely randomized design. The data were subjected to Duncan's multiple range tests and one-way ANOVA using SPSS. 22 Software. Five replicates were used for each biological treatment. Different letters indicate significant differences based on Turkey's honest significant difference (HSD) test at $P<0.05$.

3. Results

Drought reduced the growth drastically, and *T. harzianum* not only ameliorated these negative effects but also enhanced growth significantly. In the present study, shoot and root length, shoot and root dry weight, and leaf area were reduced from 52.02 to 32.5% by drought stress, while increased by *T. harzianum* up to 42.7%, respectively (Table 1). Impact of drought stress on photosynthetic pigments is shown in Table 2. And it was observed that drought stress reduced chlorophyll *a*, chlorophyll *b* and total chlorophylls by 29.1% (Table 2). Alone, *T. harzianum* inoculation proved significant and caused an increase of 15.4, 9.3, 15.04% in chlorophyll *a*, chlorophyll *b*, and total chlorophylls, respectively, and 20% in carotenoid contents depicting its importance in protecting photosynthetic apparatus (Table 2).

Proline and soluble protein was observed to enhance by 78.9 and 47.6% in drought stressed plants (Fig. 1-A and B).

Table 1 Shoot and root length, shoot and root dry weight, and leaf area in drought stressed and *Trichoderma harzianum* inoculated *Solanum lycopersicum* plants

Treatments[1)]	Shoot length (cm)	Root length (cm)	Shoot dry weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Leaf area (cm^2 $plant^{-1}$)
Irrigated (Tr–)	27.12 b	13.78 b	2.43 b	3.11 b	21.87 b
Irrigated (Tr+)	35.47 a	15.16 a	3.18 a	4.21 a	38.19 a
Drought (Tr–)	13.01 d	8.75 d	1.25 d	1.68 d	14.75 d
Drought (Tr+)	20.14 c	10.83 bc	1.76 c	2.79 bc	18.96 c

[1)] Tr–, absence of *T. harzianum* inoculated; Tr+, presence of *T. harzianum* inoculated.
Data presented are means of five replicates. Different letters indicate significant differences based on Turkey's honest significant difference (HSD) test at $P<0.05$.

Table 2 Photosynthetic pigments in drought stressed and *Trichoderma harzianum* inoculated *Solanum lycopersicum* plants

Treatments[1)]	Photosynthetic pigments (mg g^{-1} FW)[2)]					
	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll (*a*/*b*)	Chlorophyll (*a*+*b*)	Carotenoids	Total pigments
Irrigated (Tr–)	1.42 b	0.39 b	3.64 b	1.81 b	0.28 d	2.09 c
Irrigated (Tr+)	1.68 a	0.43 b	3.90 a	2.11 a	0.35 c	2.46 ab
Drought (Tr–)	0.81 d	0.21 c	3.85 a	1.02 c	0.46 b	1.48 d
Drought (Tr+)	1.28 c	0.67 a	1.91 c	1.95 b	0.67 a	2.62 a

[1)] Tr–, absence of *T. harzianum* inoculated; Tr+, presence of *T. harzianum* inoculated.
[2)] FW, fresh weight.
Data presented are means of five replicates. Different letters indicate significant differences based on Turkey's honest significant difference (HSD) test at $P<0.05$.

However, *T. harzianum* alone caused an increase of 32.08 and 26.7% in proline and protein content, respectively. While, *T. harzianum* was inoculated to drought stressed plants and caused an increase of 80.8 and 67.1% in proline and protein content, respectively (Fig. 1-A and B). However, content of phenols and flavonoid was reduced by 15.5 and 52.7% in drought-stressed plants as compared to *T. harzianum* colonized plants, which caused further increase of 17.7 and 10.4%, respectively (Fig. 2-A and B). Drought stress reduced the endogenous levels of hormones studied such as IAA, IBA, GA_1, GA_4 in tomato plant tissue (Table 3). The concentrations of ABA and SA in plant tissue stressed by drought were increased by 74.2 and 37.8%, respectively (Table 3). However, inoculation of tomato with *T. harzianum* enhanced their concentrations, e.g., IAA by 45%, GA_1 by 38.5%, and other phytohormones by 27% (Table 3) under drought stress conditions (–1.2 MPa).

4. Discussion

In the present study, *T. harzianum* resulted in significant improvement in growth of tomato plants subjected to water stress or not. *T. harzianum* caused a significant increase in growth of tomato, which may be because of its positive influence on the levels of phytohormones resulting in growth promotion. Such a significant increase in plant growth of tomato due to *T. harzianum* has been reported by Mastouri *et al*. (2012). The improvement of plant growth by *T. harzianum* under drought stress was reported by Shukla *et al*. (2015) for *Triticum aestivum* L. Drought-induced reduction in growth of plants is a cumulative effect of several factors like increased temperature and the availability of soil water, during drought, mineral uptake is hampered resulting in further reduction in water uptake by the plants (Jatav *et al*. 2014). Drought poses a negative effect on the synthesis of photoassimilates, the prime reason for their declined production is the destruction of chlorophyll pigments and the synthesis of pigment intermediates together with the decline in the uptake of important ions like magnesium which forms an important component of cholorphyll pigment molecule (Azarmi *et al*. 2011; Ahanger *et al*. 2014a). In the present investigation,

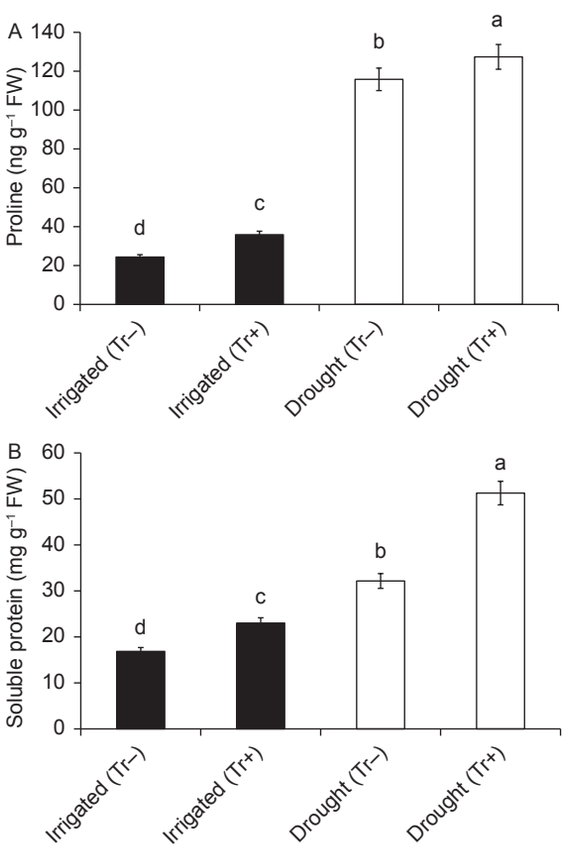

Fig. 1 Proline (A) and soluble protein (B) content in drought stressed and *Trichoderma harzianum* inoculated *Solanum lycopersicum* plants. Tr–, absence of *T. harzianum* inoculated; Tr+, presence of *T. harzianum* inoculated; FW, fresh weight. Data are means±SD, *n*=5.

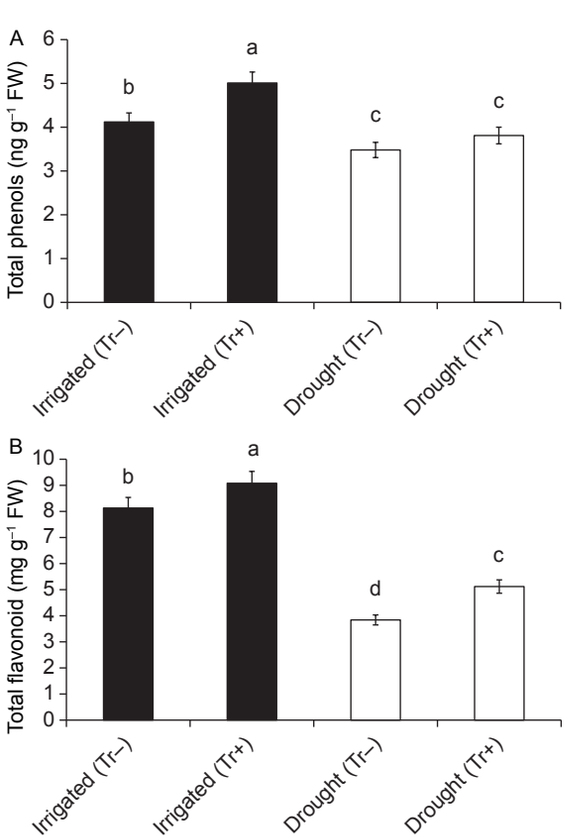

Fig. 2 Total phenols (A) and total flavonoid (B) content in drought stressed and *Trichoderma harzianum* inoculated *Solanum lycopersicum* plants. Tr–, absence of *T. harzianum* inoculated; Tr+, presence of *T. harzianum* inoculated. FW, fresh weight. Data are means±SD, *n*=5.

Table 3 IAA, IBA, GA_1, GA_4, ABA, and SA content in drought stressed and *Trichoderma harzianum* inoculated *Solanum lycopersicum* plants[1)]

Treatments[2)]	IAA (mmol L^{-1} g^{-1} FW)	IBA (mmol L^{-1} g^{-1} FW)	IAA/IBA	GA_1 (mmol L^{-1} g^{-1} FW)	GA_4 (mmol L^{-1} g^{-1} FW)	GA_1/GA_4	ABA (nmol L^{-1} g^{-1} DW)	SA (nmol L^{-1} g^{-1} DW)
Irrigated (Tr–)	3.12 b	123.81 b	0.025 b	4.27 b	7.96 b	0.536 b	879.1 d	14 871 d
Irrigated (Tr+)	5.68 a	167.86 a	0.033 a	6.95 a	10.93 a	0.635 a	1 204.3 c	16 034 c
Drought (Tr–)	1.07 d	73.11 d	0.014 c	1.38 d	3.04 d	0.453 c	3 412.2 a	23 916 a
Drought (Tr+)	2.89 c	96.45 c	0.029 b	3.07 c	5.79 c	0.530 b	2 093.8 b	18 481 b

[1)]IAA, indole acetic acid; IBA, indole butyric acid; GA, gibberellic acid; ABA, abscissic acid; SA, salicylic acid; FW, fresh weight; DW, dry weight.
[2)]Tr–, absence of *T. harzianum* inoculated; Tr+, presence of *T. harzianum* inoculated.
Data presented are means of five replicates. Different letters indicate significant differences based on Turkey's honest significant difference (HSD) test at $P<0.05$.

T. harzianum was observed to promote cholorphyll synthesis individually as well as when inoculated to drought stressed plants. Improved photosynthetic efficiency and the synthesis of photosynthetic pigments in tomato due to inoculation of *T. harzianum* has been reported earlier by Azarmi *et al*. (2011). Another important reason for *T. harzianum*-mediated enhancement in photosynthetic pigments may be due to its strong effect on the phytohormones (Resende *et al*. 2014), which was also observed in our study.

During the present study, drought stress caused accumulation of proline and soluble proteins, which were further enhanced by *T. harzianum* inoculation. Plants accumulating higher contents of free proline show increased stress tolerance (Abd_Allah *et al*. 2015). Accumulation of proline in drought stressed tomato plants corroborate with the findings of Jatav *et al*. (2012) for wheat and Manivannan *et al*. (2007) for sunflower. Shukla *et al*. (2015) have reported that priming *Triticum aestivum* L. with *T. harzianum* improved drought stress tolerance by mediating enhanced synthesis and accumulation of proline, thereby conferring tolerance to drought stress. Similar to this finding, our results also reflect increased accumulation of proline in *T. harzianum* inoculated tomato plants as compared to control plants. Proline has a significant role in ROS scavenging and maintaining the structure of proteins and membranes (Ahanger *et al*. 2014a). In addition, it leads to improvement in energy generation and storage by influencing the nitrogen metabolism (Ahanger *et al*. 2015; Hashem *et al*. 2015). Proline protects metabolic processes during stressful condition by replacing water, thereby bringing stability to important cellular structures (Zhifang and Loescher 2003).

Accumulating higher content of phenols provide protection to plants by acting as free radical scavengers and mediating the cell wall formation (Ahanger *et al*. 2015; Hashem *et al*. 2016). In the present study, increased phenol synthesis in *T. harzianum* inoculated plants resulted in improved growth in drought stressed tomato plants and thereby protecting it from oxidative stress by eliminating ROS. Increased synthesis of phenols and flavonoids have the direct effect on the antioxidant activity, besides contributing to cell wall formation which protects plants from biotic stress outbreaks (Surekha *et al*. 2014). Flavonoids serve as endogenous regulators of auxin movements for mediating developmental regulation, and *T. harzianum* inoculated plants may have exhibited precise growth hormone regulation and the photoprotection of photosynthetic pathway (Brunetti *et al*. 2013), which were also got in our study. It has been shown by several workers that plants maintaining relatively higher phenol synthesis exhibit increased antioxidant activity and therefore better growth (Ahmad *et al*. 2016). Enhanced synthesis of proline due to *T. harzianum* in tomato reported in the present study may have resulted in growth stimulation by ROS scavenging and membrane protection. *Solanum viarum* plants inoculated by *T. harzianum* exhibited higher accumulation of flavonoids, phenols, tannins and alkaloids as compared to plants inoculated with arbuscular mycorrhizal fungi (AMF) or plant growth promoting rhizomicroorganisms (PGPR), therefore confirming *T. harzianum* to be more effective in growth maintenance (Hemashenpagam and Selvaraj 2011). Significant increase in flavonoids in *T. harzianum* inoculated plants confirms its role in triggering the protective pathways in tomato under drought stress.

Tomato plants that were inoculated with *T. harzianum* showed higher endogenous levels of growth hormones. Melon plants inoculated by *Trichoderma* spp. exhibited improvement in growth and the yield potential (Martinez-Medina *et al*. 2014). Growth hormones are known for their role in plant developments and also mediate signalling under stress conditions for bringing elicitation of specific response. In present study, ABA and SA were increased due to drought stress, and may be due to *T. harzianum* have helped inoculated plants to maintain the expression of stress responsive proteins for counteracting oxidative damage of drought stress induced (Hermosa *et al*. 2012). In our study, *T. harzianum* resulted in improved growth by maintaining optimal levels of growth regulators. Normal

development and the potential to counteract the harmful effects of stresses depend on the hormone mediated signalling pathways (Pieterse *et al*. 2009). *T. harzianum* increased ABA in tomato plants and can contribute to stress adaptations like signal perception and its transduction besides its role as an antitranspirants to cut down the water losses under water deficit conditions. Enhanced synthesis of growth hormones like ABA can impart better drought stress tolerance to plants by regulating the stomatal movements in order to check water losses for maintaining the water balance. Optimal concentration of salicylic acid is important for developing stress response and the concentration may vary with the kind and the severity of stress. Salicylic acid regulates photosynthesis by upregulating antioxidant metabolism and thereby preventing the deleterious effects of drought stress and in the present study increase in salicylic acid due to inoculation of *T. harzianum* may have protected photosynthesis by acting as an important signal molecule for upregulating antioxidant system (Yuan and Lin 2008; Khan *et al*. 2015).

5. Conclusion

It can be concluded from the present study that drought affects tomato growth by altering the synthesis of chlorophyll pigments, hormone balance and hence the metabolism. However, *T. harzianum* proved to be useful in mitigating the negative effects of drought stress by modulation plant secondary metabolites. Improved osmolyte proline concentration in plant tissue and the synthesis of growth hormones by *T. harzianum* may have coordinately worked for bringing the growth regulation by protecting membranes from ROS and enhance root system accessing to more nutrients.

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