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Trichoderma Koningii enhances tolerance against thermal stress by regulating ROS metabolism in tomato (*Solanum lycopersicum L.*) plants

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ABSTRACT

Thermal stress (TS) can be detrimental to tomato crop at different growth stages and is accounted by the generation of highly fatal reactive oxygen species (ROS). In this study, the effects of *Trichoderma koningii* ITCC 5201 *(TK)* were investigated on the tolerance of tomato (*Solanum lycopersicum* L.) plants under TS. TS elevated SOD, POD, PPO PAL, and Proline activity in the present study. However, a further reduction was observed in these enzymatic activities by the application of *TK* in the stressed and treated seedlings, while an increase in the starch, protein, and total phenol content was observed in stressed and treated plants. TS affirms the increase in H_2O_2 generation and lignin deposition, but reduction in the accumulation was observed by the treatment of *TK* to tomato seeds. In conclusion, *TK* proved to be very beneficial in imparting resistance to the tomato plants against heat stress through improved modulation of antioxidants.

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KEYWORDS
T. koningii; abiotic stress; thermal stress; reactive oxygen species (ROS); antioxidant enzymes

Introduction

In plant's life cycle its vegetative advancement and reproductive development is highly influenced by exasperation of nature which is expressed in the form of various biotic and abiotic stresses. Plants repeatedly encounter a plentitude of stress conditions such as high and low temperature, flooding, salt, drought, oxidative stress, and heavy metal toxicity (Mahajan and Tuteja 2005). Abiotic stresses are major limitations responsible for shrinking agriculture production worldwide, it has been estimated that more than 50% of yield reduction is due to various abiotic stresses (Rodriguez et al. 2005; Acquaah 2007). Among the various abiotic stresses tomato plants are recurrently exposed is TS, which causes an adverse effect on the physiological functions of the plant (Wen et al. 2008). When plants are being exposed to a regime of extremely high temperature, the disruption of cellular association and cell damage take place that adversely affect plant growth and may even lead to a gradual shutting down of plant metabolic activity (Schoffl et al. 1999; Schopfer et al. 2001). Heat stress can initiate oxidative stress in plants through peroxidation of polyunsaturated fatty acids of membrane and by disturbing the membrane integrity through protein denaturation (Brueske 1980; Camejo et al. 2006). As a response to this stress condition, different reactive oxygen species (ROS) are produced in the plant system including superoxide ion, singlet oxygen, hydrogen peroxide, and hydroxyl radical, these ROS are also produced in plants during normal metabolic condition also but during stress conditions there production increases many fold, thereby posing a serious threat to plant vitality (Sharma and Dubey 2005). For controlling ROS level and to safeguard the cells under conditions of stress various ROS scavenging enzymes are present in plant tissue *viz*., catalase, superoxide dismutase, and peroxidases etc., along with these enzymes a complex of various low molecular mass antioxidants, such as glutathione, ascorbate, and tocopherols, are also present (Blokhina et al. 2003).

Plant biostimulants (PBs) could be used as an alternative for safeguarding the plants against the abiotic stresses (Bhardwaj et al. 2014) due to the enhanced tolerance induced by the associated microorganisms (Balestrini et al. 2018). PBs can competently induce a plentitude of biochemical, physiological, and molecular plant responses, which may eventually lead to an enhanced tolerance to both biotic and abiotic stress tolerance (Rouphael et al. 2018; Rouphael and Colla 2020). *Trichoderma* spp were termed as PBs, thereby making them an environment-friendly innovation (Colla and Rouphael 2015). *Trichoderma* spp. are opportunistic fungi residing in the rhizospheric region and can parasitize other fungi and also promote plant growth response. *They* colonize plant roots and establish symbiotic relationships with a wide range of host plants, as a consequence plant growth and performance frequently are enhanced (Harman 2011; Harman et al. 2004; Shoresh et al. 2010). The capabilities of a number of *Trichoderma* spp. to surmount extreme environments make their presence possible in varied geographical location (Hermosa et al. 2004). The PBs along with mitigating the stress induced by free radicals by directly scavenging and preventing ROS formation (Polo and Mata 2018) also increase the fruit yield (Francesca et al. 2020). Improvement in tolerance to water deficit, due to T22 in tomato seedlings by strengthening antioxidant defense and elevation in the activity of ascorbate and glutathione-recycling enzymes, was reported by Mastouri et al. 2012. Moreover, this result demonstrated that T22 enhances seedling vigor, increases

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the ability of colonized plants to tolerate stress by inducing a protective physiological barrier in plants against oxidative damage, and also stated significance of *Trichoderma* sp. in ameliorating heat stress in host by modulating its antioxidative network. Therefore, in this study we propose that *Trichoderma koningii (T. koningii)* plays a vital role in counteracting the harmful effects of ROS by enhancing the plant's defense arsenals and hypothesize that the elevated enzymatic activities during the TS may be the result of increased expression of the responsible genes.

Materials and methods

The experiment was conducted under laboratory and greenhouse conditions in the Department of Mycology and Plant Pathology, Institute of Agriculture Sciences, Banaras Hindu University, to elucidate the role of *Trichoderma* spp. in the alleviation of TS in plants. The experimental methods applied and the procedures adopted are discussed below.

Preparation of fungal inoculum

Trichoderma koningii (ITCC 5201) was used as a seed inoculant for this study. *T. koningii* was grown on Potato Dextrose Agar (PDA) and incubated for 6 days at 27 ± 1°C. Spores were harvested and the final concentration was adjusted to 2×10^7 CFU/ ml by sterile distilled water.

Seed treatment with T. koningii

Seeds of tomato (*Solanum lycopersicum* (L.) cv Pant Tomato 3) were sanitized for 30 s with sodium hypochlorite (1%), washed twice with sterile water, and dried under a sterile stream of air. The seeds were then coated with 2000 µl of the conidial suspension of *T. koningii*, to deposit 2×10^7 CFU per gram of seeds. Control seeds were treated with an equal amount of sterilized double distilled water. The microbial suspension was prepared in 1% CMC (carboxymethyl cellulose) and the seeds were soaked (10 h) in the suspension for coating. The microbial suspension was then drained and the seeds were dried overnight in sterile Petri dishes. Two sets of uncolonized seeds and two sets of *Trichoderma* colonized seeds were maintained for further analysis.

Green house experiment

For three consecutive days, the soil was sterilized at 15 p.s.i for 30 min in an autoclave, and the mixture (1.5 kg) was filled in each plastic pot (15 × 10 cm). The experiment comprised of the following 4 treatments: T1 (untreated and unstressed seeds), T2 (untreated seeds + heat shock), T3 (*T. koningii* treated and unstressed seeds), and T4 (*T. koningii* treated + heat shock). Five pots were used for each treatment and eight seeds were sown in each pot. The potted plants were maintained in the protected conditions (Green house) at 28/20°C with an alternate light: dark cycle: 14 h: 10 h and around 80% relative humidity and irrigated as required or at intervals of 1–2 days until partial saturation with regular watering for a growth period of 1 month.

Induction of heat stress in plants

After providing a growth period of 1 month to the plants under greenhouse condition, the plants were divided into two sets: one set of plants from the control and other set of plants inoculated with *Trichoderma* were taken for induction of heat stress inside the plant growth chamber (RXZ series climate incubator, Ningbo, China). The plants were first acclimatized inside the chamber for 3–5 days and then a heat shock was given at45°C for 5 hours in order to observe the effects of *Trichoderma* application during high-temperature stress.

Sample collection and biochemical analysis

The pots were taken out from the growth chamber after heat stress induction and 5 tomato plants were randomly uprooted from each treatment without causing any plant damage. Roots were washed with distilled water to remove the adhering soil and were further blotted dry. In a similar manner, plants were also taken out carefully from the pots in which heat stress was not given and the sample was further taken for biochemical analysis.

Biochemical analysis

(1). Polyphenol oxidase (PPO) assay

0.1 g of leaf samples was homogenized in 2 ml of chilled sodium phosphate buffer (0.1 M, pH 6.5). After centrifuging the homogenate at 16,000 g for 30 min at 4°C, the supernatant was collected, and used directly for enzyme assay. The reaction mixture consists of 0.4 ml catechol (1 mM), 0.4 ml enzyme extract, and 3 ml of sodium phosphate buffer (50 mM, pH 6.5). Control was prepared by the reaction mixture containing only substrate. Catechol was used as a substrate for PPO (EC 1.14.18.1) assay and absorbance (405 nm) was recorded (Gauillard et al. 1993). The PPO activity was expressed as the change in OD per min per g FW.

(2). Phenylalanine ammonia lyase (PAL) assay

From each treatment, 0.1 g of leaf samples was homogenized in 2 ml of sodium borate buffer (100 mM, pH 7.0; 4°C) containing 2-mercaptoethanol (1.4 mM). The homogenate was centrifuged at 16,000 g for 15 min (4°C) and the supernatant was collected, used as the enzyme source. A mixture, containing 0.2 ml of enzyme extract, 0.5 ml of borate buffer (200 mM, pH 8.7), and 1.3 ml of distilled water, was prepared, and the reaction was initiated by the addition of 1 ml of l-phenylalanine (100 mM, pH 8), incubated at 32°C for 30 min. Trichloroacetic acid (TCA, 1M), 0.5 ml was added for the termination of reaction. Activity of PAL (EC 4.1.3.5) was recorded (290 nm), as described by Brueske (1980), by the subsequent formation of trans-cinnamic acid. The PAL activity was calculated in terms of µmol per g FW (Tripathi et al. 2018).

(3). Superoxide dismutase (SOD) assay

The activity of SOD (EC 1.15.1.1) was assessed by the Fridovich (1974) method, evaluating the ability of enzyme

extract from different treatments to inhibit photochemical reduction of nitroblue tetrazolium (NBT) chloride. 0.1 g of fresh leaves from different samples were taken and homogenized in 2.0 ml of pre-chilled extraction buffer (pH 7.5) containing sodium phosphate buffer (100 mM) and EDTA (0.5 mM) in a mortar and pestle. The homogenate was centrifuged at 15,000 g (20 min; 4°C) and the supernatant was collected. The reaction mixture (3 ml) consisted of methionine (200 mM), NBT (2.25 mM), EDTA (3 mM), sodium phosphate buffer (100 mM, pH 7.8), sodium carbonate (1.M), and enzyme extract. The reaction was initiated by adding riboflavin (0.4 ml of 2 µM) and illuminating the sample tubes under two 15-W fluorescent lamps for 15 min. Using an enzyme-free reaction mixture, the control was prepared. The lights were turned off for the termination of reaction and the tubes were kept in dark until the absorbance (560 nm) was recorded. One unit of the SOD activity was defined as the amount of enzyme that reduced the absorbance to 50% compared to the control.

(4). Peroxidase (PO) assay

PO (EC 1.11.1.7) activity was assessed using the Hammerschmidt et al. (1982) method with slight modification. 0.1 g of leaf samples was individually homogenized in sodium phosphate buffer (2 ml of 100 mM; pH 7.0; 4°C), centrifuged (16,000 g for 15 min, 4°C) and the supernatant was collected used as an enzyme source. The reaction mixture was prepared by pyrogallol (1.5 ml of 50 mM), enzyme extract (0.05 ml), and H_2O_2 (0.5 ml of 1% v/v). The enzyme-free reaction mixture was used as a control. Changes in absorbance (420 nm) were measured at intervals of 30 s for 3 min. The enzyme activity was expressed as a change in U per min per g FW.

(5). Free proline content

The proline content was estimated by the Bates et al. (1973) method. 0.1 g of leaf tissue was homogenized with aqueous sulphosalicylic acid (5 ml of 3%) and the homogenate was centrifuged (10,000 g for 15 min). The filtered supernatant (2 ml) was reacted with glacial acetic acid (2 ml) and ninhydrin (2 ml, prepared by dissolving 1.25 g of ninhydrin in 30 ml warmed glacial acetic acid and 20 ml of 6 mol/l phosphoric acid) at 100°C for 1 h. The reaction was terminated in an ice bath and then brought to room temperature to stabilize the purple color of the extract. Toluene (4 ml) was added to each tube, vortexed (15–20 s), and the top aqueous layer (purple) was collected to record absorbance (520 nm). The concentration of proline samples was determined using a standard curve plotted with known proline concentrations.

(6). Protein estimation

Protein estimation was done as per Bradford method (Bradford 1976). 0.1 g of leaf samples was homogenized in sodium phosphate buffer (2 ml of 100 mM; pH 7.0) and the homogenate was centrifuged (1000 g for 5 min; 4°C). The supernatant was used as the enzyme source. The reaction mixture consisted of 3 µl protein samples, 799 µl water, and 600 µl Bradford reagent and was incubated for 10 min. Blank was prepared without any protein samples. The absorbance was estimated in a spectrophotometer at 595 nm.

(7). Sugar estimation

The amount of total soluble sugars were estimated by Phenol sulfuric acid reagent method (Dubois et al. 1951). 500 mg each of plant material sample taken from each treatment was homogenized with 10 ml of 80% ethanol and centrifuged (2000g for 20 min). The supernatant was collected separately to 1.0 ml of alcoholic extract; 1.0 ml of 5% phenol solution was added and mixed. Then 5.0 ml of 96% sulfuric acid was added rapidly. Each tube was gently agitated during the addition of the acid and then allowed to stand in a water bath at 26–30°C for 20 min. The developed characteristic yellow orange color was measured at 490 nm after setting a 100% transmission in a spectrophotometer against the blank; the calibration curve was prepared by using known glucose concentrations. The quantity of sugar was expressed as mg/g fresh weight of tissue (Meena et al. 2014).

(8). Starch estimation

Starch estimation was carried out by the McCready et al. (1950) method. The residual mass obtained after the extraction of soluble sugar from the plant material was suspended in water (5 ml) followed by the addition of perchloric acid (6.5 ml of 52%) to the residue and the mixture was stirred. The mixture was centrifuged (2000g for 20 min) and decanting and collection of supernatant was done and the complete process was replicated thrice. Supernatant was taken from each step and by adding distilled water, the final volume was made up to 100 ml. Whatmann's no. 1 filter paper was used for filtering the mixture; 1 ml of the filtrate was examined for the presence of starch following the same procedure for total soluble sugar. The amount of starch was calculated in terms of glucose equivalent and starch factor 0.9 was used for the conversion of glucose values. Starch quantity was expressed in mg per g FW.

(9). Total phenol content estimation

Total Phenol Content was determined following the method of Zheng and Shetty (2000). Leaf tissue (0.1 g) was placed in 5 ml of 95% ethanol and kept at 0°C for 48 h. Samples were separately homogenized and centrifuged (10,000 g; 10 min). To filter extract (1 ml), ethanol (1 ml of 95%), distilled water (5 ml), and Folin–Ciocalteu regent (0.5 ml of 50%) were added, and the contents were mixed properly. After 5 min., 1 ml of 5% sodium carbonate was added, the reaction mixture was kept for 1 h and the absorbance of the color developed was recorded at 725 nm. Standard curves were prepared for each assay using various concentrations of Gallic acid in 95% ethanol. Absorbance values were converted to mg GA equivalents (GAE) per g FW (Jain et al. 2012).

1 Hydrogen peroxide production

The histochemical analysis of H_2O_2 was performed, according to Thordal-Christensen et al. 1997 by DAB (3,3-diaminobenzidine) resulting in a reddish-brown staining. For the qualitative estimation of H_2O_2 leaf disks were

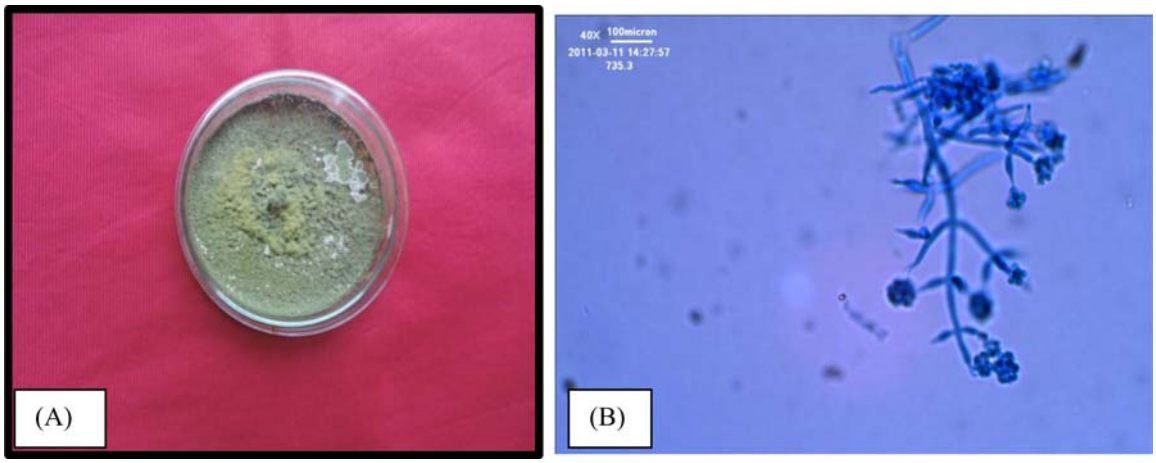


Figure 1. (A) Cultural characteristics and (B) Conidial Morphology of *T. koningii* (ITCC 5201).

immersed in DAB solution (1 mg ml^{-1}; pH 7.5) and incubated in dark for 20 h at room temperature. After incubation the leaf disks were boiled in 15 ml solution containing absolute ethanol and lactophenol (2:1) for 5 min and then rinsed with ethanol (1 ml of 50%) twice. The leaf disks were then viewed under light microscope (Nikon DS-fi1, Japan) in order to view H_2O_2 production in leaf tissues.

1 Histochemical staining (Lignification)

Examination of transverse stem sections was performed by taking the stem of the plant from all treatments, which were further fixed in ethanol (95%, v/v) followed by mounting on a slide in a solution of saturated aqueous phloroglucinol (prepared in 20% HCl) and examined by light microscope (Nikon DS-fi1, Japan). The presence of red-violet color indicates positive lignin staining (Jensen 1962).

Statistical analyses

The experiment was a completely randomized design (CRD) with three replications. Results are expressed as means of three replicates and vertical bars indicate standard deviation of the mean. Different letters indicate significant difference among treatment results taken at same time interval according to Duncan's multiple range tests at $p \leq 0.05$.

Results

The detrimental effect of high temperature was more pronounced in uninoculated stressed plants as compared to *T.koningii* (Figure 1) inoculated stressed plants. The biochemical studies indicate that *Trichoderma* inoculated plant showed a better tolerance to high-temperature stress, whereas control plants exhibited signs of a lower tolerance.

Effect on SOD and POx activities

The differential response between the treatments for heat stress and the effect of *T. koningii* on the enhancement of TS tolerance was monitored in co-relation to SOD and POx activities after the artificial induction of heat stress (45°C for 4–5 hrs). In an early plant response to TS, the Singlet oxygen generated is usually dismutated rapidly via SOD to H_2O_2 and the H_2O_2 thus produced contributes significantly toward the structural reinforcement of plant cell wall via POx activity. High-temperature stress resulted in elevated responses of SOD and PO.

SOD activity in the present study was highest under stressed and untreated conditions [Figure 2(a)]. Although its activity was significantly high in all the treatments compared to untreated and unstressed plants, its maximum activity was observed in the plants receiving heat stress only where 1.19-, 2.46-, and 2.62-fold increase in SOD activity was recorded compared to the stressed treated, unstressed treated, and untreated unstressed plants, respectively. Similar to SOD activity, the treatment not receiving the

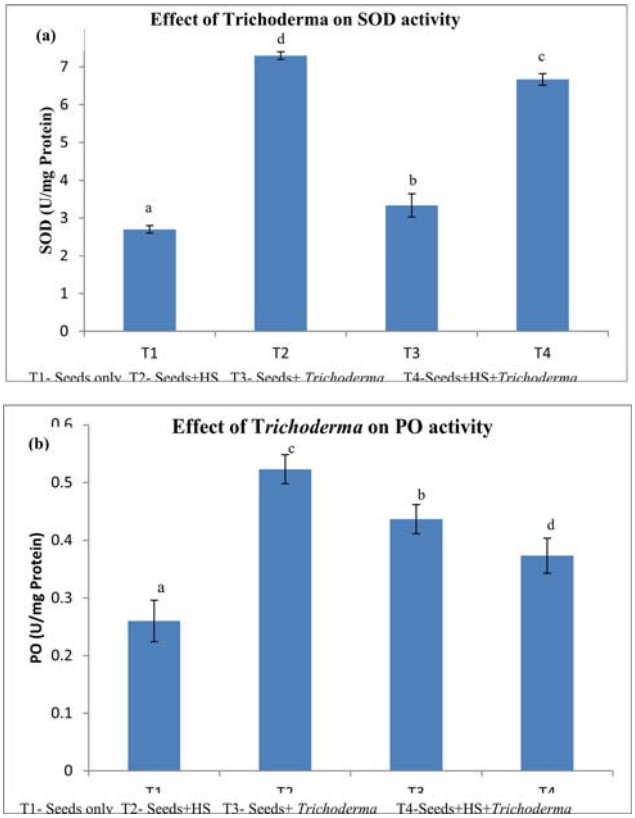
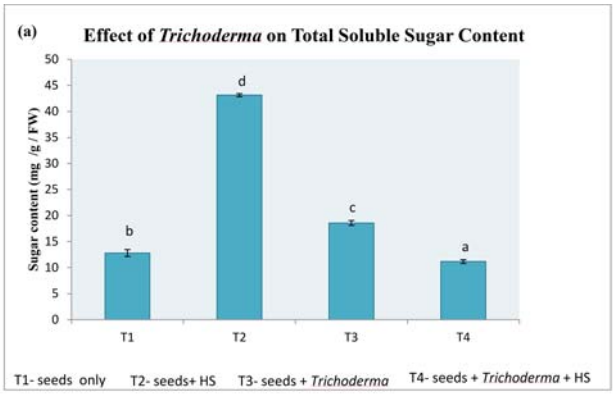


Figure 2. Changes in super oxide dismutase (a) and Peroxidase (b) activities. Different letters indicate significant difference among treatment results taken at same time interval according to Duncan's multiple range test at $p \leq 0.05$. Where, T1: Control (Only seeds were sown without giving any treatment); T2: Seed + Heat Stress; T3: Seed + *Trichoderma* treatment; T4: Seed + *Trichoderma* treatment + Heat stress.



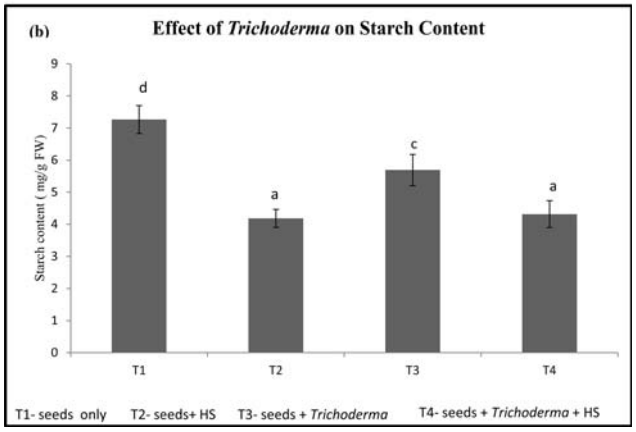


Figure 3. Changes in total soluble sugar (a) and total starch content (b) Different letters indicate significant difference among treatment results taken at same time interval according to Duncan's multiple range test at $p \leq 0.05$. Where, T1: Control (Only seeds were sown without giving any treatment); T2: Seed + Heat Stress; T3: Seed + *Trichoderma* treatment; T4: Seed + *Trichoderma* treatment + Heat stress.

T.koningii application but only HS (T2) also showed highest induction of PO activity where 1.23-, 1.33-, and 2.33-fold increase was recorded over stressed treated, unstressed treated, and untreated unstressed plants, respectively [Figure 2 (b)]. Under high-temperature stress the activity of these two enzymes was minimum in unstressed, untreated condition, while it was maximum in stressed, untreated condition.

Effect on total soluble sugar and starch content

To explicate the effect of the *T.koningii* on the sugar and starch content as a result of heat stress induction different treatments were observed in the present study. Sugar content in the present study was highest under stressed, untreated condition [Figure 3(a)]. Although its activity was significantly high in all the treatments compared to treated and stressed plants, its maximum activity was observed in the plants receiving heat stress only where 2.52-, 3.07-, and 3.58-fold increase in Sugar content was recorded compared to unstressed treated, unstressed untreated, and stressed treated plants, respectively [Figure 3(a)]. Starch content in the leaf extracts showed a dissimilar increment pattern wherein in stressed condition a lower degradation of starch into sugar was observed in treated leaf extracts [Figure 3 (b)]. Maximum starch content was observed in unstressed untreated condition which shows a 1.32-, 1.60-, and 1.6-fold increase over unstressed treated, stressed treate,d and untreated stressed plants, respectively.

Proline content

High temperature enhanced free proline content (FPC) accumulation in untreated, stressed condition as compared to treated, stressed condition where the accumulation of proline was less due to lower interception of stress. The FPC in untreated, stressed condition showed an increment of 1.96-, 2.74-, and 5.29-fold over stressed treated, unstressed treated, and untreated unstressed plants, respectively [Figure 4].

Effect on phenylpropanoid activities

To elucidate the effect of the TS on elicitation of PAL activity a defense response treatment of *T.koningii* was used in the present study. Non-pathogenic rhizosphere microbes, such as *Trichoderma* spp, are known to induce PAL, PPO, and phenolic accumulation in plants against abiotic stress. In all the treatments in the present study a consistent PAL activity was observed. Highest PAL activity was seen in stressed, untreated condition [Figure 5(a) and Figure 6] showing an increment of 1.20-, 2.46-, and 9.05-fold increase over the stressed treated, unstressed treated, and untreated unstressed plants, respectively, thereby showing that *Trichoderma* inoculation increases PAL content at the time of stress. Similarly, PPO activity was also observed to be highest in stressed untreated condition [Figure 5(b) and Figure 6] where 1.57-, 2.84-, and 3.38-fold increase was recorded over stressed treated, unstressed treated, and untreated unstressed plants, respectively. In non-treated healthy control plants very low PAL and PPO activities were detected which were significantly lower than the other treatments which remained nearly constant during the experimental period. The result of this study shows that the content of phenol in leaf extracts increases significantly in response to high-temperature stress [Figure 5(c) and Figure 6]. The total phenolic content (TPC) was highest in stressed untreated conditions, where 1.12-, 1.25-, and 7.23-fold increase was recorded over stressed treated, unstressed treated, and untreated unstressed plants, respectively, thereby indicating that in stressed, inoculated condition *Trichoderma* plays an important role in increasing phenol content in plant as compared to uninoculated, stressed condition hence, showing the effectiveness of *Trichoderma* in lowering down the impact of TS.

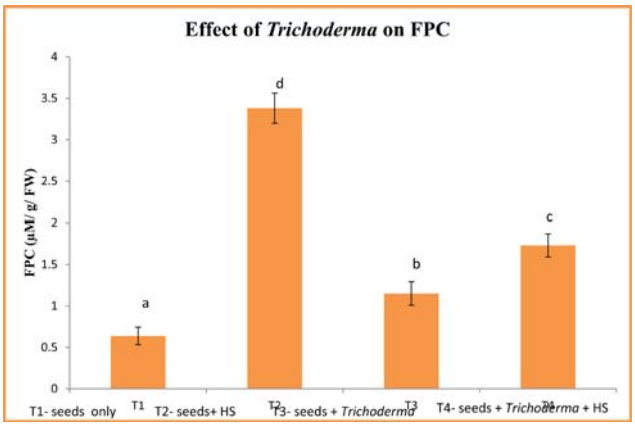


Figure 4. Changes in total free proline content. Different letters indicate significant difference among treatment results taken at same time interval according to Duncan's multiple range test at $p \leq 0.05$. Where, T1: Control (Only seeds were sown without giving any treatment); T2: Seed + Heat Stress; T3: Seed + *Trichoderma* treatment; T4: Seed + *Trichoderma* treatment + Heat stress.

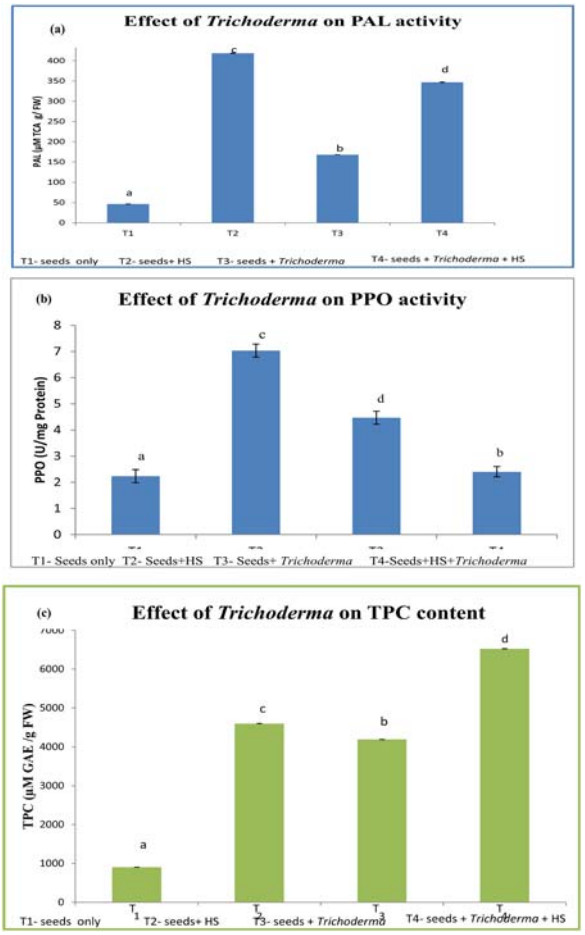


Figure 5. Changes in phenyl ammonia lyase (a), and poly phenol oxidase (b) activities and total phenolic content (c) in plants. Different letters indicate significant difference among treatment results taken at same time interval according to Duncan's multiple range test at $p \leq 0.05$. Where, T1: Control (Only seeds were sown without giving any treatment); T2: Seed + Heat Stress; T3: Seed + *Trichoderma* treatment; T4: Seed + *Trichoderma* treatment + Heat stress.

Protein content

High temperature leads to protein denaturation; as a result, protein content gets reduced when heat shock was given to untreated plants, as observed in the leaf extracts of untreated plants, while the protein content was increased in the case of *Trichoderma*-treated stressed leaf extracts, which showed 2.91-, 3.90-, and 5.85-fold increase over treated unstressed, untreated unstressed, and stressed untreated plants, respectively [Figure 7]. It signifies that *Trichoderma* has a pivotal role in maintaining protein content even in high-temperature condition.

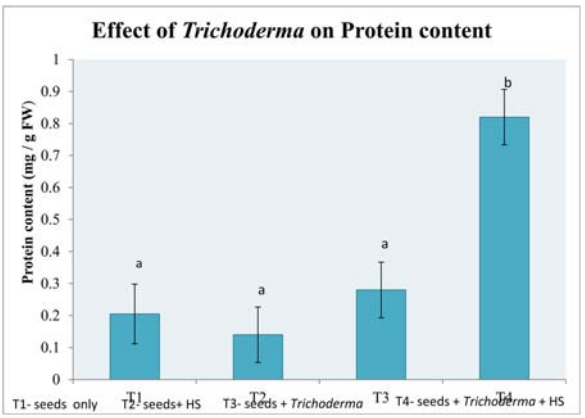


Figure 6. Changes in protein content in plants. Different letters indicate significant difference among treatment results taken at same time interval according to Duncan's multiple range test at $p \leq 0.05$. Where, T1: Control (Only seeds were sown without giving any treatment); T2: Seed + Heat Stress; T3: Seed + *Trichoderma* treatment; T4: Seed + *Trichoderma* treatment + Heat stress.

Effect on hydrogen peroxide generation

DAB is readily taken from the cut portion of the leaves and is distributed throughout the leaves. The hydrogen peroxide formed in the leaves can be easily detected as reddish-brown color visible to naked eye. In order to ensure the formation of visible polymer, the use of DAB uptake in *in situ* hydrogen peroxide study requires the presence of PO activity. A simple test for the required PO activity involves tissue exposure to DAB and hydrogen peroxide. DAB polymerization was studied in treated and untreated leaves with heat stress in *Trichoderma* to provide data based on H_2O_2 detection for DAB sensitivity. DAB polymerization was observed after 12 hrs of incubation [Figure 8]. The effect of *Trichoderma* inoculation and heat stress on peroxidase activity was determined on leaf tissues. The peroxidase level was found highest in stressed untreated condition, and a lower H_2O_2 content was observed in stressed treated condition, which clearly shows that *Trichoderma* lowers down the production of H_2O_2.

Effect on lignin deposition

Significant variation in the transverse section of stem from different treatment was observed through histochemical staining. Maximum deposition of lignin was seen in untreated stressed, followed by treated stressed [Figure 8]. A comparatively lower lignin deposition was observed in the case of treated unstressed. Lowest level of lignin deposition was seen in the case of untreated unstressed plants; this emphasizes the role of *Trichoderma* in lignin deposition at the time of heat stress, thereby strengthening the plant toward stress.

Discussion

Treatment of seeds with PBs for countering the effect of abiotic stress, along with promoting plant defense, has been previously reported (Rady et al. 2019). Temperature plays an imperative role in the plant life cycle, starting from the very first process of germination to flowering, pollination, fruit set, quality of fruit, seed production, and seed storage (Sawhney and Polowick 1985). A marked reduction in the number of flowers along with a drop in fruit set has been reported in the case of tomato when the temperature goes beyond 30°C (Sato et al. 2000). Campobenedetto et al. (2020) reported that seed treatment of cucumber seedling with PBs KIEM® promotes germination and seedling growth under heat stress conditions. *Trichoderma* strain *T. koningii* was taken for the purpose and was studied for its potentials in managing stress. The results showed that *Trichoderma* isolate used in this study, restricted or lowered down the production of ROS in the plants, thereby lowering the harmful effect caused by them on the plant's metabolism. Plant roots are colonized by *Trichoderma* spp. which contributes to a symbiotic relationship with a wide variety of host plants (Harman 2011; Harman et al. 2004; Shoresh et al. 2010).

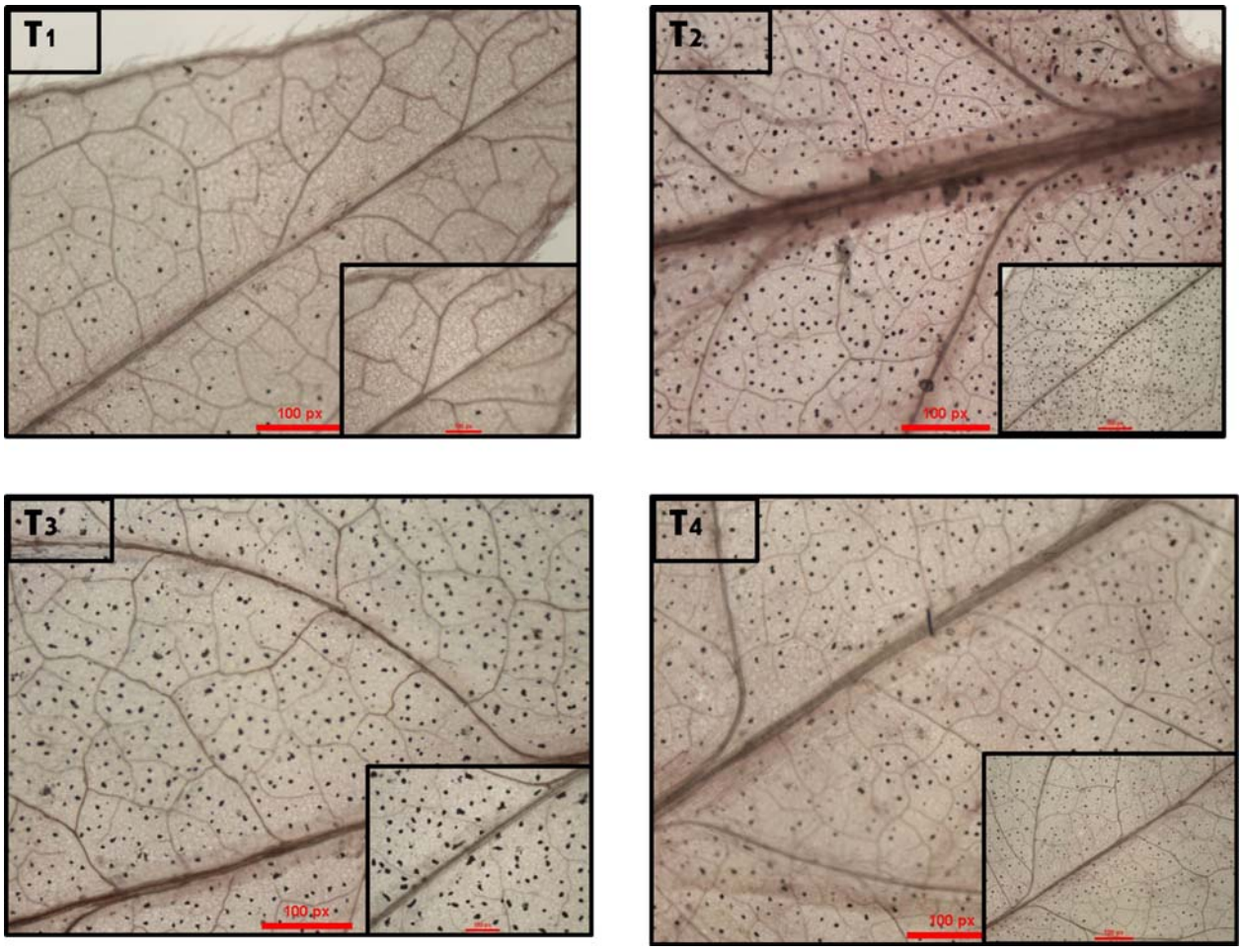


Figure 7. Visualization of hydrogen peroxide detected by DAB staining: Detections have been done on four-week-old tomato plant's leaves treated with *Trichoderma* and provided with subsequent heat treatment and were then further compared with the control. T1 (untreated and unstressed seeds), T2 (untreated seeds + heat shock), T3 (*T. koningii* treated and unstressed seeds) and T4 (*T. koningii* treated + heat shock).

Plants contain an array of cellular mechanism to defend themselves against a wide range of abiotic stress. Priming the plants with beneficial microorganism like *Trichoderma* may accelerate cellular defense response in plants so that they can withstand the stress in a more pronounced way. A comparatively lesser information is present regarding the host mechanism that affixes the model of *Trichoderma* root colonization for regulating signaling pathways that lead to the initiation of varied defense and developmental responses. It is presumed that initiation of signaling cascade, which leads to the activation of varied physiological and biochemical defense response in the plant that includes programmed cell death, callose deposition, formation, and increase in the concentration of ROS and secondary metabolites, is firstly recognized by microbe associated molecular pattern (MAMP) (Shoresh et al. 2010).

In this study, leaf extract analysis showed a higher activity of PO and SOD in stressed and untreated plants compared to stressed and treated plants after the induction of heat stress. Gulen and Eris (2004) demonstrated significantly increased total and specific PO activities due to high temperature which is consistent with the studies reporting the increased PO enzyme activity in response to different types of abiotic stress. Hydrogen peroxide generation was also confirmed by DAB staining. Interestingly, in the present studies the production of hydrogen peroxide increases with the temperature which was further lowered down by *Trichoderma* inoculation. A reduction in hydrogen peroxide level due to the treatment of cucumber seeds with PBs KIEM® was also reported by Campobenedetto et al. 2020, depicting a potential role played by the PBs in avoiding the accumulation of ROS.

Trichoderma strain selected in the study reduced accumulation of free soluble sugar under heat stress in comparison to untreated control where a comparatively higher level of soluble sugar was observed. An increased content of starch was found in leaf extracts from untreated control plants in comparison to treated plants. On comparing the stressed and unstressed condition, the starch content was higher in unstressed condition. The results of the present studies are in agreement with those of Wallwork et al. (1998) where barley grains from heat-stressed plants accumulated less starch than the control plants due to the reduced conversion of sugar to starch. Sheoran and Saini (1996) suggested that stress could inhibit the deposition of starch in pollen; either by decreasing the availability of assimilates or by impairing the enzyme activity involved in starch biosynthesis and they also demonstrated that under stressed conditions, the level of sugar increases in plant leaves. Accumulation of total soluble sugars (TSS) under heat stress has been implicated in the establishment and maintenance of thermotolerance (Wahid et al. 2007; Rizhsky et al. 2004).

Free proline content shows a marked increase under high temperatures, about 1.5-fold increases in FPC was found in high temperature-treated leaves of mulberry (Chaitanya et al. 2001). Proline accumulation in high temperature-

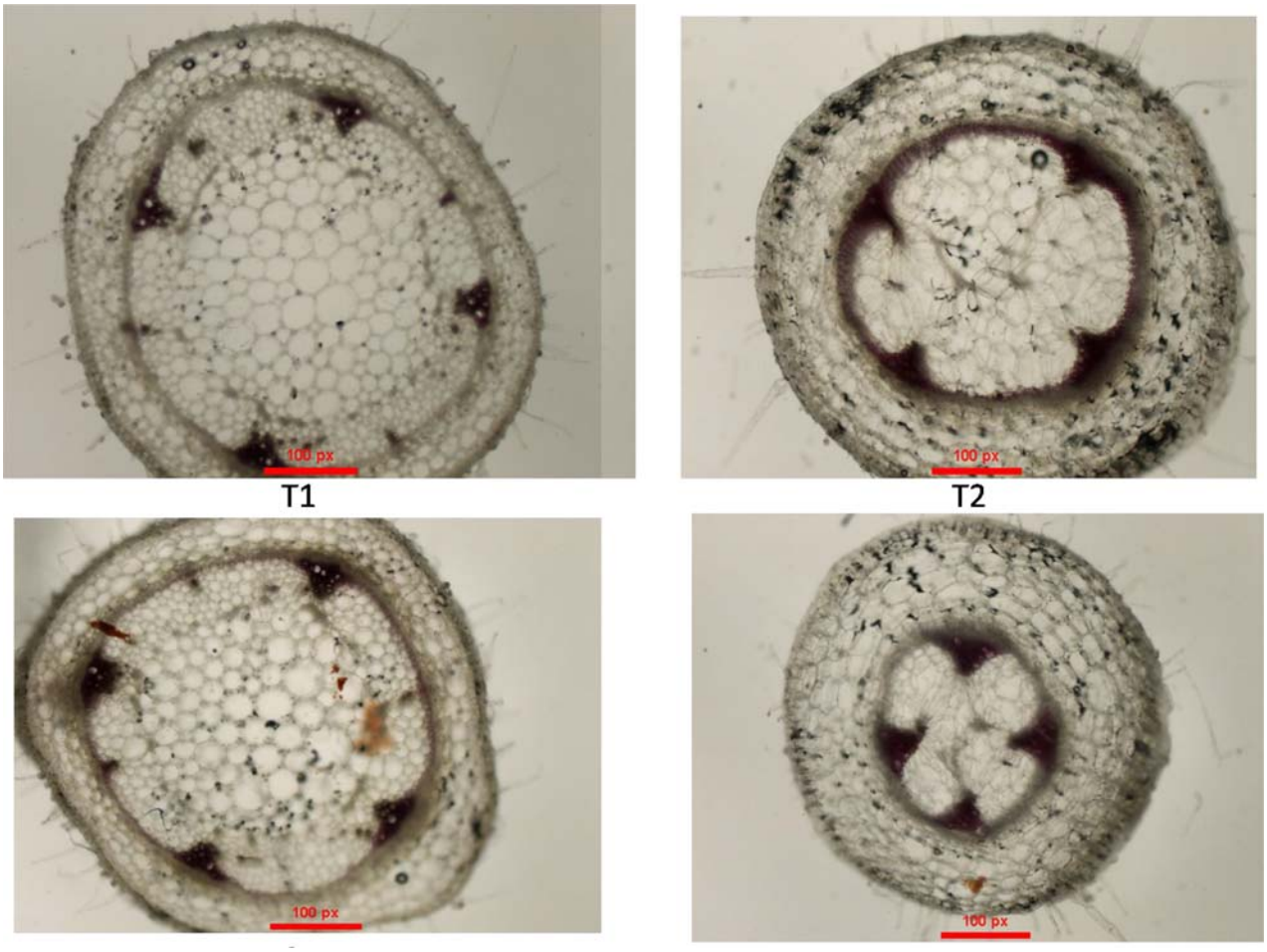


Figure 8. Influence of different treatments on lignification in tomato stem by histochemical staining. T1 (untreated and unstressed seeds), T2 (untreated seeds + heat shock), T3 (*T. koningii* treated and unstressed seeds) and T4 (*T. koningii* treated + heat shock).

stressed plant is due to its enhanced synthesis and decreased oxidation of proline. It is reported that enhanced amino acids, especially the proline content, stabilize subcellular structure and scavenge free radicals (Bohnert et al. 1995). In the present study also the maximum level of FPC was observed in untreated stressed condition, while the level of FPC significantly decreases in treated stress condition; it can be concluded as *Trichoderma* treatment lowers down the effect of high temperature stress by significantly reducing the stress perception at the membrane level of the cell and also by reducing ROS generation, thereby stabilizing cellular environment.

The production of different phenolic compounds, such as flavonoids and phenylpropanoids, is stimulated due to TS (Dixon and Paiva 1995). Treating the host by *Trichoderma* is accounted for an increase in the level of Phenyl Ammonia Lyase activity that acts as a chief enzyme of the phenylpropanoid pathway, thereby catalyzes the transformation, by deamination, of L-phenylalanine into trans-cinnamic acid, that is the primary mediator for the synthesis of phenolics (Levine et al. 1994). As a consequence of abiotic (high and low temperatures, UV-B light, wounding, etc.) stresses, an increase in PAL activity and accumulation of many phenolics were observed (Solecka and Kackperska 2003; Sgarbi et al. 2003). Wen et al. (2008) reported that an increase in PAL activity may be involved in enhancing high-temperature stress resistance. The above results thereby confirm that changes in the activities of PAL and other enzymes are involved in phenylpropanoid biosynthesis, and the accumulation of various phenolics could be an early step of plant response to stress. In another study, gene *Pal1* was found to be upregulated by *Trichoderma* which encodes for PAL (Shoresh et al. 2010). In the present study, analysis of leaf extracts shows a considerably higher activity of PAL under stressed condition in treated and control conditions, which thereby shows that *Trichoderma* enhances the plant defense response to high temperature by maintaining a higher PAL level.

PPO is a ubiquitous copper-containing enzyme which utilizes molecular oxygen to oxidize common orthodiphenelic compounds such as caffeic acid and catechol to their respective quinines (Constabel and Barbehenn 2008). A significant increase in the activity of PPO along with the temperature was reported by Rivero et al. (2001). The results observed in the present study are in agreement with the findings of various workers, the level of PPO was higher in stressed condition as compared to the unstressed condition, but interestingly the PPO level gets reduced in *Trichoderma* inoculated plants under TS condition.

A significant increase in total phenolic content at 35°C was reported by Rivero et al. (2001). Accumulation of phenolics in plant leaves in response to TS was caused by the activation of PAL (Nozolillo et al. 1990). Singh et al. (2003) observed that seed treatment with *T. viride* enhanced the TPC in chickpea plants. In the present study, there was a considerable increase in TPC in leaf extracts from treated and stressed condition as compared to untreated and stressed condition which clearly indicates the efficiency of

Trichoderma in elevating TS. It was also confirmed by histochemical staining of the transverse section of tomato stem, that with the increase in temperature an increase in the total phenol content is also observed, which can be directly correlated by lignin deposition in the vascular bundles of plant stem.

Lignin, a polymer of phenylpropanoid compound, is constitutively present in plants. However, its content and composition is known to change when plants are exposed to various stresses. An increase in lignification is often observed in response to biotic and abiotic stresses experienced by the plants and is considered among one of the mechanisms adopted by the plant as its defense response owing to its antimicrobial and nondegradable nature (Rogers and Campbel 2004). However, results in the present study showed variation in lignin deposition in tomato under the TS. This variation is attributed to the role of *T. koningii* in stimulating the lignification process in an enhanced level.

A decrease in the total protein content under heat stress condition was also reported, which may be due to protein denaturation and protein synthesis inhibition at higher temperatures, since higher temperature injury was often attributed to protein denaturation (Levitt 1980 Gulen and Eris 2004). The results of this study were consistent with those found by various workers; they also demonstrated that the protein content was significantly reduced under stress condition compared to unstressed condition, which is consistent with the findings of the this study. Reduction in total soluble protein content in mulberry leaf under heat stress was also reported by Chaitanya et al. (2001).

3,3′-diaminobenzidine (DAB) is a commonly used chemical for the detection of localized H_2O_2 *in planta* which is quite difficult owing to its inimitable property of being extremely metabolically active (Reth, 2002). In the presence of peroxidase, it polymerizes to produce a reddish-brown precipitate due to contact with H_2O_2 and thereby acting as a significant marker for the detection of peroxide accumulation (Dıaz-Vivancos et al. 2006). The use of DAB-mediated tissue printing for localization of peroxidase in plants was also reported by Spruce et al. (1987). The present study thus demonstrates that *T.koningii* treatment used in the study can act significantly for the enhancement of defense response in plants toward the TS by the induction of the defense-related biochemical changes.

Conclusion

An augmentation in the activity of the antioxidant enzymes, phenylpropanoids, sugar content, and proline content was observed in the stressed untreated plants in comparison with the stressed treated plants. Our data show that plants respond to TS and the accompanying oxidative stress by increasing the activity of antioxidant enzymes, which may prove deleterious to plant. This increased content in the tomato plants was observed to be more in stressed untreated plants as compared to stressed treated plants, wherein *T.koningii* played a pivotal role in alleviation in TS tolerance by regulating the production of ROS and protecting the plant cells from oxidative damage. These biochemical responses can be used as appropriate markers for assessing the efficacy of treatment in plants when grown under stressed condition. A relationship between changes in antioxidative activities and phenylpropanoid activities in the stressed plants and the degree of susceptibility to the heat stress studied is evident, which clearly indicates that the *T.konigii* treatment improves TS tolerance in plants by elevating the levels of cellular plant defense response. Thereby, it can be further said that the use of these beneficial microbes can not only increase the crop regime toward higher temperature but also help the farmers to sustain their crop and its yield during the high-temperature stress.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical statement

The work was done in compliance with the ethical standards.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

RT and CK performed the experiments and drafted the manuscript. CK and RT coordinated the work and provided valuable comments.

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