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Trichoderma mediated seed biopriming augments antioxidant and phenylpropanoid activities in tomato plant against *Sclerotium rolfsii*

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Abstract
Sclerotium rolfsii Sacc. is the most destructive soilborne fungal pathogen causing collar rot disease in tomato crop. For management of collar rot disease several chemical fungicides are using that cause hazardous impacts on agroecosystem as well as human health. In the present study, *Trichoderma* spp. are used for management of collar rot disease in tomato plants. Four *Trichoderma* strains namely T. pseudokoningii BHUR2, T. harzianum BHUP4, T. viride BHUV2, T. longibrachiatum BHUR5 were applied by seed biopriming method. Bioprimed seeds were sown in earthen pots filled with sterilized soil and S. rolfsii inoculation was given after 25 days of sowing. Tomato plants primed with *Trichoderma* pseudokoningii BHUR2 were found healthier as compared to other bioprimed plants upon challenged with S. rolfsii. T. pseudokoningii BHUR2 primed tomato plants augmented anti-oxidative enzymes activity including superoxide dismutase (SOD) and peroxidaes (POx), phenylalanine ammonia lyase (PAL) and total phenol content (TPC) in response to defense mechanism against S. rolfsii infection. The application of T. pseudokoningii BHUR2 is an ecofriendly approach to manage collar rot disease in tomato plants.

Keywords: *Trichoderma* pseudokoningii, *Sclerotium rolfsii*, Collar rot, Tomato, Seed biopriming, Antioxidant

1. Introduction
Vegetables play a major role in human dietary and provide various essential nutrient, carbohydrates, vitamins and amino acids. After China, India taking leads as second largest producer of vegetables and holds approximately 15% share of vegetable production (Srivastava *et al.*, 2010) [38]. Vegetable growers use a wide range of agrochemical like fungicides to protect their crops from the damage by various phytopathogens. Definitely these agrochemicals helps growers to improve their production but the major downside of these hazardous agrochemicals is that they can be accumulate in the plant system and during the course of consumption by humans it may create human health problems. These chemicals also disturb the soil health, soil microbial diversity and contaminate the soil. Tomato (*Solanum lycopersicum* L; Synonym: *Lycopersicum esculentum* Mill.) is one of the most widely grown vegetable crop which gain a tremendous popularity over the last century. It is cultivated in almost every part of the world and consumed as fresh or processed purposes (Naika *et al*., 2005) [26]. The crop area of tomato is continuously increasing and the consumption quantity also enhanced by 3% annually average rate (Abedin *et al*., 2018) [1]. It is second important vegetable crops in the world after potato with worldwide production reaching almost 210 million tons in 2017 (Mohamed *et al*., 2017). China, the leading producer of tomatoes, accounted for 31% of the total production after that India got lead as the second highest producer in the world (Costa and Heuvelink, 2018) [5]. Tomato production is affected by many fungal and bacterial diseases (Jones *et al.*, 2014) [19]. *Sclerotium rolfsii* Sacc. is the soilborne fungal pathogen which is causing collar rot or stem rot (Punja, 1985; De Curtis, 2010) [28, 6]. This disease causes approximately 30% crop loss in both field and greenhouse conditions and sometime under conducive condition it may be reaches up to 95% (Suriyagamon *et al*., 2018) [39]. Use of fungicides as seed treatment to protect the crop from disease is a normal practice, which has also found effective. But the harmful effects of these fungicides on agro-ecosystem generate a need of safer alternative method to protect plants. Thus, the utilization of different biological control agents (BCAs) for plant protection purpose begins and they have showed the potential for this disease management (Abhilash *et al.*, 2016; Singh, 2016) [2, 20]. Biocontrol agents also help plant system to cope up with abiotic stresses i.e. drought, salinity, heat etc and

improve their growth promoting attributes as well as nutritional status (Keswani *et al*., 2016a, 2016b; Singh *et al*., 2016) [20]. Biopriming of seeds with these beneficial microorganisms is a new approach of seed treatment in which seed hydration is combined with microorganism inoculation. The most frequently used microorganisms in biopriming include *Trichoderma* spp., *Pseudomonas flourescens*, *Bacillus* spp., *Glomus* spp., and *Agrobacterium radiobacter* etc. These microbes secrete a large number of chemically diverse antimicrobial secondary metabolites, volatile compounds and hydrolytic enzymes *viz.,* celluloses, lipases, chitinases, proteases etc., which help them to control pathogen growth (Shrivastava *et al*., 2010; Harman, 2011; Harman *et al*., 2012; Kumar *et al*., 2012). The application of these microbes in biopriming process showed better plant growth promotion and increase tolerance to biotic as well as abiotic stresses (Harman, 2004; Singh *et al*., 2013) [20]. In the present study, an *in vitro* experiment was conducted to evaluate the effect of *Trichoderma* mediated seed biopriming in tomato i) for protection against *Sclerotium rolfsii* ii) effect on plant growth parameters and; iii) effect on antioxidant and phenylpropanoid activities

2. Materials and Methods

2.1 Collection and isolation of pathogen S. rolfsii

Collar region tissue of infected tomato (*Solanum lycopersicum*) plants and resting structure sclerotia present nearby collar region were collected from Horticultural fields and Agricultural farms of Banaras Hindu University (B.H.U.), Varanasi, India (25.28 N, 82.95 E). Thereafter, sclerotia were surface sterilized by using 1% of sodium hypochlorite (NaOCl) for 3 min followed by three successive thorough rinses with sterilized distilled water (SDW) and put on sterilize blotter paper for removal of excess moisture. Sterilized sclerotia were placed on the petriplates contained potato dextrose agar (PDA) medium and incubated at 26±2˚C for five days.

2.2 Biocontrol agent *Trichoderma*

The *Trichoderma* strains namely BHUP4 (MH730446), BHUR2 (MH729058), BHUR5 (MH731276) and BHUV2 (MH729057) used in this study obtained from Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindus University, Varanasi, Uttar Pradesh, India which were previously isolated from different agro-ecosystems and stored at 4˚C.

2.3 Preparation of *Trichoderma* spore suspension

Trichoderma spores were taken from seven days old culture and harvested aseptically in phosphate buffer saline (PBS; 20mM sodium phosphate, 150mM NaCl, pH 7.4), filtered by using sterilized muslin cloth to prepare stock spore suspension. Thereafter, one ml of the stock spore suspension was mixed in 9 ml of saline and OD was observed at 600 nm using UV1 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Spore concentration of was also counted with the help of hemocytometer (Singh *et al*., 2016) [20]. The diluted suspension was at 10000 rpm for 10 min and supernatant was replaced by equal amount of 1.0% of sterilized CMC (carboxymethyl cellulose) solution. The suspension was vortexed to obtain a homogeneous solution for seed biopriming.

2.4 Seed biopriming with prepared spore suspension

Tomato seeds (cv. Kashi amrit) were surface sterilized by 1% NaOCl solution for 1 min, followed by rinse with SDW thrice and dried under a sterile air stream on pre-sterilize blotting paper (Jain *et al*., 2012) [16]. The dried seeds were put on the sterilize petriplates and soaked through prepared spore suspension, while only CMC treated seeds serves as control. The excess amount of suspension was drained out and treated seeds kept in incubator at 28±2˚C with maintain 98% relative humidity for 24 h. (Singh *et al*., 2013; Jain *et al*., 2015) [20, 18].

2.5 Pot trial

Two sets of each treatment with three replications were maintained in greenhouse conditions for evaluating the performance of *Trichoderma* spore suspension in protection of plants from disease as well as plant growth promotion, antioxidant and phenylpropanoid activities. Treatments which were used in this study describe in table 1. All these experiments were conducted in greenhouse of Department of mycology and Plant Pathology, Banaras Hindu University, Varanasi, Uttar Pradesh, India (25˚15’ 59” N, 82˚59’30”E).

Table 1: Treatments used in the experiment

Treat-ment	Description (all treatment challenged with pathogen *S. rolfsii)*
T_1	Non-primed
T_2	Seed biopriming with *Trichoderma* BHUR2 spore suspension
T_3	Seed biopriming with *Trichoderma* BHUR5 spore suspension
T_4	Seed biopriming with *Trichoderma* BHUP4 spore suspension
T_5	Seed biopriming with *Trichoderma* BHUV2 spore suspension

The experiment was conducted in earthen pots (3 litre). Potting mixture consisted of sand and soil (1:1 ratio) was filled in the autoclavable polypropylene bags and sterilized for three consecutive days at 15lb pressure for 30 min in an autoclave. Ten air dried bioprimed seed were sown in each pot at an approximately depth of 1.5 cm. After 25 days of sowing (DAS), plants were challenged with the pathogen *S. rolfsii*. The pathogen *S. rolfsii* was mass cultured on corn meal-sand media (Corn seed 250g, washed send 750g, distilled water 250ml) at 26±2˚C for 15 days. The colonized culture of pathogen was blended for homogenization of inoculum before application in pots. Prepared inoculum (active fungal mycelium and sclerotia) was inoculated at the rate of 20g per pot on the top soil of the pots and covered with sterilized potting mixture. Irrigation was done manually in every alternate day to maintain adequate moisture level in the pots.

2.6 Assessment of mortality

Mortality observations were taken after 15 days of pathogen inoculation (DPI) as per method Shokes *et al*., (1996) [32]. During the course of infection, the collar region of plant gets flaccidity which indicates disease initiation. The disease severity was estimated on the basis of individual plants scoring on a 0-5 visual scale of increasing severity (Latunde-Dada, 1993) [24] and the percent disease severity was calculated according to formula Erkilic *et al*. (2006) [8].

$$\%DS = \frac{\Sigma(n \times v)}{N \times V} \ 100$$

Where; n = score of infection according to scale, v = number of seedlings per category, N = total number of seedlings were screened, V = highest score for infection

2.7 Assessment of plant growth parameters

Five random plant samples were uprooted after 25 days of inoculation (DAI) and washed thoroughly by placing them on sieve (1mm diameter) under running tap water to remove adhering soil particles from roots. Thereafter, plants were subjected for drying by placing them on two layers of blotting papers. Fresh weight (FW) of shoot and root were taken and then oven dried at 80˚C for determined dry weight (DW). Shoot length (SL), root length (RL), number of lateral roots (LR) were estimated by using Image J software (Schneider *et al*., 2012) [30].

2.8 Estimation of anti-oxidative enzyme activities

Leaves of tomato plants from all the treatments were collected randomly at 24, 48, 72 and 96 h after pathogen inoculation and washed separately by placing them on sieve (1mm dia) under running tap water and used for further estimation of anti-oxidative enzyme activities.

2.8.1 Superoxide dismutase (SOD) activity

Superoxide dismutase activity was estimated by using Fridovich method (1974). 0.1g of leaf sample was homogenized with extraction buffer (2.0 ml; 0.1M phosphate buffer containing 0.5 mM EDTA at pH 7.5) in a pre-chilled mortar and pestle. The homogenate was centrifuged at 15,000 rpm for 20 min at 4˚C. The reaction mixture was consisted of 200 mM methionine 2.25 mM nitroblue tetrzolium chloride (NBT), 3 mM EDTA, 100 mM phosphate buffer (pH 7.8), 1.5 sodium carbonate enzyme extract (200 µl) and the final volume was maintained to 3 ml. Add 2 M riboflavin (0.4 ml) for initiation of reaction and placed the tubes under the light of two 18W fluorescent lamp for 15 min. After 10 min, termination of reaction was done terminated by switching off the light and the tubes were kept in dark. For control, a complete reaction mixture was taken without enzyme extract. The optical density (O.D.) was recorded at 560 nm using UV1 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.8.2 Peroxidase (POx) activity

Peroxidaes activity was estimated spectrophotically with slight modification in Hammerschmidt method (Hammerschmidt *et al*., 1982) [10]. 0.1 g of leaf tissue from each treatment was taken and homogenized in ice cold 0.1 M phosphate buffer (5.0 ml; pH 7.0) at 4˚C. Homogenate was centrifuged at 16,000 rpm at 4˚C for 15 min, pellet was discarded and supernatant used as enzyme extract. The reaction mixture was contained 0.05 M pyrogallo (1.5 ml), enzyme extract (50 µl) and 1% H_2O_2 (0.5 ml). The absorbance was recorded at 30s interval for 3min in 420 nm. The enzymatic activity was expressed as U min/g fresh weight.

2.9 Assessment of phenol content in plant

2.9.1 Assessment of phenylalanine ammonia lyase (PAL) in plant

Phenylalanine ammonia lyase (PAL) activity was performed by using Brueske (1980) [4] method. Leaf samples (1.0 g) from each treatment were taken and homogenized in ice cold 0.1M sodium borate buffer (5.0 ml; pH 7.0) with adding 1.4 mM mercaptoethanol followed by centrifuge at 16,000 rpm at 4˚C for 15 min. Thereafter, 0.2 ml of supernatant was taken in test tube as enzyme extract and add 0.5 ml of borate buffer (pH 8.7), 1.3 ml of distilled water to complete the reaction mixture. For initiate the reaction, 0.5 ml of l-phenylalanine (0.1mM, pH 8.7) was added in each tube and incubates at 32˚C for 30 min. The reaction was terminated by 0.5 ml of trichloroacetic acid (1 M). The absorbance was recorded at 290 nm and PAL activity was measured in terms of amount of t-cinnamic acid (t-CA) formed. The activity was expressed as M TCA g per fresh weight.

2.9.2 Assessment of total phenol content (TPC) in plant

Total phenol content was determined by Zheng and Shetty (2000) method. Leaf samples (0.1 g) were taken and placed in to a glass vials (10 ml size) which contain of 5ml of 95% ethanol. For maximum extraction vials was stored at 0˚C for 48 hours. Thereafter, sample was homogenized and centrifuged at 15,000 rpm for 10 min. 1 ml of the supernatant was taken out into a new glass vial and mix with 1 ml of 95% ethanol and 5 ml of SDW. Thereafter, 0.5 ml of 1 N Folin-Ciocalteau reagent was added and after 5 min 1 ml of 5% Na_2CO_3 was added and incubate at room temperature for 60 min. The absorbance was recorded at 725 nm. Standard curve prepared by using various concentration of gallic acid (GA) in 95% ethanol. Absorbance values were converted to mM Gallic acid equivalent (GAE) g per fresh weight.

2.10 Statistical analysis

Experiments were repeated once using a completely randomized design. The data are expressed as the mean of three independent replications ± standard deviations. All means were compared by DMRT ($P \leq 0.05$), using SPSS version 16.

3. Results & Discussion

3.1 Assessment of mortality

Three days after challenge inoculation of tomato plants with *S. rolfsii*, water soaked lesions had developed on the collar region in the control plants. All the bioprimed treatments showed a significant reduction in disease severity over the non-primed pathogen challenged control (Fig. 1a). Lowest disease severity was recorded in T2 (10.08%) followed by T4 (13.38%), T5 (16.56%) in respect of non-primed pathogen challenged control. It has been reported that *Trichoderma* spp. are potential BCA for the management of *S. rolfsii* (Elad *et al*., 1980; Papavizas, 1983; Tsahouridou and Thanassoulopoulos, 2002) [7, 27, 40] by its ability to produces various enzymes and secondary metabolites that affect the growth of pathogen. *Trichoderma* also uses some other mechanism like antibiosis, mycoparasitism and competition to colonize with *S. rolfsii* sclerotia and mycelium (Harman *et al*., 2006; Singh and Singh, 2012; Kumar *et al*., 2012) [20].

3.2 Assessment of plant growth promoting traits

Biopriming of tomato seeds with different *Trichoderma* strains showed enhancement in all the plant growth promoting traits in comparison to non-primed pathogen challenged control at 25 days of inoculation. The germination of tomato seeds were highest in T2 followed by T4, T5 where 1.24, 1.15 and 1.11 fold increase recorded in seed germination respectively, in respect of non-primed pathogen challenged control (Fig. 1b). All the plants primed with *Trichoderma* spore suspension showed increment in their shoot and root

length at 25 DAI in comparison of untreated pathogen control plants. At 25 DAI, shoot length and root length were found maximum in T2 which was about 2.88 and 3.16 fold increased respectively as compared to the non-primed control plants (Fig. 2). Similarly, fresh and dry weight of shoot and root were enhanced in all bioprimed treatment in comparison to non-primed control plants (Fig. 3, Fig. 4). The fresh and dry weight of shoot increased by 3.88 and 5.87 fold respectively in T2 as compared to control plants. The root fresh and dry weight found significantly higher in T2 (4.07, 12.21), followed by T4 (3.27, 7.44) and T5 (2.54, 4.67) fold as compared to control plants. The increment in lateral root numbers was observed in T2 with an increase of 2.39 fold followed by T4 (2.12 fold), T5 (1.85 fold) as compared to non-primed pathogen challenged control (Fig. 5). Various reports showed that *Trichoderma* have a stimulatory effect on plant growth due to the increment in plant nutrient use efficiency and modification of the soil conditions (Harman, 2000; Jain *et al*., 2012) [16]. It also produces various secondary metabolites, volatiles and plant hormones which promote the plant shoot and root growth that ultimately enhances the photosynthetic efficiency (Benítez *et al*., 2004; Singh *et al*., 2017; Kurrey *et al*., 2018) [3, 15, 23]. Colonization of *Trichoderma* strains in roots and soil system helps in solubilization of minerals such as Mn, Fe, Cu, Zn and rock phosphate (Yedidia *et al*., 2000; Singh *et al*., 2013) [42, 17]

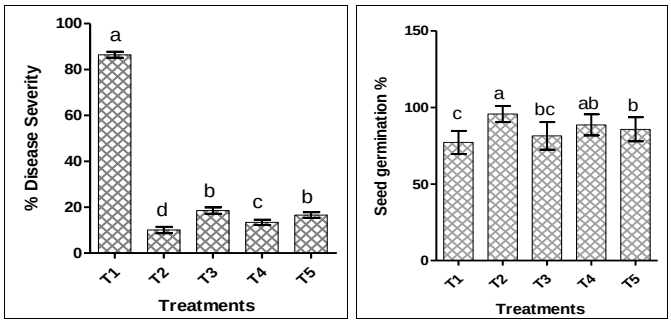

Fig 1: Effect of *Trichoderma* spp. seed biopriming on a) disease severity and b) seed germination. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

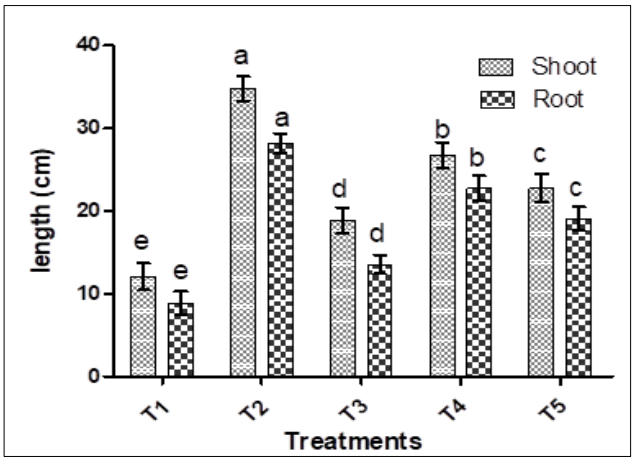

Fig 2: Effect of *Trichoderma* spp. biopriming on shoot and root length. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

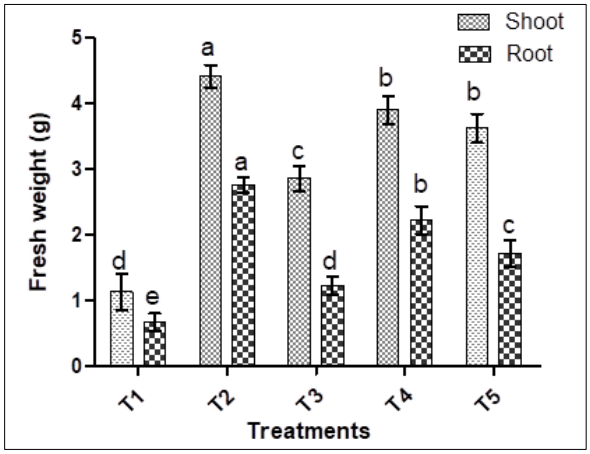

Fig 3: Effect of *Trichoderma* spp. biopriming on shoot and root fresh weight. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

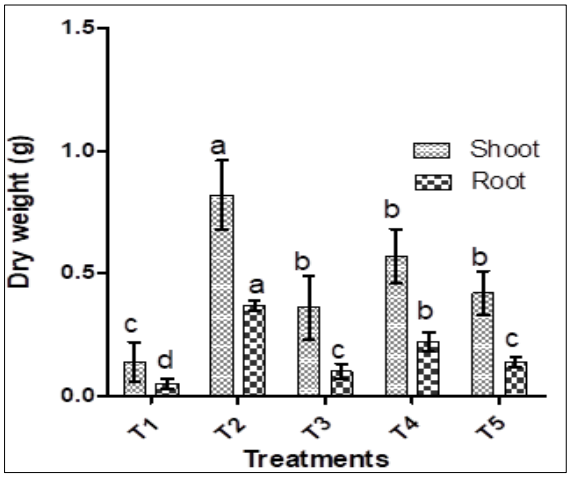

Fig 4: Effect of *Trichoderma* spp. biopriming on shoot and root dry weight. Results are expressed as means of three replicates and vertical bars indicate standard deviations of. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

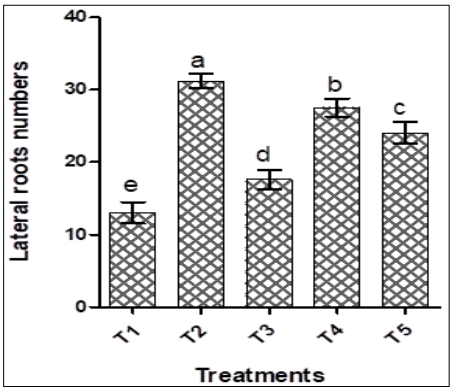

Fig. 5 Effect of *Trichoderma* spp. biopriming on lateral root number. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

3.3 Estimation of anti-oxidative enzyme activities

SOD and POx activities consistently increased and reached higher level at 72h in all primed plants. After inoculation of pathogen, an increase was observed in POx at 48 h in T2

(2.06 fold) followed by T4 (1.76 fold) and T5 (1.52 fold) as compared to pathogen challenged control (Fig. 6). The POx activity was recorded to be significantly higher in T2 when compared with other treatments. A gradual declination in POx activity was observed in all treatments at 96 h while non-primed pathogen challenged plants showed a sharp declination in POx activity at 72 h. Similarly, SOD activity was found higher in T2 with 3.37 fold increase at 72 h as compared to non-primed pathogen control plants (Fig. 7). The enzymes POx and SOD work together with other enzymes of the ascrobates-glutathione cycle to promote scavenging of free radicals (Hemandez *et al*., 2001) and have been reported to induce with microbial inoculation in plants (Vaishnav *et al*. 2013; Jain *et al*. 2013) [17].

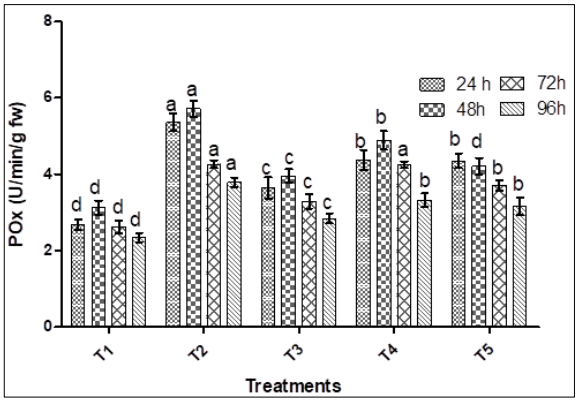

Fig. 6 Effect of *Trichoderma* spp. biopriming on POx activity at different time interval. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

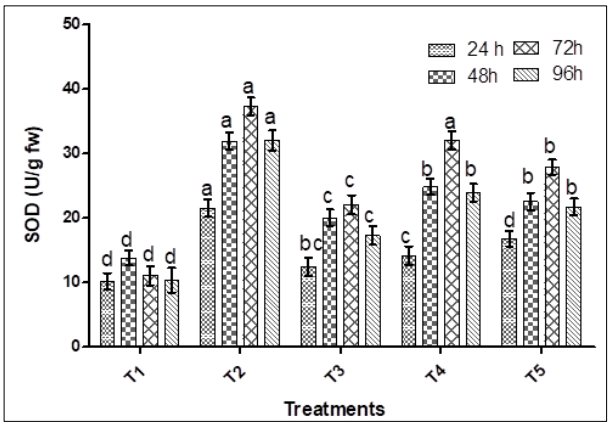

Fig. 7 Effect of *Trichoderma* spp. biopriming on SOD activity at different time interval. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

3.4 Assessment of phenol content in plant

PAL level was increased significantly in all treatments after 24h of pathogen challenge and thereafter the activity started decline. The PAL activity in T2 was 2.98 fold higher than the T1 plants at 72 h (Fig. 8). Similarly, TPC was detected increased in all the treatments at 48 h compared to non-primed pathogen challenged (Fig. 9). The highest TPC was observed in T2 (4.01 fold) followed by T4 (3.23 fold) and T5 (1.99 fold). PAL activity helps in rapid reorganization of phytopathogen which ultimately potentiates the accumulation of disease resistance factors like phenols, lignin and phytoalexine. Plants phenolics are the natural products that formed due to activation of phenylpropanoid pathway that ultimately play role in plant growth promoting microbes mediated induced systemic response (Sarma *et al*., 2002; Mandal and Mitra, 2007) [25].

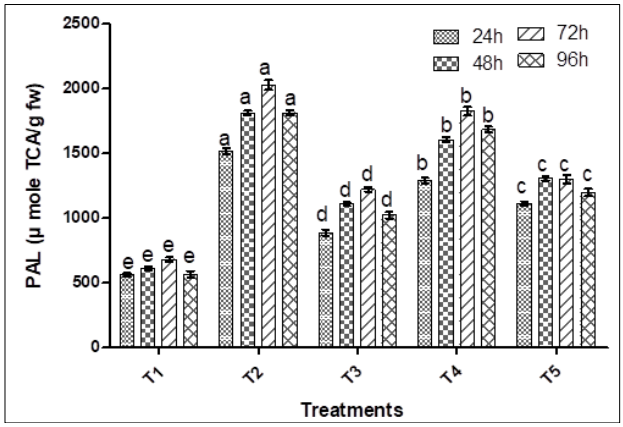

Fig 8: Effect of *Trichoderma* spp. biopriming on PAL activity at different time interval. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$.

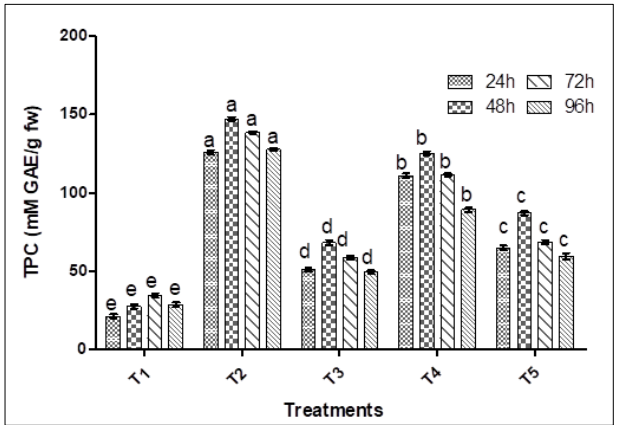

Fig 9: Effect of *Trichoderma* spp. biopriming on TPC at different time interval. Results are expressed as means of three replicates and vertical bars indicate standard deviations. Letters indicate significant differences among treatments according to Duncan's multiple range test at $P \leq 0.05$

4. Conclusion

The use of biocontrol agent for management of *S. rolfsii* disease is a better and safer way rather than the use of chemicals that pollute our ecosystem and affect the human health. As our results suggested that the *Trichoderma pseudokoningii* BHUR2 is most effective strain to promote tomato plant growth under *S. rolfsii* challenged condition, this is strain may be commercialize as potential biocontrol agent only after a large filed trials.

5. Acknowledgments

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