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Supprssion of Fusarium wilt in chickpea by biofortified vermicompost

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Abstract

Chickpea (*Cicer arietinum*) is a widely cultivated and versatile fruit. Chickpea production faces various constraints, both biotic and abiotic stresses. Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *ciceri*, is a devastating disease that affects chickpea plants worldwide, causing significant economic losses in chickpea cultivation. Inthis study the potential use of biofortified vermicompostfor wilt management was investigated. The biological control agents *viz*. *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Bacillus subtilis* were used to fortified the vermicpompost. Data revealed that the Chickpea plants treated with vermicompost fortified with *Trichoderma* showed maximum root length (14.95 cm) after 15 days of sowing followed by T-2 (11.25 cm) and T-3 (9.85 cm). Maximum dry weight was observed in plants treated with vermicompost fortified with *Trichoderma*. After 15 DAS, 3.15 g dry weight was in T-1 followed by T-2 and T3. PAL levels increased significantly in all treatments up to 48 h, followed by a decline in its activity. Maximum PAL activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 48 h followed by T2, T3 and T4. At 48 h T1 showed 3.6 fold increases in PAL activity in comparison to control. PO levels increased significantly in all treatments up to 72 h, followed by a decline in its activity. Maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 72 h. Maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 72 h followed by T2, T3 and T4.

Keywords: - *Biofortified Vermicompost, Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis*

Introduction

Chickpea, scientifically known as *Cicer arietinum*, is a versatile legume that has been cultivated for thousands of years and holds a significant place in global agriculture and culinary traditions. Belonging to the Fabaceae family, chickpeas are commonly consumed in various forms across different cuisines worldwide, from salads and soups to spreads and snacks. Renowned for their nutty flavor and dense nutritional profile, chickpeas are not only a staple food but also hold immense importance in terms of health, sustainability, and cultural heritage. Chickpeas are a rich source of plant-based protein, dietary fiber, vitamins, and minerals. They provide essential nutrients such as iron, folate, phosphorus, and manganese,

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contributing to overall health and well-being. Their nutritional density makes them a valuable component of vegetarian and vegan diets, aiding in meeting daily nutrient requirements.

Incorporating chickpeas into the diet offers numerous health benefits. The high fiber content aids in digestion, promotes satiety, and helps regulate blood sugar levels, reducing the risk of diabetes and cardiovascular diseases. Additionally, chickpeas contain antioxidants and anti-inflammatory compounds that may help combat oxidative stress and inflammation in the body, potentially lowering the risk of chronic diseases like cancer and arthritis. Chickpeas play a vital role in sustainable agriculture. As legumes, they have the unique ability to fix atmospheric nitrogen through symbiotic relationships with soil bacteria, enriching the soil and reducing the need for synthetic fertilizers. Their cultivation also contributes to crop rotation strategies, improving soil health and reducing pest and disease pressures in agroecosystems.

Chickpea production faces various constraints, both biotic and abiotic stresses. Among abiotic stresses temperature, draught, soil condition, nutritional deficiency and imbalance are very significant. Chickpea plants are also attacked in both field and nursery by various plant pathogenic microorganism and insects. Among the biotic stresses fusarium wilt, dry root rot, Aschochyta blight, collor rot, cause considerable losses in chickpea. Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *ciceri*, is a devastating disease that affects chickpea plants worldwide, causing significant economic losses in chickpea cultivation (Srinivas *et al.*, 2019). It colonizes the vascular system of the plant, obstructing water and nutrient flow and leading to wilting and eventual death of the plant. The fungus enters the plant through the roots and spreads via the xylem vessels, clogging them with fungal mycelium, which restricts water uptake. It produces spores that persist in the soil, contributing to its long-term presence in affected areas (Basco*et al.*, 2017). Symptoms of Fusarium wilt typically start with wilting and yellowing of lower leaves, progressing upwards. As the disease advances, the entire plant wilts, leaves turn yellow or brown, and the plant eventually dies. Browning of vascular tissues in the stem may be visible upon close inspection. Fusarium wilt can lead to significant yield losses, especially in regions where susceptible chickpea cultivars are grown continuously or where the pathogen is prevalent in the soil.

Integrated disease management, involving a combination of resistant cultivars, cultural practices, biological control, and soil management techniques, offers the most effective and sustainable approach in managing Fusarium wilt. Given the persistent nature of Fusarium wilt and its ability to survive in soil for extended periods, a holistic approach that combines various strategies tailored to the specific conditions of each farming system is crucial for effective management of this destructive disease in chickpea crops.

Plant diseases pose significant challenges to global agriculture, affecting crop productivity and food security. Traditional methods of disease management often involve the use of synthetic chemicals, which can have adverse effects on the environment and human

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health. In recent years, vermicompost has emerged as a promising eco-friendly alternative for plant disease management (Yatoo *el al*., 2021).

Materials and methods

Experimental site

The *in vitro* experiments were conducted in the Laboratory of Faculty of Agricultural Sciences and Allied Industries, Rama University, Kanpur, India. The *in vivo* experiments were carried out in the polyhouse and agricultural field of the same department, where chickpea crop was raised in pots (15 x 10 cm) and field and all physical precautions were kept in view in order to protect the crop from the external damage. The site of the experiment was unaltered during experimentation period.

Collection of diseased samples from different districts of Uttar Pradesh

Survey for incidence of fusarium wilt from different zones of Eastern Uttar Pradesh and collection of diseased samples

Random method of survey was carried out to record the severity of wilt in chickpea. The survey was conducted during 2022-23 in five districts of Uttar Pradesh *i.e* Kanpur, Prayagraj, Lucknow, Unnao and Etawah. The observations on stage of crop and disease severity were recorded on the rating scale of (1-9). Chickpea plants showing typical wilt symptoms were collected separately in paper bags and brought to the laboratory for isolation of associated pathogen and further investigations.

Isolation of the pathogen

The pathogen was isolated on PDA medium from infected root of chickpea plant. A small portion of diseased tissue along with a portion of adjacent healthy tissue were cut into small pieces (3 to 5 mm in length) and then surface sterilized with 0.1% $HgCl_2$ for 30 sec. The pieces were then rinsed thrice with sterilized distilled water. Sterilized and rinsed pieces were inoculated aseptically on sterilized Petriplates containing PDA medium. The inoculated Petriplates were incubated at 20- 25 ^{0}C for 5-6 days. When the fungal colony developed, a small cut was made on mycelium with cork borer and was transferred on another Petriplate containing PDA medium to obtain pure culture. The mycelial bit was also transferred to fresh PDA slants in order to store it for future use.

Maintenance and storing of the pathogen

The pure culture of the pathogen *F. oxysporum* was maintained on PDA slants throughout the period of investigation by periodic sub culturing on fresh media and stored in a refrigerator at 4 ^{0}C.

Pathogenecity test

Chickpea seeds were surface sterilized by using 1% sodium hypochlorite for 30 sec and was rinsed twice with sterilized distilled water and then air-dried. Soil mixture containing sandy loam soil, and farmyard manure (2:1) was autoclaved for 30 min at 15 lbs pressure for three consecutive days. Half amount of soil was also mixed with crushed mycelial powder of *F. oxysporum*. The seeds were sown in 15 × 10 cm^2 pots under greenhouse conditions.

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Untreated seeds sown in pathogen infected and pathogen uninfected soil served as positive and negative controls, respectively.

Preparation of Vermicompost

Temple, farmyard and kitchen wastes were used as feedstock in the present study. The temple wastes mainly consisted of *Aegle marmelos* leaves, *Datura stramonium*, *Tagetes erecta* and *Hibiscus rosasinensis* flowers. The offerings were collected from different temples in the city but the bulk from two temples namely ''ISKON'' and ''JK Temple'' which receive most of the devotees. The kitchen wastes were collected from the cafeteria of hostels in the Rama University which had major share of fruit and vegetable peels (60–75%) and the remainder consisted of used tea leaves, flour, rice, bread, noodles, cooked vegetables and potatoes. Flesh, bones, fat, egg shells, etc. were not included in the kitchen waste as they are not easily degradable and can be toxic to earthworms. The yard wastes mainly consisted of dried deciduous leaves (a mixture of *Mangifera indica*, *Saraka asoka*, *Syzygium cumini*, *Tamarindus indica* and various grasses). Six opaque, rectangular plastic boxes with dimensions measuring 340 cm × 160 cm × 60 cm, were used for vermicomposting and 45 holes, each of diameter 0.65 cm, were drilled at the base of the container for allowing proper exchange of gases. The experiment was set up in a randomized complete block design with three replications of each type of feedstock. Each feed stocks were kept for 2–3 days and thoroughly mixed before placing in the plastic boxes to avoid clumping and compaction of the substrates following addition of water in each of the three feedstock, mature cow dung was added at a ratio of about 1:7 to provide an instant source of food to the earthworms. Finally the boxes were covered with a layer of soil for decomposition. Adult *clitellate* worms, *Eisenia fetida*, ranging in length from 4 to 8 cm were added at the rate of 1.5 kg/m^2 through the developed cracks after 15 days of partial decomposition of waste to prevent worms from the thermophilic reaction occurring during composting. The moisture content of the feedstock was adjusted to 70 ± 10% at the start of vermicomposting and maintained throughout the period of vermicomposting by periodic sprinkling of water. Watering was stopped when the VC was ready as indicated by uniform dark brown to black coloured granular structure. Three days later the compost along with worms was harvested and the worms were removed by sieving (≤2 mm) (Singh *et al*., 2013).

Biological management of fusarium wilt of chickpea using biofortified vermicompost

Source of BCAs used and viability test

The biological control agents used in this study *viz*. *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Bacillus subtilis* were obtained from the culture repository of Plant Health Clinic Laboratory of the Faculty of Agricultural Sciences and Allied Industries, Rama University, Kanpur, India.

***In vitro* Efficacy of BCAs against pathogen**

The antagonistic ability of selected BCAs against the pathogen was studied *in vitro* following a dual culture assay as described by Verma *et al*. (2007). A nine mm disc (plug) of 15 days old cultures of *F. oxysporum* f. sp. *ciceri* were cut with a sharp cork borer from the growing edge of the culture plate. The cut block was placed on PDA medium 1 cm away from the edge of the plate. 9 mm disc of biocontrol agent namely *T. harzianum* isolate was

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placed at opposite end of the Petri plate. PDA plates inoculated with the pathogen alone served as the control and incubated at 25±2°C.

Similarly, the *in vitro* antagonistic ability of the bacterial isolates was studied using a dual culture assay described by Azadeh *et al*. (2010). A 9 mm plug of the *F. oxysporum* f. sp. *ciceri* was placed at the centre of a Petri plate containing PDA, then the test bacterial isolate was streaked 3 cm away from the fungal plug at both the sides towards edge of the plate by a loop loaded with 48 h old bacterial culture. The plates were incubated at 28± 2^0C for 7 days and the inhibition zone was measured from the edge of mycelium to the bacterial streaks, when the control plates showed full growth (Shanmugam *et al*., 2011). Per cent inhibition over control was calculated as per the following formulae given by Whipps (1997):

$$PI = \frac{C - T}{C} \times 100$$

Where, PI = per cent inhibition over control C = Growth of test pathogen with absence of antagonist (mm). T = Growth of test pathogen with antagonist (mm)

Microbial fortification of vermicompost

The three BCAs *viz*. *T. harzianum*, *P. fluorescens* and *B. subtilis* used in this study were chosen because of their compatibility and ascertained ability to reduce the soilborne diseases in various crops (Singh *et al*., 2013). All these selected BCAs were used to fortify the vermicompost individually. 1L of 2 days old bacterial cultures grown in NB with CFU count approximately 2×10^8 was thoroughly mixed with 25 kg of freshly prepared vermicompost in separate trays while 1L of 5 days old *T. harzianum* culture grown in PDB with CFU count approximately 4×10^7 was used to fortify other separate vermicompost tray (25 kg each). Trays were kept under shade and covered with dark polythene sheet for 10 days for acclimatization of BCAs.

Experimental details

Chickpea seeds were surface sterilized by using 1% sodium hypochlorite for 30 sec and was rinsed twice with sterilized distilled water and then air-dried.

Table1 Combinationof treatmentsused for conducting experiment

Treatment No.	Treatment
T_1	Vermicompost +*Trichoderma harzianum*+ Pathogen
T_2	Vermicompost + *Bacillus subtilis* + Pathogen
T_3	Vermicompost + *Pseudomonas flourescens* + Pathogen
T_4	Vermicompost + Pathogen
T_5	Control (Only vermicompost)

Pot experiments

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Plastic pots of 15 cm ×10 cm were used to conduct the plant growth promotion and antagonistic potentials of fortified vermicompost against *F. oxysporum* f. sp. *ciceri*. Soil was autoclaved for 30 min at 15 psi for three consecutive days. Pots were filled with soil mixture containing sterile soil and microbially fortified vermicompost in the ratio of 1:1 (w/w) (1.5 kg pot^{-1}). In the first three treatments, vermicompost was fortified individually with *T. harzianum*, *B. subtilis*, and *P. fluorescens* cultures as described above. Fourth treatment contained only vermicompost (positive control), while the fifth treatment contained only soil (Negative control).

Pathogen inoculation

The spore suspension of inoculum was prepared by pouring 20 ml of sterile distilled water in each culture plate of 5-7 days old fungal mycelium and then gently scraped using spore harvester. The concentration of conidia was adjusted to $2\text{-}3\times10^{7}$ conidia ml^{-1} using haemocytometer.5 ml of prepared spore suspension was used to inoculate each seedling in all five treatments using soil drenching method. Before inoculation, the roots were slightly severed (wounded) by inserting a needle, 1 cm away from the stem. Root severing was done to ensure pathogen penetration through roots. Observations were recorded on wilt symptoms for up to 5 weeks.

Biochemical Analysis

Biochemical analysis for determination of different antioxidants and ROS (H_2O_2) in the leaves of chickpea plants at different time intervals after pathogen inoculation was performed according to the method of Singh *et al*. (2013). The enzymatic assays namely phenylalanine ammonia-lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), superoxide dismutase (SOD) and total phenol content (TPC) was performed after 0, 24, 48, 72 and 96 h pathogen inoculation as described by Jain *et al*. (2012).

Superoxide dismutase (SOD) assay

SOD (EC 1.15.1.1) activity was assayed following the method of Fridovich (1974) by measuring the ability of enzyme extract from samples to inhibit photochemical reduction of nitroblue tetrazolium (NBT) chloride. Fresh leaves (0.1 g) from each of the treatments were homogenized in 2.0 ml of extraction buffer (0.1 mol l^{-1} phosphate buffer containing 0.5 mmol l^{-1} EDTA at pH 7.5) in a prechilled mortar and pestle. The homogenate was centrifuged at 15 000 g for 20 min at 4 ºC. The reaction mixture contained 200 mmol l^{-1} methionine, 2.25 mmol L^{-1} NBT, 3 $mmol^{-1}$ L EDTA, 100 mmol L^{-1} phosphate buffer (pH 7.8), 1.5 mol L^{-1} sodium carbonate and enzyme extract. The final volume was maintained to 3 ml. Reaction was started by adding 2 l mol L^{-1} riboflavin (0.4 ml), and the tubes were illuminated with two 15 W fluorescent lamps for 15 min. Reaction mixture without enzyme served as control. The reaction was terminated by putting the light off and keeping the tubes in dark until the absorbance was recorded at 560 nm. One unit of the SOD activity was defined as the amount of enzyme reducing the absorbance to 50% in comparison to control lacking enzyme.

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Phenylalanine ammonia-lyase (PAL) assay

Leaf sample of 0.1 g from each of the treatments was homogenized in 2 ml of (0.1 mol l^{-1} sodium borate buffer (pH 7.0; 4 ºC) containing 1.4 m mol L^{-1} ß-mercaptoethanol and centrifuged at 16000 rpm at 4 ºC for 15 min. The supernatant was used as enzyme source. To the reaction mixture containing 0.2 ml of enzyme extract, 0.5ml of 0.2 mol L^{-1} borate buffer (pH 8.7) and 1.3 ml of water were added. The reaction was initiated by the addition of 1ml of 0.1 mol L^{-1} phenylalanine (pH-8.7) and incubated for 30 min at 32 ºC. The reaction was terminated by addition of 0.5 ml of trichloroacetic acid (TCA, 1 M). PAL (EC 4.1.3.5) activity was measured following the formation of trans-cinnamic acid at 290 nm as described by Brueske (1980) and was expressed in terms of µmol L^{-1} TCA per g fresh weight (FW).

Total phenolic content (TPC) assay

The TPC 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 ºC for 48 h. The samples were homogenized individually and centrifuged at 13000 rpm for 10 min. To 1 ml of the supernatant, 1 ml of 95% ethanol and 5 ml of sterile distilled water and 0.5ml of 50% Folin–Ciocalteau regent were added, and the content was mixed thoroughly. After 5 min, 1 ml of 5% sodium carbonate was added, the reaction mixture was allowed to stand for 1 h and the absorbance of the colour developed was recorded at 725 nm. Standard curves were prepared for each assay using various concentrations of gallic acid (GA; Sigma-Aldrich-27645) in 95% ethanol. Absorbance values were converted to mg GA equivalents (GAE) g^{-1} FW.

Polyphenol oxidase (PPO) assay

Leaf samples (0.1 g) were homogenized with 2 ml ice cold phosphate buffer (0.1 mol l^{-1}, pH 6.5). The homogenate was centrifuged at 16000 rpmfor 30 min at 40^{C} and the resulting supernatant thus obtained was used directly in the enzyme assay. The reaction mixture contained 0.4 ml catechol (1m mol L^{-1}) in 3 ml sodium phosphate buffer (0.05 mol L-1; pH 6.5) and 0.4 ml enzyme extract. Reaction mixture containing only substrate served as control. Catechol was used as substrate for PPO (EC 1.14.18.1) and increase in absorbance was recorded at 405 nm (Gauillard *et al.*,1993). The linear portion of the activity curve was used to express PPO enzyme activity as change in O.D. min^{-1} g^{-1} FW.

Peroxidase (PO) assay

PO (EC 1.11.1.7) activity was assayed by the method of Hammerschmidt *et al.* (1982), with slight modification. Leaf samples (0.1 g) were homogenized separately in 2 ml of 0.1 mol l^{-1} phosphate buffer (pH 7.0), at 4^{0}C, centrifuged at 16000 x *g* at 4^{0}C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml pyrogallol (0.05 mol L^{-1}), 0.05 ml enzyme extract and 0.5 ml H_2O_2 (1% v/v). Reaction mixture without the enzyme served as control. The changes in the absorbance at 420 nm were recorded after 30s intervals for 3 min. The enzyme activity was expressed as change in the U min^{-1} g^{-1} FW.

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Determination of Disease Incidence

The disease incidence was recorded on a scale of 0–4 referring to the degree of wilt as reported by Song *et al*. (2004) where scale zero refers to healthy plant without any wilt symptoms. On the other hand scale four refers to complete wilted plants. The scale 1, 2 and 3 refers to different degrees of wilt which indicates the scale of disease severity. The scale 1- plant showed yellowing of leaves and wilting ranging from 1-20%; scale 2- plant showed yellowing leaves and wilting ranging from 21-40%; scale 3- plant showed yellowing leaves and wilting ranging from 41-60%. Scale 4- is when all leaves become yellow as an indication of complete infection. Disease incidence is a parameter which includes disease percentage and disease severity according to Song *et al*. (2004) as given below:

$$\text{Disease incidence (\%)} = \frac{\sum \text{scale} \times \text{number of plants infected}}{\text{highest scale} \times \text{total number of plants}}$$

Results

Isolation, purification and maintenance of *F. oxysporum* isolates

The isolates purified and identified as *F. oxysporum* based on morphological and cultural characters using the descriptions given by C.M.I (1970). The isolates were designated serially from Fol 1 to Fol 20. The purified isolates were maintained in PDA slants and stored at 4°C for further use.

Test of pathogenicity of *F. oxysporum* isolates

` After isolation and purification of *F. oxysporum* from the collected samples, they were subjected to the pathogenicity tests on susceptible genotype of chickpea cultivar 'Pant gram -4' through soil inoculation method. Out of 20 isolates of *F. oxysporum* tested for pathogenicity, 10 isolates showed typical wilt symptoms like drooping and wilting of lower leaves. Plants showed yellowing of the lower leaves, occasional formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of the remaining leaves, and finally death of the plant. Thus, 10 isolates showed positive result for Koch's postulate while the remaining 10 isolates failed to prove Koch's postulate indicating their non-pathogenicity to chickpea. Those 10 isolates which showed positive results for Koch's postulate were selected for further study. The selected isolates were named as Fol 1, Fol 2, Fol 6 Fol 8, Fol 9, Fol 12, Fol 16, Fol 17, Fol 19 and Fol 20 (Table 2). The colony characteristics of these isolates were studied .These selected isolates were further studied for per cent disease incidence (PDI) through 'soil inoculation' method. The results of pathogenicity are presented in Table 2.

Table 2 Test of pathogenicity of *F. oxysporum* isolates collected from different districts of Uttar Pradesh

S. No.	Isolate name	Root rot symptoms	Koch postulates
1	Fol 1	+ve	+ve

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2	Fol 2	+ve	+ve
3	Fol 3	-ve	-ve
4	Fol 4	-ve	-ve
5	Fol 5	-ve	-ve
6	Fol 6	+ve	+ve
7	Fol 7	-ve	-ve
8	Fol 8	+ve	+ve
9	Fol 9	+ve	+ve
10	Fol 10	-ve	-ve

Study of the effect of selected isolates of *F. oxysporum* on chickpea in pots

The studies of per cent disease incidence (PDI) of 10 selected isolates of *F. oxysporum* were studied by soil inoculation methods in pots under greenhouse conditions. The data were recorded from 30 DAI to 120 DAI (Table 3). It is evident from Table 3, none of the isolate showed PDI up to 30 DAI while four isolates i.e. Fol 2, Fol 5, Fol 6 and Fol 8 recorded PDI of 19.52%, 16.43%, 16.21% and 18.55%, respectively at 60 DAI. All the ten isolates recorded different levels of PDI at 90 DAI. Maximum PDI *i.e* 35.55% was recorded in treatment with Fol 2 followed by 30.12% PDI by Fol 8 and similarly the values of PDI decreased with other isolates. From the observations of Table 3, Fol 2 isolated from Kanpur, was found to be the most aggressive amongst the ten isolates of *F. oxysporum*, and thus was selected as test pathogen for carrying out further experiments.

Table 3 Effect of selected *F. oxysporum* isolates on disease incidence of chickpea through soil inoculation method**.** Results are expressed as mean of triplicates ± S.D.

Name of the isolates	**Per cent disease incidence (%)**				
	Days after inoculation				
	30	**60**	**90**	**105**	**120**
Control	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
Fol 1	0 ±0	0 ±0	22.63 ±5.25	46.95 ±8.22	70.25 ±13.41
Fol 2	0 ±0	17.52 ±5.25	34.55 ±7.33	52.85 ±8.33	88.63 ±14.33
Fol 3	0 ±0	0 ±0	21.75 ±6.38	41.32 ±9.75	64.85 ±14.33
Fol 4	0 ±0	15.43 ±5.25	31.12 ±6.25	46.51 ±8.22	80.25 ±10.25
Fol 5	0 ±0	0 ±0	20.40 ±8.45	30.42 ±9.25	61.70 ±12.33
Fol 6	0 ±0	0 ±0	25.52 ±6.38	40.77 ±14.33	70.35 ±12.25
Fol 7	0 ±0	14.21 ±6.38	26.31 ±6.25	40.63 ±14.33	72.53 ±13.55
Fol 8	0 ±0	19.55 ±6.78	21.11 ±7.22	43.36 ±8.55	76.52 ±13.11
Fol 9	0 ±0	0 ±0	22.55 ±8.11	36.11 ±5.85	63.47±10.12
Fol 10	0 ±0	0 ±0	21.18±6.22	38.56 ±5.22	64.36 ±13.11

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***In vitro* Efficacy of *BCAs* against Pathogen**

The above described BCAs were evaluated for antagonistic activities against *F. oxysporum*f. sp. *ciceri* after 4 days in dual culture assay. Table 4 shows that the bioagents significantly reduced the radial growth of *F. oxysporum* f. sp. *ciceri*. *T. harzianum* showed more antagonistic activity than *B. subtilis* and *P. fluorescens* against the radial growth of *F. oxysporum* f. sp. *ciceri*.

Table 4 Effect of bioagentson the growth of *F. oxysporum* f. sp. *ciceri*

Microbial strain	**Radial growth (cm)**	**Inhibition Percentage (%)**
B. subtilis	1 ± 0.1^{b}	87.76 ± 1.66^{c}
P. fluorescens	1.46 ± 0.02^{c}	82.85 ± 0.2^{b}
T. harzianum	0.47 ± 0.15^{a}	94.15 ± 1.69^{d}
Control	9.0 ± 0^{d}	0.0 ± 0^{a}

Effect of biofortified vermicompost on defense related enzymes in chickpea plants challenged with *F. oxysporum* f. sp. *ciceri*

Phenylalanine Ammonia Lyase (PAL)

PAL levels increased significantly in all treatments up to 48 h, followed by a decline in its activity. Maximum PAL activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 48 h followed by T2, T3 and T4. At 48 h T1 showed 3.6 fold increases in PAL activity in comparison to control. At the same time T2, T3 showed 3.5 and 2.5 fold increase in PAL accumulation when compared to control. Plants from non fortifiedvermicompost also showed higher PAL accumulation in comparison to the control. At 48 h plant only with vermicompost (T4) showed 2.3 fold increase in PAL accumulation when compared to control (Figure 1).

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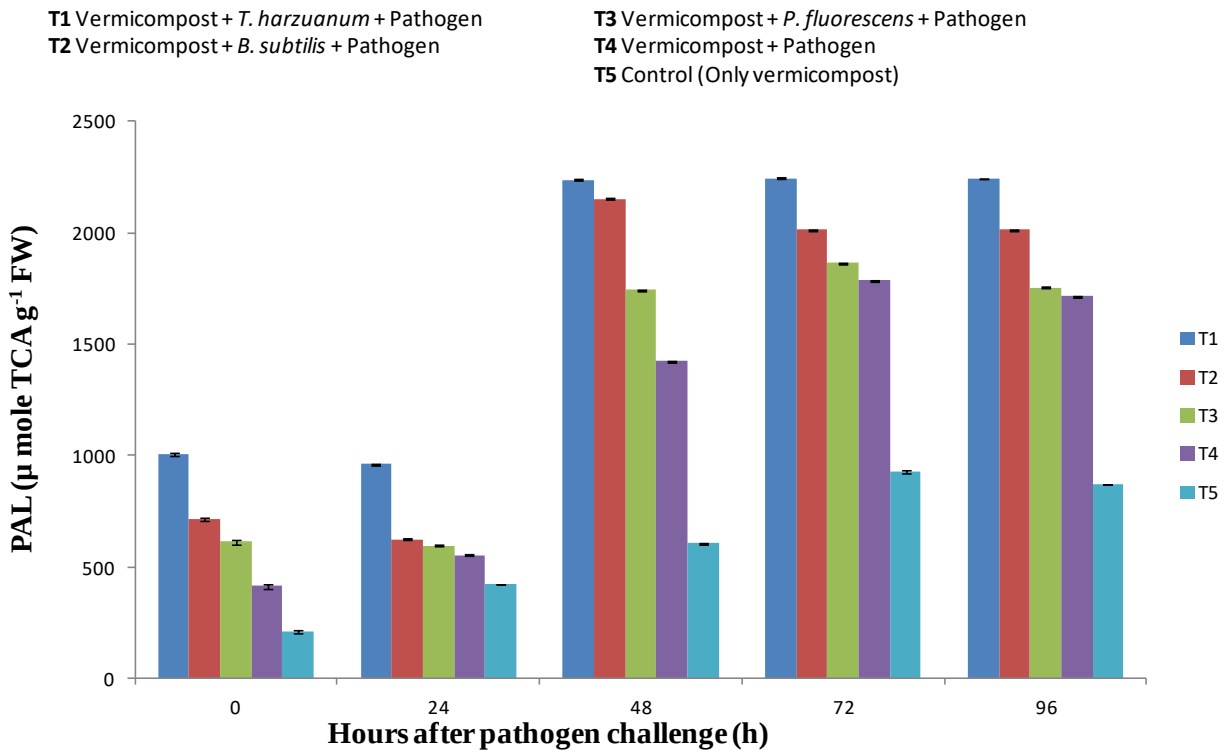

Figure 1 PAL activity at different time intervals in chickpea raised from seeds sown in soil amended with biofortified vermicompost challenged with *F. oxysporum*.

Peroxidase (PO)

PO levels increased significantly in all treatments up to 72 h, followed by a decline in its activity. Maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 72 h followed by T2, T3 and T4. At 72 h T1 showed 8.2 fold increase in PO accumulation in comparison to control. At the same time T2 and T3 showed 7.5 and 4.5 fold increase in PO activity in comparison to control. Plants from only vermicompost also showed higher PO accumulation in comparison to the control. At 48 h and 72 h plant only with vermicompost (T4) showed 3.09 and 2.3 fold increase in PO accumulation when compared to control (Figure 2).

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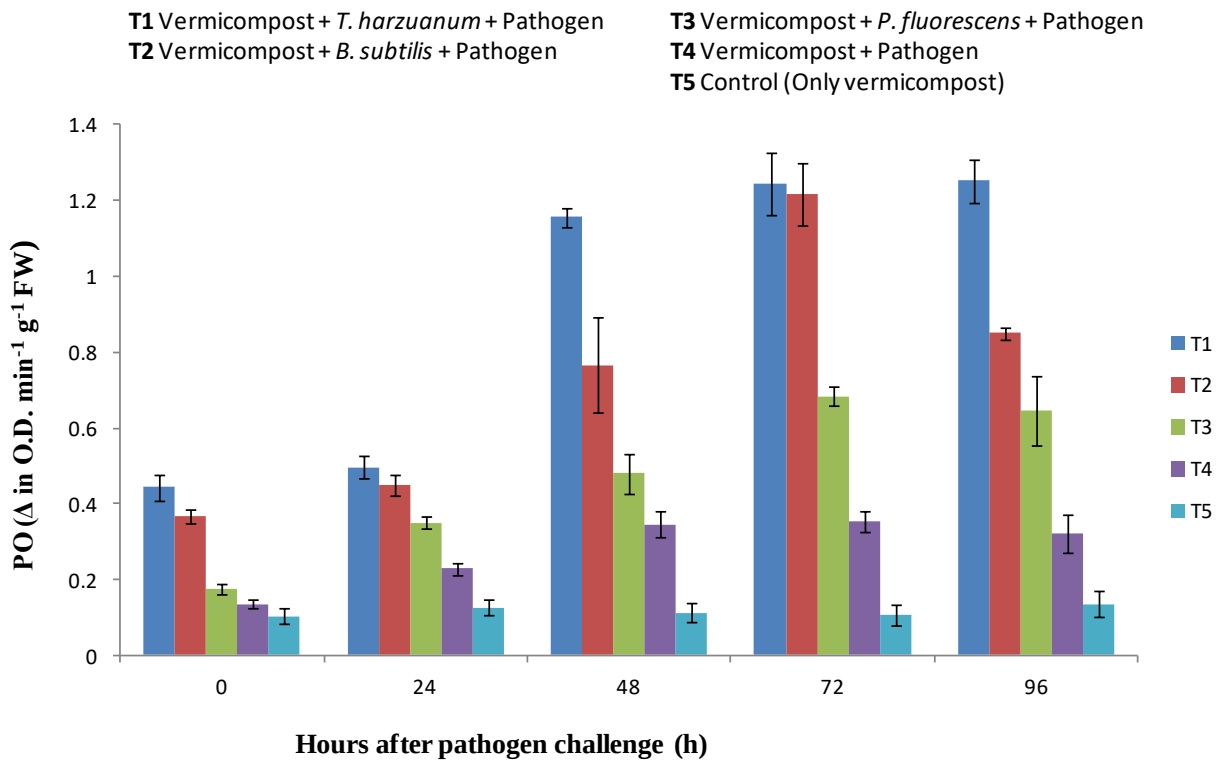

Figure 2 Effect of microbial fortified vermicompost on PO activity.

Polyphenol oxydase (PPO)

PPO levels increased significantly in all treatments up to 72 h, followed by a decline in its activity. Maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 72 h followed by T2, T3 and T4. At 72 h T1 showed 5.4 fold increase in PPO accumulation in comparison to control. At 72 h plant only with vermicompost (T4) showed 2.3 fold increases in PPO accumulation when compared to control (Figure 3).

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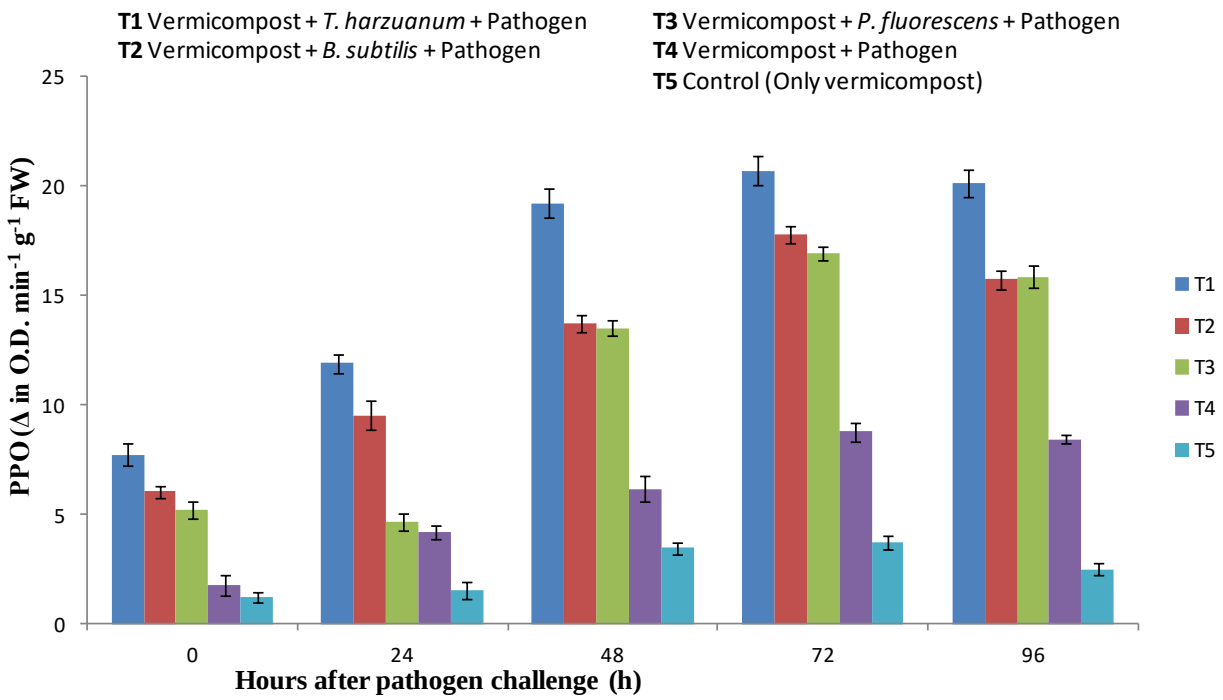

Figure 3 Effect of microbial fortified vermicompost on PPO activity**.**

Total phenol content (TPC)

The TPC followed a similar trend that of PAL with maximum increment at 48 h in T1 followed by a sharp decline in its activity. The amount of TPC content shows significant variation among different treatments. The highest phenolic content was observed at 48 h in T1. The total phenolic content was higher by 6.5, 5.4, 3.9 and 3.8 fold in T1,T2, T3 and T4 respectively, in comparison to control at 48 h (Figure 4).

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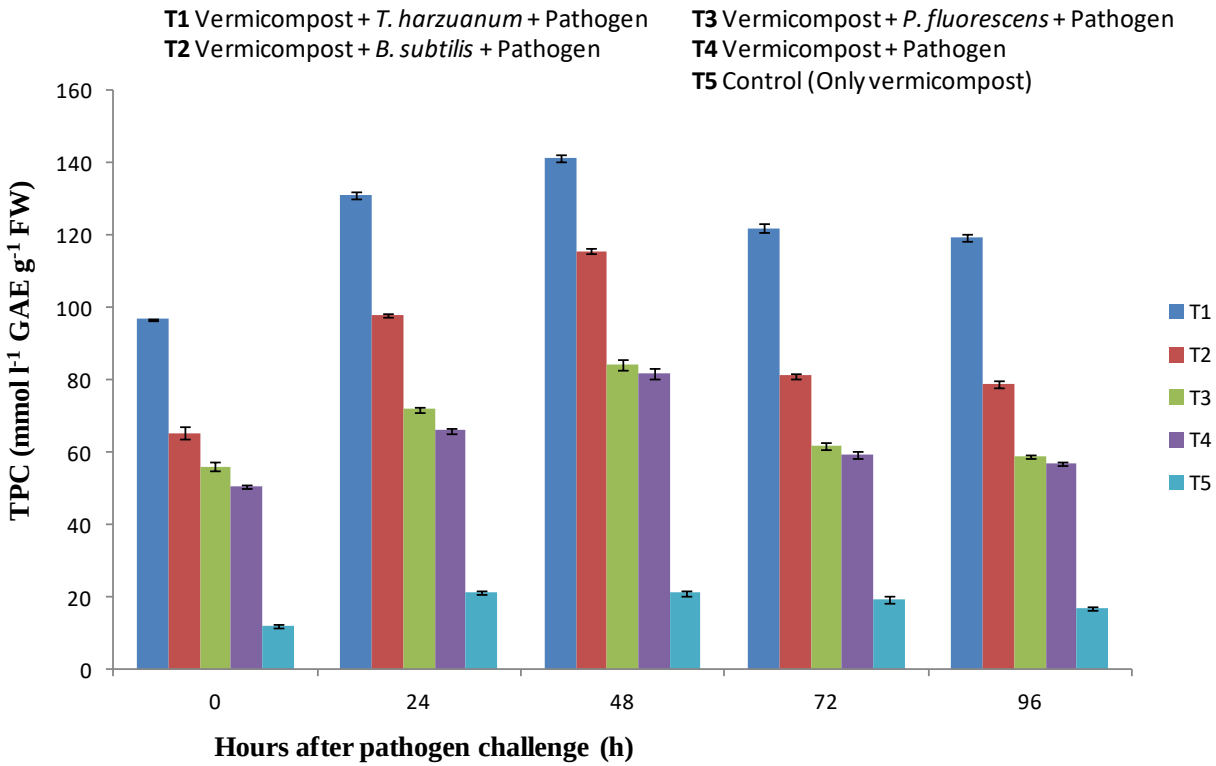

Figure 4 Effect of microbial fortified vermicompost on TPC**.**

The BCAs *viz*. *Bacillus*, *Pseudomonas* and *Trichoderma* used in the present study are well known to control *F. oxysporum* (Cherkupally*et al*., 2017; Bisen*et al*., 2015; Bisen*et al*., 2016; Jain *et al*., 2012). It is also evident from the results of this study that these rhizospheric strains showed very high levels of antagonistic activity against the test pathogen. Production of cell wall degrading enzymes, HCN and siderophores can be correlated with their antagonistic activity against test pathogen. Several studies have demonstrated that production of siderophores, HCN and lytic enzymes by *Pseudomonas*, *Bacillus* and *Trichoderma* strains resulted in effective control of plant pathogens (Mokhtar *et al*., 2013; Harman *et al*., 2004).

Discussion

Effect of microbial fortified vermicompost on growth parameters of chickpea crop

It is widely accepted that use of composts and vermicomposts as soil amendments could improve soil nutrient status, promote soil health and improved most of the characteristics of crop plants compared with synthetic fertilizer. The introduction of biofortified vermicompost led to the significant results in terms of yield enhancement and reduction of disease incidence. The results presented in this study showed that there was a clear difference in growth promotion in chickpea plants grown in microbial fortified vermicompost as well as in vermicompost alone. Highly significant variations were observed in root length, shoot length and dry weight among the treatments. Results of the current study are in accordance with the report of Wang *et al*. (2017) who reported that the application of vermicompost in addition with other bioinoculants in chickpea promoted growth. In their

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study using different treatments, it was found that maximum growth in chickpea plants was obtained where a combination of different treatments i.e. vermicompost, *Bacillus pumilus*, *Trichoderma* and a mycorrhizal fungus *Glomus mosseae* were used. Similar results were also reported by Bachman and Metzger (2008) who stated positive effect on productivity enhancement and nematode management through vermicompost and bio-pesticides in brinjal. The findings of this study are also in agreement with experimental findings which showed that vermicompost or its combination with *Pseudomonas fluorescens* based biopesticide have played a vital role in promoting growth in chickpea (Bora and Deka, 2007).

Effect of fortified vermicompost on activity of defense related enzymes in chickpea

Plants contain a range of mechanisms to protect themselves against invading phytopathogens. Treatment with biofortified vermicompost may accelerate cellular defense responses which is most economical substitute are only expressed when plant is challenged with pathogen. Results from the present investigation indicated that chickpea plants with biofortified vermicompost exhibited higher levels of defense related enzyme activity and accumulated phenols in leaves when challenged with *F. oxysporum*. ISR develops systemically in response to colonization of plant roots by beneficial microorganisms from vermicompost and selected BCAs (van der Ent *et al*., 2009). Such cellular responses also include an early oxidative burst and a stronger upregulation of defense genes (Jain *et al*., 2012). Induction of defense proteins and enzymes in the present study can be correlated as a defense response triggered against pathogen invasion in chickpea. The results obtained demonstrate that treatment with biofortified vermicompost led to many fold increase in the activities of defense related enzymes such as PAL, PO, PPO, SOD and phenols, suggesting their role in disease resistance. Maximum enzyme activities were recorded in the plants treated with vermicompost fortified with *T. harzianum*.

Phenols have diverse roles in plants defense, such as cell wall strengthening, antimicrobial activity and synthesis of signaling compounds salicylic acid (Nawrocka *et al*., 2018). Maximum PAL activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 48 h. Our findings are in agreement with the studies conducted on suppression of damping off diseases in psyllium by *Bacillus subtilis* and vermicompost. Amooaghaie *et al*. (2018) reported that vermicompost and *B. subtilis* induced systemic resistance through nitric oxide (NO) signaling. Their combined application triggered the accumulation of defense related enzymes including β-1,3-glucanase, PAL, PPO and also effectively reduced lipid peroxidation in psyllium leaves. To some extent, the increased activity of PAL is directly related to the increased antimicrobial activities and therefore greater degree of host plant resistance to pathogen is achieved.

The enzymes SOD and PO, work together with other enzymes of the ascorbate–glutathione cycle to promote scavenging of free radicals (Jain *et al*., 2012). SOD is part of a group of antioxidative enzymes catalyzing the dismutation of O^{2-} to H_2O_2 and O_2 that are most important to ameliorate the damage caused by oxidative stress (Rao *et al*., 2015; Camejo*et al*., 2016). PO enzyme catalyzes the reduction of H_2O_2*via* transport of electrons to various donor molecules, which is linked to broad range of physiological processes, including

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lignification, auxin metabolism, cross-linking of cell wall proteins and defense against phytopathogens (Sarma*et al*., 2015). In the present study, analysis of plants after pathogen infection indicated that the PO levels increased significantly in all treatments up to 72 h, followed by a decline in its activity. Maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 72 h. Similar results were also found in previous study where vermicompost biofertilizer was found to induce maximum PO and SOD activity in greenhouse cucumber after 72 h of *Pythium aphanidermatum* challenge (Sabbagh and Valizadeh, 2016). Following treatment with biofrotified vermicompost, increased PO activity in treated plants, as observed in may lead to accumulation of lignin which is important physical barrier to check the pathogen invasion.

Enhanced PPO activities in plant tissues against phytopathogens and insect pests have been reported in several beneficial plant–microbe interactions (Jain *et al*., 2012). To some extent, increased activity of PAL and PPO is directly proportional to the increased antimicrobial activity and decrease in accumulation of toxic oxidation products and therefore greater degree of resistance to pathogen is achieved. In this present study maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*at 72 h. An increased PPO activity in all the plants treated with fortified vermicompost is also an indicator of increased pathogen tolerance. An increased level of defense related enzymes PAL, PO, PPO in plants treated with *B. subtilis* and vermicompost under the stress generated by *F. oxysporum* in *Plantago pysillium* (Amooaghaie *et al*., 2018). Our results are also in agreement with Bosco *et al*. (2017) where increased PAL, PO and PPO activity was recorded in chickpea when treated with biofortifiedvermicompost against fusarium wilt.

Increased activity of defense related enzymes in the plants treated with biofortified vermicompost may be considered as a part of the response of the host cells to pathogen which is useful in check the development of the fungus without causing further damage to the surrounding tissues and may partially account for the observed delay in symptom development.

Conclusion

The three BCAs *viz. T. harzianum, P. fluorescens*and *B. subtilis* were chosen because of their compatibility and ascertained ability to reduce the soilborne diseases in various crops and used to fortify the vermicompost individually. Effect of biofortified vermicompost on plant growth and disease suppression was recorded. The influence of fortified vermicompost on the growth characters was clearly observed after 15 days of transplanting. All treated plants showed significant improvement in root length in comparison to the control. Chickpea plants treated with vermicompost fortified with *Trichoderma* showed maximum root length (14.95 cm) after 15 days of sowing. Chickpea plants treated with vermicompost fortified with *Trichoderma* showed maximum shoot length at every interval. Maximum shoot length 57.5 cm was observed in T-1 followed by T-2 (45.13 cm) and T-3 (42.25 cm) after 15 DAS. Plant dry weight was recorded at 15, 45, 60 and 90 days after sowing. Maximum dry weight was observed in plants treated with vermicompost fortified with *Trichoderma*.

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Biochemical analysis of plants from each treatment was done to evaluate the effect of biofortified vermicompost on defense related enzymes. PAL levels increased significantly in all treatments up to 48 h, followed by a decline in its activity. Maximum PAL activity was recorded in leaves from plants grown in vermicompost fortified with *T. harzianum*(T1) at 48 h followed by T2, T3 and T4. At 48 h T1 showed 3.6 fold increases in PAL activity in comparison to control. PO levels increased significantly in all treatments up to 72 h, followed by a decline in its activity. Maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 72 h followed by T2, T3 and T4. At 72 h T1 showed 8.2 fold increase in PO accumulation in comparison to control. PPO levels increased significantly in all treatments up to 72 h, followed by a decline in its activity. Maximum PO activity was recorded in leaves from plant grown in vermicompost fortified with *T. harzianum*(T1) at 72 h followed by T2, T3 and T4. At 72 h T1 showed 5.4 fold increases in PPO accumulation in comparison to control. The TPC followed a similar trend that of PAL with maximum increment at 48 h in T1 followed by a sharp decline in its activity. The amount of TPC content shows significant variation among different treatments. The highest phenolic content was observed at 48 h in T1.

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