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Biochemical Mechanism of Native Fungal Bioagents in the Management of Root-Knot Nematode *Meloidogyne incognita* on Tomato

M. Annapurna*, B. Bhagawati and Kurulkar Uday

Department of Nematology, Assam Agricultural University, Jorhat, Assam, India

**Corresponding author*

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A B S T R A C T

Analysis of defense-related enzymatic activities of the fungal bioagents *viz*., *Trichoderma viride*, *T. harzianum*, *Pochonia chlamydosporia* and *Purpureocillium lilacinum* against root-knot nematode *Meloidogyne incognita* on Tomato were carried out and revealed that all the tested fungal bioagents have the ability to induce defense-related enzymatic activity against *M. incognita* which resulted in the increase in the plant growth parameters like shoot height, shoot weight, root length, root weight after 15, 30 and 45 days after inoculation (DAI) and decrease in the nematode multiplication on the tomato and in the soil as compared to the untreated control after 30 and 45 DAI. However, among the tested bioagents, *T. harzianum* not only showed the highest biochemical activity of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and total phenol content but also showed increase in the plant growth parameters of tomato and decrease in the nematode multiplication on tomato as well as in the soil.

Introduction

Root-knot nematode attack not only more than two thousands of plant species but also caused five percent of global crop loss (Hussey and Janssen, 2002). An avoidable yield loss of tomato due to *M. incognita* was recorded to the tune of 13.20 percent in Assam (Anon., 2013). The application of chemical nematicides will become prohibited due to not only the increase of resistance in the target pathogen but also caused the environmental hazard. To reduce such causes, bioagents are found to be an increase in the attention and use of such bioagents offer an effective, safe, persistent and natural durable protection against crop pest (Anita and Samiyappan, 2012). However, many natural enemies attack *Meloidogyne* spp. in the soil (Kok *et al.*, 2001) and such enemies can be used as bioagent for the effective management of *Meloidogyne* spp. (Karssen *et al.*, 2006). Among them, fungi are unique natural enemies for managing the nematodes in soil (Mark *et al.*, 2010).

The root - beneficial bioagents association either showed antagonistic activity towards pathogen or induces defensive enzymes (Kavitha *et al.*, 2013) which impart in the improvement of plant growth parameters and reduce the multiplication of target pathogen (Harman *et al.*, 2004). However, the efficacy of bioagents varies from species to species (Irving and Kerry, 1986). So, one of the means

of increasing potentiality of bioagents is to use native biocontrol agents (Singh *et al.,* 2013). The potential benefits and fit fall must be examined so that effective native biocontrol agent (s) can be utilized. Hence, a study was undertaken on the induction of biochemical mechanism of native fungal bioagents in the management of root-knot nematode *M. incognita* on tomato.

Materials and Methods

Source and maintenance of *M. incognita* and fungal bioagents

M. incognita egg masses were obtained from Experimental plot, Department of Nematology, Assam Agricultural University (AAU), Jorhat-13 and pure culture were maintained on Tomato in pots in the Net house, Department of Nematology, AAU, Jorhat-13. Pure culture of biocontrol agents *viz.*, *Trichoderma viride*, *T. harzianum* and *Pochonia chlamydosporia* and *Purpureocillium lilacinum* were obtained from Department of Plant Pathology, AAU., Jorhat-13 and were maintained on Potato Dextrose Agar (PDA) at Post Graduate Laboratory, Department of Nematology AAU., Jorhat -13.

Nematode inoculums

For nematode reproduction, the most susceptible variety of tomato (cv. Pusa Ruby) was used as the host plant. 25 days old tomato plants were transplanted into pots containing 1 kg sterilized soil with finely dried cow dung and sand in the ratio of 2:1:1, respectively.

One week after transplantation, the plants were each inoculated with approximately 1,000 freshly hatched second stage juveniles (J_2s) of *M. incognita* added to holes in the soil around the stem of each plant. The plants were kept in a green house at 25± 2°C and watered as needed.

Mass culture of bioagents

For mass culture of *T. harzianum*, *T. viride*, *P. chlamydosporia* and *P. lilacinum*, 1kg vermicompost was put into polypropylene bags. The bags were plugged with non-absorbent cotton and autoclaved at 121^{o}C temperature for 30 minutes.

Each bag containing the sterilized medium was inoculated with 1ml of each of the liquid formulation of bioagent under aseptic conditions and was incubated at 25± 2^{o}C for 15days.

Pot experiment

The experiment was conducted in the net house of the Department of Nematology, AAU Jorhat-13 during winter season of 2016-2017. The pots (1kg capacity) were arranged in a completely randomized design with five replications for each treatment. All the pots were transplanted with 25 days old seedlings of tomato. The pots receiving the treatments with bioagents were inoculated with second stage juveniles of *M. incognita* @ 1J_2/cc soil as also 15 days old culture of bioagents grown on vermicompost @ 2% (w/w). Two control treatments *viz*., *M. incognita* alone (@ 1J_2/cc soil) and uninoculated and untreated control.

Treatment details are as follows.

$T_{1=}$ *M. incognita* @ 1 J_2/cc of soil + *T. viride* @ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost).

$T_{2=}$ *M. incognita* @ 1 J_2/cc of soil + *T. harzianum* @ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost).

$T_{3=}$ *M. incognita* @ 1 J_2/cc of soil + *P. chlamydosporia* @ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost)

$T_{4=}$ *M. incognita* @ 1 J_2/cc of soil + *P. lilacinum* @ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost).

$T_{5=}$ *M. incognita* @ 1 J_2/cc of soil alone.
$T_{6=}$ Uninoculated and Untreated control.

Observations

Observations on defense enzymatic activities *viz*., peroxidase, polyphenoloxidase, phenylalanine ammonia lyase and total phenol content were taken at 15, 30 and 45 days after inoculation. Furthermore plant growth parameters like fresh shoot and root length, fresh shoot and root weight and nematode multiplication like number of galls and egg masses per root system and final nematode population in 250cc soil at 30 and 45days after inoculation were recorded.

Biochemical analysis of root samples

Root samples were collected from each treatment at 15, 30 and 45 days of inoculation and further processed to study the enzymatic activities induced by the bioagents. The detail methodology for each activity is described below.

Assay of peroxidase (PO)

Root samples (1gm) maintained at -70 °C were homogenized in 2ml of 0.1 M sodium phosphate buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 16,000 rpm at 4 °C for 15 min and the supernatant was used as enzyme source. The reaction mixture consists of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 percent H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2 °C). The changes in absorbance at 420 nm were recorded at 30s intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min^{-1} mg^{-1} protein (Hammerschmidt *et al.*, 1982).

Assay of polyphenol oxidase

Root samples (1gm) were homogenized in 2ml of 0.1 M sodium phosphate buffer (P^H 6.5) and centrifuged at 16,000 rpm for 15 min at 4^{o}C and the supernatant was used as enzyme source. The reaction mixture consisted of 2ml of the enzyme extract and 1.5ml of sodium phosphate (P^H 6.5). To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm $min^{-1}mg^{-1}$ protein (Mayer *et al.*, 1965).

Assay of phenylalanine ammonia lyase (PAL)

Root samples (1gm) were homogenized in 3 ml of ice-cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2- mercaptoethanol and 0.1 gm of insoluble polyvinyl pyrrolidone. The extracts were filtered through cheese cloth and the filtrate will be centrifuged at 16,000 rpm for 15 min. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L – phenylalanine to trans-cinnamic acid at 290 nm. Samples containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L - phenylalanine in the same buffer for 30 min at 30^{o}C. The amount of Trans – cinnamic acid synthesized was calculated. Enzyme activity was expressed as nmol trans–cinnamic acid min^{-1} mg^{-1} protein (Dickerson *et al.*, 1984).

Estimation of total phenols

Root samples (1gm) were homogenized in 10 ml of 80 per cent methanol and agitated for 15 min at 70°C (Zieslin and Ben – Zaken, 1993). 1ml of the methanolic extract was added to 5ml of distilled water and 250 µl of Folin – Ciocalteau reagent (1N) and the solution was kept at 25^{o}C. The absorbance of the developed blue colour was measured using a

spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol mg^{-1} protein.

Statistical Analysis

Results obtained were treated statistically by applying probability using one way analysis of variance for treatments. Statistical analyses were performed using Web Based Agricultural Statistics Software Package WASP 2.0.

Results and Discussion

All the bioagents *viz., T. viride, T. harzianum, P. chlamydosporia* and *P. lilacinum* showed greater influence for induction of defense enzymatic activities against *M. incognita* in tomato (Table 1). The peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) activities and total phenol content in the roots of tomato were found to be significantly increased in all the treatments after 15, 30 and 45 DAI as compared to the controls (Figure 1, 2, 3 and 4), the maximum being recorded in T_2 *i.e., M. incognita* @ 1 J_2/cc of soil + *T. harzianum* @ 20gm/plant. In this treatment the PO activity was recorded to be 5.00, 5.07 and 5.27 mg, PPO activity was recorded to be 1.34,1.66 and 1.90 mg, PAL activity was recorded to be 22.56, 24.01 and 25.52 nM and total phenol content was recorded to be 1.64, 1.70 and 1.87 mg at 15, 30 and 45DAI respectively (Table 1). In respect of other bioagents increased PO, PPO, PAL activity and total phenol content were recorded in *T. viride* followed by *P. chlamydosporia* and *P. lilacinum.*

The least PO, PPO, PAL activity and total phenol content was recorded in the T_5 (3.15, 3. 27 and 3.39 mg) followed by T_6. However, all the treatments were found to be significantly different from each other. Govindappa *et al.,* (2010) observed high peroxidase activity in the fungal bioagent *T. harzainum* treated roots of *Carthamus tinctorius* infected by *Macrophomina phaseolina* over control. Devrajan and Sreenivasan (2002) also reported that synthesis of biochemicals like peroxidase and polyphenol oxidase (catechol oxidase) in fungal bioagent *P. lilacinum* treated roots of *Musa* sp. cv. Robusta infected with *M. incognita.* Deepa *et al.,* (2014) studied the biochemical mechanism of biocontrol agents like, *T. harzianum*, *T. viride* and *P. chalmydopsoria* against citrus nematode *Tylenchulus semipenetrans* on *Citrus limonia* and explored the induction of plant defense enzymes *viz.*, peroxidase, polyphenoloxidase, phenylalanine ammonia lyase and total phenols by these bioagents.

They observed profound influence of these bioagents in the induction of these defense enzymes in citrus roots infected by *T. semipenetrans* wherein the fungal bioagent, *T. harzianum* was observed to show highest enzymatic activities as compared to other fungal bioagents thus confirming the results of the present investigation.

As far as plant growth parameters *viz*., fresh shoot height, fresh shoot weight, fresh root length and fresh root weight is concern, at 15 DAI maximum shoot height, shoot weight, root length, root weight were recorded in the treatment with untreated and uninoculated control (T_6) and it was significantly different from rest of the treatments (Table 2).

Among the tested bioagents, maximum plant growth parameters like fresh shoot height, fresh shoot weight, fresh root length and fresh root weight were recorded in the treatment, *M. incognita* + *T. harzianum* (T_2), followed by T_1 (*M. incognita* +*T. viride*), T_3 *(M. incognita* + *P. chlamydosporia*) and T_4 (*M. incognita* +*P. lilacinum*) (Table 2; Figure 5 and 6). Similar trend of improvement in plant growth parameters was recorded at 30 DAI and 45 DAI.

Table.1 Activity of phenols and defense enzymes in tomato roots treated with fungal bioagents and inoculated with *M.incognita*

Treatments	Peroxidase (PO) (change in absorbance m^{-1} mg^{-1} protein)			Polyphenol oxidase (PPO) (change in absorbance m^{-1} mg^{-1} protein)			Phenylalanine ammonia lyase (PAL) (nmoltranscinnamic acid m^{-1} mg^{-1} protein)			Phenol (mg/g fresh root)		
	15DAI	30DAI	45DAI	15DAI	30DAI	45DAI	15DAI	30DAI	45DAI	15DAI	30DAI	45DAI
T_1	4.84	4.91	5.05	1.32	1.59	1.81	20.15	20.55	21.17	1.51	1.63	1.76
T_2	5.00	5.07	5.27	1.34	1.66	1.90	22.56	24.01	25.52	1.64	1.70	1.87
T_3	4.11	4.28	4.39	1.26	1.43	1.66	16.49	17.71	18.85	1.31	1.43	1.59
T_4	3.98	4.04	4.16	1.23	1.37	1.57	13.63	15.48	16.79	1.22	1.30	1.53
T_5	3.15	3.27	3.39	0.57	0.66	0.70	10.48	10.57	10.75	0.80	0.90	1.09
T_6	2.60	2.67	2.83	0.30	0.36	0.43	6.05	7.14	7.28	0.32	0.40	0.49
S.Ed (±)	0.04	0.04	0.05	0.01	0.02	0.02	0.18	0.24	0.24	0.02	0.02	0.02
C.D.(0.05)	0.10	0.09	0.11	0.02	0.04	0.04	0.37	0.51	0.51	0.03	0.03	0.04

$T_{1=}$ *M. incognita* @ 1 J_2/cc of soil + *T. viride* @ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost), $T_{2=}$ *M. incognita* @ 1 J_2/cc of soil +*T. harzianum*@ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost), $T_{3=}$ *M. incognita* @ 1 J_2/cc of soil +*P. chlamydosporia*@ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost), $T_{4=}$ *M. incognita* @ 1 J_2/cc of soil +*P. lilacinum*@ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost), $T_{5=}$ *M. incognita* @ 1 J_2/cc of soil alone and $T_{6=}$ Uninoculated and Untreated control

Table.2 Effect of fungal bioagents on plant growth parameters of tomato infected by *M. incognita*

Treatments	Fresh Shoot Length(cm)			Fresh Shoot weight(gm)			Fresh Root Length(cm)			Fresh Root weight(gm)		
	15 DAI	30DAI	45DAI	15 DAI	30DAI	45DAI	15DAI	30 DAI	45DAI	15DAI	30 DAI	45DAI
T_1	21.11	46.02	67.98	6.42	11.87	16.93	12.58	18.84	38.66	6.72	8.66	13.14
T_2	21.31	46.48	69.15	7.01	12.12	17.46	12.59	20.12	38.82	7.06	9.07	14.42
T_3	20.64	44.48	65.48	5.87	10.99	16.25	11.34	16.88	35.40	5.94	7.87	12.26
T_4	18.73	43.18	64.53	5.53	10.53	15.82	10.56	16.22	33.48	5.44	7.65	11.82
T_5	16.78	35.23	61.09	5.16	8.83	13.41	7.38	13.14	25.28	5.03	5.44	9.18
T_6	22.48	48.06	69.19	7.65	12.32	17.76	13.97	20.29	39.06	7.58	10.06	14.81
S.Ed (±)	0.23	0.50	0.77	0.08	0.12	0.18	0.14	0.21	0.46	0.10	0.11	0.21
C.D. at 0.05	0.48	1.05	1.58	0.16	0.26	0.39	0.29	0.45	0.88	0.20	0.23	0.44

Details of treatments: $T_{1=}$ *M. incognita* @ 1 J_2/cc of soil + *T. viride* @ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost), $T_{2=}$ *M. incognita* @ 1 J_2/cc of soil +*T. harzianum*@ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost), $T_{3=}$ *M. incognita* @ 1 J_2/cc of soil +*P. chlamydosporia*@ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost), $T_{4=}$ *M. incognita* @ 1 J_2/cc of soil +*P. lilacinum*@ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost), $T_{5=}$ *M. incognita* @ 1 J_2/cc of soil alone and $T_{6=}$ Uninoculated and Untreated control

Table.3 Effect of fungal bioagents on nematode multiplication of *M. incognita* on tomato

Treatments	No of Galls		No. of egg masses		Nematode population /250 cc soil	
	30 DAI	45 DAI	30 DAI	45 DAI	30 DAI	45 DAI
T_1	39.06 (6.29)	56.95 (7.58)	27.90 (5.33)	46.28 (6.84)	241.30 (15.55)	258.06 (16.08)
T_2	35.74 (6.02)	53.96 (7.38)	24.60 (5.01)	41.36 (6.47)	232.97 (15.28)	240.99 (15.54)
T_3	43.72 (6.65)	73.97 (8.63)	33.02 (5.79)	64.00 (8.00)	267.80 (16.38)	289.86 (17.04)
T_4	47.11 (6.90)	79.06 (8.92)	36.46 (6.08)	67.89 (8.27)	288.84 (17.01)	309.61 (17.61)
T_5	91.85 (9.61)	112.92 (10.65)	54.11 (7.39)	86.54 (9.33)	441.76 (21.03)	471.25 (21.72)
T_6	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)	0.00 (0.70)
S.Ed (±)	0.09	0.10	0.07	0.11	0.10	0.11
C.D. at 0.05	0.19	0.20	0.15	0.23	0.21	0.24

Figure in parenthesis are square root transform value before analysis.

Details of treatments: $T_{1=}$ *M. incognita* @ 1 J_2/cc of soil + *T. viride* @ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost), $T_{2=}$ *M. incognita* @ 1 J_2/cc of soil +*T. harzianum*@ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost), $T_{3=}$ *M. incognita* @ 1 J_2/cc of soil +*P. chlamydosporia*@ 2% enriched vermicompost (@ 1ml of formulation/Kg vermicompost), $T_{4=}$ *M. incognita* @ 1 J_2/cc of soil +*P. lilacinum*@ 2% enriched vermicompost (@ 1ml of formulation /Kg vermicompost), $T_{5=}$ *M. incognita* @ 1 J_2/cc of soil alone and $T_{6=}$ Uninoculated and Untreated control

Fig.1 Peroxidase activity induced by fungal bioagents in tomato roots at 15, 30 and 45DAI

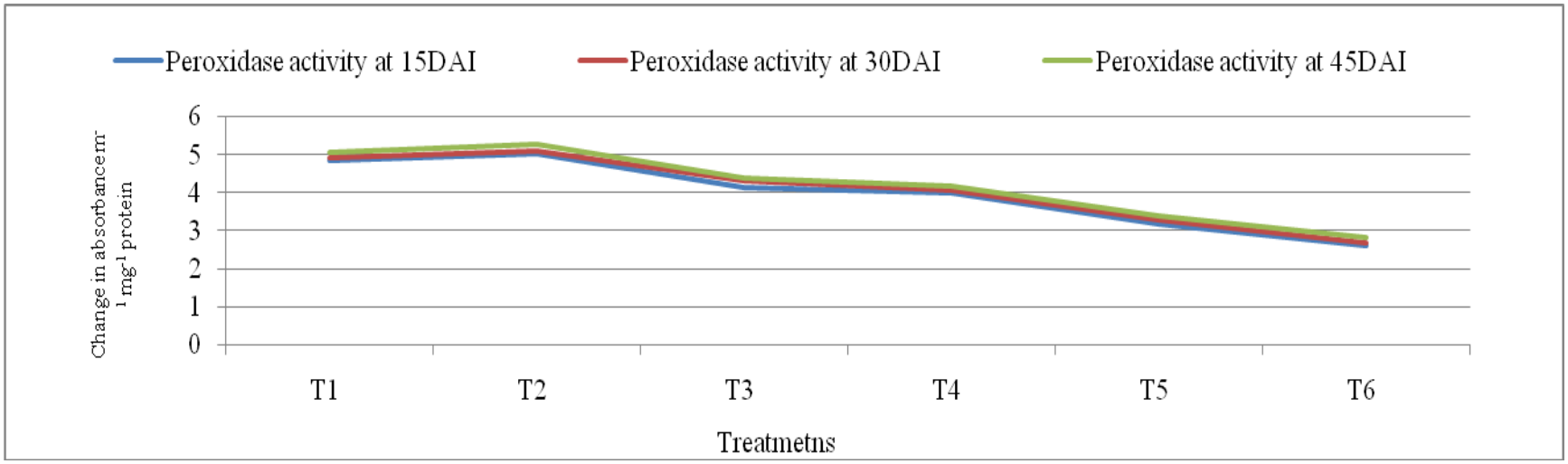


Fig.2 Ploypheno1 oxidase activity induced by fungal bioagents in tomato roots at 15, 30 and 45 DAI

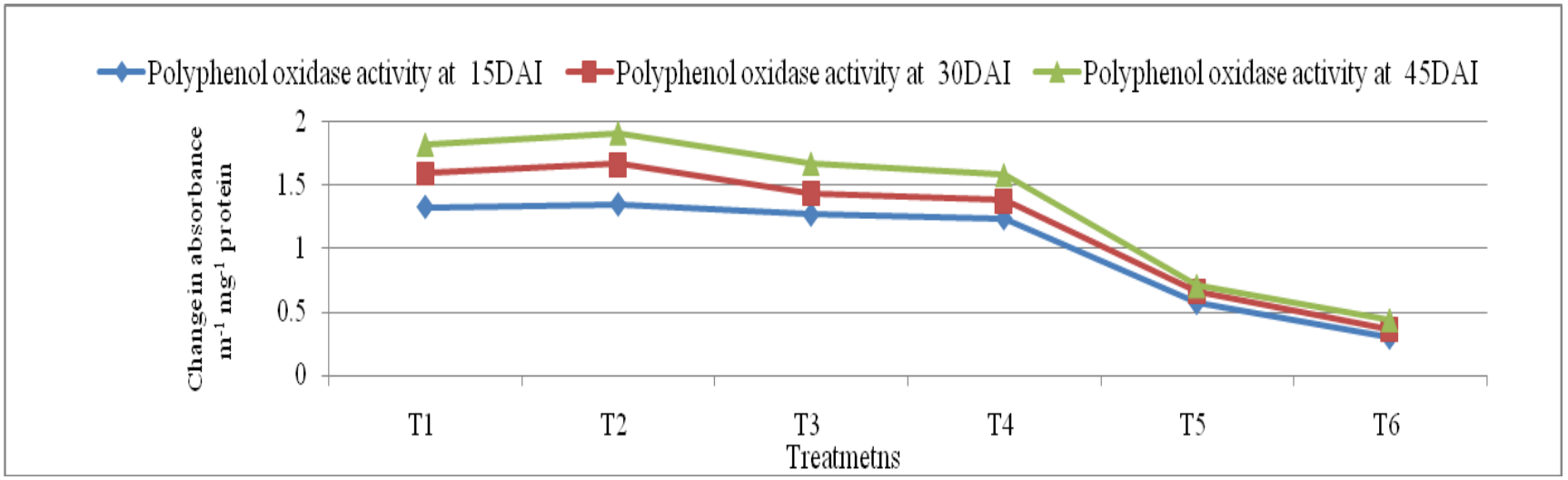


Fig.3 Phenylalanine ammonia lyase activity induced by fungal bioagents in tomato roots at 15, 30 and 45 DAI

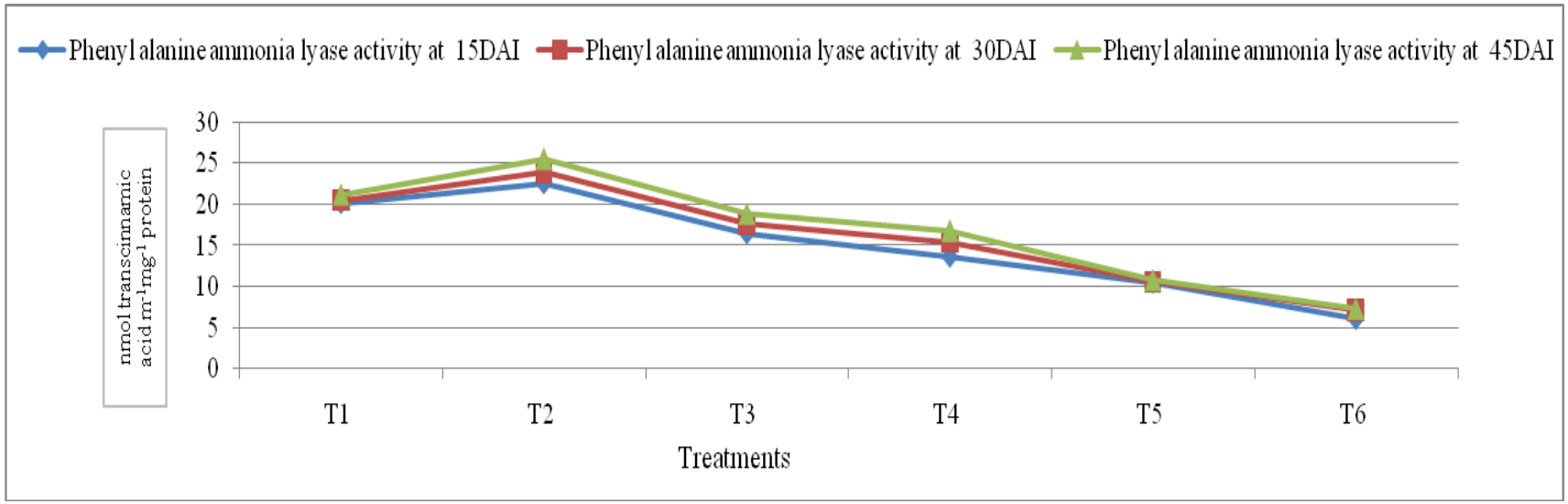


Fig.4 Activity of total phenols induced by fungal bioagents in tomato roots at 15, 30 and 45 DAI

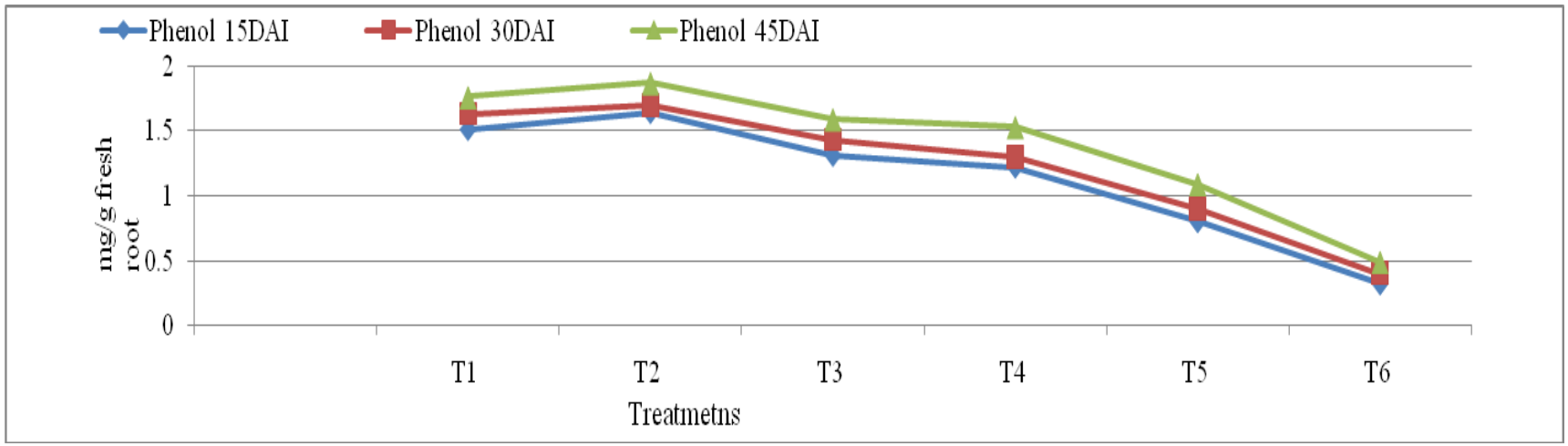


Fig.5 Effect of fungal bioagents on fresh shoot and root length of tomato infected by *M. incognita* after 15, 30 and 45DAI

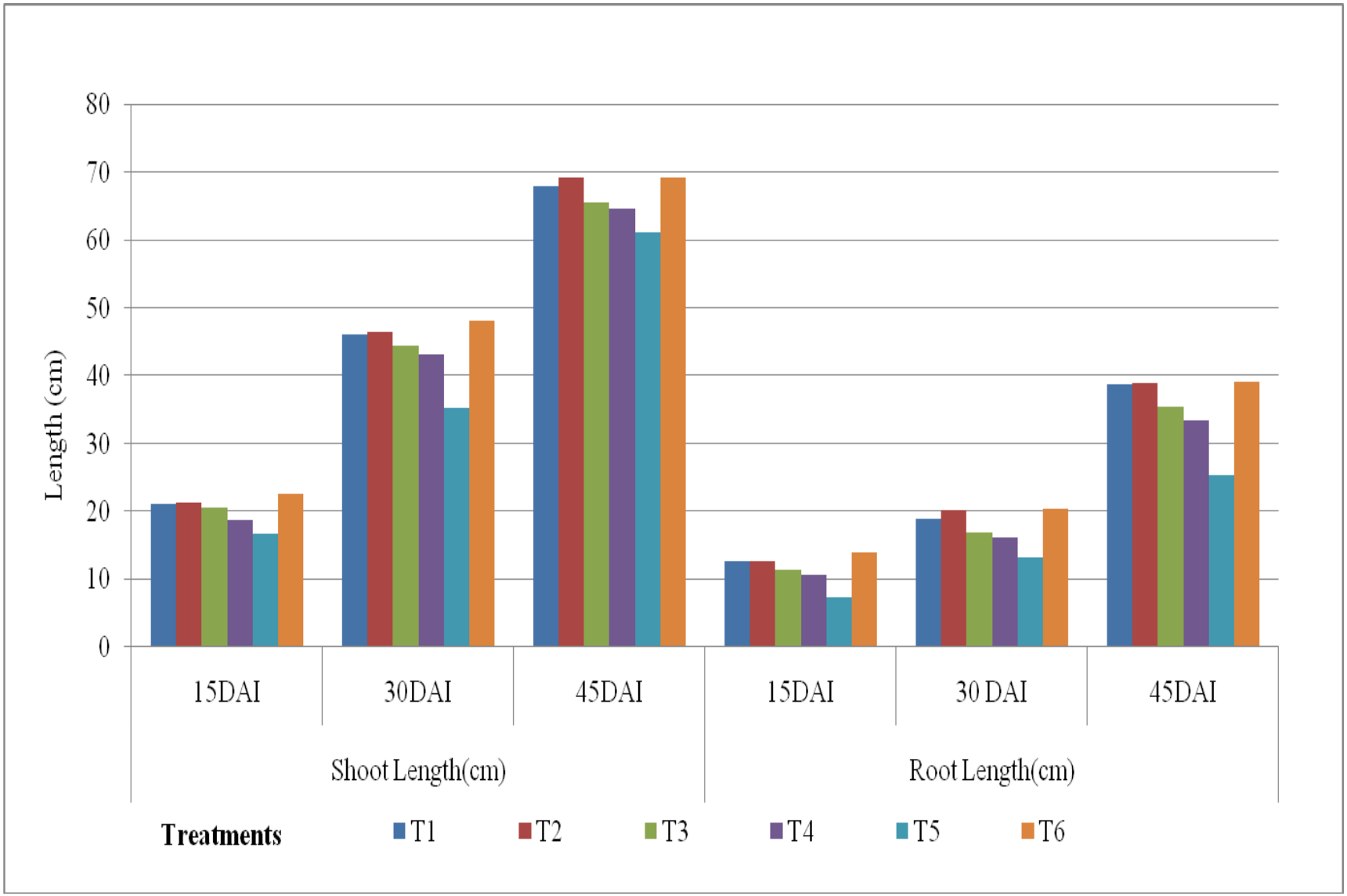


Fig.6 Effect of fungal bioagents on fresh shoot and root weight of tomato infected by *M. incognita* after 15, 30 and 45DAI

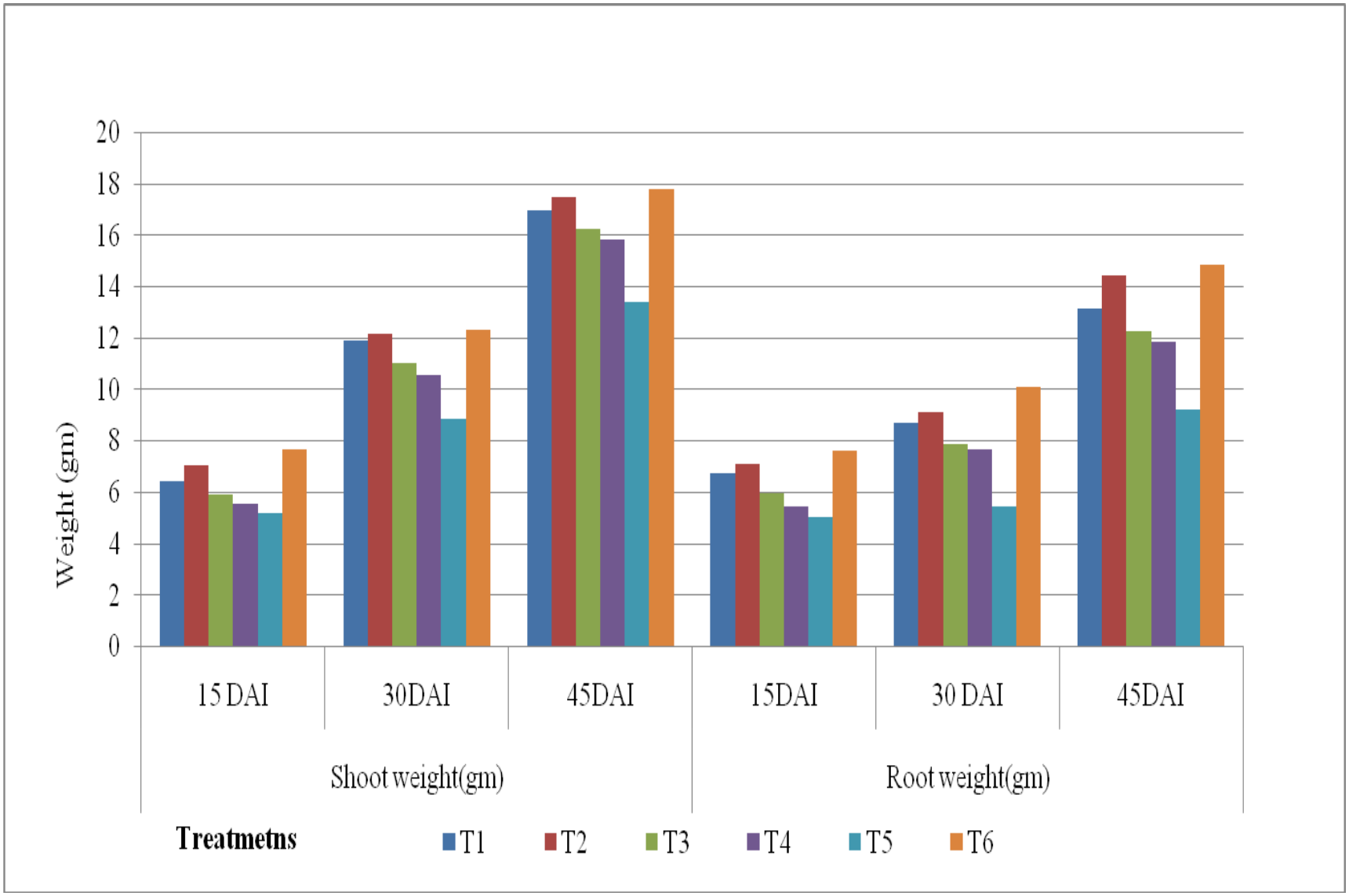


Fig.7 Effect of fungal bioagents on nematode multiplication of *M. incognita* on tomato after 30DAI

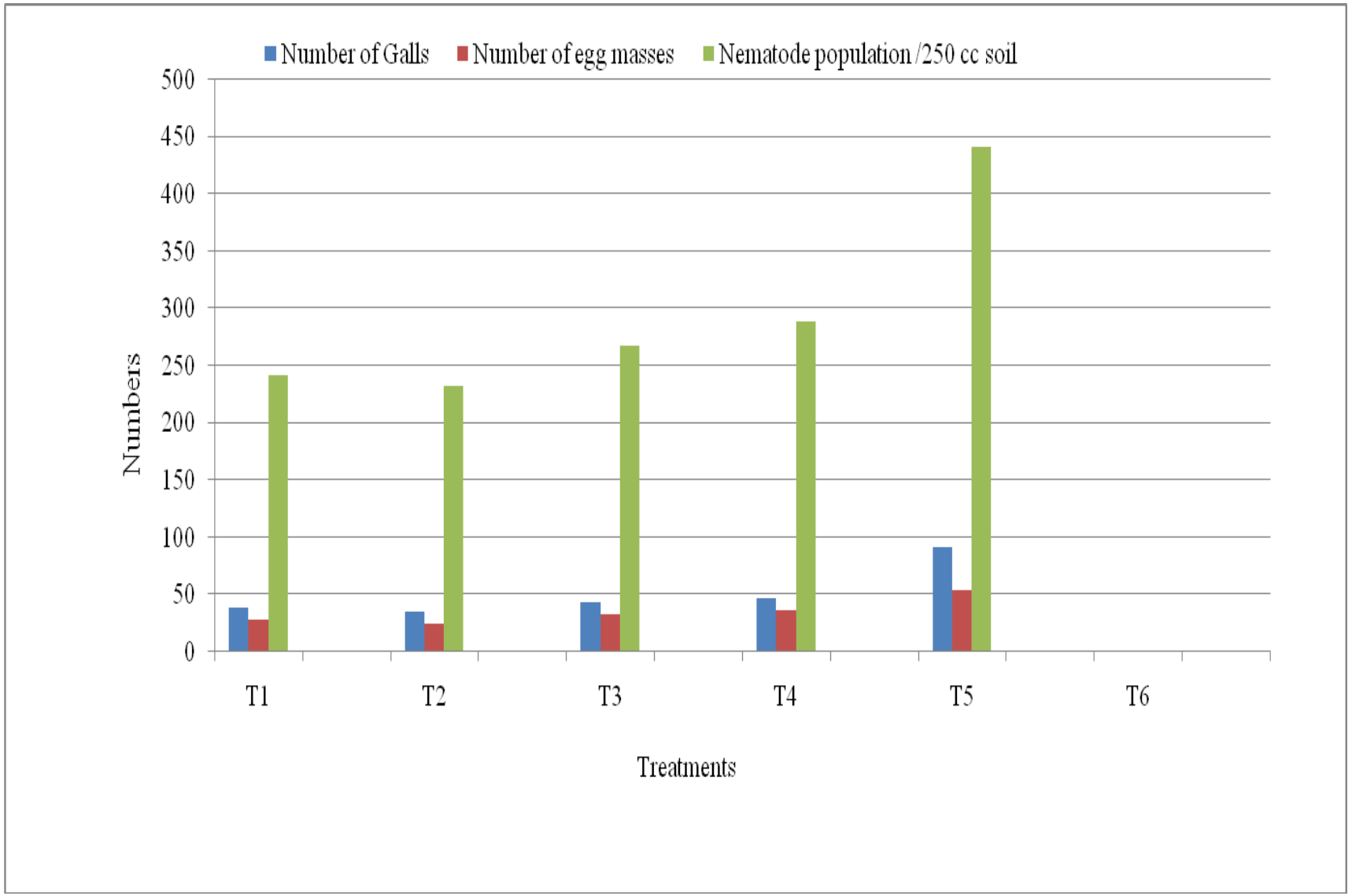
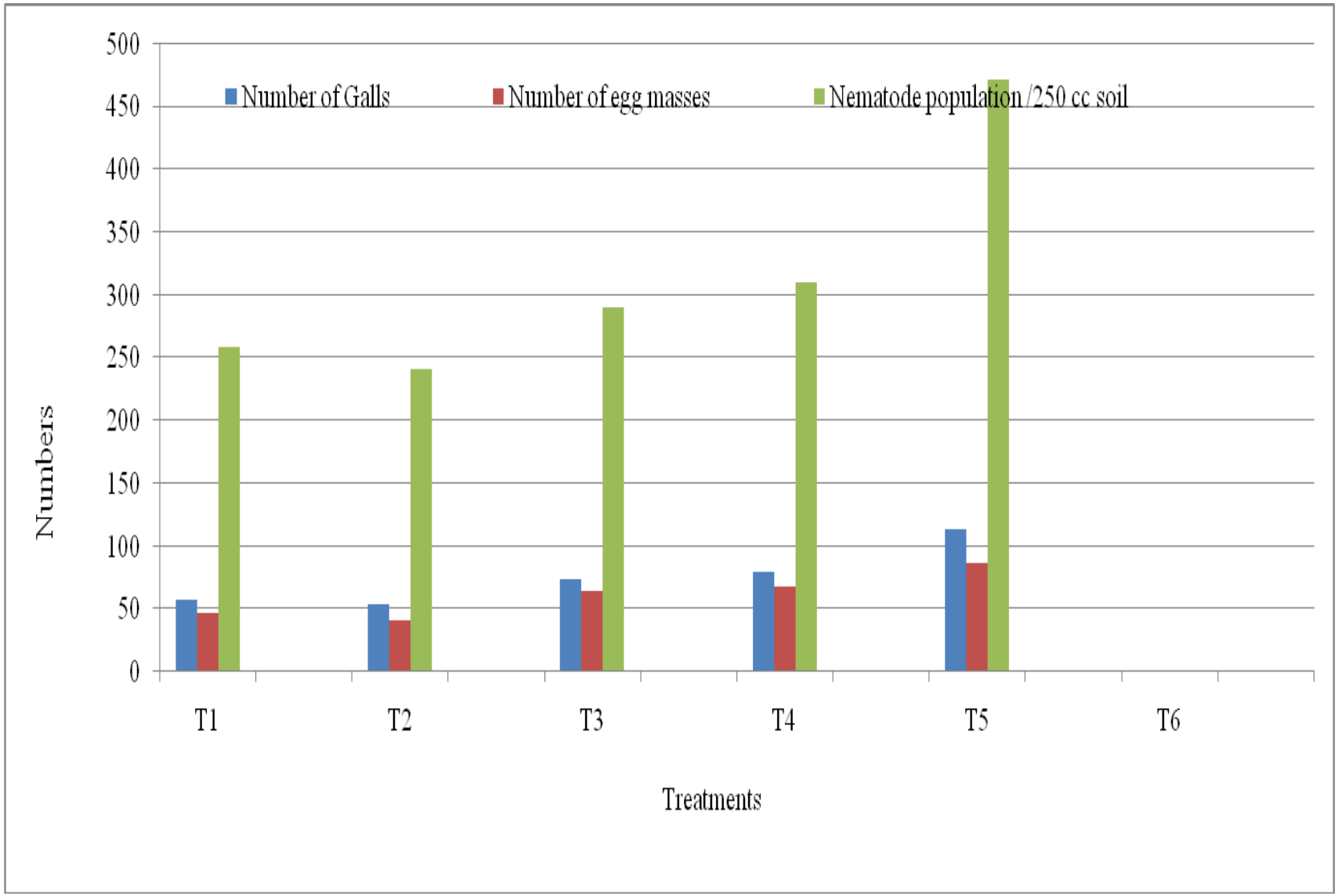


Fig.8 Effect of fungal bioagents on nematode multiplication on tomato infected by *M. incognita* after 45 DAI



Some fungal bioagents including *Trichoderma* spp. are more rhizopsheric competent and have their direct influence on either plants growth or induction of plant defensive activity against pathogens (Shoresh *et al.,* 2010, Hermosa *et al.,* 2012, Brotman, 2013). Naserinasab *et al.,* (2012) observed that application of *Trichoderma* spp found to be improve the plant growth parameters through increases in the enzymatic activities in treated *Lycopersicon esculentum* which ultimately reduced the biotic potentiality of plant parasitic nematode, *M. incognita* (Siddiqui and Akhtar, 2009) and support the result of the present investigation. All the treatments with fungal bioagents significantly reduced the number of galls and egg masses per root system and final nematode population in soil as compared to control (nematode alone) at 30 and 45 DAI (Table 3). The minimum number of galls and egg masses per root system and final nematode population in soil was recorded in the treatment T_2 (*T. harzianum*) and maximum was recorded in T_5 (nematode alone) at 30 and 45 DAI. Among the tested bioagents, *T. harzianum* was found to be most effective in reducing the nematode infection and multiplication followed by *T. viride*, *P. chalmydosporia* and *P. lilacinum* (Table 3; Figure 7 and 8). The fungal bioagent *T. harzianum* showed their biofefficay against *M. incongita* in respect of reducing their reproduction rate as compared to untreated control (Khan and Haque, 2011). However, the similar result also reported by Deepa *et al.,* (2014) who recorded the reduction in final nematode population in citrus plants treated with *T. harzianum*, *T. viride* and *P. lilacinum*. Similarly, Lal and Rana (2013) recorded lowest number of galls, egg masses and final nematode population of *M. incognita* in tomato plants treated with *T. harzainum* as seed treatment and/or soil application. The reason behind in increase in the plant growth parameters of tomato and decrease in the nematode multiplication on the host and in the soil in present investigation is might be due to that all the tested bioagents have ability to showed increased in the PO, PPO, PAL activity and total phenol content after 15, 30 and 45 DAI and it indicates that all the tested bioagents have capacity to induce resistance mechanism through release of such biochemicals which showed antagonistic activity toward pathogen *M. incognita* and enhanced the plant growth parameter as compared to control. Among the tested bioagents, *T. harzianum* was found to be more virulence in term of release of biochemicals *viz.*, PO, PPO, PAL and total phenol content in inoculated tomato plant which results in increased plant growth parameters and decreased nematode multiplication on tomato and in the soil.

Hence, the study revealed that the tested native fungal bioagents like *Trichoderma viride*, *T. harzianum*, *Pochonia chlamydosporia* and *Purpureocillium lilacinum* has ability in the improvement of plant growth parameters of tomato and decrease in the nematode multiplication in soil by release of defense enzymes like PO, PPO, PAL and total phenol content in tomato root against infected pathogen *M. incognita*.

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