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Induction of Defence Enzymes using Bio-Agents in Tomato Infected with Root-Knot Nematode, *Meloidogyne incognita*

B.S. CHANDRAWAT[1]*, A.U. SIDDIQUI[1], S.S. BHATI[1] AND VINOD SAHARAN[2]

[1]Department of Nematology, [2]Department of MBBT, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur-313001 (Rajasthan), India
**Corresponding author;* E-mail:brijrcanemat@gmail.com

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ABSTRACT: An experiment was carried out in root-knot nematode infested soil (2 J_2/g soil) to assess the induction of defence enzymes PO, PPO, PAL and SOD by bio-agents against *Meloidogyne incognita* in a susceptible variety of tomato. Bio-agents *Trichoderma viride, T. harzianum, Pochonia chlamydosporia* and *Paecilomyces lilacinus* each @ 2g and 4g per kg soil and *Pseudomonas fluorescens* @ 4g/kg soil (standard check) were added to soil. Application of bio-agents increased the level of PO, PPO, PAL and SOD in tomato roots. Among all the treatments application of *T. viride* and *P. fluorescens* @ 4g/kg soil were found to be the best treatments to enhance enzymatic activity. Among enzymes the PO was found highest in tomato roots during different times of observation followed by SOD, while PPO and PAL were observed in low quantity. The enzyme activity showed gradual increase from 7 DAI onwards to 28 DAI in plant roots treated with bio-agents as compared to untreated ones. The lowest enzyme activity was recorded at 60 DAI in untreated control plant roots. *T. viride* @ 4g/kg soil was found to improve plant growth of tomato as also reducing number of galls per plant (20.50), number of egg masses per plant (14.17), number of eggs per egg mass (80.00), nematode population/200cc soil (180.33) and total nematode population (2036.00) in tomato and was at par with *P. fluorescens*.

Key words: Bio-agents, defence enzymes, root-knot nematode, tomato

Among plant parasitic nematodes, the root-knot nematodes are a severe constraint and causing major economic damage to vegetable production including tomato around the world (Anwar *et al*., 2007; Williamson and Hussey, 1996). The formation of galls by root-knot nematode grossly affect nutrient partitioning and water uptake in the host (Anwar and Van Gundy, 1993) thus affecting yield of the crop (Reddy, 1985 and Anwar and McKenry, 2012). In order to obtain effective control, nematicides are often applied at higher doses, which may be costly, uneconomical and phyto-toxic and may cause residue problems which may create ecological disturbance in the nature. However, the use of nematode resistant varieties remains the most viable option.

Plant resistance is one of the eco-friendly options for the management of nematode diseases. A series of biochemical and physical reactions occur in plants in response to root-knot nematode infection. Plants synthesize certain compounds that are toxic to root-knot nematode. Resistance is usually associated with hypersensitive reaction (HR), a rapid and localized cell death in the infected plant in response to nematode attack. Reactive oxygen species (ROS) play an important role in plant defence and during pathogen attack, levels of ROS detoxifying enzymes like peroxidase (PO) and catalase (CAT) are often suppressed in resistant plants (Klessing *et al.*, 2000). As a result, plants produce more ROS and accumulation of these components leads to HR in plant cells. For example, hydrogen peroxide plays a major role in triggering HR in incompatible interactions. Antioxidant enzymes such as PO, PAL, PPO, SOD, POD and CAT are considered to be the main protective enzymes engaged in the removal of free radicals and activated oxygen species (Blokhina *et al.*, 2003, Devi *et al.*, 2000; Chawla *et al.*, 2013). Oxidative enzymes such as PO, PAL, PPO, SOD and CAT are reported to be involved in the mechanism of disease resistance.

So far substantial work has been done on various aspects of *M. incognita* on tomato, however, there is not much information available on role of bio-agents in determining the defence enzymes against root-knot nematode, *M. incognita*, thus, the present investigation was undertaken.

MATERIAL AND METHODS

The studies were conducted at Rajasthan College of Agriculture, MPUAT, Udaipur. The experiment was laid out in pot filled with root-knot nematode infested soil having 2 J_2/g of soil obtained from the pure culture plots of Department of Nematology, RCA, Udaipur. Utmost care was taken right from sowing to till harvest of the experiment for proper growth and development of plants.

The experiment was carried out in 6" earthen pots filled with 1 kg steam sterilized soil inocultaed with *M. incognita* @ 2 J2/g soil. The inoculation of J2 was done on one month old seedling of tomato (1 seedling/pot). Talc–based formulations of *T. viride*, *T. harzianum, P. chlamydosporia* and *P. lilacinus* were added to soil each @ 2g and 4g per kg soil. Each treatment was replicated three times. Untreated and standard check (*Pseudomonas fluorescens* @ 4g/kg soil) was also maintained for comparison. So in all there were ten treatments.

Assessment of the induction of defence enzymes, peroxidase, polyphenol oxidase, phenylalanine lyase and superoxide dismutase by bio-agents against *M. incognita* in a susceptible variety of tomato was done on every 7days interval after transplanting (7, 14, 21, 28 and 60) days after transplanting. The experiments were harvested 60 days after transplanting. Observation on enzyme analysis and various growth parameters *viz*., fresh root and shoot weight, shoot and root length were recorded whereas for studying nematode infestation, the plant tissues were stained in 0.1% acid fuchsin in lacto phenol at 80ºC for 2-3 minutes (McBeth *et al.*, 1941). Then after gentle wash, roots were kept in clear lactophenol for 24 hrs and then examined under microscope for nematode infection and observation of number of galls/plant, number of egg masses/plant and number of eggs/egg mass were recorded. Final soil population/200 cc soil and total population were also calculated.

ENZYME ANALYSIS

Determination of peroxidase (PO) enzymes in tomato roots

The method proposed by Hammerschmidt *et al.* (1982) was adopted for assaying the activity of peroxidase (EC 1.11.1.7). A 20% homogenate was prepared in 0.1M phosphate buffer (pH 7.0) from the root parts of the plant. The homogenate was centrifuged at 16,000 rpm at 4°C for 15 min and the supernatant was used for the assay. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 per cent 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

Determination of polyphenol oxidase (PPO) enzymes in tomato roots

Polyphenol oxidase (EC 1.10.3.1) activity was estimated simultaneously by the method of Mayer *et al.*, (1965). The enzyme extract was prepared by homogenizing 0.5 g of plant tissue in 2.0 ml of the extraction medium 0.1M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 16,000 rpm for 15 min at 4 °C and the supernatant was used for the assay. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 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.

Determination of phenylalanine ammonia lyase (PAL) enzymes in tomato roots

The activity of phenylalanine ammonia lyase (EC 4.3.1.5) was measured following the method of Dickerson *et al*., (1984). Root samples (1g) 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 g of insoluble polyvinyl pyrrolidine. The extract was filtered through cheese cloth and the filtrate was 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. A sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 m-1. Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} protein

Determination of super oxide dismutase (SOD) enzymes in tomato roots

SOD was assayed according to the method of Beauchamp and Fridovich (1971). The activity of SOD (EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of NBT. The roots (0.5g) were homogenized with 3.0ml of potassium phosphate buffer, centrifuged at 2000 rpm for 10 minutes and the supernatants were used for the assay. The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 1.0 mM EDTA and 20 µl enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes 30 cm below 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with black cloth. Non-illuminated tubes served as control. The absorbance at 560 nm was read. One unit of SOD is the amount of extracts that gives 50% inhibition rate of NBT reduction.

RESULTS AND DISCUSSION

The enzymatic activity of selected bio-agents was assayed in tomato roots infested with the root- knot nematode *M. incognita*. Application of bio-agents increased the level of PO, PPO, PAL and SOD in tomato roots. Among all the treatments application of *T. viride* and *P. fluorescens* @ 4g/kg soil was found to be the best treatments to enhance enzymatic activity. Among enzymes, the PO was found highest in tomato roots during different times of observations followed by SOD, while PPO and PAL were observed in low quantity. The enzyme activity showed gradual increase from 7 DAI onwards to 28 DAI in plant roots treated with bio-agents as compared to untreated ones. The lowest enzyme

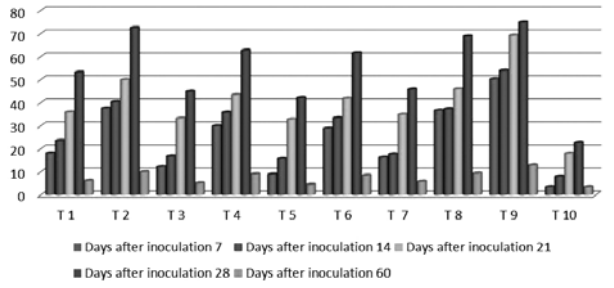

Fig. 1. Effect of bio-agents on peroxidase activity in tomato roots infected with *M. incognita*

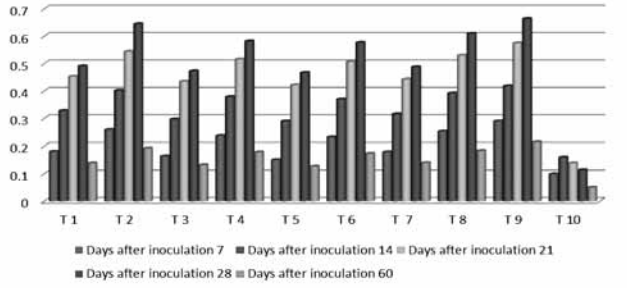

Fig. 2. Effect of bio-agents on polyphenol oxidase activity in tomato roots infected with *M. incognita*

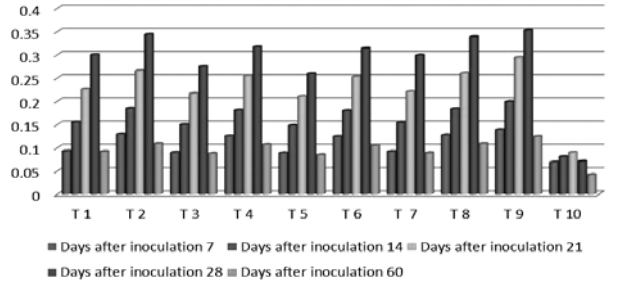

Fig. 3. Effect of bio-agents on phenylalanine ammonia lyase activity in tomato roots infected with *M. incognita*

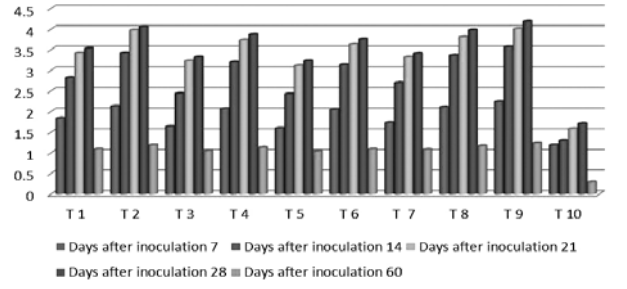

Fig. 4. Effect of bio-agents on super oxide dismutase activity in tomato roots infected with *M. incognita*

Table 1: Effect of bio-agents on plant growth characters of tomato infected with *M. incognita*

Treatments		Shoot length (cm)			Shoot weight (g)			Root length (cm)			Root weight (g)		
		Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled
T 1	*T. viride* @ 2 g/kg soil	28.57	28.43	28.50	27.40	27.17	27.28	34.40	34.60	34.50	24.23	24.13	24.18
T 2	*T. viride* @ 4 g/kg soil	34.13	34.38	34.26	34.90	35.00	34.95	43.17	43.48	43.33	29.43	29.67	29.55
T 3	*T. harzianum* @ 2 g/kg soil	27.03	26.90	26.97	25.57	25.30	25.43	31.03	31.18	31.11	22.20	22.33	22.27
T 4	*T. harzianum* @ 4 g/kg soil	31.17	31.39	31.28	32.20	32.83	32.52	41.27	41.40	41.33	26.67	26.83	26.75
T 5	*P. chlamydosporia* @ 2 g/ kg soil	26.27	26.42	26.34	23.27	23.48	23.38	29.93	30.08	30.01	20.37	20.87	20.62
T 6	*P. chlamydosporia* @ 4 g/ kg soil	30.93	30.80	30.87	29.77	29.50	29.63	39.07	38.90	38.98	25.67	25.98	25.83
T 7	*P. lilacinus* @ 2 g/kg soil	27.60	27.80	27.70	26.37	26.37	26.37	32.20	32.30	32.25	23.87	24.05	23.96
T 8	*P. lilacinus* @ 4 g/kg soil	32.53	32.90	32.72	32.83	33.07	32.95	42.60	42.42	42.51	27.51	27.63	27.57
T 9	*P. fluorescens* (Standard check) @ 4 g/kg soil	36.93	36.82	36.88	36.43	36.25	36.34	44.90	45.10	45.00	30.18	30.38	30.28
T 10	Control	10.37	10.22	10.29	4.50	4.27	4.38	8.37	8.58	8.48	2.50	2.67	2.59
SEm±		0.212	0.234	0.220	0.25	0.235	0.22	0.22	0.275	0.24	0.19	0.197	0.17
CD at 5%		0.624	0.689	0.648	0.743	0.695	0.660	0.653	0.811	0.714	0.546	0.582	0.509
CV		1.283	1.414	1.331	1.60	1.49	1.42	1.11	1.37	1.21	1.38	1.46	1.28

Note: (1) Data are average value of three replications, (2) Initial inoculums level 2 J2/g soil.

Table 2: Effect of bio-agents on nematode reproduction of tomato infected with *M. incognita*

Treatments	No. of galls/ plant			No. of egg masses / plant			No. of eggs and larvae / egg mass			Larval population / 200cc soil			Total population		
	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled
T 1	25.33	26.33	25.83	21.67	21.00	21.33	102.67	100.33	101.50	232.33	227.33	229.83	3389.67	3247.33	3318.50
T 2	20.33	20.67	20.50	13.67	14.67	14.17	79.33	80.67	80.00	182.00	178.67	180.33	1994.67	2077.33	2036.00
T 3	28.33	29.00	28.67	23.67	24.67	24.17	114.00	117.33	115.67	296.00	302.00	299.00	4180.00	4405.33	4292.67
T 4	22.67	21.67	22.17	16.33	17.00	16.67	95.00	93.67	94.33	206.00	199.00	202.50	2582.67	2578.67	2580.67
T 5	32.33	31.67	32.00	26.33	27.33	26.83	127.67	129.00	128.33	308.33	312.00	310.17	4905.67	5087.00	4996.33
T 6	24.67	24.33	24.50	19.33	19.00	19.17	101.00	97.00	99.00	219.00	215.33	217.17	3048.33	2918.33	2983.33
T 7	26.67	25.33	26.00	22.00	21.67	21.83	106.33	105.00	105.67	271.67	269.00	270.33	3697.00	3620.33	3658.67
T 8	22.33	22.00	22.17	15.67	14.67	15.17	83.67	82.33	83.00	195.00	191.67	193.33	2286.00	2166.00	2226.00
T 9	18.67	18.00	18.33	9.33	8.67	9.00	70.67	68.67	69.67	160.33	158.00	159.17	1461.00	1384.67	1422.83
T 10	61.67	63.33	62.50	44.67	47.33	46.00	209.67	211.33	210.50	981.00	989.00	985.00	14274.33	14955.00	14614.67
SEm±	0.73	0.73	0.60	0.98	0.97	0.81	1.59	1.86	1.45	1.57	3.14	2.04	154.53	160.54	123.51
CD at 5%	2.16	2.14	1.77	2.88	2.85	2.39	4.69	5.48	4.28	4.64	9.28	6.01	455.85	473.59	364.36
CV	4.47	4.45	3.68	7.95	7.75	6.54	2.52	2.97	2.31	0.89	1.79	1.16	6.40	6.55	5.08

Note:(1) Data are average value of three replications, (2) Initial inoculums level 2 J2/g soil.

activity was recorded at 60 DAI in untreated control plant roots. (Fig. 1, 2, 3 & 4).

Among all the treatments application of *T. viride* @ 4g/kg soil was found to be the best treatments to improve plant growth characters of tomato as well as in reducing number of galls per plant (20.50), number of egg masses per plant (14.17), number of eggs per egg mass (80.00), nematode population/200cc soil (180.33) and total nematode population of *M. incognita* (2036.00) in tomato. However, standard check *P. fluorescens* @ 4g/kg soil was found to be superior in improving plant growth as well as in reducing nematode population (Table 1 & 2).

Application of a talc-based formulation of bio-agents as soil treatment significantly reduced the root-knot nematode population in tomato roots. This is in agreement with the report that the level of infestation of *M. incognita* on tomato was reduced with fewer galls and egg masses in the root following root dipping with *P. fluorescens* strain (Pf l) (Santhi and Sivakumar, 1995). The studies on induced defence mechanisms revealed significant accumulation of PO, PPO, PAL and SOD in treated tomato plants inoculated with *M. incognita*. Accumulation of these enzymes began seven days after inoculation with the nematode and gradually increased up to 28 days. Among all the treatments application of *T. viride* @ 4g/kg soil was found to be the best treatments to enhance enzymatic activity. However, standard check *P. fluorescens* @ 4g/kg soil was found equally effective to increase the enzyme activity against *M. incognita* in tomato roots. Chen *et al.* (2000) reported greater activities of PO and PPO in cucumber root tissues treated with *Pseudomonas corrugate* challenged with *P. aphanidermatum*. Anita *et al*. (2004) studied accumulation of defence enzymes, *viz.* peroxidase, polyphenol oxidase, chitinase, phenylalanine ammonia lyase and catalase, in tomato root tissue treated with *P. fluorescens* isolate Pfl in response to invasion by the root knot nematode *M. incognita*. Activities of all the enzymes were significantly higher in bacterized tomato root tissues. Duan *et al.* (2011) showed that the *A. niger* could reduce the root-knot index and nematode populations, compared with untreated control in tomato. The activities of defense enzymes were enhanced significantly, such as phenylalanine ammonia, polyphenol oxidase, peroxidase, superoxide and catalase. Mohamed and Abo-Elyousr (2011) proved that application of different bio-control agents (*P. fluorescens, P. lilacinus* and *P. guilliermondii*) not only had a lethal effect on nematode, but also enhanced the plant growth, supplying many nutritional elements and induced the systemic resistance in plants.

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