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Bio-agents: A source for initiation of defence enzymes in chilli infected with root-knot nematode, *Meloidogyne incognita*

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Abstract
The plant parasitic nematodes are major problem in chilli. Out of these, root-knot nematodes have been considered as severe constraint to chilli production. Root knot nematode particularly *Meloidogyne incognita* is widely distributed and ranked most destructive pathogen on vegetable crops in the world. Nematode infection in plant roots cause stress and in response to the infection a series of biochemical and physical reactions occur in plants. 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. As a result, plants produce more ROS and accumulation of these components leads to HR in plant cells. Oxidative enzymes such as PO, PAL, PPO, SOD and CAT are reported to be involved in the mechanism of disease resistance. This research aimed to examine the effect of the application of bio-agents to the induction of defence enzymes peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and super oxide dismutase **(**SOD) against *M. incognita* in chilli. This research was conducted with Completely Randomized Designs (CRD) using bio-agents *Trichoderma viride*, *Trichoderma harzianum*, *Pochonia chlamydosporia* and *Paecilomyces lilacinus* each @ 2g and 4g per kg and *Pseudomonas fluorescens* @ 4g/kg were added to soil. The results showed that the level of PO, PPO, PAL and SOD was increased in chilli roots with the application of all bio-agents as compared to untreated control but *P. fluorescens* and *T. viride* @ 4g/kg soil was found to be the best treatments. The level of PO and SOD were found highest while, PPO and PAL were found lowest in chilli roots during different time of observations i.e. 7th, 14th, 21th, 28th and 60th DAT. The enzyme activity showed gradual increase till 28 DAT in treated roots as compared to untreated ones but gradually decrease at 60 DAT. Among all the treatments application of *P. fluorescens* and *T. viride* @ 4g/kg soil was found to be the best treatments to improve plant growth and decrease number of galls and egg masses per plant, number of eggs per egg mass, nematode population/200cc soil and total nematode population over other treatment.

Keywords: Chilli, root-knot nematode, bio-agents, defence enzymes

Introduction
Chilli (*Capsicum frutecens*) belongs to family Solanaceae. Chilli is grown for its pungent fruits, which are used both green and ripe red form (the latter in the dried form) to add pungency and taste to the food. A large number of chilli varieties are available in India and most of them have gained popularity and are under commercial cultivation. Its high demand makes it a commercial commodity. The conditions required for growing these crops also suites to many pathogens. Hence the production of chilli is tremendously reduced by pest and diseases which are considered as major biological constraints to low productivity. The diseases caused by fungus, bacteria, virus, insect pests, nematodes and poor crop management specially lack of crop rotation practice opportunities due to small land holdings.

Among the plant parasitic nematodes root-knot nematode (*Meloidogyne incognita*), reniform nematode (*Rotylenchulus reniformis*), lesion nematode (*Pratylenchus penetrans*) and stunt nematode (*Tylenchorhynchus brassicae*) are major problem. Out of these, root-knot nematodes have been considered as severe constraint. Root knot nematode particularly *M. incognita* is widely distributed and ranked most destructive pathogen on vegetable crops in the world [1, 2, 3, 4]. These are endo-parasitic migratory-vascular feeders where during feeding they induce the formation of “galls” as well as the development of “giant cells" on the roots of their hosts. These alterations grossly affect nutrient partitioning and water uptake in the host [5]. Crop losses due to nematode infection estimated up to 46 per cent [6, 7].

12.2 % loss recorded in chilli crop [8] by plant parasitic nematodes. A national loss due to this nematode pest in chilli was worked out to be 12.85 per cent and in monetary terms has been worked out to the tune of 210 million rupees [9]. Several control strategies, such as host plant resistance, rotation with non-host crops, sanitation, destruction of residual crop roots and discriminating use of nematicides, have been reported to effectively keep the root knot nematode population below damaging threshold level [10].

Uses of nematicides although proved to be effective, quick in action, more reliable, easily adoptable and widely used at present time. In order to obtain effective control, nematicides are often applied at higher doses, which may be costlier, uneconomical, phyto-toxic and may cause residue problems which may create ecological disturbance in the nature. 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 [11]. 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 [12, 13, 14]. While, 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 chilli. However, there is not much information available on determining the role of bio-agents on defence enzymes activity in chilli plants against root-knot nematode, *M. incognita*. Keeping this in view, the present investigations were undertaken to assess the potential of bio-agents against root-knot nematode *M. incognita* to evolve eco-friendly and economically faceable methods for the management of nematodes in chilli.

Materials and Methods

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, Rajasthan College of Agriculture, Udaipur. Utmost care was taken right from sowing to till harvest of experiment for proper growth and development of plants.

I. Pots filling and transplanting

6" size earthen pots were washed, cleaned and disinfected before use by rinsing them with 4 per cent formalin solution. Pots were filled with 1 kg root knot nematode infested soil. Some space (0.5-1.0") from the above was left unfilled for watering. Talc–based formulation of *T. viride*, *T. harzianum*, *P. chlamydosporia* and *P. lilacinus* each were added @ 2g and 4g per kg soil and *Pseudomonas fluorescens* @ 4g/kg soil. Each treatment was replicated three times. Uniform size chilli seedlings were transplanted in pots.

II. Harvest

Assessment of the induction of defence enzymes PO, PPO, PAL and SOD by bio-agents against *M. incognita* in chilli roots was done on 7th, 14th, 21th, 28th and 60th day after transplanting. The plants were uprooted completely after 60 day after transplanting. Observation on enzyme analysis and various growth parameters *viz*., fresh root and shoot weight, shoot and root length were recorded without delay whereas for studying nematode infestation, the plant tissues were stained in 0.1% acid fuchsin in lacto phenol at 80ºC for 2-3 minutes [15]. Then after gentle wash, roots were kept in clear lacto phenol 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.

III. Estimation of soil population

The soil samples (200 cc) collected from the experimental pots were processed using Cobb's sieving and decanting technique [16] followed by Baermann's funnel technique [17]. After 24 hrs the suspension was drawn in a beaker. The volume of suspension was made to 100 ml and then after thoroughly bubbling 10 ml of suspension was drawn with the help of a pipette and poured over a counting dish for counting. Population count was done under a stereoscopic binocular microscope.

IV. Enzyme analysis

1. Determination of peroxidase (PO) enzymes in chilli roots.

The method proposed by Hammerschmidt *et al*., (1982) [18] 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.

2. Determination of polyphenol oxidase (PPO) enzymes in chilli roots

Polyphenol oxidase (EC 1.10.3.1) activity was estimated simultaneously by the method of Mayer *et al.*, (1965) [19]. 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.

3. Determination of phenylalanine ammonia lyase (PAL) enzymes in chilli roots

The activity of phenylalanine ammonia lyase (EC 4.3.1.5) was measured following the method of Dickerson *et al*., (1984) [20]. 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.

4. Determination of super oxide dismutase (SOD) enzymes in chilli roots

SOD was assayed according to the method of Beauchamp and Fridovich (1971) [21]. 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 3.0 ml 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 the rate of NBT reduction.

Results and Discussion

1. Plant growth characters and nematode reproduction

Data presented in table- 1& 2 showed that all bio-agents significantly increased the plant growth characters of chilli and decrease root-knot nematode reproduction as compared to untreated check. Among all the treatments application of *T. viride* @ 4g/kg soil was found to be the best treatments followed by *P. lilacinus* and *T. harzianum* @ 4g/kg soil to improve plant growth characters of chilli [*i.e.* shoot length (20.07 cm, 19.25 cm, 18.05 cm), shoot weight (9.05 g, 7.82 g, 6.43 g), root length (33.95 cm, 32.00 cm, 30.91 cm) and root weight (28.55 g, 27.30 g, 26.14 g)]. As regard to nematode reproduction, *T. viride* @ 4g/kg soil proved best treatment in reducing number of galls per plant (21.67), number of egg masses per plant (12.67), number of eggs per egg mass (81.67), nematode population/200cc soil (114.83) and total nematode population of *M. incognita* (1610.50). However, standard check *P. fluorescens* @ 4g/kg soil was found the best treatment to improving plant growth characters as well as in reducing nematode population over other treatment.

Table 1: Effect of bio-agents on plant growth characters of chilli 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	13.97	14.20	14.08	5.42	5.52	5.47	27.38	27.47	27.43	23.70	23.83	23.77
T 2	*T. viride* @ 4 g/kg soil	20.20	19.93	20.07	9.17	8.93	9.05	33.80	34.10	33.95	28.43	28.67	28.55
T 3	*T. harzianum* @ 2 g/kg soil	12.60	12.78	12.69	4.63	4.53	4.58	24.87	25.03	24.95	21.10	21.30	21.20
T 4	*T. harzianum* @ 4 g/kg soil	17.97	18.13	18.05	6.37	6.50	6.43	30.98	30.83	30.91	26.27	26.02	26.14
T 5	*P. chlamydosporia* @ 2 g/kg soil	12.10	12.00	12.05	4.42	4.33	4.38	23.53	23.40	23.47	19.50	19.35	19.43
T 6	*P. chlamydosporia* @ 4 g/kg soil	16.13	16.00	16.07	5.90	6.02	5.96	29.95	29.78	29.87	25.13	25.00	25.07
T 7	*P. lilacinus* @ 2 g/kg soil	13.23	13.37	13.30	5.10	5.18	5.14	25.93	26.10	26.02	22.80	22.97	22.88
T 8	*P. lilacinus* @ 4 g/kg soil	19.13	19.37	19.25	7.77	7.87	7.82	32.10	31.90	32.00	27.37	27.23	27.30
T 9	*P. fluorescens* (Standard check) @ 4 g/kg soil	22.50	23.13	22.82	10.37	10.52	10.44	36.13	36.47	36.30	29.65	29.93	29.79
T 10	Control	5.83	5.70	5.77	2.67	2.60	2.63	10.43	10.30	10.37	2.98	2.83	2.91
SEm±		0.191	0.187	0.175	0.11	0.077	0.08	0.33	0.278	0.30	0.27	0.263	0.26
CD at 5%		0.565	0.551	0.517	0.335	0.226	0.235	0.966	0.821	0.878	0.799	0.776	0.779
CV		2.158	2.091	1.968	3.18	2.14	2.23	2.06	1.75	1.87	2.07	2.01	2.01

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 chilli infected with *M. incognita*

Treat-ments	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	29.33	28.67	29.00	19.67	21.00	20.33	119.00	121.67	120.33	187.33	189.00	188.17	3274.33	3502.33	3388.33
T 2	22.00	21.33	21.67	12.33	13.00	12.67	81.00	82.33	81.67	115.67	114.00	114.83	1579.33	1641.67	1610.50
T 3	32.67	33.00	32.83	21.00	22.00	21.50	123.00	126.33	124.67	208.33	212.00	210.17	3624.67	3840.67	3732.67
T 4	24.67	23.67	24.17	15.67	15.00	15.33	96.67	95.00	95.83	126.67	125.67	126.17	2150.67	2054.67	2102.67
T 5	34.67	35.33	35.00	22.67	23.33	23.00	128.67	130.67	129.67	222.33	219.33	220.83	4029.33	4145.00	4087.17
T 6	27.00	27.67	27.33	17.33	18.67	18.00	110.33	108.33	109.33	129.67	131.00	130.33	2560.00	2674.67	2617.33
T 7	31.00	30.33	30.67	20.33	19.67	20.00	121.67	124.33	123.00	195.00	193.33	194.17	3448.00	3412.00	3430.00
T 8	23.33	24.00	23.67	14.00	13.00	13.50	86.67	85.33	86.00	119.33	120.67	120.00	1810.33	1714.67	1762.50
T 9	20.33	19.67	20.00	8.67	8.00	8.33	72.67	71.33	72.00	107.33	106.00	106.67	1166.33	1100.33	1133.33
T 10	50.67	52.00	51.33	35.33	36.33	35.83	203.67	206.33	205.00	876.67	882.00	879.33	11579.67	11908.00	11743.83
SEm±	0.70	0.82	0.73	0.81	0.95	0.81	1.14	1.38	0.94	1.31	1.87	1.53	107.19	123.39	105.75
CD at 5%	2.06	2.43	2.15	2.39	2.80	2.39	3.37	4.07	2.78	3.86	5.51	4.50	316.20	364.01	311.97
CV	4.10	4.83	4.27	7.52	8.65	7.45	1.73	2.07	1.42	0.99	1.41	1.15	5.27	5.94	5.14

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

Table 3: Effect of bio-agents on peroxidase (PO), poly phenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and super oxide dismutase (SOD) activity in chilli roots infected with *M. incognita*.

Specific activity of Enzymes (umol/min/gm)																				
DAI	7	14	21	28	60	7	14	21	28	60	7	14	21	28	60	7	14	21	28	60
Treat-ments	**PO**					**PPO**					**PAL**					**SOD**				
T 1	12.857	31.607	45.422	55.432	8.994	0.201	0.316	0.462	0.572	0.201	0.122	0.177	0.253	0.309	0.102	1.91	2.94	3.48	3.58	1.10
T 2	27.716	41.334	55.065	73.167	23.627	0.281	0.420	0.546	0.700	0.231	0.140	0.201	0.283	0.348	0.121	2.23	3.46	3.94	4.11	1.21
T 3	11.25	30.028	41.475	47.875	7.302	0.193	0.305	0.451	0.556	0.190	0.117	0.172	0.246	0.300	0.096	1.76	2.72	3.27	3.39	1.06
T 4	23.740	38.120	51.569	70.911	21.569	0.264	0.409	0.533	0.648	0.219	0.134	0.196	0.279	0.339	0.115	2.09	3.27	3.74	3.91	1.18
T 5	10.375	28.646	40.432	45.958	6.400	0.180	0.302	0.444	0.553	0.181	0.111	0.171	0.244	0.285	0.094	1.70	2.51	3.15	3.27	1.02
T 6	21.484	37.584	47.481	69.981	19.877	0.256	0.406	0.529	0.617	0.215	0.131	0.194	0.278	0.249	0.112	2.07	3.18	3.63	3.84	1.17
T 7	11.757	30.902	43.562	51.118	8.430	0.198	0.312	0.458	0.562	0.193	0.119	0.175	0.249	0.304	0.099	1.83	2.81	3.39	3.48	1.08
T 8	25.996	39.953	53.768	71.842	22.951	0.276	0.417	0.537	0.674	0.226	0.137	0.198	0.282	0.345	0.119	2.16	3.39	3.82	4.04	1.19
T 9	33.552	67.133	70.714	77.622	33.383	0.302	0.451	0.559	0.723	0.278	0.153	0.210	0.303	0.356	0.146	2.25	3.60	4.04	4.20	1.25
T 10	4.342	7.951	20.300	31.550	5.075	0.120	0.193	0.172	0.128	0.074	0.078	0.085	0.092	0.080	0.046	1.08	1.32	1.67	1.74	0.24
SEm±	0.001	0.003	0.002	0.003	0.002	0.005	0.003	0.002	0.002	0.003	0.004	0.003	0.003	0.002	0.002	0.004	0.003	0.004	0.003	0.004
CD at 5%	0.004	0.008	0.007	0.008	0.006	0.015	0.010	0.005	0.006	0.008	0.012	0.008	0.009	0.006	0.007	0.012	0.009	0.013	0.010	0.011
CV	0.01	0.01	0.01	0.01	0.02	3.95	1.61	0.68	0.64	2.30	5.74	2.77	2.21	1.17	3.92	0.38	0.18	0.22	0.16	0.60

2. Enzyme determination

The enzymatic activity of selected bio-agents was assayed in chilli roots infested with the root- knot nematode *M. incognita.* The results showed increased level of enzyme activity in chilli plants roots treated with bio-agents as compared to untreated control. The all bio-agents was showed enhancement in enzyme activity on different dose but the maximum enzyme activity was recorded with *T. viride* followed by *P. lilacinus* and *T. harzianum* @ 4g/kg soil and *P. fluorescens* @ 4g/kg soil was at par. while, minimum was observed in untreated control plant. The level of PO and SOD were found highest while, PPO and PAL were found lowest in chilli roots during different time of observations i.e. 7th, 14th, 21th, 28th and 60th DAT. The enzyme activity showed gradual increase from 7th day after transplanting (DAT) to 28th DAT in treated roots as compared to untreated ones but it drastically decreased at 60 DAT. During the different time of observation the highest enzyme activity was showed 28th day in all treated plants as well as untreated plants but the difference can clearly see in treated and untreated plant all the time.

The result of our findings indicated that application of bio-agents as soil treatment significantly reduced the root-knot nematode *Meloidogyne incognita* population in chilli roots. Root galling due to *M. incognita* infection was less in chilli plant roots treated with bio-agents as compared to untreated inoculated control. This is in agreement with the findings that *Trichoderma viride* is an effective bioagent for the control of root-knot nematode in chilli [22]. The effective concentration of bioagents *T. viride* as seed dresser was found to be 8 g/kg seed. Studies on induced defence mechanisms revealed significant accumulation of PO, PPO, PAL and SOD in treated chilli plants infected with *M. incognita.* Accumulation of these enzymes start seven days after inoculation with the nematode and gradually increased up to 28 days then it will start decreasing. Increased enzyme activity was also found in plants inoculated with the nematode alone, but it was less as compare to treated plants. Among all the treatments application of *P. fluorescens* and *T. viride* @ 4g/kg soil was found to be the best treatments to enhance enzymatic activity against *M. incognita* in chilli roots. These results were in agreement with those of other workers who studied on enzyme analysis in nematode infected different crops treated with bio-agents. Cucumber root tissues treated with *Pseudomonas corrugate* showed greater activities of PO and PPO [23]. The activities of defence enzymes (*viz.* peroxidase, polyphenol oxidase, chitinase, phenylalanine ammonia lyase and catalase) were significantly higher in bacterized tomato root tissues challenged with the nematode [24]. Endophytic bacteria were able to suppress the number of galls and nematode population in roots of pepper. They were able to induce systemic resistance on pepper through increased salicylic acid and peroxidase contents in roots [25]. *Pseudomonas fluorescens* is an efficient biocontrol for nematode management in the chilli crops and also, it clearly indicates that biocontrol were more effective in providing a prolonged nematode attack resistance to the chilli crops [26]. The tomato plants treated with *A. niger* could reduce the root-knot index and nematode populations, compared with untreated control. The activities of defense enzymes (*viz.* phenylalanine ammonia, polyphenol oxidase, peroxidase, superoxide and catalase) were enhanced significantly [27]. 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 [28]. The level of enzymatic activity (PO, PPO, PAL and SOD) was increased in the tomato roots by application of bio-agents (*T. viride, T. harzianum, P. chlamydosporia, P. lilacinus* and *P. fluorescens*) [29]. All the bio-agents found suitable for increase plant growth character of tomato and reduce nematode population of *M. incognita* in tomato. Among all the treatments application of *Pseudomonas fluorescens* and *Trichoderma viride* was found to be the best treatments [29].

The study concludes that bio-agents are effective option for management of root knot nematode in chilli, it clearly indicates that bio-agents were more effective in increase plant growth and reduce nematode infection in plants. Bio-agents also play major role in changes the level of defence enzyme in chilli plants and providing a prolonged resistance against nematode attack to the chilli plants as compared to the control.

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