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Response of Defence Related Enzymes in Tomato Treated with Oil-Cakes against Root-knot Nematode, *Meloidogyne incognita*

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A B S T R A C T

Plants produce a spacious range of biologically active molecules which have antagonistic properties against plant parasitic nematodes. The peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and superoxide dismutase (SOD) are the most defense enzymes produce by plants during nematode infection. An experiment was conducted to assess the response of defence enzymes PO, PPO, PAL and SOD in tomato roots when treated with oil cakes (castor, mahua, karanj and mustard @ 2.5 and 5.0 q/ha and neem cake @ 5.0 q/ha as a standard check) against *M. incognita*. The enzymatic activity was assayed in tomato roots and results revealed that application of oil-cakes increased the level of defense enzymes with exposure of time. Among all, the application of mustard cake @ 5.0 q/ha was found best to enhance enzymatic activity from 7 days after inoculation onwards to 28 DAI as compared to untreated check. The lowest enzyme activity in among enzymes was recorded at 60 DAI in untreated control plant roots. Among enzymes the PO was found highest followed by SOD during different time of observations, while PPO and PAL were observed in low quantity. As regard to nematode reproduction, mustard cake @ 5.0 q/ha soil proved best treatment followed by castor and mahua @ 5.0 q/ha. However, standard check neem cake @ 5.0 q/ha was found the best treatment to increasing enzymatic activity, improving plant growth characters as well as in reducing nematode population over other treatment.

Introduction

Root-knot nematode, *Meloidogyne incognita* infects tomato crop (Alam *et al.*, 1975; Khan *et al.*, 1978) and causing major economic damage to agricultural vegetable production including tomato around the world (Sasser, 1980; Jones *et al.*, 1991; Williamson and Hussey, 1996; Fourie and McDonald, 2000; Anwar *et al.*, 2007).Damage to tomato crop due to nematodes has been documented by various workers. Bhatti and Jain (1977) and

Sharma and Baheti (1992) estimated the crop losses up to 46 per cent. Reddy (1985) estimated 39.77 per cent loss in tomato field. The tomato yield losses ranging from 32 to 40% due to root knot nematode has been reported (Anwar and McKenry, 2012). Arya (1957) found its infestation on tomato in Rajasthan.

Plant endo-parasitic nematodes, spend a major part of their life cycles embedded in the roots of a host plant and are therefore exposed to a variety of host defense responses (Jones *et al.,* 2007). Reactive oxygen species (ROS) including superoxide radical (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are often detected in plant–pathogen interactions and are associated with symptom development. Plants have acquired the relevant protective defense mechanisms to maintain the lowest possible levels of ROS inside the cell during stress circumstance (Wojtaszek, 1997). However, under stressful conditions, their formation might increase to excess the antioxidant scavenging capacity, thus creating oxidative stress by reaction and damage to all bio-molecules (Halliwell and Gutteridge, 1999). In addition, ROS are highly reactive to membrane lipids, protein and DNA. 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. Alterations of plant enzymes mainly peroxidase, poly-phenol oxidase, catalase, superoxide dismutase and protease in the tissues of nematode infected, susceptible and resistant varieties were extensively studied. Such alterations differed in susceptible and resistant cultivars (Molinari, 1995; Sharma, 1993; Zacheo *et al.,* 1987). The root-knot nematode infection increased peroxidase activity, phenol content, polyphenol oxidase activities, phenylalanine ammonia lyase, tyrosin ammonia lyase in roots of cotton, coffee, chick pea, banana and rice (Gregory and Michael, 1978; Mazzafera, 1989; Mishra and Mohanty, 2007; Patel *et al.,* 2001; Sundararaju and Suba, 2006; Xu *et al.,* 2008) In tomato, numerous reports in literature illustrate the influence of the root-knot nematode on roots phenol content, lipid peroxidation, antioxidants and peroxidase activities as defense mechanism against nematode infection (El-Sherif and El-Wakil, 1991; Kuzniak and Sklodowska, 2001). Polyphenol oxidase activity also increased by 16-24% in resistant cultivars and by 12-18% in the susceptible one (Hasan and Saxena, 1997). Likewise, peroxidase activity increased in tomato resistant cultivars up to 5 times than that in healthy plants as measured 10 days after inoculation by *M. incognita* and decreased thereafter to normal levels within few days. Also, *M. incognita* resistant varieties of tomato had significantly higher peroxidase (Shukla and Chakraborty, 1988, Zacheo *et al.,* 1993), Polyphenol oxidase (PPO) and indole acetic acid (IAA) (Nagesh *et al.,* 1998) activity than the susceptible varieties. However, few reports illustrate the role of organic matter in improving defense mechanisms of plants against the root-knot nematodes infection.

The present study was carried out to investigate the defense-related enzyme

responses (PO, PPO, PAL and SOD) of tomato treated with oil-cakes against the root-knot nematode *Meloidogyne incognita.*

Materials and Methods

The experiment was laid out in pot filled with root-knot nematode infested soil having 2 juvenile per gram soil obtained from the pure culture plots. Utmost care was taken right from sowing to till harvest of experiment for proper growth and development of plants.The details of material required and methods applied were as follows:

Preparation and maintenance of pure culture of *M. incognita*

Tomato plants infected with *M. incognita* were uprooted from the pure culture plots of the department and brought to the laboratory. Egg masses, collected from the infected roots and kept in distilled water in cavity block at laboratory temperature for hatching. Freshly hatched 2^{nd} stage juvenile were then inoculated on one month old tomato plants already grown and maintained in 6" sized earthen clay pots filled with steam sterilized soil to obtained adequate pure population of *M. incognita* on the plants and in soil to carry out further experiments.

Disinfection and filling of pots

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 infested soil. Some space (0.5-1.0") from the above was left unfilled for watering. Castor, mahua, karanj and mustard oil-cakes were added to soil each @ 2.5 and 5.0 q/hasoil. Each treatment was replicated three times. Untreated and standard check (neem cake @ 5.0 q/ha soil) was also maintained for comparison.

Transplanting and after care

Uniform size seedlings were transplanted in pots, one healthy plant in each pot was maintained. Care was taken right from sowing till harvest of experiments. To avoid insect damage, spray of Malathion (0.05%) was given as and when required. Weeds were uprooted from experimental pots. The pots were randomly rotated to eliminate the effect of sun and shade. The appropriate amount of water was provided throughout the course of experimentation.

Harvest

Assessment of the response of defence enzymes PO, PPO, PAL and SOD in tomato roots treated with oil-cakes against *M. incognita* was done on every 7days interval after transplanting *i.e.*7, 14, 21, 28 and 60 days after transplanting. The experiment was harvested 60 days after transplanting, while harvesting, the care was taken to avoid losses of both roots and nematodes in adhering soil. 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 (McBeth *et al.,* 1941). 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.

Estimation of soil population

The soil samples (200 cc) collected from the experimental pots were processed using Cobb's sieving and decanting technique (Cobb, 1918) followed by Baermann's funnel technique (Christie and Perry, 1951). After 24 hrs the suspension was drawn in a beaker

from the funnel and kept for some time to allow the nematode to settle down. The excess water was gently poured out of the beaker without disturbing the nematodes already settled at bottom.

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.

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 the rate of NBT reduction.

Results and Discussion

Plant growth and nematode reproduction

Data presented in Table 1 and 2 reveal that all tested oil-cakes applied 2.5 and 5.0 q/ha are remarkably improve plant growth and reduce gall formation, nematode population, number of eggs, egg production capacity as compared to untreated check. Difference in plant growth improvement and nematode reductions were can be seen.

Among all the treatments application of mustard cake @ 5.0 q/ha was found the best treatments to improve plant growth character, reducing number of galls per plant, number of egg masses per plant, number of eggs per egg mass, nematode population/200cc soil and total nematode population of *M. incognita* in tomato.

However, standard check neem cake @ 5.0 q/ha was found the best treatment to improving plant growth characters as well as in reducing nematode population over other treatment.

Determination of defence enzymes

Peroxidase (PO), Polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL) and Super oxide dismutase (SOD) were determined in roots of infected tomato plants and those treated with oil-cakes. Results in Table-3 manifested that application of oil-cakes increased the level of PO in tomato roots. Among all the treatments application of mustard cake @ 5.0 q/ha was found to be the best treatments to enhance enzymatic activity. However, standard check Neem cake @ 5.0 q/ha was found the best treatment to increase the enzyme activity against *M. incognita* in tomato roots.

The enzyme activity showed gradual increase from 7 DAI onwards to 28 DAI in plant roots treated with oil-cakes as compared to untreated ones. The lowest enzyme activity in among enzymes was recorded at 60 DAI in untreated control plant roots. Among enzymes the PO was found highest in tomato roots during different time of observations followed by SOD, while PPO and PAL were observed in low quantity.

The result presented here shows that application of oil-cakes increase the enzymatic activity in tomato roots, increase plant growth and reduce the nematode population.

This effect of oil-cakes may possibly be attributed to their high contents of certain active compounds which are very effective and target specific to controlling pests of the various crops. Akhtar and Malik (2000), Siddiqui and Alam (2001), they have reported that phenols, amino acids, aldehydes and fatty acids are release from neem which is antagonistic to root knot nematodes.

Table.1 Effect of oil-cakes 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	Castor cake @ 2.5q/ha.	26.63	26.37	26.50	24.90	25.10	25.00	28.37	28.70	28.53	19.33	20.10	19.72
T 2	Castor cake @ 5q/ha.	31.87	31.20	31.53	32.23	32.08	32.16	38.80	39.13	38.97	27.20	27.38	27.29
T 3	Mahua cake @ 2.5q/ha.	25.93	25.57	25.75	23.87	23.98	23.93	27.27	27.45	27.36	18.50	19.03	18.77
T 4	Mahua cake @ 5q/ha.	30.17	30.43	30.30	30.90	31.13	31.02	37.43	37.10	37.27	26.10	25.95	26.03
T 5	Karanj cake @ 2.5q/ha.	24.40	24.73	24.57	22.00	22.17	22.08	26.83	27.10	26.97	18.33	18.50	18.42
T 6	Karanj cake @ 5q/ha.	28.43	28.77	28.60	29.13	28.95	29.04	35.93	36.27	36.10	24.93	24.72	24.83
T 7	Mustard cake @ 2.5q/ha.	27.40	27.63	27.52	25.97	26.30	26.13	29.83	30.17	30.00	20.67	21.42	21.04
T 8	Mustard cake @ 5q/ha.	33.10	33.43	33.27	33.97	34.17	34.07	40.67	41.00	40.83	28.90	29.23	29.07
T 9	Neem cake (Standard check) @ 5q/ha.	34.53	34.95	34.74	35.57	35.75	35.66	42.70	43.37	43.03	30.47	30.73	30.60
T 10	Control	10.80	11.13	10.97	3.63	3.77	3.70	8.97	8.60	8.78	3.20	3.10	3.15
SEm±		0.183	0.361	0.238	0.17	0.216	0.18	0.29	0.313	0.25	0.42	0.436	0.41
CD at 5%		0.541	1.065	0.702	0.500	0.638	0.530	0.865	0.922	0.732	1.251	1.287	1.202
CV		1.163	2.280	1.505	1.12	1.42	1.18	1.60	1.70	1.35	3.37	3.43	3.22

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

Table.2 Effect of oil-cakes on *M. incognita* nematode reproduction on tomato plant

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	Poole d	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled	Ist Year	IIst Year	Pooled
T 1	29.33	30.00	29.67	21.33	22.33	21.83	116.00	114.00	115.00	280.00	284.00	282.00	3874.00	3964.00	3919.00
T 2	21.33	20.67	21.00	14.67	14.00	14.33	87.33	86.00	86.67	203.00	202.00	202.50	2296.00	2216.00	2256.00
T 3	30.67	30.33	30.50	23.00	23.33	23.17	128.00	130.00	129.00	303.67	305.33	304.50	4463.00	4561.33	4512.17
T 4	22.67	22.00	22.33	16.00	14.67	15.33	94.33	95.67	95.00	220.00	217.00	218.50	2610.00	2489.67	2549.83
T 5	32.00	32.33	32.17	26.00	26.33	26.17	132.67	134.33	133.50	348.33	352.33	350.33	5190.33	5301.33	5245.83
T 6	23.33	22.67	23.00	18.33	17.67	18.00	99.67	98.67	99.17	232.67	229.67	231.17	2990.33	2892.00	2941.17
T 7	28.33	29.00	28.67	20.33	21.33	20.83	110.00	112.00	111.00	260.00	263.00	261.50	3537.33	3704.33	3620.83
T 8	19.67	20.00	19.83	13.67	13.33	13.50	80.00	79.33	79.67	191.00	192.67	191.83	2049.67	2022.00	2035.83
T 9	18.33	17.67	18.00	11.67	11.00	11.33	74.33	75.67	75.00	179.33	174.00	176.67	1764.33	1703.33	1733.83
T 10	65.00	66.67	65.83	52.00	53.67	52.83	211.00	215.00	213.00	908.33	920.00	914.17	15511.00	16130.33	15820.67
SEm±	0.69	0.61	0.60	0.68	0.81	0.69	1.61	1.45	1.35	5.01	3.23	3.88	133.18	124.08	124.68
CD at 5%	2.05	1.79	1.76	2.00	2.39	2.05	4.75	4.28	4.00	14.77	9.51	11.45	392.89	366.03	367.81
CV	4.13	3.61	3.56	5.41	6.45	5.54	2.46	2.20	2.06	2.77	1.78	2.15	5.21	4.78	4.84

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

Table.3 Effect of oil-cakes on peroxidase (PO), poly phenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and super oxide dismutase (SOD) activity in tomato 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	14.520	25.375	34.877	41.842	8.092	0.207	0.363	0.516	0.598	0.160	0.115	0.180	0.255	0.303	0.101	1.76	2.77	2.99	3.36	0.98
T 2	31.494	40.827	47.171	58.280	18.919	0.273	0.418	0.559	0.710	0.229	0.144	0.194	0.290	0.342	0.117	2.10	3.34	3.63	3.77	1.13
T 3	12.800	21.823	33.750	39.614	7.302	0.196	0.359	0.505	0.576	0.149	0.112	0.178	0.241	0.299	0.098	1.74	2.56	2.86	3.25	0.90
T 4	30.704	39.840	45.986	56.137	17.171	0.265	0.411	0.552	0.704	0.221	0.141	0.192	0.279	0.331	0.115	2.01	3.23	3.52	3.73	1.09
T 5	11.024	20.582	33.524	38.402	6.541	0.186	0.354	0.497	0.567	0.144	0.109	0.174	0.234	0.292	0.097	1.66	2.43	2.70	3.19	0.86
T 6	28.562	38.092	32.480	54.501	15.479	0.255	0.407	0.548	0.700	0.217	0.138	0.189	0.285	0.326	0.114	1.97	3.12	3.41	3.69	1.02
T 7	15.366	25.968	39.163	42.885	9.078	0.215	0.369	0.524	0.623	0.168	0.118	0.183	0.263	0.314	0.102	1.80	2.86	3.09	3.48	0.99
T 8	33.862	42.885	47.960	59.407	20.751	0.281	0.421	0.571	0.718	0.237	0.146	0.196	0.295	0.348	0.118	2.14	3.40	3.73	3.86	1.15
T 9	36.084	50.272	71.334	73.167	29.971	0.299	0.464	0.611	0.765	0.275	0.156	0.211	0.302	0.366	0.130	2.18	3.49	3.96	3.99	1.24
T 10	4.511	9.530	17.340	23.515	3.834	0.109	0.169	0.180	0.130	0.080	0.072	0.084	0.094	0.076	0.045	1.115	1.29	1.605	1.79	0.21
SEm±	0.022	0.016	0.015	0.003	0.003	0.002	0.002	0.001	0.002	0.001	0.026	0.038	0.055	0.069	0.021	0.404	0.625	0.705	0.744	0.212
CD at 5%	0.065	0.047	0.044	0.009	0.010	0.006	0.005	0.002	0.005	0.002	0.077	0.113	0.162	0.203	0.062	1.191	1.845	2.079	2.194	0.625
CV	0.17	0.09	0.06	0.01	0.04	1.43	0.80	0.20	0.53	0.60	4.61	3.59	2.69	1.92	5.65	0.35	0.20	0.19	0.17	0.35

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

Similarly, some researchers have reported that use of organic amendments, composts, neem, poultry, inorganic fertilizers and nematicide (nemacur 10 G)] reduced number of galls, nematode reproduction, fecundity increased Plant chemicals (MDA contents, $H2O_2$, glutathione, Ascorbic acid, Soluble protein) and increased antioxidant substances (SOD, APOX, CAT, PAL, Glutathione-S-transferase) over control in tomato (Hossam *et al.,* 2012) fruit extract of neem induces defense response through enhanced activities of phenylalanine ammonia-lyase, tyrosine ammonia-lyase, polyphenol oxidase, peroxidase in tomato (Bhuvaneshwari and Paul, 2012). Goel and Paul (2014) evaluated that neem extract significantly reduced disease severity in the treated plants by inducing activities of PO and Lipoxygenase. Xiao, Z. (2016) found vermin-compost could significantly suppress root pests via modulating soil properties as well as plant defences, particularly for the susceptible plant. Findings of parihar *et al., (*2015) is that use of *Pochonia chlamydosporia*, oil cakes of neem, mustard and cotton were effectively suppressed the nematode population and kept the infection at significantly low levels. These are capable of producing secondary metabolites, which have an allelopathic effect and induces plant resistance against the invasion of nematodes.Findings of Resha and Savita Rani (2015) shows the application of various parts of neem (seeds, leaves) altered the physiology of host plant and developed the strong defensive mechanism of the root against nematodes, it increases plant growth and juvenile's mortality, reduction in egg hatching and root knot formation.

In present study thus it may be concluded that changes in defence enzymes after infection are related to defence action of plant, because abnormal metabolites are produced in contiguous un-infected tissues. Such metabolites collected in infected tissues and are harmful to parasites and suppress their growth and penetration. The metabolites released from the chemical constituents of oil-cakes stirred the plant cells to release abnormal metabolites which revolt the nematodes from the un-infected plant cells. So, the use of neem, castor, mahua, karanj and mustard oil-cakes stimulated and changes the physiology of plant cells and tissue to induce the defence enzymes, revolt the nematode parasites.

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