



Screening of anti-nematode potential through inhibition of egg hatching in plant-parasitic nematode *Meloidogyne javanica*

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ABSTRACT

Plant-parasitic nematodes have emerged as nature's most successful among all parasites known till today. These animals have been reported from all terrains of all ecosystems. Their capability to survive on a wide diversity of the host plants, circumvent host plant defence is a few of several of their secrets making them most successful of all known parasites. Among various groups of plant-parasitic nematodes, endo-parasitic nematodes are the most damaging one and also difficult to control. *Meloidogyne* spp. are commonly known as root-knot nematodes. Our inability to control them is primarily due to our poor understanding of the biology of these plant parasites. Due to the availability of the complete genome sequence of few *Meloidogyne* species, biotechnological interventions are used to unravel the secrets of their success. Chemical controls of these nematodes are extensively reported in the literature. Due to the environmental toxicity associated with these chemicals, and restrictions on the use of chemicals against nematodes led to screening and development of eco-friendly management strategies. The present study was conducted to screen nematotoxic properties of Neem (*Azadirachta indica*), Jatropha (*Jatropha curcas*), Kachnar (*Bauhinia variegata*), Bel (*Aegle marmelos*) and Eucalyptus (*Eucalyptus globules*) leaf extracts against root-knot nematode *Meloidogyne javanica* *in vitro*. The aqueous extracts were used against the hatching of the nematode eggs, movement of second stage juveniles (J2) and the viability of the J2 in increasing concentration of the bioactive compound. The eggs were treated with various concentrations of the selected extracts for different time periods ranging from 24h to 6 days. A significant inhibition of egg hatching and increase in the mortality of the nematode juvenile in few of the aqueous extracts were recorded. Reduced egg hatching and increased mortality of the nematode juveniles could be the indicators of the presence of anti-nematode potential in the selected plant leaves. The results from the study can pave the way for the development of eco-friendly management strategies for plant-parasitic nematodes.

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INTRODUCTION

Plant parasitic nematodes (PPN) are one of the successful multicellular organisms which parasitize almost all plants [1-3]. In order to feed on various plants, PPNs have diversified during evolution some 120-135 million years ago [4,5]. Due to diversification, nematodes have got an edge over other organism in terms of their suitability to survive on any environmental conditions [6]. Phylogenetic studies of nematode genome have provided evidences supporting the evolution of parasitism on various occasions during evolution in plant-parasitic nematodes [6]. Similar trend was followed by animal parasitic nematodes as well [7-9]. This observation strongly suggests acquisition of favourable gene during evolution of

nematodes [10,11]. Nematodes have acquired all possible niches in any ecosystem [12-14]. Root-knot nematodes follow a typical life cycle [15-17], a clear sexual dimorphism [18-20]. For example in *Meloidogyne*, male nematode remains vermiform throughout its life and feeds only from outer surface of the plant [21-23] and the female is tear drop shaped and remains inside the plant root [24-26]. After fertilization, embedded female lays eggs in gelatinous matrix, egg masses can be seen on the surface of the roots. Each egg mass of *Meloidogyne* contains 400-700 eggs [27,28]. First moulting takes place inside the egg, larva (J2) which comes out of the egg, represents infective stage of *Meloidogyne*. The root-knot nematodes take 28 days to complete its life cycle under favourable environmental conditions [29-33]. Plant parasitic nematodes are serious pest on plants which cause significant damage to

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crop plants [34-36]. These nematodes have developed special strategies to parasitize the plants and these strategies also make them feed on various parts of plants. As mentioned in earlier studies about the nature of damage caused by plant parasitic nematodes and other secondary infections, plants suffer significant damage in field causing huge economic loss. Significant amount of money is being lost due to the damages caused by these nematodes to crop plants. Plant-parasitic nematodes exhibit serious biotic stress on plants, due to which growth and yield are significantly affected [37,38]. Wilting of the plant is the most common symptom of the secondary infection followed by nematode attack [39-41]. It has also been reported that nematode damage to plant parts invites secondary pathogens, which in turn ameliorates the damage. Over several years, careful estimation of the damage caused by different nematodes has been carried out through a series of co-ordinated projects. Estimates from India projected 21.3 percent of the crop loss which amounts to 1.58 billion USD annually. As per an estimate, PPNs cause \$173 billion loss annually worldwide. The estimate of the loss from India also raises serious concerns. It warrants significant efforts in management of plant-parasitic nematodes. Nematode causing damage to several varieties of crop plants has been accessed in great details. *Meloidogyne spp* causing damage to rice plants has been shown to be most economically damaging nematode.

Presently there are several methods used to control the damage caused by plant-parasitic nematodes. Method employed to control the damage caused by PPNs aim to keep the population of nematode below certain threshold in fields. The control methods currently employed includes chemical control using nematicides. Biological control of PPNs is based on the concept of “enemies enemy is friend” [42]. Varieties of fungi [43] and bacteria [44,45] are used as biological weapon against PPNs. Other biological agents have also been explored for their ability to control the damage caused by plant-parasitic nematodes. Physical control, regulatory control and cultural control methods have also been employed to limit the damage caused by PPNs. Scientists also have put forward a theory of controlling the plant-parasitic nematode not by any single method but by a combination of several methods. This approach is a part of integrated pest management (IPM) [46,47]. Among several management methods, chemical control method is widely used by farmers in the field [47]. On one hand, chemical based management strategies provide quick fix solutions but on the other hand, toxicity associated with these chemicals seriously affects the environment and also the non-target species. There are growing concerns among several scientists that this indiscriminate use of chemical based nematicides will lead to development of resistance in nematodes [48-50]. Among various groups of nematodes, endo-parasitic nematodes are most damaging one. The control strategies target endo-parasitic nematodes such as root-knot nematodes and cyst nematode. Due to these reasons, it becomes imperative to look for alternate methods for management of these nematodes beyond the chemical control approach. Therefore it is the need of the hour to develop eco-friendly management techniques.

Various parts of plants have been shown to possess anti-nematode potential [51]. Certain studies have used extracts

from plants to control the damage caused by PPNs both *in-vivo* and *in-vitro* [52]. The idea to controlling plant-parasitic nematode using plant based products mooted on finding of presence of secondary metabolites as natural defence molecules in plants, which when scaled up can prove a better source for nematode management. This idea further strengthens the development of plant based products for nematode control. Earlier studies have revealed the presence of anti-nematode potential in various plants [53]. These bio-based control approach is easy to formulate and safe to use in fields. Several species of root-knot nematodes has been tried to control using plant based formulations [54-56]. Isolates of root-knot nematodes behave differently to different botanicals. Therefore, it will be of significant interest to look for more number of plants with anti-nematode properties. The objective of this study was to screen leaves of five selected plants for their anti-nematode potential against root-knot nematode *M. javanica*. Hatching of nematode eggs in presence of different concentrations of leaf extract over a period of time was used as measure to identify the presence of anti-nematode potential in selected plants.

MATERIALS AND METHODS

All the procedures related to collection of root-knot nematode, identification of root-knot nematode, maintenance of nematode stocks on susceptible tomato plant *Lycopersicon esculentum* var. Pusa Ruby, preparation of leaf extracts, hatching of nematode eggs, treatment of nematode eggs (hatching) in presence of active compound, monitoring of hatching, inhibition of hatching (if any) over selected period of time was carried out using following standard protocol.

Maintenance of Nematode

Root-knot nematodes used in this study were isolated from tomato plants grown locally in Agra. Roots of the infected tomato plants were washed gently in running tap water to remove larger mud particles. The fine mud particles adhered to the root were removed by keeping the root in a bucket full of water for two hours and then washing again under running tap water. Individual egg masses were hand-picked using light microscope and a laboratory forceps. Corresponding females were dissected out under the microscope and kept in water and immediately taken for perennial pattern cutting and identification. Perennial pattern and identification was done following the procedure described earlier [57]. Egg masses corresponding to females of *Meloidogyne javanica* were collected for hatching. Individual egg masses were put in 35 mm glass petriplate filled with water. This assembly was kept at 28°C in biological incubator for 24 hours. Thirty egg masses individually were used for hatching. Next day 100µl water was taken out from each petriplate using glass pipette. Water from each petriplate was monitored under microscope for their number and viability of the active juveniles (J2). Fifteen millilitre of water containing active J2 were used for infecting the roots of freshly grown tomato plants.

Maintaining *Meloidogyne javanica* Infection on Tomato Plants

Tomato plants are particularly susceptible against infection by root-knot nematodes. Seeds of tomato *Lycopersicon esculentum* var. Pusa Ruby were obtained from national seed corporation Beej Bhawan, Pusa Complex, IARI, New Delhi. Soil and sand mixture in ratio of 3:1 was sterilized in pressure cooker in several small batches and filled in mud pots for growing nursery. Fifty tomato seeds were sown in one big pot for three weeks and watered using tap water. After three weeks, individual plantlets were transferred in single pots containing sterilized soil and sand mixture each and allowed to grow for two more days. Soil near the roots of plants was removed and fifteen millilitre of water containing active juveniles was poured directly above and near the root. The roots were then covered with soil. All the plants were shifted in shade following infection. Watering of the plants was avoided for first three days and then the plants were watered as and when needed. Infection of the plants was allowed for forty five days. Following the time period, the infected plant was dugout and as per the standard protocol described above, the perennial pattern was cut and the corresponding female species was identified. Once identified as *Meloidogyne javanica*, the pure culture of this particular nematode was maintained in tomato plants throughout the year.

Collection of Eggs from Infected Tomato Plants

Meloidogyne javanica population maintained on Pusa Ruby variety of tomato, eggs masses were extracted using laboratory forceps and surgical needles. In case of heavily galled roots, removal of egg masses was done as described by Hussey and Barke [58]. In brief, galled roots were cut in small pieces and then the pieces were kept in 0.1 percent solution of sodium hypochlorite. This mixture was then blended in kitchen blender for two minutes at intervals of few seconds. Suspension of the eggs, thus obtained, was passed through series of sieves of various pore sizes nested one above the other. 100 mesh size sieve was mounted over 400 and 500 mesh size sieve. The suspension from the blender was passed through the assembly of these sieves. The assembly was kept under gently running tap water for one minute. The content of the 400 mesh size and 500 mesh size sieve was collected in a glass beaker (Borosil). Individual content from each of the sieves was further taken for examination under microscope. Eggs were then separated out and collected in petriplate with water. All the egg masses used for hatching were laid over a filter paper with wire gauze over a glass petriplate filled with water. Assembly was arranged such that it kept the filter paper wet all the times. This assembly was maintained at 28°C in incubator for 24 hours. Juveniles thus obtained were used for maintaining the stocks of the nematode, and the egg masses obtained from 400 and 500 mesh sieve were used in screening of plant extracts.

Preparations of Botanical Leaf Extracts

Leaves from five selected plants Neem (*Azadirachta indica*), Jatropha (*Jatropha curcas*), Kachnar (*Bauhinia variegata*), Bel (*Aegle marmelos*) were collected from the campus of Govt.

PG college Fatehabad, Agra Uttar Pradesh, India (Latitude 27.022492 Longitude 78.307349) and Eucalyptus (*Eucalyptus globules*) was collected from outside the college perimeter wall (Latitude 27.035450 Longitude 78.285230). The collected leaves were washed thoroughly under running water and kept on filter paper for drying under shade. Ten grams of each plant leaves was mixed with 100 ml of distilled water and grounded in kitchen blender at high speed. Suspension obtained was filtered through filter paper (Whatman No. 2). The similar procedure was repeated with all other leaves. Filtrate was centrifuged using clinical centrifuge at one thousand rpm for ten minutes. The extract was removed and the pellet was dissolved in one ml of distilled water. This preparation was taken as hundred percent stock solution. This stock solution was further diluted five, ten and twenty times with distilled water. Control experiment was done using only the distilled water without any plant extracts.

Screening of Anti-nematode Potential

Screening of anti-nematode potential in leaves of selected plants was carried out in laboratory conditions. From the stock solution, dilutions of 5%, 10% and 20% were made and these were mixed with the egg masses (15 egg mass in each sterilized petriplate with 10 ml of the selected concentration of the leaf extracts) and the incubation was done at 28°C in a biological incubator. Hatching of the nematode egg was allowed till the end of sixth day. Hatching was monitored at 24hrs, 48hrs 72hrs, 96hrs and on 6th day. Each experiment was conducted in triplicate. After the specified time intervals, numbers of juveniles were counted using nematode counting slides. Anti-nematode potential of the leaf extract was observed in terms of delay in hatching of the nematode eggs. In batch kept till 6th day, number of dead juveniles were also counted and recorded. Presence and absence of the anti-nematode potential from the selected plants was assessed as percentage of the juvenile nematode that was active at the time of monitoring. Number of dead juveniles was also counted in each set.

Mortality Study with Juveniles

The J2s were transferred in a 30 mm glass petriplate and then allowed to stay in various concentration of the leaf extract. Their mobility was monitored under light microscope initially and after the incubation period of 96 hours, mortality of the juveniles was recorded (Table 2).

Screening of anti-nematode potential represents the presence of the anti-hatching compounds in the selected leaves and its various concentrations. Presence of active compound reported in leaf is extrapolated based on the number of juveniles hatched out from the egg masses (15 egg masses were used with each concentrate in triplicate). Eucalyptus leaf extract (06 days of treatment) inhibited the hatching of the egg masses over incubation period and also with increasing concentration of the leaf extract. Numbers of juveniles in 20% of the leaf extract significantly declined. Similar trend was observed with 20% concentration of Bel, Jatropha and Neem. Number of juveniles in each of these treatments was recorded as 54, 48 and 18 (06 days of treatment). With 10% of the extract and

96 hours of incubation, number of juvenile recorded as 206, 125, 187, 70, and 40 in Eucalyptus, Bel, Kachnar, Jatropha and Neem respectively. This trend was observed with all the extracts except Kachnar. The observations thus, confirm the presence of nematode control activity in the leaves of the selected plants which hinders with hatching of the *Meloidogyne javanica* eggs. Extract from the neem plant demonstrated strong anti-nematode activity and its extract significantly reduced the hatching of the eggs. High concentration of neem extract had nematicidal properties as well.

As described in Table 1, screening of anti-nematode potential in selected leaf extract provided evidence of the presence of the anti-nematode compounds. Various concentrations of the leaf extract inhibited the hatching of *Meloidogyne javanica* eggs to significant level. In order to further verify the results obtained from the screening experiment, mortality studies were performed with juveniles (J2) of *M. javanica*. In case of the Eucalyptus, the percentage of mortality was recorded as 40.9%. In case of Bel, Kachnar, Jatropha and Neem, the mortality of the juveniles were recorded as 11.9%, 25%, 62.2% and 86.6% respectively. From the above observations, it is clear that Jatropha and Neem leaf extract have strongest anti-nematode potential (Table 2).

Statistical Study

Data have been expressed as the mean ± standard error (S.E.) and statistical analysis was carried out by employing Student’s t-test and analysis of variance by one way ANOVA (Dunnnett’s Multiple Comparison Test). A conventional $p < 0.05$ was taken as evidence of significant differences and $p < 0.01$ was considered as highly significant while $p > 0.05$ was not significant (Monte Carlo approach). All the experiments were conducted in triplicate with appropriate control.

The LC₅₀, LC₉₀ Values of Mortality

The LC ₅₀ , LC ₉₀ values of Mortality					
S.No	Name of the plant	LC ₅₀ (mg/ml)	LC ₉₀ (mg/ml)	Regression	Regression equation
1.	Eucalyptus	42.07	86.91	0.92	Y = 0.93+2.05X
2.	Bel	17.62	53.18	0.20	Y = 0.20+3.19X
3.	Kachnar	24.10	62.83	0.36	Y = 0.36+3.25X
4.	Jatropha	15.62	36.44	0.49	Y = 0.49+3.92X
5	Neem	14.99	31.37	1.84	Y = 1.85+0.85X

As evident, all the extract from the selected plants exhibited the anti-nematode potential in leaf extracts, however, Neem leaf extract led to suppression in hatching of nematode eggs

(Table 1) as compared to the untreated control groups. This activity was most noticeable at the dose of 20 gm/100ml. Neem leaf extract at 5 gm/100ml as well as 10 gm/100ml also exhibited the presence of anti-nematode potential, but this activity was quite significant in 20 gm/100ml. All the standard parameters with respect to screening analysis and regression analysis of the data showed the statistical significance ($p < 0.05$) of the experiment.

DISCUSSION AND CONCLUSIONS

Various studies over the years have documented utilization of several resources for management of plant-parasitic nematodes. These resources also include waste materials [59]. Neem based formulations are the products which has been most relied upon. A review of plant based products used for nematode control has been documented earlier [60]. Scientists have tried collagen amended soil for controlling the menace of plant-parasitic nematodes. Metabolites from higher fungi were also utilized. Anti-nematode potential of products such as serpentine was also tried. Tannic acid, fungal toxins, fatty ester, sea weeds, mustard oil, compounds from well known plants in developed world, rape seed, activity of phenolic compounds was also tested for its efficacy for nematode control. Secondary metabolites and the role of essential oil were also explored [61]. Products such as manure have also been tried as management option. Biological control of plant-parasitic nematode has been studied in great detail as to scale up the product for future use. Potential of bacteria is also used for management of nematodes [62]. The utilization of fungi as bio control agent has been described in details [63]. Since most of the plant-parasitic nematodes feed on the roots of the plants, screening of rhizosphere bacteria for nematode control was also described in literature [64]. Resistant varieties of crop plant offer natural ways of resisting nematode infection. A list of plants known to have exhibiting some form of resistance is listed [65]. Sustainable approaches for management of nematode alone and also in combination of other pathogen is also described in detail [66]. Transgenic approaches are also being under study since 2006 for their potential in controlling the infection by plant-parasitic nematode. Transgenic approaches are based primarily on better understanding of the nematode biology.

As described earlier, the environmental toxicity associated with the use of chemical nematicides led to its ban in many countries. Quest to search for alternative control strategies have led to exploration of several options starting from waste materials to advanced molecular approach. Chemical moieties

Table 1: Number of J2 observed after incubating for specified time

S.No	Name of the plant	Concentration of leaf extracts											
		5%				10%				20%			
		24h	48h	96h	144h	24h	48h	96h	144h	24h	48h	96h	144h
1	<i>Eucalyptus</i>	134	224	317	416	128	163	206	254	90	134	128	91
2	<i>Bel</i>	90	136	227	301	108	135	125	111	65	87	63	54
3	<i>Kachnar</i>	111	187	254	325	121	145	187	229	108	156	235	270
4	<i>Jatropha</i>	70	103	165	238	65	74	70	64	42	58	52	48
5	<i>Neem</i>	81	90	105	132	54	51	40	38	43	43	28	18

Table 2: Evaluation of mortality studies in presence of leaf extract from selected plants in various concentrations

S.No	Name of the plants	Percent mortality of <i>M. Javanica</i> J2 at different concentration of the leaf extract			
		5%	10%	20%	100%
1	<i>Eucalyptus</i>	6.7	12.4	26.7	40.9
2	<i>Bel</i>	7.7	8.5	7.7	12
3	<i>Kachnar</i>	10.2	19.4	16.7	25
4	<i>Jatropha</i>	36	47.4	58.8	62.3
5	<i>Neem</i>	23.7	47.4	60	86.7

of the selected leaf extract need further characterization for commercial production of bio-active compound. Based on the result of this screening of anti-nematode potential in five selected plants, it can safely be concluded that plant extracts offers effective and cheap source for controlling the menace of root-knot nematode in field. This experiment shows that neem possesses strongest anti-nematode potential against root-knot nematode, which is followed by Eucalyptus, Jatropha, Bel and Kachnar. Plant based formulations provide a clear approach for managing root-knot nematode. Detailed studies are required with more number of plants and also with different types of nematode (based on their feeding habits) for listing number of plants having natural anti-nematode properties.

AUTHORS CONTRIBUTION

Research design, study, analysis of the data, interpretation of the findings and writing of the manuscript was done jointly by SS and BCY.

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