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In vitro effect of botanicals against rice root knot nematode *Meloidogyne graminicola*

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Abstract
This research was conducted to find out the effect of aqueous extracts of different Botanicals against the rice root-knot nematode, *Meloidogyne graminicola* in laboratory and Screen house conditions at CCSHAU, Hisar. Aqueous extracts from plant leaves were screened for nematode mortality against second stage juveniles (J2) and egg hatchability of *M. graminicola.* The nematode juveniles and eggs were exposed to 48h and 10 days to different dilutions (100%, 50% and 25%) of botanical extracts respectively. All the treatments significantly increased the percentage of mortality at 48 h. of exposure in aqueous extracts concentration (100%, 50% and 25%) as compared to control (Nematode alone). At 48 h. (100% concentration) the maximum mortality percent was found in Neem (*Azadirachta indica*) 89.00%, whereas in egg hatching experiment with the dilutions as 1:10, 1:20, 1:30, 1:40 and 1:50 were prepared by diluting stock solution. The Neem *(Azadirachta indica*) at 1:20 (v/v) dilution significantly reduced the egg hatchability and it was followed by Cauliflower (*Brassica oleracea var. botrytis*) and Cabbage (*Brassica oleracea var. capitata*).

Keywords: Botanicals, M. graminicola, *in vitro*, rice

Introduction
Rice (*Oryza sativa* L.) is the world's most important food crop of Asian origin. It is an indispensable source of calorie for almost half of the world population with in Asia. Nematodes are small unsegmented worms live in water, soil, plants and animals. Plant parasitic nematodes are mostly microscopic. It causes significant damage for almost all crops. Global crop loss caused by plant parasitic nematodes is more than $100 billion annually Khan *et al*., (2008) [8]. Root knot nematodes, *Meloidogyne* spp, are the major plant parasitic nematodes attacking most of the crops. It is a major problem in green houses and nurseries.
Root-knot nematode, (*Meloidogyne* spp) are among the serious and wide spread nematode pests attacking various economically important crops. Reduction in the quantity and quality of the crop produces results in decline in net profits. Several control practices of nematode management *viz* cultural methods, physical control, biological control, chemical control, use of resistant varieties etc. have been found highly effective against different nematodes. But each has its own merits and demerits but farmer mainly relied on chemical nematicides because it effectively kills nematodes in soil. Chemical control, though gives quick control but these chemicals are not only expensive but also highly toxic residual effect of chemical on the environment and particularly on non-target organisms (Anastasiadis *et al*., 2008) [1].
Therefore there is an urgent need to replace pesticides with alternative means of control that are less toxic and more eco friendly. Other convential methods for nematode control like cultural, physical, biological and plant resistance, which too have technical and operational limitations of one kind or other. The use of non-hosts and antagonistic plants having allelopathic effect offers a promising area for combating and managing root-knot nematodes. Plant parts and their products have been found to posses nematicidal and nematostatic properties against plant parasitic nematodes. Nematicides of plant origin contains iso-thiocyanates, thiophenics, glucosides, alkaloids, phenolics and fatty acids in their different plant parts which are fatal to nematodes.
A number of organic components of plant origin, including oil-seed cakes, chopped plant parts and plant extracts have been used as nematode control agents (Tiyagi *et al.,* 2009) [14, 15]. In recent days, interest has been shifted in discovering nematostatic compounds of the plant origin (Chitwood, 2002) [4, 11]. In nature, plants produce a number of secondary metabolites to defend themselves against various pests, diseases and nematodes. In India, huge numbers of plants are available which have nematicidal properties. These botanicals offer an alternate strategy for the nematode management due to their facile biodegradability, selective toxicity to target organisms and ecofriendly nature.

Different plant extracts have been tested by different scientists for their nematicidal properties (Netscher and Sikora, 1990; Kaur *et al* 2012) [9, 7]. Botanical pesticides are readily available in many places and are often cheaper than their synthetic counterparts. Furthermore, crude extracts of the botanicals are easy to prepare by farmers.

The use of botanical extracts for controlling *Meloidogyne* is becoming appealing because of the growing problem of environmental pollution arising from the use of persistent pesticides. There has been a de-registration of some hazardous nematicides. Increasing pressure is on farmers to use non-chemical pest control methods that do not pollute the environment. This emphasis the need for new methods of control such as the use of plant extracts. Efficacy of various plant extracts in nematode control has been studied by Chavan *et al*., (2013) [2], *In vitro* toxicity (LC_{50}) of methanol, ethyl acetate and hexane extracts of *Crotalaria juncea* on *M. graminicola* J_2 indicated that methanol extract was 1.04 times more toxic to J_2s of *M. graminicola* compared to hexane and ethyl acetate extracts. Nematicidal effect of garlic has been reported, but was phytotoxic. Aqueous extracts from fresh leaves of *Calotropis procera, Azadirachta indica, Clerodendrum inerme* and *Lantana camara*. All the extracts reduced the hatching of egg-masses but the maximum reduction occurred in *Calotropis procera* and the least in *Lantana camara*. Maximum mortality of 2nd stage juveniles was observed in leaf extracts of *Azadirachta indica* and least in *Calotropis procera* by Chedekal, (2013) [3]. Studies on the identification and use of local plant materials for the control of nematodes, or integrated with other methods of control, are current areas of research in plant nematology. So the objective of this study was to evaluate the effect of some botanicals on *M. graminicola* on rice *in vitro* conditions.

Material and Methods

Experimental site

The experiment was carried out in the laboratory and screen house of the Department of Nematology, CCS Haryana Agricultural University, Hisar. Hisar lies between latitude 29.1oN, longitude 75.7oE at an altitude of 215.0 m above sea level.

Preparation of Botanical Extracts

The leaves of different selected plants *viz*. Neem (*Azadirachta indica*), Cauliflower (*Brassica oleracea var. botrytis*) and Cabbage (*Brassica oleracea var. capitata*). Leaves of 10 gram each were washed under running tap water and added 100 ml distilled water and grinded in blender for 3 minutes. The mixture was allowed to stand 3 h. filtered through Whatman No. 2 filter paper then the extracts of all leaves kept for centrifugation in centrifuge machine at 1000 rpm for 10-15 minute. The supernatant was removed and were taken as stock solution (100%). The dilutions of concentration were prepared with distilled water according to the larval mortality and egg hatching of *M. graminicola*.

Obtaining eggs and J2 of *M. graminicola*

M. graminicola populations were obtained from rice roots grown in culture pots. Eggs of M. *graminicola* were extracted from infected rice roots either by teasing galled roots with needles or by using a modification of the Hussey-Barker method (Hussey & Barker 1973) [6] wherein galled root pieces were placed in 0.1 per cent NaOCl solution and processed in a waring blender at 20 sec intervals for 3 min. The water suspension bearing the free eggs was passed through a bank of sieves – 100 mesh (pore size 150 µm) nested over 400 mesh (38 µm) and 500 mesh (26 µm) sieves. The contents of the sieves were washed thoroughly with running water to remove the chlorine. Finally the residues of 400 and 500 mesh sieves were collected in a beaker. The contents of the beaker were examined under a stereo zoom microscope for the presence and density of eggs. The J2 were obtained by further pouring egg suspension over 4-ply facial tissue paper mounted on a piece of moulded wire net. The assembly was fixed in a Petri-plate and fresh water was added. The water was removed from the Petri-plate daily to collect the hatched J2 and replaced with fresh water. The assembly was maintained at 24 ± 1° C in a BOD incubator until the J2 hatched. Freshly hatched J2 were used for the experiments according to the treatments.

Mortality test of nematode larvae

The evaluation was carried out in small Petri dishes. There were three botanicals and replicated three times. The Petri dishes with distilled water was taken as control. Freshly hatched 100 J2 (1 ml) of *M. graminicola* were suspended in l ml of 100%, 50% and 25% of each leaf extracts which was prepared from the stock solution. All the Petri dishes were kept at ambient temperature 25(±) 1^0C. in BOD incubator. After 48 h. of incubation, all dead and alive 2nd stage juveniles (J2) were counted with the aid of counting dish under stereomicroscope. The dead juveniles attained the shape of straight line and the mortality was ensured by touching the juvenile with a fine needle. The ratio of dead nematodes/number of total nematodes expressed the percentage mortality.

Larval Mortality was calculated by Following Formula (Ahmed *et al*., 2005) recorded

$$\text{Mortality (\%)} = \frac{\text{Number of larvae killed}}{\text{Total No. of larvae}} \times 100$$

Testing for egg hatching: Hatching experiment was carried out in small Petri dishes. There were five dilutions (1:10, 1:20, 1:30, 1:40 and 1:50) and replicated three times. The Petri dishes with distilled water was taken as control. A suspension of 1 ml (100 eggs) of *M. graminicola* in water was prepared. The eggs were suspended in l ml of 1:10, 1:20, 1:30, 1:40 and 1:50 of each leaf extracts which was prepared from the stock solution. All the Petri dishes were kept at ambient temperature 25 (±) 1 ^{0}C. in BOD incubator. Hatching was observed after 10 days. All data collected were subjected to OPSTAT analysis at 5%.

Results and Discussion

Mortality test

Percentage of *M. graminicola* viable J2 was decreased with increased concentrations of botanicals at 48 hrs observed in Table 1 and Fig. 1. All the treatments significantly increased the percentage of mortality at 48 hrs. of exposure in different plant extracts concentration (100%, 50% and 25%) as compared to control (Nematode alone). At 48 hrs. (100% concentration) the maximum mortality percent was found in Neem (*Azadirachta indica*) 89.00% as compared with Cauliflower (*Brassica oleracea var. botrytis*) and Cabbage (*Brassica oleracea var. capitata*) and control (water alone) 4.67%. Highest larval mortality was observed at 100% (with mean of 59.50) botanical extract as compared to 50 and 25% concentrations. Using plant extracts in controlling plant

parasitic nematodes has shown by several authors (Satyal *et al*., 2012) [12]. A similar type of results was found in *in vitro* studies of Dongre and Simon (2013) [5], studied the Nematicidal activity of extracts from plants was assayed against *Meloidogyne graminicola*. In laboratory assayas extracts from Neem (*Azadirachta indica*), Bael (*Aegle marmelos*), Jatrofa (*Jatropha curcas*), Eucalyptus (*Eucalyptus globus*), Sahjan (*Moringa oleifera*), Ber (*Ziziphus mauritiana*), Sarifa (*Annona reticulate*), Congress grass (*Parthenium argentatum*) were most effective in killing the nematode. *In vitro* studies 25%, 50% and 100% concentrations of leaf extracts significantly reduced second stage juvenile of mortality after 24 and 48 (hrs.). The plant leaf extracts of Bael (88.53%) and Neem (80.31%) exhibited highly promising mortality 80-88% after 48 (hrs.) exposures at 100% concentration. A similar type of experiment was carried out by Singh *et al*., (2015) [13] evaluated the Aqueous extracts of leaves of botanicals i.e. *Argemone maxicana, Azadirachta indica, Calotropis procera, Datura inoxia, Delonix regia, Parthenium hysterophorus, Moringa oleifera, Saraca asoka* and *Withania somnifera* plants were tested against root-knot nematode, *Meloidogyne incognita* under *in-vitro* trials. Undiluted crude leaf extracts of *A. maxicana, A. indica* and *P. hysterophorous* exhibited 78, 62 and 70 larvae inhibition from eggs and 100, 100 and 60% mortality (out of hatched juveniles), respectively whereas *Purpureocillium lilacinum* (check) exhibited 79% inhibition of egg hatch and 31% mortality. Another *in-vitro* trial on mortality of tested nematode was observed up to 72 h with a revival test for 24 h. After revival 100% mortality was recorded with *A. indica* followed by *A. maxicana* (90%) and *P. hysterophorous* (56%) whereas *P. lilacinum* killed only 38% *M. incognita* larvae.

Egg hatching test: All plants extracts showed inhibitory effect on egg hatching. The rate of hatching was inversely proportional to concentration of extracts with exposure period, as it decreased with increase in concentration. The maximum hatching of eggs was observed in 1:50 while lowest rate at 1:10 dilution in all extracts tested (Table 2). Among aqueous extracts, extracts obtained from Neem showed most inhibitory effect followed by Cauliflower and Cabbage respectively (Fig. 2). Minimum hatching (50%) was recorded with Neem followed by Cauliflower and Cabbage after 10 days at 1: 20 concentration of botanicals. The maximum egg hatching was recorded in water (95%). It is revealed from Table 2 that all three factors *viz*, aqueous extracts, their concentrations and exposure time significantly affected hatching individually. The present investigation are in adjustable conformity with the finding of Pavaraj *et al*., (2012) [10], who while testing Ten different plants which were collected from in and around Sivakasi area. The plants were shade dried and powdered. The plant extracts were prepared by soxhlet apparatus using methanol as a solvent. Methanol extracts of ten plants were screened for egg hatchability and nematicidal activity against second stage juveniles of *M. incognita* in the laboratory. There was a gradual decrease in egg hatching with increase in extract concentration. *Nepeta cataria, Couroupita guianensis* and *Pentanema indicum* were found to be most effective in reducing egg hatching. Similarly, Umar and Adamu (2014) [16] also reported the nematicidal potential of different concentrations of leaf extract of *Euphorbia heterophylla, Richardia brasiliensis and Scoparia dulcis* contained in Petri dishes in the laboratory. Egg hatch inhibition was observed over a period of 96 hrs. against second stage juveniles of *M. javanica*. The results of the study showed that the extracts inhibited egg hatch. While evaluating the nematicidal properties of 15 plant and their various parts *viz. Albizia amara, Aristolochia bracteata, Tagetes erecta, T. patula, Origanum majorana, Azadirachta indica, Butea monosperma* and *Calotropis gigantea* leaves, *Acorus calamus* roots, *Allium sativum* bulbs, *Citrullus lanatus, Areca catechu* and *Annona reticulate* seeds, and *C. gigantea* and *Carica papaya* latex against the root-knot nematode, *M. incognita* egg masses by Saravanapriya *et al*. (2004) [11] reported that the seed extract of *A. catechu* showed highest inhibition rate at 0.1 per cent concentration. The latex of *C. papaya* caused 98.22 and 100 per cent hatching inhibition at 1.0 and 10.0 per cent concentrations, respectively. The latex of *C. gigantea* also caused 100 per cent inhibition at 10.0 per cent concentration.

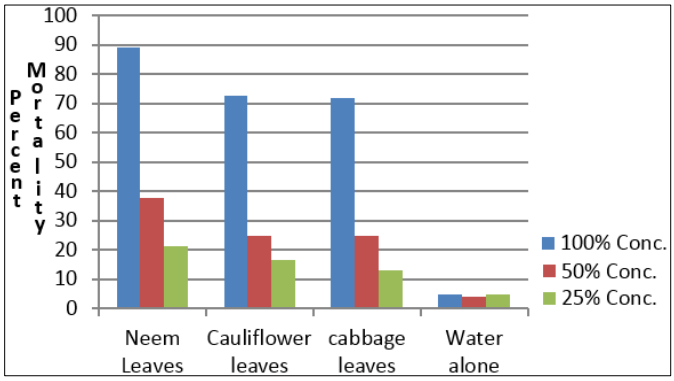

Fig 1: Effect of Botanicals leaf extracts on larval mortality of *Meloidogyne graminicola*

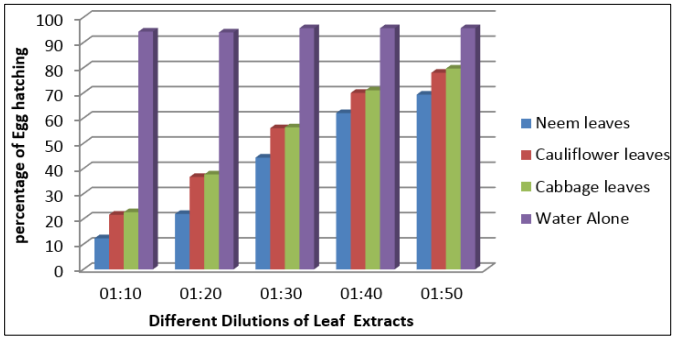

Fig 2: Effect of Botanicals extracts on Egg hatching of *Meloidogyne graminicola*

Table 1: Effect of Botanicals extracts on larval mortality of *Meloidogyne graminicola*.

Dilutions	Botanicals				
	Neem leaves	Cauliflower leaves	Cabbage leaves	Water Alone	Mean
100%	89.00	72.67	71.67	4.67	59.50
50%	37.67	24.67	24.67	4.00	22.75
25%	21.33	16.67	13.00	4.67	13.92
Mean	49.33	38.00	36.44	4.44	

C.D. at 5% level

Botanicals: 2.31; Dilutions: 2.00; Botanicals x Dilutions: 4.01

Table 2: Effect of Botanicals extracts on hatching of *Meloidogyne graminicola*.

Dilutions	Botanicals				
	Neem leaves	Cauliflower leaves	Cabbage leaves	Water Alone	Mean
1:10	12.33	21.67	22.67	94.33	37.75
1:20	22.00	36.67	37.67	94.00	47.58
1:30	44.33	56.00	56.33	95.67	63.08
1:40	62.00	70.00	71.00	95.67	74.67
1:50	69.33	78.00	79.67	95.67	80.67
Mean	42.00	52.47	53.47	95.07	

C.D. at 5% level

Botanicals: 1.56; Dilutions: 1.74; Botanicals x Dilutions: 3.48

Conclusion

Among all plant leaf extracts, Neem leaves showed maximum larval mortality (89.0%) at 100% concentration and also most inhibitory effect on hatching compared to cauliflower and cabbage at 1:10 dilution. Among all the dilutions of plant leaf extracts, the minimum hatching was obtained in 1:10 dilution.

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