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Research Article

Zoology for Novel drug discovery

Nematode Extract-Induced Resistance in Cowpea against *M.Incognita*

Avijit Mukherjee*

Department of Zoology, Netaji Mahavidyalaya, Arambagh, Hooghly-712601, West Bengal, India

Abstract: Every plant in the world has a special defence mechanism to protect themselves from their enemies. In India, total crop losses annually 10-40% by plant-parasitic nematodes. These plant parasitic nematodes are roundworms, which are microscopic in nature. Although there are hundred different kinds of plant parasitic nematodes infect plants, root-knot nematode (*Meloidogyne incognita*) is one of the serious pest for cowpea plants. They manage to evolve sophisticated defence mechanism for their own protection and also creates interrelationship with the roots of their host plant to form giant cells. They also have the capacity to sense and respond to chemical signals of host plant and by this way they orient themselves within the roots and enhances own survival. Present investigation was carried out to establish the biocontrol potentiality of nematode extract (*Meloidogyne* sp.) on *Vigna unguiculata* (cowpea) L.walp variety infected with *Meloidogyne incognita* (Kofoid& White) Chitwood nematode. The result of in vitro laboratory bioassay showed that application of nematode extract safe for second-stage juveniles (J_2) of *M. incognita*. The result of in vivo test revealed that nematode extract increased growth of inoculated plants in terms of shoot length, shoot weight and root length as compared with inoculated untreated plants. Application of nematode extract showed reduction in root gall number and number of nematode eggs in inoculated roots. In nematode extract treated plants PAL (Phenylalanine ammonia lyase) activity generally increased in the roots which may interfere with infected juveniles at the time of root penetration. Although there are several current management practices have been identified for plant nematode control such as use of botanicles, cultural practices, physical methods, chemical nematicides etc but application of nematode extract is one of new method which ultimately reduces management cost and enhances crop production.

Keywords: Defence Mechanism, Nematode, PAL activity, Root gall, Management Practices

***Corresponding Author**

Avijit Mukherjee , Department of Zoology, Netaji Mahavidyalaya, Arambagh, Hooghly-712601, West Bengal, India

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1. INTRODUCTION

Phytoparasitic nematodes are one of the most devastating crop pests to control. Historically, management of nematode-induced crop damage has been achieved with the utilization of crop rotation, plant resistance and other cultural practices, or chemical nematicides. Generally two groups of chemical nematicides predominate: low-molecular-weight soil fumigants and contact carbamates or organophosphates. Traditional chemical control methods using the nematicides available for the last few decades is in a declining status internationally. Health and hazard concerns which have elicited close scrutiny by regulatory agencies have resulted in increased restrictions or prohibitions of use. Plants successfully resist invasion from an array of pathogens by inducing plant responses. Plants have existing some physical and chemical barriers to ward Off pathogen attack. During the battle between plants and pathogens, there are two possible outcomes. One possibility is that the susceptible plant succumbs to the pathogen, leading to disease. The other possibility is that the pathogen can be recognized by the plant species, when the particular plant disease resistance gene (R) product detects the corresponding avirulence (avr) gene derived signal from the pathogen [1,2,3]. A hypersensitive response (HR) is one example which triggers the plant to combat pathogens. The hypersensitive response causes necrosis of plant tissue around the site of infection which serves to limit the spread of the infection throughout the plant. The HR is characterized by rapid, localized cell death at the site of infection[3]. Additional evidence also suggests that signal molecules produced from dying cells are involved in the induction of a variety of defense-related genes [3,4]. In addition to the HR, a secondary defense response is triggered in a plant that renders uninfected parts of the plant resistant to a broad range of pathogens. This phenomenon, called systemic acquired resistance (SAR)[5] is associated with increased levels of salicylic acid (SA) in the whole plant[6]. Associated with SAR is the early increased expression of pathogenesis related genes that molecular markers for resistance response[5]. In the view of plant parasitic nematodes which generally inhabit the 'O' horizon of soil, their management practices with nematode extract is one of the new methods identified in our laboratory. About 2000 plant species are susceptible to plant nematode infection and they cause approximately 5% of global crop loss[7]. Cowpea *Vignaunguiculata* (L.walp) is an important vegetable crop and is infected by root-knot nematode, *Meloidogyne incognita* in tropical and subtropical countries like India [8].Nematologists around the world are working to identify and to manipulate natural enemies of nematodes, so that they can be used as biological control agents. Several types of bacteria and fungi have been isolated from nematode populations that were apparently being kept at low levels by natural enemies. Several culture–dependent methods were established to isolate nematode-associated soil bacteria and it was discovered that J_2 of *M.hapla* established a very specific subset of bacterial community from different soils[9]. Under the influence of salicylic acid (SA), which acts as an endogenous signal for the activation of certain plant defense responses, including pathogenesis-related (PR) gene expression and establishment of greater resistance[10]. Phenyl-propanoid pathway is the source of endogenous SA concentration in plants and is most probably synthesized from trans-cinnamic acid catalysed by phenylalanine ammonia lyase (PAL). The active dispersal of plant parasitic nematodes is in a limited condition, but this is compensated by passive dispersal with manoeuvre of infested plant products, soil and also water. Preventing entry of nematodes in the uninfested area is a new challenge. In this experiment we discuss the possibility of nematode control with nematode extract in cowpea plants as a new research area.

2. MATERIALS AND METHODS

2.1. Test materials

Cowpea plants with *Meloidogyne incognita* (Kofoid & White) Chitwood infection were collected from a cowpea farm in Santiniketan, Birbhum, West Bengal, India and authenticated through Department of Botany. A single egg mass were used to establish a population on Cowpea *Vigna unguiculata* (L.walp) variety in the Glass house at Visva Bharati University, WB, India.

2.2. In vitro test with salicylic acid on M.incognita juveniles

Nematode (*Meliodogyne incognita*) eggs were extracted from infected roots of tomato plant using 0.5% sodium hypochloride (Naocl) solution, and then 4 minutes vigorously shaken.[11] Active $J_{2(}$stage juveniles) obtained from egg masses were kept in cavity blocks containing sterile tap water, each block containing 110 + 20 juveniles. To assess the e□ect of SA, the sterile tap water was removed by pipette and immediately replaced by 1 ml of 10 mM SA. Two cavity blocks containing only sterile tap water served as the negative controls. Mortality of the nematodes at room temperature (26 + 28C) was recorded hourly for 6 h.

2.3. Phototoxicity test

Salicylic acid and nematode extract were applied separately as a foliar spray on cowpea cv. L walp plants at the concentration of 2.76 mg/plant and 2 mg/plant respectively. The treated plants were observed up to 15 days to find any toxic effects such as yellowing or wilting of leaves but they did not show any toxic e□ects due to the spraying of SA and nematode extract. Cowpea *Vigna unguiculata* (L.walp) plants susceptible to *M. incognita* wereused for the experiment.

2.4. In vivo glass house bioassay

Aseptically germinated seeds of cowpea cv. L.walp were sown, one per pot, in pots (32 cm diam.) containing a sterilized mixture of clay soil and compost (2:1 v/v). The pots were divided into six groups of 10 each. The groups were: non–inoculated, untreated; inoculated, untreated; non–inoculated and treated with SA; inoculated and treated with SA; non-inoculated and treated with nematode extract; inoculated and treated with nematode extract. In foliar spray groups, SA and NE were applied as a foliar spray by an atomizer, each plant receiving 2.76 mg/plant and 2 mg/plant respectively. Plants in the non-inoculated untreated and inoculated untreated groups received an equal volume of distilled water spray. After 24h, inoculated groups were inoculated with 2700±150 *M. incognita* $J_{2.}$ Treatments were repeated 3 days after inoculation at the same dose. The plants were regularly watered and the experiment was conducted outdoors at an ambient atmospheric temperature (28 ± 2^0C) and humidity (73± 3%).

2.5. *Phenylalanine ammonia lyase (PAL) extraction*

At 4th days after inoculation, PAL was extracted from the roots of cowpea plants. From each group the plants were uprooted and their fresh roots were separately taken, mixed and chopped into pieces. One hundred mg of roots from each group were homogenized in 25mM borate – HCL buffer, pH 8.8, 5mM 2- Mercaptoethanol (400µl-1) at 4°C. Following centrifugation at 12000rpm for 20min at 4°C, the supernatant was used as enzyme source. The activity of PAL was assessed using the method of Brueske[12] according to Sadasivam and Manickam[13] by measuring release of trans-cinnamic acid from L- Phenylalanine. Data were analysed by ANOVA (*P*=0.05) and means are presented with the standard error of the difference between means of ten replicates, followed by Duncan's new multiple range test to compare means[14]

2.6. *Plant growth and nematode assessments*

On the 40th day after inoculation, shoot length, shoot weight, length of longest root, root weight, number of root galls and number of eggs per g of root were recorded. Nematode eggs were extracted from the roots with the sodium hypochlorite method [7]. Three samples of root pieces were taken at random from each plant and the total protein in each sample was determined by the Folin-phenol method[15].

3. STATISTICAL ANALYSIS

Statistical analysis (by one way analysis of variance, ANOVA) was performed to test differential effects among the treatments using MS-Excel software. P values are obtained from the ANOVA table. The value p<0.001 implies a significant difference among the treatments at 0.1% level and means are presented with the standard error of the difference between means of ten replicates. Data shows significant difference between control (NU, IU) and treated groups (N SA, I SA. N NE and I NE).

4. RESULTS

Nematodes survived as well in 2mg NE in *vitro* as in the controls, with only 2.3% and 3.2% mortality after 6h. SA and nematode extract increased growth of inoculated plants in terms of shoot length, shoot weight and root length as compared with inoculated untreated plants (Table I). There were fewer galls and eggs in roots of SA and nematode extract treated ones than in inoculated untreated plants. Root mass was greater in inoculated untreated plants compared to the non-inoculated plants. PAL activity increased in the roots of cowpea sprayed with nematode extract and SA, irrespective of nematode inoculation. Root protein content was greater in inoculated treated plants compared with inoculated untreated groups.

Table I. Plant growth, protein content and nematode infestation of cowpea sprayed with SA and nematode extractat 24h before and 72h after inoculation with 2700±150 second stage juveniles of *M. incognita* (40 days after inoculation)

Treatment*	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Root galls	Eggs per g root	PAL activity in root (µg/g)	Root protein conc. (mg/g)
NU	147	28.4	26.2	5.8	-	-	13.6	5.23
IU	104.2	17.4	19.8	13.0	230	787	19.4	4.7
N SA	109.2	22.4	22.6	8.0	-	-	19.3	6.3
I SA	121.6	24.0	22.2	9.8	188	663.3	18.3	5.23
N NE	126.6	25.4	22.4	9.4	-	-	19.3	6.3
I NE	138.4	27.2	22.2	9.0	166.2	574.6	18.3	5.63
SED	3.29	2.48	1.78	1.97	7.57	1.924	0.146	0.141
P	<0.001	0.002	0.048	0.037	<0.001	<0.001	<0.001	<0.001

Dashes(-) indicate no root galls or eggs in this group. NU, non–inoculated untreated; IU, inoculated and untreated; NSA, non–inoculated and treated with SA at 2.76 mg/plant; ISA, inoculated and treated with SA at 2.76 mg/plant; NNE, non-inoculated and treated with nematode extract at 2 ml / plant; INE, inoculated and treated with nematode extract at 2 ml / plant. Means of seven replicates.

5. DISCUSSION

In controlling plant parasitic nematodes, detection and identification of them is the first priority because proper controlling involves checking their spreads to other normal plant species. Any crop which is contaminated with nematodes may show only patches of damage but their number and infective property are much more throughout the whole plant. They also secrete some substances through their own style which is present at the anterior of the worm and by this secretion, infection rate increases and it also helps in the formation of host feeding cells. This secretory activity also helps them proper parasitism with the host plant[16]. These nematodes are generally harmful to crop growth and development depending on population density. There are several practices identified to reduce the effect, such as biocontrol bacteria[17], essential oils such as citral, menthol[18]etc but treatment with nematode extract is a new era. In the present study, it has been demonstrated that cowpea plants treated with nematode extract enhanced resistance against infection by *M.incognita* and improved plant growth. It appears from the data that treatment with NE was more effective than SA treatment. Infected roots had greater enzyme activity in all the plants studied. Treatment of host plants with NE at the time of root penetration reduced subsequent nematode reproduction assessed at 40 days after inoculation. We showed that NE did not kill infective juveniles *in vitro*. Since fewer juveniles were recovered from nematode extract-treated roots at 40 days after inoculation, it may be that enhanced PAL activity interfered with J_2 at the time of root penetration. Treatment of host plants with nematode extract at the time of root penetration reduced subsequent nematode reproduction, assessed 40 days after inoculation. In this experiment we found that nematode extract treated plants effectively increased resistance to nematodes. It is, therefore, likely that improved growth and reduced protein content of NE-treated nematode-infected plants was a result of the reduction in root-knot reproduction in NE-treated plants.

6. CONCLUSION

In conclusion, it is evident that complete elimination of nematodes is not possible; the main goal is to manage their population below damaging levels. It is still not understood the interacting mechanism between nematode extract-root/leaf interaction leads to induce systemic resistance against plant-parasitic nematodes but it has been suggested in plants nematode-extract-induced systemic resistance triggers a signal transduction pathway that is different from other common pathogen or bacterial or chemical induced pathways. This nematode extracts synergistically and independently enhances plant immune response which protects them from parasitic infections.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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