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Trigonella foenum-graecum (fenugreek)-mediated suppression of *Meloidogyne javanica* in mungbean

TAYYABA ZIA, IMRAN ALI SIDDIQUI*, SYED SHAHID SHAUKAT*, SYED NAZARUL-HASNAIN

Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan
*Soil Biology and Ecology Laboratory, Department of Botany, University of Karachi, Karachi-75270, Pakistan

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Summary

Soil amendments with powdered seeds of *Trigonella foenum – graecum* (fenugreek) caused soil suppressiveness against *Meloidogyne javanica*. Decomposed seeds of fenugreek caused marked reduction in nematode population densities and subsequent root-knot development as compared to the aqueous extract of the seeds indicating that some indirect factors are involved in the suppression of root-knot nematode. Both decomposed seeds and aqueous extracts enhanced plant height and fresh weights of shoot whereas root growth remained uninfluenced. Changes in fungal communities associated with nematode control were studied by comparing population numbers of fungi in the soil and in internal root tissues (endorhiza) in non-amended and fenugreek-amended soils. *Acremonium* sp., *Chaetomium globosum, Fusarium solani, Macrophomina phaseolina* and *Rhizoctonia solani* were found to colonize inner root tissues of mungbean. *Acremonium* sp., *C. globosum* and *F. solani* were isolated in a relatively higher frequency from roots growing in the amended soils while *M. phaseolina* and *R. solani* colonized greatly in roots growing in non-amended soil. Of the fungi isolated from soils, *Penicillium brefaldianum* caused maximum juvenile mortality of *M. javanica* whereas *F. solani* caused greatest inhibition of egg hatch.

Key words: Organic amendments, saprophytic fungi, root-infecting fungi, root-knot nematode, fungal diversity

INTRODUCTION

Organic amendments, including animal manures, composts and green manures, are commonly used in agricultural systems to recycle nutrients and energy as well as improve soil conditions for plant growth (Hadar et al., 1992; Muchovej and

Pacovsky, 1997). Amendments provide energy and nutrients to soil, drastically changing the environment for the growth and survival of crops and microorganisms (Drinkwater et al., 1995). Some organic amendments suppress certain soil-borne plant pathogens and/or the diseases they cause, and several have been effectively used for the control of plant – parasitic nematodes (Rodríguez-Kábana, 1986; Ali et al., 2001).

Trigonella foenum-graecum (fenugreek) is a native to the area extending from Eastern Mediterranean to Central Asia and Ethiopia and frequently cultivated in Pakistan, India and China (Morton, 1990). Seeds of fenugreek have been reported for their pharmaceutical properties in treating certain human diseases such as diabetes and hypocholesterolaemia (Stark and Madar, 1993; Ali et al., 1995). Although, we have demonstrated that aqueous, methanolic and chloroform extract of fenugreek cause heavy mortality of the juveniles of *Meloidogyne javanica in vitro* (Zia et al., 2001), its exact role in suppressing nematode population in soil is unknown.

The aims of the present study were to evaluate i) the effects of decomposed seed material on infectivity of *M. javanica*; ii) the effect of aqueous extract of the seed material on infectivity of *M. javanica*; iii) fenugreek-mediated changes in the fungal community structure and as a consequence suppression of *M. javanica* in mungbean and iv) fenugreek-mediated impact on growth of mungbean.

MATERIALS AND METHODS

Fenugreek seeds and nematodes:

Trigonella foenum-graecum L. (fenugreek) seeds were purchased from the local market, cleaned, dried and finally powdered in an electric grinder. *Meloidogyne javanica* (Treub.) Chitwood. was obtained from pure cultures maintained on roots of eggplants (*Solanum melongena* L.). The entire root system was dipped in water and soil was removed gently without detaching egg sacs. Eggs were extracted by vigorous shaking of infested roots in a 1% sodium hypochlorite solution for three minutes. The resulting suspension was then passed through a range of different mesh sieves. The eggs were collected on a fine sieve (38 µm) and washed in tap water to remove all traces of sodium hypochlorite before use. Hatched juveniles of *M. javanica* were obtained by placing the eggs in sterile distilled water for 3 days at 28°C.

Effects of decomposed seeds:

Unsterilized sandy loam soil, pH 8.1 was mixed with powdered seeds of fenugreek to make 0, 1.0 or 3% concentrations and put into 8 cm diam. plastic pots at 350 g/pot. The soil was watered daily to allow for decomposition of the material. Three weeks after soil amendments eight mungbean *Vigna radiata* (L.) Wilczek seeds were sown in each pot and after germination four seedlings were maintained in each pot. One week after seedling emergence, the roots were infested with 2000 freshly hatched juveniles of *M. javanica* by making three holes around the seedlings. Each treatment was replicated four times and pots were arranged in a randomized block design.

Effects of seed extract:

To determine whether decomposition was a requisite for the release of toxic principles for the suppression of root-knot nematode, in another experiment soil was drenched with 0, 25 or 50 ml extract (200 g/500 ml water = stock solution). Rest of the procedure was the same as described above.

The experiment was terminated 45 days after the addition of nematode and plant growth parameters (plant height and fresh weight of shoot) were recorded. The number of galls developed on the entire root system was also counted under a low magnification (x 6). To determine the nematode penetration, a one g root sample after through washing with tap water was stained in 0.25% acid fuchsin with lactic acid and macerated in an electric grinder for 45 seconds. The macerate was suspended in 100-ml water, and *M. javanica* females and juveniles in 5 samples of 5 ml each were counted with the aid of a low power microscope (x 6). For the assessment of nematode populations, soil of a treatment (from all the replicates) was mixed thoroughly in a plastic container and 5 samples of 50 g were used for the extraction of nematodes using modified Baermann funnel technique.

In a previous experiment, whereas aqueous extract of the seeds was ineffective, decomposed seed material caused significant suppression of nematode population densities and consequent root-knot development in mungbean. These results lead us to the conclusion that either direct (continuous release of the toxic principles) or indirect (change in the fungal community structure in the amended soil) mechanisms were involved. Experiment of decomposed fenugreek seed was repeated and the same protocol was followed as described earlier. Apart from growth and disease parameters, a change in fungal community structure in soil and inner root tissues in both amended and non-amended soil was also assessed. Root samples were divided into two equal portions and one portion was used to estimate nematode populations in the roots while the remaining part was used to assess fungal root colonization.

Isolation of fungi from roots:

To determine the fungal colonization, roots after washing in running tap water were cut into 5 mm long pieces, surface sterilized thoroughly with 1% $Ca(OCl)_2$ and placed onto PDA plates supplemented with penicillin and streptomycine sulfate to avoid bacterial contamination. The plates were incubated at room temperature for 5 days and percentage colonization of the fungi was calculated as follows:

$$\% \text{ Colonization} = \frac{\text{Number of root pieces colonized by a fungus}}{\text{Total number of root pieces}} \times 100$$

(In this experiment, data on nematode population densities, root-knot disease and growth parameters were almost similar to those of the previously described experiment, therefore, they are not presented).

Isolation and enumeration of fungi from soil:

To determine the total fungal populations, at each sampling time 10 g of the moist soil was taken and added to 250 ml Erlenmeyer flasks containing 100 ml sterile distilled water and stirred for 2–4 mins. A serial dilution was prepared and 0.1 ml aliquots of dilutions from 10^3 were plated onto PDA and Czapek's Dox agar medium supplemented with penicillin and streptomycin sulphate to avoid bacterial contamination. One week after incubation at 28° C, the total fungal counts in the plates were recorded.

Nematicidal and hatch reducing activity of the isolated fungi:

Isolated fungi were tested for their nematicidal activity against *M. javanica.* The fungi were grown in 250 ml Erlenmeyer flask containing 100 ml Czapek's Dox liquid medium. One week after incubation at 28° C, the extract was filtered through Whatman No. 1 filter paper and the filtrate collected in a beaker prior to use. One ml of the fungal extract with one ml of surface sterilized *M. javanica* juvenile (35–50 juveniles/ml) was placed in a glass cavity slide and kept at 28° C. There were three replicates for each fungal filtrate. After 48 h incubation period, the number of dead and surviving juveniles were counted and the mean percent mortality calculated. The efficacy of the extracts on hatching of *M. javanica* egg masses was also determined by adding two medium size egg masses of equal size into glass cavity slides. Three such slides for each fungal filtrate were kept at 28° C and total number of hatched juveniles were counted after 120 h. Nematicidal and hatch inhibiting activity of the filtrate was compared with that of Czapek's Dox broth without any fungal culture.

Statistical analysis:

Data were subjected to one way analysis of variance (ANOVA) followed by least significant differences (LSD). Treatment means were also compared using Duncan's multiple range tests in accordance with Sokal and Rohlf (1995). Fungal populations were transformed to log10 (×+1) before the analyses.

Diversity indices:

The general species diversity of the fungal communities was measured by the popular Shannon-Weiner information theory function.

$$H' = -\sum_{i=1}^{s} p_i \log p_i$$

Where p_i is the proportion of total number of colonies N belonging to the it species (Shannon and Weaver, 1963). The variance of general diversity H' was calculated in accordance with Magurran (1988), as follows:

$$\text{Var}(H') = \frac{\sum p_i (\log p_i)^2 - (\sum p_i \log p_i)^2}{N} + \frac{S-1}{2N^2}$$

The general diversity incorporates two components of diversity: species richness, which expresses the number of species (S) as a function (ratio) of total number of individuals (N), and equitability, that measures the evenness of allotment of individuals among the species.

Species richness was simply determined by the number of species $\hat{S}$ (Magurran, 1988). Equitability component of diversity and its variance were measured in accordance with Pielou (1975):

$$J' = H'/H'_{max}$$

The equitability index J' is the ratio between observed (H') and maximal diversity (H'_{max}). Variance of equitability was estimated as:

$$Var(J') = Var(H')/(\log S)^2$$

RESULTS

Soil amendment with powdered fenugreek-seeds significantly ($p<0.001$) reduced nematode population densities in soil, nematode penetration rates and root-knot development in mungbean (Table 1). The greatest suppression of nematode population densities in soil (>36%), nematode invasion (>56%) and subsequent galling (>53% compared to the controls) was achieved following application with 3% fenugreek-seeds. Soil amendment with 1% fenugreek-seeds gave maximum plant height whereas 3% fenugreek-seeds produced greatest fresh weight of shoots. Plants grown in non-amended soils had the greatest fresh weights of roots.

Table 1.

Effect of decomposed *Trigonella foenum-graecum* (fenugreek) seeds on the development of root-knot infection, nematode populations in soil and root and growth of mungbean.

Treatments	Galls/root system	Root-knot nematode Populations		Plant height (cm)	Shoot weight (g)	Root weight (g)
		250 g soil	1 g root			
Trigonella 0%	82	3102	144	17.3	2.3	1.3
Trigonella 1.0%	45	2396	74	19.8	2.9	1.1
Trigonella 3.0%	38	1975	62	19.3	3.3	1.0
$LSD_{0.05}$	11	386	16	1.1	0.4	0.5

Only 50 ml aqueous extract of fenugreek seeds caused significant ($p<0.05$) reduction in galling (>15%) and nematode invasion (>19%) compared to the untreated controls. On the other hand aqueous extract at both the concentrations failed to reduce

nematode population densities in soil (Table 2). Aqueous extract applied at 25 ml gave maximum ($p<0.05$) plant height and fresh weight of shoot while root weight was not significantly influenced by the extract.

Table 2.

Effect of aqueous extract of *T. foenum-graecum* (fenugreek) seeds on the development of root-knot infection, nematode populations in soil and root and growth of mungbean.

Treatments	Galls/root system	Root-knot nematode populations		Plant height (cm)	Shoot weight (g)	Root weight (g)
		250 g soil	1 g root			
Trigonella 0 ml Ss	76	2682	128	17.6	1.9	1.1
Trigonella 25 ml Ss	82	2667	137	19.1	2.5	0.9
Trigonella 50 ml Ss	64	2481	116	18.6	2.4	1.0
$LSD_{0.05}$	10	281	17	0.9	0.4	0.2

Table 3.

Effects of soil amendments with *T. foenum-graecum* (fenugreek) seeds on soil fungal community structure [Log cfu (x+1)].

Fungal species	Trigonella (%)								
	0			1.0			3.0		
	Sampling time (days)								
	0	15	73	0	15	73	0	15	73
Acremonium sp.	0	0.34	0	0	0.49	1.41	0	0	0.99
A. fumigatus	0	0.34	0.34	0	0	0	0.34	0	0.49
A. niger	0.49	1.06	1.43	0.34	1.26	2.25	0.49	0.44	2.15
Aspergillus sp.	0	0	0	0	0.34	0.49	0	0	0
Chaetomium globosum	0	0	0.34	0.34	0.44	1.26	0.44	0.34	0.49
Cladosporium herbarum	0	0.34	0.34	0	0.34	0	0	0.34	0
F. oxysporum	0.34	0.49	0.84	0.34	0.56	0.56	0	0.56	1.06
F. solani	0.34	0.56	1.26	0.49	1.38	1.43	0.49	0.84	1.59
Fusarium spp.	0	0.34	0	0	0	0.49	0	0.34	0
Paecilomyces varioti	0	0	0	0	0.44	0.84	0	0	0.34
Penicillium brefaldianum	0	0	0.34	0	0.34	0	0	0	0
Penicillium sp.	0.49	0	0.34	0.34	0	0	0	0.34	0.34
Rhizopus stolonifer	0.49	0.56	0.84	0.44	0.99	1.26	0.34	1.26	1.84

In general, soil amended with 1% fenugreek showed greater number of fungi with high colony forming units as compared to soils treated with 3% fenugreek or kept untreated (Table 3). Aspergilli and Fusaria were most dominant represented each with three species. *A. niger* was isolated with highest frequency from all type of soil at all the times. *Paecilomyces varioti* and a *Penicillium* sp., which were isolated from the amended soils, were absent in the non-amended soils. Likewise, an *Aspergillus* sp., isolated from 1% fenugreek amended soils was not recovered from 3% amended or non-amended soils. In general, fungal species diversity (H') decreased with the passage of time in controls as well as treatments (Table 4). With respect to the controls, in soil treated with 1% fenugreek, species diversity (H') increased slightly but decreased in 3% fenugreek amended soils. Equitability (J') was highest in controls. Species richness (S') increased slightly in 1% fenugreek over the controls but in 3% fenugreek, it equalled the values for the controls.

Table 4.

General diversity H', equitability (J'), and the number of species S in *T. foenum-graecum* amended soils at various sampling periods. Var (H') = variance of H'; Var (J') = variance of J'.

Diversity	Trigonella (%)								
	0			1.0			3.0		
	Sampling time (days)								
	0	15	73	0	15	73	0	15	73
H'	1.624	1.715	1.447	1.766	1.700	1.310	1.600	1.499	1.284
Var (H')	0.017	0.042	0.019	0.045	0.016	0.004	0.026	0.036	0.003
J'	1.009	0.824	0.658	0.985	0.738	0.596	0.994	0.721	0.584
Var (J')	0.006	0.009	0.004	0.014	0.003	0.001	0.010	0.008	0.0008
$\hat{S}$	5	8	9	6	10	9	5	8	9

Table 5.

Percentage colonization of the fungal species isolated from inner root tissues of mungbean growing in soil amended with *T. foenum-graecum* (fenugreek) seeds.

Fungal species	Colonization (%)		
	T. foenum-graecum (%)		
	0	1.0	3.0
Acremonium sp.	0	3.1	7.8
Chaetomium globosum	0	12.5	12.5
Fusarium solani	20.3	32.8	34.3
Macrophomina phaseolina	39.0	35.9	12.5
Rhizoctonia solani	43.7	23.4	21.8
$LSD_{0.05}$	14.2	11.8	8.7

Acremonium sp., *Chaetomium globosum*, *Fusarium solani*, *Macrophomina phaseolina* and *Rhizoctonia solani* were found to colonize inner root tissues of mungbean (Table 5). *Acremonium* sp., C. *globosum* and *F. solani* were isolated in a relatively higher frequency from roots growing in the amended soils while *M. phaseolina* and *R. solani* colonized greatly in roots growing in non-amended soil. *Acremonium* sp. and *C. globosum* were isolated only from the inner root tissues growing in the amended soils. Of the fungi isolated and tested for their activity against *M. javanica*, *Penicillium brefaldianum* caused maximum (68%) juvenile mortality followed by *F. solani* producing 51% juvenile's deaths (Table 6). F. solani caused greatest inhibition in egg hatch followed by *C. globosum*.

Table 6.

Percentage deaths and number of hatched juveniles of *M. javanica* by the extracts of fungal species isolated from soil amended with *T. foenum-graecum*.

Fungal species	Mortality(%)	Hatched juveniles
	Incubation period (hour)	
	48	120
Czapek's dox broth	3	311
Acremonium sp.	41	263
Aspergillus fumigatus	21	222
A. niger	37	181
Aspergillus sp.	18	285
Chaetomium globosum	32	168
Cladosporium sp.	9	321
Fusarium oxysporum	29	193
F. solani	51	156
Fusarium spp.	17	251
Paecilomyces varioti	46	188
Penicillium brefaldianum	68	235
Penicillium sp.	15	277
Rhizopus stolonifer	21	301
$LSD_{0.05}$	10.1	24.7

DISCUSSION AND CONCLUSION

Our results indicate that amendment of soil with fenugreek caused soil suppressiveness to root-knot nematodes and resulted in changes in the fungal communities of

the soil, rhizosphere and endorhiza. We suggest that fungal species, especially endophytes, which were exclusively suppressed or markedly promoted by the fenugreek seed amendment, might contribute to the observed suppressiveness of *M. javanica*. Following fenugreek-amendment, numbers of fungi with nematicidal activity were especially favored due to this amendment. In a previous report Shaukat and Siddiqui (2001) found an increased number of fungi both in the rhizosphere and root suppressive to *M. javanica* following soil amendment with powdered shoot of *Lantana camara*, a tropical weed.

Isolation of microfungi from the rhizosphere of mungbean yielded a broad fungal spectrum dominated by genera and species rather widespread and frequently found in agricultural soils, rhizospheres, and roots of crop plants (Barron 1968; Booth, 1971; Domsch et al., 1980; Gerlach, 1982). This fungal spectrum overlaps the one obtained by Hong (1969), Girlanda et al., (2001) and Shaukat and Siddiqui (2001). No *Oomycetes* (*Pythium* and *Phytophthora*) were isolated in this study, despite the fact that they can grow on the laboratory medium used. This is in accordance with the fact that disease pressure is usually low in Karachi soils. A high temperature during mungbean cultivation might have had a negative role on fungal survival and colonization. Furthermore, soil amendment with fenugreek might also resulted in an increased number of antibiotic producing bacteria particularly pseudomonads which are known to reduce fungal infection in a number of economically important crops (Keel et al., 1992). *Macrophomina phaseolina* and *Rhizoctonia solani* that were not recovered from the soils (either amended or non-amended) also isolated from root interiors most likely due to the fact that specialized isolation techniques and selective medium are normally used for the isolation of these two fungi which were not employed in this study.

In the present study, populations of *F. oxysporum* and *F. solani* were markedly altered following soil amendment with fenugreek. Both these fungi are highly pathogenic causing severe damage to a number of crops and are controlled by the application of organic amendment. However, the majority of soil fungi are not pathogenic, and a large number of them may even be beneficial to plants and/or contribute positively to ecosystem functioning. Indeed, nonpathogenic saprotrophic microfungi perform key ecological roles in the soil ecosystem through decomposition of organic matter, nutrient cycling, natural control of plant pathogens, and a myriad of other functions (Cooke and Rayner, 1984; Curl and Truelove, 1986; Dix and Webster, 1995). Common rhizosphere fungi are well documented as decomposers of cellulose and hemicellulose (*Trichoderma, Penicillium,* and *Fusarium*), as well as chitin (Paecilomyces) (Domsch et al., 1980). The ability of certain saprotrophic *Aspergillus* and *Paecilomyces* strains to protect against pathogenic fungi and nematodes through competition, parasitism and antagonism is also well known (Siddiqui et al., 2000; Siddiqui et al., 2001). In this context, it is surprising that saprotrohic rhizosphere fungi have been largely neglected as nontarget, beneficial resident microorganisms potentially affected by an organic amendment, especially when the latter produce nematicidal and antifungal metabolites with a relatively broad range of action.

The results reported here indicate that fenugreek amendment resulting in suppression of root-knot nematode is not only associated with changes in the fungal community structure in the soil and rhizosphere but also with changes in the fungal community within the plant tissue. This strongly suggests that the physiology of a plant growing in fenugreek-amended soil differs from one grown in the absence of fenugreek. Hence, possibly organic amendments can be used to manipulate the soil micro-flora and induce desired changes in the endophytic microflora to the advantage of the plant.

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Ograniczenie występowania *Meloidogyne javanica* na roślinach na *Vigna radiata* pod wpływem *Trigonella foenum – graecum*

Streszczenie

Dodanie do gleby zmielonych nasion powodowało jej ujemne działanie na *Meloidogyne javanica*. Wodny ekstrakt z nasion był mniej skuteczny niż rozłożone nasiona, co dowodzi, że działanie na nicienia mają składniki pośrednie . Badano zmiany w populacjach grzybów związane ze zwalczaniem nicieni, porównując liczbę populacji grzybów w glebie i w tkankach korzeni w warunkach gleby kontrolnej i traktowanej *Trigonella*. Rozłożone nasiona kopru włoskiego powodowały silniejszą redukcję populacji nicieni i w konsekwencji – występowania narośli na korzeniach w porównaniu do wodnych ekstraktów z nasion. Niezależnie od sposobu zastosowania, nasiona kopru stymulowały wzrost (wysokość i świeżą masę pędów) roślin *Vigna radiata* (L.)Wilczek, ale wzrost korzeni nie ulegał zmianie. Wewnętrzne tkanki korzeni tej rośliny były kolonizowane przez *Acremonium coenophialum, Chaetomium globosum, Fusarium solani, Macrophomina phaseolina* i *Rhizoctonia solani. A. coenophialum, C. globosum* i *F. solani* były izolowane ze względnie dużą

częstotliwością z korzeni roślin rosnących w glebie z dodatkiem nasion kopru, podczas gdy *M. phaseolina* i *R. solani* kolonizowały głównie korzenie rosnące w glebie bez tego dodatku. Z grzybów wyizolowanych z gleby, *Penicillium brefaldianum* powodował maksymalną śmiertelność form juwenilnych, podczas gdy *F. solani* hamował głównie wylęgi jaj.