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EVALUATION OF *ASPERGILLUS* SPECIES FOR THE BIOCONTROL OF *MELOIDOGYNE JAVANICA* IN MUNGBEAN

by

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Summary. Five species of *Aspergillus* (*A. flavus*, *A. terreus*, *A. tamarii*, *A. niger* and *A. nidulans*) were tested for parasitic and mycotoxic activity against *Meloidogyne javanica* in laboratory and pot experiments. In a laboratory assay, *A. terreus* and *A. nidulans* parasitized *M. javanica* females. Culture filtrate of *A. niger* markedly inhibited egg hatching and caused juvenile mortality. These toxic activities were retained when the filtrate was heated. The activities of *A. niger* were probably due to non-proteinaceous compound(s). In a glasshouse evaluation, conidial suspensions and the respective culture filtrates of some *Aspergillus* species significantly reduced soil populations of *M. javanica* and root galling and increased plant height and shoot fresh weight of mungbean. *A. niger* in combination with *Pseudomonas aeruginosa*, a plant growth-promoting rhizobacterium, significantly reduced root-knot development and nematode population densities, and increased plant growth.

A high density of microbes including fungi, bacteria and nematodes are present in the rhizosphere. Fungi and plant-parasitic nematodes in the rhizosphere may exhibit synergistic or antibiotic interactions (Jorgenson, 1970; Powell, 1971). Many soilborne fungi are known to produce secondary metabolites (Wyllie and Morehouse, 1978; Zuckerman *et al.*, 1994). Those produced by fungi are of interest because of the possible effects exerted during plant parasitism and their potential as natural pesticides (Zuckerman *et al.*, 1994; Ciancio, 1995). Of the various soil inhabiting fungi, *Aspergillus* species are very common in agricultural and non-agricultural soils and are reported as minor parasites of plant-parasitic nematodes (Khan *et al.*, 1994; Amer-Zareen *et al.*, 2000). Filtrates produced by some soilborne fungi, including *Aspergillus*, are known to be toxic or lethal to a variety of plant-parasitic nematodes (Alam *et al.*, 1973; Mani and Sethi, 1984; Cayrol *et al.*, 1989; Amer-Zareen and Zaki, 2000; Siddiqui *et al.*, 2000).

This study reports on the parasitic and mycotoxic effects of five *Aspergillus* species on *Meloidogyne javanica* (Treub) Chitw. and on their impact on the population density of *M. javanica* and the consequent root-knot development in mungbean.

Materials and methods

Aspergillus nidulans (Eidam) Wint and *A. terreus* Thom., originally isolated from females of *M. javanica*, *A. flavus* Link and *A. niger* Van Tieghem from juveniles of the same nematode species and *A. tamarii* Kita from an egg of *M. javanica* (Amer-Zareen *et al.*, 2000) were used in the present study. To study the effect of *Aspergillus* species on parasitism, *M. javanica* fe-

males were obtained from infected tomato plants by teasing out the galls using sterilized needles, surface disinfected with 0.5% $Ca(OCl)_2$ and then five females were transferred onto 2% water agar plates amended with penicillin (100,000 units/l.) and streptomycin sulphate (0.2 g/l.). The plates were inoculated with the test fungi individually at the centre of each plate. The plates containing 2% water agar without the fungal culture served as controls. After a 7 day incubation period, 10 ml sterile distilled water was added in each plate and females were collected. The plates were incubated at room temperature and structures of emerging fungi were compared with that of the test fungi.

Culture filtrates were obtained by growing the fungi on Czapek's liquid medium at 28±1 °C in an incubator. After a week, mycelial mats were removed and the media were filtered through two layers of Whatman No. 1 filter paper. The filtrate thus obtained was collected in a beaker and kept at 6 °C prior to use. To study the effects of culture filtrates of *Aspergillus* species on egg hatching of *M. javanica*, two medium sized egg masses with 2 ml of the culture filtrate of an *Aspergillus* species were transferred to a 1 cm diam. cavity glass slide. The egg masses placed in Czapek's liquid medium served as controls. Each treatment was replicated three times and the cavity glass slides were arranged in a randomized complete block design. The number of hatched juveniles were counted after 48 h. The egg masses were then transferred into cavity glass slides containing 2 ml sterile distilled water to ascertain whether the egg masses kept in the culture filtrate had been temporarily or permanently inactivated. The juveniles were counted again after a further 48 h period.

To study the effects of culture filtrate of *Aspergillus* species on mortality of *M. javanica*, two ml of each filtrate were poured in a glass cavity slide and about 30-40 second stage juveniles of *M. javanica* were placed in each glass slide. Juveniles kept in freshly prepared liquid medium served as controls. Treatments were replicated three times and dead nematodes in each cavity slide were counted after 24 and 48 h. The nematodes were considered to be dead when they did not move on probing with a fine needle (Cayrol *et al.*, 1989). Since culture filtrate of *A. niger* caused the highest mortality of *M. javanica* juveniles, its culture filtrate was further diluted to 1/10, 1/100 and 1/1000 by adding the required amount of sterile distilled water. A similar set of cavity glass slides containing the culture filtrate was also subjected to boiling. Nematicidal activity of the culture filtrate was assessed as described above.

Unsterilized sandy-loam soil of pH 8.1 (organic matter 0.3%, water holding capacity 37.8%), obtained from the experimental field of the Crop Diseases Research Institute (CDRI), Karachi was screened through a 2 mm mesh sieve to eliminate stones. The soil had natural populations of *Tylenchorhynchus curvus* Williams, *Pratylenchus thornei* Sher *et* Allen and *Aphelenchus avenae* Bastian but not that of *Meloidogyne* spp. In addition, the soil also contained several pathogenic-fungi including *Macrophomina phaseolina* (Tassi) Goid., *Rhizoctonia solani* Khuen and *Fusarium* spp. The soil was filled in 8 cm diam. plastic pots at 350 g/pot. The upper 3 cm soil was removed and conidial suspensions of *A. niger* (cfu $2.8x10^7$ ml^{-1}); *A. nidulans* (cfu $2.8x10^7$ ml^{-1}); *A. terreus* (cfu $3.2x10^7$ ml^{-1}) and *A. tamarii* (cfu $1.7x10^7$ ml^{-1}) prepared in 25 ml water were drenched in each pot. In another similar set, culture filtrates of the *Aspergillus* spp., were drenched at 25 ml per pot. After treatment, eight mungbean, *Vinga radiata* (L.) Wilczek, seeds were sown in each pot and covered with soil. After germination only four seedlings were retained in each pot. One week after seedling emergence, soil in each pot was inoculated with 2000 freshly hatched second stage juveniles of *M. javanica* by making three holes around the seedlings. Treatments were replicated four times and pots were arranged in a randomized complete block design. The experiment was terminated 45 days after nematode inoculation and growth parameters such as plant height and

fresh weight of shoot and root were recorded. Numbers of galls induced by *M. javanica* on the entire root system were estimated. To determine the nematode invasion, the roots of plants after thorough washing in running tap water were blotted dry, reweighed and wrapped in a muslin cloth. The roots were dipped in boiling 0.2% acid fuchsin-lactic acid solution. After 3-5 minutes, the roots were removed and washed in running tap water to remove the excess stain and homogenized in an electric grinder for 1 min. The number of nematodes that had penetrated the roots were counted and penetration on a per gram fresh root weight basis calculated. The number of second stage juveniles in the soil were estimated using a modified Baerman funnel technique. To determine the amount of parasitism by *Aspergillus* species, ten females from each treatment were randomly selected, surface sterilized with 0.5% $Ca(OC1)_2$ and plated onto 0.8% water agar. The plates were incubated at room temperature and after three days the rate of fungal parasitism was estimated.

Since *Paecilomyces lilacinus*, an egg parasite of root-knot nematode, in combination with *Pseudomonas aeruginosa*, a plant growth-promoting rhizobacterium, has shown promising results in the control of *M. javanica* on various crop plants (Perveen *et al.*, 1998), an experiment was carried out to examine the combined efficacy of *A. niger* alone or incombination with *P. aeruginosa* (Schroeter) Migula, in the control of *M. javanica* in mungbean. The soil was excavated to depth of 2 cm and a 25 ml conidial suspension of *A. niger* ($4.2x10^{-7}$ cfu ml^{-1}) or aqueous cell suspension of *P. aeruginosa* ($2.5x10^{8}$ cfu ml^{-1}) was drenched separately in 8 cm diam. plastic pots containing 350 g soil. In another similar set, *A. niger* and *P. aeruginosa* were drenched together (in this co-inoculation experiment the concentration of each component was half its individual concentration). Soil drenched with 25 ml sterile distilled water served as a control. Pots were arranged in a randomized complete block design with four replicates of each treatment. Subsequently, eight mungbean seeds were sown in each pot and after germination the seedlings were thinned to four per pot. One week after seedling emergence the soil in each pot was infested with 2000 freshly hatched second stage juveniles of *M. javanica*. Plants were harvested 45 days after nematode inoculation and plant growth parameters, nematode population densities and number of galls produced on the entire root system were counted as described earlier.

The data were subjected to analysis of variance (ANOVA). Treatment means were compared using either Duncan's multiple range test or least significant difference (LSD) (Sokal and Rohlf 1995).

Result and discussion

Of the *Aspergillus* species tested, only *A. terreus* and *A. nidulans* were parasitic on *M. javanica* females *in vitro*. *A. terreus* caused 33% infection whereas 17% females were parasitized by *A. nidulans* (Table I). Apparently the degree of parasitism caused by *Aspergillus* species to nematodes in general has not been examined previously.

Aspergillus species significantly ($p<0.001$) inhibited egg hatching and caused mortality of *M. javanica* juveniles. Fewest juveniles hatched in the culture filtrate of *A. niger* (Table II). Even

TABLE I - *Colonization of* Aspergillus *species on females of* Meloidogyne javanica *in vitro.*

Species	% parasitism
A. niger	0 a
A. flavus	0 a
A. nidulans	0 a
A. nidulans	17 b
A. tamarii	0 a
A. terreus	33 c

Means followed by the same letters are not significantly different at $p\leq0.05$ according to Duncan's multiple range test.

TABLE II - *Effects of culture filtrates* of Aspergillus *species on egg hatching of* M. javanica.

Species	Number of eggs hatched		Total no. of eggs hatched	Inhibition % over control
	Culture filtrate	Distilled water*		
Czapek's broth	91 a	108 a	199	–
A. flavus	75 ab	65 bc	140	29.6
A. terreus	66 ab	71 bc	137	31.1
A. tamarii	83 ab	91 ab	174	12.5
A. niger	19 c	48 c	67	66.3
A. nidulans	62 b	71 bc	133	33.1

* After a 48-h hatching period in culture filtrate, the egg masses were transferred to sterile distilled water; means followed by the same letters are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

after transfer to distilled water, the egg hatching was minimum for *A. niger* compared to other *Aspergillus* species and the controls. The permanent inactivation of egg hatching ranged between 13% (*A. tamarii*) to 66% (*A. niger*).

Pure culture filtrate of *Aspergillus* species caused significant ($p<0.001$) mortality of *M. javanica* juveniles. Significantly greater ($p<0.01$) mortality was caused at 48 h of exposure to culture filtrate than at 24 h. All juveniles were killed in the culture filtrate of *A. niger* and fewest in filtrate from *A. terreus* (Table III). A test of culture filtrate of *A. niger* at various dilutions resulted in a gradual decrease in mortality with decrease in filtrate concentration (Table IV). Variable effects of the fungal filtrates on egg hatching and mortality of *M. javanica* observed in the present study could presumably be due to the varied nature of toxic metabolites produced by different species. Differences between *Aspergillus* spp., with respect to juvenile mortality could also be due to differences in pH of the filtrate. When pure culture filtrate of *A. niger* was boiled, it retained its effectiveness, causing 100% juvenile mortality. Similarly, Zuckerman *et al.*, (1994) found that the culture filtrate of *A. niger* after boiling for 5 min retained its nematicidal activity against second stage juveniles of *M. javanica* and *Caenorhabditis elegans*. This thermostability of the culture filtrate indicates the non-proteinaceous or non-glyco-

TABLE III - *Effects of culture filtrates of* Aspergillus *species on mortality of* M. javanica.

Species	Mortality % Exposure time (hours)	
	24	48
Czapek's broth	0 d	0 d
A. flavus	20 bc	27 c
A. terreus	6 cd	10 cd
A. tamarii	11 cd	15 cd
A. niger	100 a	100 a
A. nidulans	30 b	51 b

Means followed by the same letters are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

TABLE IV - *Effects of various concentrations of culture filtrates of* Aspergillus niger *on mortality of* M. javanica *after 48 hours.*

Treatments	Mortality %
Czapek's broth (100%)	0 a
Czapek's broth (1/10)	0 a
Czapek's broth (1/100)	0 a
Czapek's broth (1/1000)	0 a
A. niger (100%)	100 d
A. niger (1/10)	81 b
A. niger (1/100)	37 c
A. niger (1/1000)	4 a

Means followed by the same letters are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

proteinaceous nature of the active nematicidal compound(s).

The conidial suspensions of *Aspergillus* species exhibited better biocontrol and subsequently growth promoting effects compared to their respective culture filtrates. The conidial suspensions of *A. flavus* and *A. nidulans* and the culture filtrates of *A. niger* and *A. nidulans* significantly ($p<0.05$) increased plant height and the fresh weight of shoots of mungbean compared to the controls. Interestingly, root weight was significantly ($p<0.05$) increased by the culture filtrates of *A. flavus*, *A. terreus* and *A. tamarii* and by the conidial suspensions of *A. niger* and *A. nidulans*. *Aspergillus* species significantly reduced galling rates ($p<0.01$), and nematode population densities in soil ($p<0.05$) and root ($p<0.01$). The greatest reduction in root-knot development (51%), nematode populations in soil (43%) and roots (52%) was achieved following soil drenches with the conidial suspension of *A. niger*. That the culture filtrate of *A. flavus* increased the population of *M. javanica* in soil and roots (Table V) is also of significant importance. In a previous report, seed treatment with some rhizosphere bacteria increased infection levels of *Heterodera schachtii* in sugar beet (Oostendorp and Sikora, 1989). Under glasshouse conditions, none of the *Aspergillus* species parasitized *M. javanica* females. Interestingly, no egg production was observed in the controls as well as in the treated plants. Therefore, whether parasitism was the active mechanism in the suppression of root-knot nematode remains uncertain. By contrast, in a previous study (Siddiqui *et al.*, 2000), *A. terreus* was found to parasitize 5% of the eggs of *M. javanica* in egg plant while the females continued to produce egg masses. Such a difference could be explained on the basis of differences in the root exudates of mungbean and egg plant affecting the fungal populations in the rhizosphere.

Thus the possible mechanism could be the production of fungal exometabolites that might have reduced the reproductive potential of the females. However, culture filtrate of some species might have lost activity due to dilution and leaching of mycotoxins resulting from the daily watering of the pots. On the other hand

TABLE V - *Effects of conidial suspension (CS) and culture filtrates (CF) of* Aspergillus *species on growth, root-knot development and nematode population in soil and roots of mungbean.*

Treatments	Plant height (cm)	Shoot weight (g)	Root weight (g)	Galls/ root system	Nematode population	
					Soil 250 cc	Root 1 g
Control	14.8	0.47	0.33	65	2390	131
A. flavus (CS)	18.6	0.66	0.48	48	1900	106
A. flavus (CF)	16.8	0.60	0.56	63	2770	143
A. terreus (CS)	16.3	0.53	0.45	43	1980	89
A. terreus (CF)	16.8	0.58	0.55	73	2190	102
A. tamarii (CS)	16.4	0.53	0.43	50	1950	83
A. tamarii (CF)	15.7	0.69	0.61	71	2450	128
A. niger (CS)	16.6	0.58	0.56	32	1670	63
A. niger (CF)	17.5	0.69	0.44	45	1750	84
A. nidulans (CS)	19.6	0.73	0.58	37	1950	79
A. nidulans (CF)	18.1	0.77	0.49	49	2050	92
LSD 0.05	2.3	0.16	0.21	12	550	19

TABLE VI - *Effects of* A. niger *alone or in combination with* Pseudomonas aeruginosa *on growth, root-knot development and nematode population in soil and roots of mungbean.*

Treatments	Plant height (cm)	Shoot weight (g)	Root weight (g)	Galls/ root system	Nematode population	
					Soil 250 cc	Root 1 g
Control	18.2	0.46	0.55	58	2730	89
A. niger (AN)	20.6	0.59	0.67	42	2030	61
P. aeruginosa (PA)	20.6	0.71	0.47	38	2010	57
AN + PA	24.4	0.80	0.72	32	1340	53
LSD 0.05	1.5	0.13	0.15	6	326	20

the spore suspension in the soil does not readily leach out and presumably provides the continuous release of the toxins.

A. niger in combination with *P. aeruginosa* gave better results with respect to the root-knot development compared to the application of either antagonist alone. *A. niger* and *P. aeruginosa* used together significantly reduced nematode population in soil ($p<0.001$; >44%) and roots ($p<0.05$; >50%) and subsequent root-knot disease severity ($p<0.001$; >44%) compared with the controls. *A. niger* used with *P. aeruginosa* also significantly increased plant height ($p<0.001$; >34%), fresh weight of shoot ($p<0.01$; >73%) and roots ($p<0.01$; >30%) compared to untreated controls (Table VI).

Since *Aspergillus* spp., are known to be opportunistic pathogens of human beings, it is unlikely that they could be developed as a biological control agent but the nature of the nematicidal effect is worthy of further investigation.

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