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EFFECT OF *VERTICILLIUM CHLAMYDOSPORIUM* AND *PSEUDOMONAS AERUGINOSA* IN THE CONTROL OF *MELOIDOGYNE JAVANICA* ON TOMATO

by
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Summary. Efficacy of *Verticillium chlamydosporium* as a biological control agent of *Meloidogyne javanica*, the root-knot nematode, was tested under laboratory and field conditions. An ethyl acetate and hexane extract of *V. chlamydosporium* was lethal to *M. javanica* juveniles. *V. chlamydosporium* parasitized *M. javanica* eggs whereas the female was unaffected. Neither egg nor *M. javanica* female were infected with *Pseudomonas aeruginosa*. *V. chlamydosporium* did not colonize the inner root tissue of tomato whereas *P. aerugiosa* did. *V. chlamydosporium* and *P. aeruginosa* applied together had better biocontrol and promoted plant growth compared with either antagonists alone or the untreated controls.

Verticillium chlamydosporium is a facultative parasite of cyst and root-knot nematodes (Kerry, 1975) and has given effective population control of *Meloidogyne* spp., (Leij and Kerry, 1991). Similarly, *Pseudomonas* species are aggressive colonizers of roots and shoots and have a broad spectrum of antagonism to root pathogens (Sharma and Nowak, 1998; Siddiqui *et al.*, 1999). A combination of *Pseudomonas aeruginosa* and *Paecilomyces lilacinus* (an egg parasite of cyst and root-knot nematodes) have shown better control of *M. javanica* on various crops compared with their separate use (Perveen *et al.*, 1998). Therefore experiments were carried out to examine the effects of *P. aeruginosa* on the efficacy of *V. chlamydosporium* in the control of *M. javanica*, the root-knot nematode in tomato.

Materials and methods

Egg masses of *Meloidogyne javanica* (Treub) Chitw. obtained from a pure culture maintained on egg plants, *Solanum melongena* L., in a glass house were placed in distilled water and incubated at room temperature for 24 hr. After hatching, juveniles were collected and a suspension of juveniles in distilled water was prepared.

The culture filtrate of *Verticillium chlamydosporium* Goddard (KUCC-839) was prepared by growing the fungus in Czapek's liquid medium for 15 days in dark at 24 °C. The culture filtrate was extracted with ethyl acetate or hexane (1:2) and concentrated on a rotary vacuum evaporator (Eyla) under reduced pressure at 37 °C. Dilutions of 1.0, 0.1, and 0.01 mg/ml of the extracts were prepared in their respective solvents, transferred into watch glasses, 1 ml per watch glass, and left for 48 hr., to evaporate the organic solvent. Two ml of the juvenile suspension were added to each watch glass (15-25 juveniles per watch glass). Juveniles kept in evaporated ethyl acetate or hexane served as control. There were three replicates of each treatment. Watch glasses were incubated at room temperature (25±30 °C) and observation on the mortality of juveniles was re-

corded at 24, 48 and 72 hr. intervals. The experiment was repeated twice.

Biological control potential of *V. chlamydosporium* alone or in combination with *Pseudomonas aeruginosa* (Schroeter) Migula (isolate IE-6), a plant growth-promoting rhizobacterium, was investigated in 2x1 meter microplots at the Department of Botany, University of Karachi, in a sandy-loam soil (sand 70%: silt 19%: clay 10%; pH 8.1). *V. chlamydosporium* was grown on Corn Meal Agar medium for two weeks at 24 °C. *P. aeruginosa* was multiplied on King's B medium for five days at 25±30 °C. Antagonists were scraped from the medium surface using a sterilized bent glass rod after adding 10 ml sterile distilled water. After removing the soil to a depth of 12 cm, a 300 ml suspension of microbial antagonists was drenched separately in 1.5 m furrow. The inoculum of *V. chlamydosporium* contained 3.5×10^7 conidia + chlamydospores ml^{-1} and *P. aeruginosa* 3.2×10^8 cfu ml^{-1}. In another set, soil was drenched with a mixture of both *V. chlamydosporium* and *P. aeruginosa* (150+150 ml per 1.5 m furrow). Soil drenched with sterile distilled water served as control. After drenching, the surface was covered with soil and six, three-week-old tomato (*Lycopersicon esculentum* Mill.) seedlings cv SUN 6002 were planted. One week after planting, the seedlings were inoculated with 1000 juveniles (less than one-week-old). The juveniles were suspended in 25 ml water which was poured into three holes made around the roots. Each treatment was replicated three times and the plots were randomized. Plants were watered as needed and the experiment was terminated 90 days after nematode inoculation. Data on plant height, root length, fresh weight of shoot and root, number of galls and egg mass and nematode population density in soil and root were recorded. Nematode populations in the soil were estimated using a modified Baermann funnel technique. To determine nematode invasion, roots of each plant were cut into 5 mm segments and mixed thoroughly. This sample was divided into three equal portions. One portion was blotted dry and 1 g root was dipped for 3 mins. in boiling acid fuchsin + lactic acid. Roots were macerated in an electric grinder for 45 seconds. The macerate was suspended in 100 ml water and *M. javanica* females and juveniles in five samples of 5 ml were counted with the aid of low power microscope.

To determine fungal establishment in the roots, 5-mm-long root pieces from one of the remaining samples were plated (five pieces per plate) onto PDA plates containing penicillin (100,000 units/l) and streptomycine sulphate (0.2 g/l) after surface disinfestation with 1% $Ca(OCl)_2$ for three mins. After one week incubation at 24 °C, any fungal growth was identified. The bacterium was re-isolated from the inner root tissue by a modified method of Pillay and Nowak (1997) in which 1 g roots were surface sterilized with 1% $Ca(OCl)_2$ for 1 min., rinsed twice with sterile distilled water, submerged for 30s in 15% H_2O_2 and again rinsed twice in sterile distilled water. The tissue was then macerated in 10 ml of 0.1 M $MgSO_4$ (buffered to pH 6.5) plus 0.02% Tween 20. Ten fold serial dilutions were prepared and 50 µl aliquots from the appropriate dilution were plated onto KB medium. Plates were incubated at room temperature for 48 hr. and the numbers of cfu was recorded.

For the re-isolation of the biocontrol agents, ten egg-masses per treatment were randomly selected and crushed in a drop of 0.01% sodium hypochlorite solution to dissolve the gelatinous matrix. Eggs were washed in distilled water, dispersed in 3 ml water and 0.5 ml of each suspension was plated onto 0.8% water agar and KB medium to observe the parasitism by *V. chlamydosporium* and *P. aeruginosa*, respectively. The dishes were incubated at the desired temperatures as described earlier. After three days, 100-200 eggs on each dish were examined and the growth of fungus, if found, was identified. For bacterial infection, dishes were observed after two days under Ultra Violet light at 360 nm since the bacterium produces a fluorescent pigment (Bashan *et al.*, 1993). Ten hand

picked females after surface sterilization with 0.5% $Ca(OCl)_2$ were washed thoroughly and plated onto 0.8% water agar and KB medium to observe the parasitism by *V. chlamydopsorium* and *P. aeruginosa*, respectively.

Data were analysed and subjected to one way analysis of variance (ANOVA) followed by Standard Error of the Difference between means (SED) according to Gomez and Gomez, (1984).

TABLE I - *Nematicidal activity % of different concentrations of ethyl acetate and hexane fractions of* Verticillium chlamydosporium *on* Meloidogyne javanica.

Treatment	Concentration	Exposure time (days)		
		1	2	3
Ethyl acetate	Control	0 b	0 c	4 c
	1 mg/ml	40 a	51 a	55 a
	0.1 mg/ml	14 b	27 b	34 b
	0.01 mg/ml	11 b	21 b	31 b
Hexane	Control	0 c	1 c	4 c
	1 mg/ml	64 a	77 a	83 a
	0.1 mg/ml	31 b	40 b	52 b
	0.01 mg/ml	16 bc	29 b	38 b

Mean followed by the same letter in each column are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test.

Results and discussion

Ethyl acetate and hexane extracts of *V. chlamydosporium* caused significant ($p<0.05$) mortality of *M. javanica* juveniles. There was a progressive increase in mortality of juveniles as the concentrations of extracts and exposure period were increased. The hexane fraction was more effective than ethyl acetate extract. Most mortality (83%) was obtained after 72 hours in the hexane extract (1 mg per ml); 1 mg/ml of ethyl acetate extract resulted in 55% juvenile mortality (Table I). The result of the present study would indicate that active compound responsible for the nematicidal activity was hexane soluble and hence may be non-polar in nature.

Soil application with *V. chlamydosporium* alone or in combination with *P. aeruginosa* significantly ($p<0.05$) enhanced the biomass of tomato foliage. A *V. chlamydosporium-P. aeruginosa* combination resulted in maximum plant height, root length and fresh weight of shoot whereas untreated control plants had the greatest fresh weight of root (Table II).

The greatest reduction in nematode multiplication and galling were achieved when *V. chlamydosporium* and *P. aeruginosa* were applied together followed by the *P. aeruginosa*

TABLE II - *Effects of* Pseudomonas aeruginosa *and* V. chlamydosporium *on the development of root-knot infection and populations of* M. javanica.

Treatment	Plant height (cm)		Plant fresh wt. (g)		No. of galls/root system	Egg mass per root system	Nematode population	
	Shoot	Root	Shoot	Root			Soil 250 cc	Root g^{-1} fresh wt.
Control	28.0	13.5	19.9	5.8	90	62	1010	201
V. chlamydsporium (VC)	35.8	15.5	38.9	4.3	65	31	917	142
P. aeruginosa (IE-6)	40.1	17.1	38.5	3.7	42	26	782	105
VC + IE-6	42.3	19.0	43.1	3.8	35	24	760	98
SED	3.3	3.9	6.5	0.6	9	8	35	21
Significance level (p)	*	N.S.	*	*	**	**	**	**

**, $P<0.01$; *, $P<0.05$; N.S., non-significant.

treatment alone. *V. chlamydosporium* used alone also significantly ($p<0.05$) reduced nematode multiplication and consequently gall induction (Table II). When *V. chlamydosporium* was used alone, 7% of the eggs were found infected with the fungus. Application of *P. aeruginosa* did not exert any adverse effect on fungal parasitism and resulted in 10% egg infection. *V. chlamydosporium* was not found to infect *M. javanica* females. Neither eggs nor females were found to be infected with *P. aeruginosa* (Table III). *P. aeruginosa* was re-isolated from the inner root tissue of tomato. There was no inhibitory effect of *V. chlamydosporium* on bacterial endo-root colonization. *V. chlamydosporium* was not found to colonize inner root tissues (Table III).

TABLE III - *Effects of* V. chlamydosporium *on infection of* M. javanica *egg and root colonization by* P. aeruginosa.

Treatment	Egg infection %	Root colonization [(log cfu g^{-1} fresh weight) + 1]
Control	0	0
Verticillium chlamydosporium (VC)	7	–
Pseudomonas aeruginosa (IE-6)	–	5.15
VC + IE-6	10	5.28
S.E.D.	0.9	0.05
Significance level (P)	**	***

**, $P<0.01$; ***, $P<0.001$.

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