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Nematicidal effect of certian bio-agents against root-knot nematode, (*Meloidoygne incognita*) [(kofoid and white) chitwood] on tomato

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Abstract
The results of the gram reaction and biochemical tests for the identification of the native isolates of *P. fluorescens* and *B. cereus* revealed that all the isolates produced similar results with regard to gram staining (negative), motility (positive), starch hydrolysis (negative), gelatin liquefaction (positive), fluoresent pigmentation (positive) and produced IAA, sidrophore and hydrogen cyanide with little variation. Among the isolates pf5 and Bc6 produced maximum quantity of IAA. All isolates of *Paecilomyces lilacinus,* colony characters were observed in similar. The morphological characters of *M. incognita* were observed similar. The culture filtrates of *P. fluorescens*, *B. cereus* and *P. lilacinus* at all conc. caused root-knot nematode mortality and reduced egg hatch count. Maximum inhibition of egg hatch was observed with *P. lilacinus* at 40 per cent conc. followed by *P. fluorescens* and. *B. cereus*. The culture filtrate of *P. liacinus* isolates was found to posses nematicidal action on second stage juveniles (J2) of *M. incognita*.

Keywords: Tomato, *P. fluorescens, B.cereus, P. lilacinus, Meloidoygne incognita*

Introduction
In India, tomato (*Lycopersicon esculentum* L.) is severly affected by the root-knot nematode, *Meloidoygne* spp., having a yield loss up to 46% (Bhatti & Jain, 1977; Jain& Bhatti1978) [4, 14], 39.70% (Reedy1985a, b) and 61%(Nirmala Devi &Tikoo,1992) [23]. The root-knot nematode, *Meloidoygne incognita* and *M.javanica* are the most perdominant and widely prevalent species inflicting serious losses in tomato. The chemical means of nematode control such as soil fumication, application of various nematicides and pesticides ect pose enoromus threat to both soil fauna as well as human being. One of the promising alternatives is the use of some of bio control agents to observe the possibility of ther nematicida\nematostatice properties for the nematode problem. The present investigation was, therefore, undertaken to highlight the nematicidal properties of some bio-control agents for managing infection caused by root-knote nematode, *Meloidoygne incognita* on tomato.

Material and Methods
Isolation of bacterial antagonists
Rhizosphere colonizing *Pseudomonas* sp. and *Bacillus* sp. were isolated from soil adhering on fresh roots of tomato plants collected from 10 different locations. The strains were isolated from collected soil samples using serial dilution technique. One ml of suspension from 10^{-5} and 10^{-6} dilution was pipetted out and poured into Petri plate. Later, selective media (King's B for *Pseudomonas* and nutrient agar for *Bacillus*) were poured, rotated and incubated at room temp. 28 ± 2ºC for 24 h for the isolation of bacterial antagonists (*Pseudomonas* sp. and *Bacillus* sp.) respectively. Bacterial isolates were identified by morphological characters and gram staining. The isolates of *Pseudomonas* sp. were designated as Pf_1 to Pf_{10} and *B. cereus* were designated as Bc_1 to Bc_{10}.

Biochemical tests for *P. fluorescens* and *B. cereus*
For the identification of *P. fluorescens*, certain biochemical tests were conducted according to Bergey's Manual for Determinative Bacteriology (Breed *et al*., 1989) [5].

Gram Staining
A loopful of bacterial culture was transferred on a clean slide, a smear was made which was air-dried and heat fixed. The smear was flooded for one min. with ammonium oxylate crystal violet. Excess strain was poured off and the slide was washed in a gentle steam of water. Lugol's iodine solution was applied and allowed to remain for one min. decolorized with 95 per cent ethyl alcohol. The smear was washed in gentle stream of water and counter stained with safranin for 30 seconds. The Gram negative cells appeared red in colour and Gram positive cells appeared violet in colour (Cyrabree and Hindshill, 1975) [7].

Motility
Semisolid agar medium was prepared and the bacterial isolates were incubated at 30ºC for 72 h. and observed for motility. The distance of growth from the point of stab showed motility.

Starch Hydrolysis
Filter paper was dipped in a day old culture suspension, was placed on Petri dishes containing starch agar medium, and were incubated for two days. The plates were then flooded with one per cent iodine solution. A colourless halo around the growth and blue colour in the rest of the plates showed utilization of starch by the microorganism (Stolpe and Godkeri, 1981) [32].

Gelatin Liquefaction
Filter paper discs were dipped in a day old culture suspension and were placed on Petri dishes containing gelatin nutrient agar medium. The Petri dishes were incubated at 30ºC for two days and then flooded with 12.5 per cent $HgCl_2$ solution. The development of yellow halo around the growth indicated utilization of gelatin (Stople and Godkeri, 1981) [32].

Fluorescent Pigment
The test tubes containing sterilized King's B medium were inoculated with the isolate of *Pseudomonas* sp., incubated for five days and observed. Yellowish green fluorescent pigment observed under UV light (366 nm) indicated positive results.

Estimation of IAA
Indole acetic acid (IAA) in the methanol fraction was determined by employing Salper reagent (Gordon and Paleg, 1957) [11]. To 1.5 ml dist. water in a test tube 0.5 ml of methanol residue was mixed, four ml fresh Salper reagent was rapidly added, kept in complete darkness for one h. and read in spectrophotometer at 535 nm. From a standard curve prepared with known conc. of IAA, the quality of IAA in the filtrate was calculated (1 division = 0.307 µg of IAA).

Production of Siderophore
Effective endophytic *P. fluorescens* isolates were grown in King's B broth for three days at room temperature (28± 3°) centrifuged at 3000 g for ten min. and the supernatants were collected. The pH of the supernantant was adjusted to 2.0 with dilute HCL and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. Five ml of ethyl acetate fraction was mixed with five ml of Hathway's reagent (1.0 ml of 0.1 M Fe Cls in 0.1 N Hcl to 100 ml dist. water plus 1.0 ml of potassium ferricyanide). The absorbance for dihydroxy phenol was read 700 nm in a spectrophometer (Reeves *et al.,* 1989). A standard curve was prepared using dihydroxybenzoic acid. The quantity of siderophore synthesized was expressed as N mol dihydroxybenzoic acid / ml of culture filtrate. Three replications were maintained for each treatment.

Production of hydrogen cyanide (HCN)
Effective endophytic *P. fluorescens* isolates were grown at 28± 3° on a rotary shaker in Tryptic soya Broth (TSB). Filter paper (what man No.1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 28± 3° for 48 hr. the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evoled. The colour was eluted by placing the filter paper in a clean test tube containing 10 ml of dist. water and absorbance was measured at 625 nm (Sadasivam and Manickam, 1992). Three replications were maintained for each treatment.

Endospore staining of *Bacillus cereus*
Isolates were smeared on a glass slide, air-dried and heat fixed. Then the smear was flooded with malachite green (5% aqueous). The slide was heated under the steam. It was counterstained with safranin (0.5% aqueous) after washing in tap water. Finally the smear was washed with disti. water and blot dried. The slide was examined microscopically under oil immersion objective (Aneja, 1993) [1].

***Paecilomyces lilacinus* native isolates**
P. lilacinus was isolated from soil collected from different location in Tamil Nadu by dilution plate technique (Waksman, 1952) [34] using PDA medium. Single colony was selected from the Petri dishes and re-streaked on PDA slants and stored at 4ºC for further studies. The isolates were designated as Pl_1 to Pl_{10}.

Root-knot nematode
Meloidogyne incognita was isolated from soil and roots collected from Dharmapuri districts of Tamil Nadu. Morphological characters of female, male and juveniles were studied and confirmed with the help of descriptions outlined by Whitehead (1968) [35] and Williams (1973) [36].

In vitro* efficacy of biocontrol agents against *Meloidogyne incognita
P. fluorescens native isolates were grown on King's B broth, *B. cereus* on NA broth and *P. lilacinus* on PDB in 250 ml conical flask. The flasks were incubated for ten days at 28 ± 2ºC for *P. lilacinus* and seven days at 28 ± 2ºC for *P. fluorescens*, *B. cereus*. The contents were passed through Whatman No.42 filter paper and filtrates were used within 24 h. Four conc. *viz*., 10, 20 and 40 of culture filtrates of the antagonists were prepared. Each treatment was replicated four times with a suitable control. About 200 J2 of *M. incognita* were collected from infested tomato plants grown in culture pots. These juveniles (J2) were exposed to filtrate dilutions for 24, 48 and 72 h. in nine cm dia. Petri plates and observations on immobilized/killed nematodes and total larval hatch count were recorded periodically.

Result and Discussion
Biochemical tests for *Pseudomonas fluorescens*
The results of the gram reaction and biochemical tests performed for the identification of the native isolates of *P. fluorescens* designated as Pf_1 to Pf_{10} revealed that all the isolates produced similar results with regard to gram staining

(negative), motility (positive), starch hydrolysis (negative), gelatin liquefaction (positive), fluorescent pigmentation (positive) and produced IAA, sidrophore and hydrogen cyanide with little variation. Among the isolates Pf_5 produced maximum quantity (3.5) of IAA followed by Pf_6, Pf_1 and Pf_2 (3.4, 3.3, and 3.2 respectively) in the decreasing order of merit. The isolate Pf_5 recorded maximum production of sidrophore and HCN). (Table.1)

Biochemical test for *Bacillus cereus*

The results of the gram reaction and biochemical performs for the identification of the isolates of *Bacilles cereus* designated as Bc_1 to Bc_{10} revealed that all the isolates produced similar results with regard to gram staining (Positive) motility (Positive), starch hydrolysis (Negative), gelatin liquefaction (Positive) Florescent pigmentation (Positive), Endospore (Positive), produced IAA, sidrophore and hydrogen cyanide with slight variation. Among the isolates Bc_6 produced maximum quantity (2.9) of IAA followed by Bc_4 and Bc_9 in the decreasing order of merit (Table 2).

Paecilomyces lilacinus

The colony characters of native isolate of *P. lilacinus* revealed (Table 3) fast growth with dull white and powdery texture mycelium. The conidia were hyaline, ovoid to fusoid and formed in long chains. The colony characters of *P. lilacinus* observed in the present study are similar to those reported by the earlier workers (Barnett and Hunter, 1972; Sutton *et al*., 1998; Lorene, 1995) [3, 33].

Meloidogyne incognita

The female of *M. incognita* (Table 4.) are endo-parasitic, having spherical body with projecting neck. Males and juveniles are clearly annulated with cone shaped lateral view. *M. incognita* measured an average female length of 618 µm, male length of 1583

Table 1: Biochemical test for *Pseudomonas flourescens*

S.No	Parameters	Isolates of *P.flourescens*									
		Pf_1	**Pf_2**	**Pf_3**	**Pf_4**	**Pf_5**	**Pf_6**	**Pf_7**	**Pf_8**	**Pf_9**	**Pf_{10}**
1	Gram staining	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
2	Motility	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
3	Starch hydrolysis	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
4	Gelatin liquefaction	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
5	Fluorescent pigment	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
6	Estimation of IAA (µg/ml)	3.3	3.2	3.0	3.0	3.5	3.4	3.0	3.0	3.0	3.0
7	Sidoerophose production (Hydroxmate)	0.87	0.82	0.82	0.84	0.84	0.86	0.84	0.85	0.82	0.82
8	Hydrogen cyanide production	8.11	7.98	7.98	8.07	8.19	8.16	8.07	8.05	7.98	7.98

Table 2: Biochemical test for *Bacillus cereus*

S.No	Parameters	Isolates of *B. cereus*									
		Bc_1	**Bc_2**	**Bc_3**	**Bc_4**	**Bc_5**	**Bc_6**	**Bc_7**	**Bc_8**	**Bc_9**	**Bc_{10}**
1	Gram staining	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
2	Motility	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
3	Starch hydrolysis	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
4	Gelatin liquefaction	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
5	Fluorescent pigment	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
6	Estimation of IAA (µg/ml)	2.3	2.0	2.1	2.7	2.0	2.9	2.0	2.1	2.5	2.0
7	Sidoerophose production (Hydroxmate)	0.69	0.67	0.62	0.71	0.60	0.76	0.60	0.62	0.67	0.63
8	Endospore staining	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive

Table 3: Studies on the general character of *Paecilomyces lilacinus* native isolates

Isolates	General characters		Conidia (µm)	
	Mycelium colour	**Conidia colour**	**Length**	**Breath**
Pl_1	Initially white and became pale yellow	Hyaline	5.6 - 6.2	1.9-2.3
Pl_2	Hyaline to yellow	Hyaline to bright colour	4.9-6.1	1.8-2.2
Pl_3	Hyaline	Red or Violet colour	5.1-6.0	1.7-2.1
Pl_4	White to Pale yellow	Hyaline to dark coloured	5.1-5.9	1.6-2.0
Pl_5	Hyaline	Hyaline or rarely dark	5.5-6.1	1.8-2.2
Pl_6	Initially white and became pale yellow	Hyaline	5.6-6.2	1.9-2.3
Pl_7	Hyaline	Red or Violet colour	5.1-6.0	1.7-2.1
Pl_8	Hyaline to yellow	Hyaline to bright colour	4.9-6.1	1.8-2.2
Pl_9	Hyaline	Rarely dark colour	5.5 - 6.1	1.8 - 2.3
Pl_{10}	Hyaline to yellow	Hyaline or bright colour	4.9-6.1	1.8-2.2

Table 4: Studies on the general character of *Meloidogyne incognita* native isolates

Stage	General characters	Length (µm)
Female	Females are endoparasitic, body spherical with projecting neck, posterior cuticular pattern displays with striage closely spaced, very wavy to zig zag especially dorsally and laterally.	512-725 (618.5)
Male	Males are clearly annulated cone shaped.	1109-1954 (1531.5)
Juvenile	Juveniles head not off set truncate cone shape in lateral view and sub-hemispherical in dorso-ventral view	338-406 (372)

Mortality and hatching of *M. incognita*

***Pseudomonas fluorescens* native isolates**

The data presented in table 5 indicated that the culture filtrate of *P. fluorescens* at all the conc. caused root-knot nematode mortality and reduced egg hatch count. Maximum inhibition of egg hatch was observed with isolate Pf_5 of *P. fluorescens* (90.53) at 40 per cent conc. followed by 20 per cent conc. (170.62). It was also observed that with an increase in conc. of culture filtrate, a corresponding decrease in hatch count occurred. The 40 per cent conc. of culture filtrate also exhibited nematicidal action by increasing nematode mortality (61.40 per cent of 72 h. of observation).

***Bacillus cereus* native isolates**

The results of the present study (Table 6) clearly showed that the culture filtrate of *B. cereus* was found to be toxic to *M. incognita* and mortality has occurred after 24 h exposure. The highest mortality (56.33) was observed after 72 h exposure in 40 per cent conc. of culture filtrate which also recorded a larval hatch count of 95.50. As the exposure time increased, there was an increase in mortality of root-knot nematode.

***Paecilomyces lilacinus* native isolates**

Data depicted in (table 7) indicated that the culture filtrate of *P. lilacinus* isolates at all the conc. exhibited larvicidal effect. The culture filtrate was found to possess nematicidal action on second stage juveniles (J2) of *M. incognita*. The percentage of juvenile mortality increased with increasing exposure time at all conc. of culture filtrates

Table 5: Effect of culture filtrates of *P. fluorescens* on mortality and hatching of *Melodogyne incognita*

T.No	Culture filtrate (conc.)	*Per cent nematode mortality after (h)				*Total egg hatch count	Per cent hatch inhibition
		24	48	72	Mean		
T_1	10	14.16	19. 16	29.30	20.87	398.39	73.38
T_2	20	26.36	34.23	45.33	35.31	170.62	31.43
T_3	40	37.50	48.50	61.40	49.13	90.53	16.67
T_4	Control	2.26	2.40	2.66	2.44	542.93	-
Mean		20.07	26.07	34.67	26.94	300.62	-
SE (d)						3.3648	-
CD (1%)						11.2905	-

	T	H	TH
SE (d)	1.300	1.126	2.253
CD (1%)	3.658	3.168	6.336

* Mean of four replications

Table 6: Effect of culture filtrates of *B. cereus* on mortality and hatching of *Meloidogyne incognita*

T. No	Culture filtrate (conc.)	*Per cent nematode mortality after (h)				*Total egg hatch count	Per cent hatch inhibition
		24	48	72	Mean		
T_1	10	12.46	16.42	25.87	18.26	400.69	74.01
T_2	20	23.40	31.48	43.47	32.79	175.51	32.42
T_3	40	33.51	45.33	56.33	45.06	95.50	17.64
T_4	Control	2.29	2.43	2.69	2.48	541.43	
Mean		17.92	23.92	32.09	24.64	303.28	
SE (d)						3.5932	
CD (1%)						12.0569	

	T	H	TH
SE (d)	1.297	1.123	2.247
CD (1%)	3.649	3.160	6.321

* Mean of four replications

Table 7: Effect of culture filtrates of *P. lilacinus* on mortality and hatching of

T. No	Culture filtrate (conc.)	*Per cent nematode mortality after (h)				*Total egg hatch count	Per cent hatch inhibition
		24	48	72	Mean		
T_1	10	18.48	27.70	31.00	25.73	248.47	45.85
T_2	20	32.52	43.52	56.26	44.10	114.42	21.11
T_3	40	43.42	55.33	78.50	59.08	48.26	8.90
T_4	Control	2.29	2.41	2.68	2.46	541.97	
Mean		24.18	32.24	42.11	32.84	238.28	
SE (d)						4.3661	
CD (1%)						14.6504	

	T	H	TH
SE (d)	1.722	1.491	2.982
CD (1%)	4.843	4.194	8.388

* Mean of four replications

While the hatch count was increased with decreasing conc. of culture filtrate. The fungal filtrates at 40 per cent conc. also exhibited strong nematicidal action.

Nematicidal property of culture filtrates of *P. lilacinus* (Jatala *et al.,* 1981; Zaki, 1994; Oduor and Woudo, 1995; Cayrol *et al.,* 1989; Khan and Goswami, 2000; Goswami *et al.,* 2001; Goswami and Archana Mittal, 2004) [6, 12] and *P. fluorescens* (Gokte and Swarup, 1988, Oostendorp and Sikora, 1989; Santhi and Sivakumar, 1995; Seenivasan and Lakshmanan, 2001; Jayakumar *et al.,* 2001; Dietter Haas and Genevievedefago, 2005; Krishnaveni, 2005; Jonathan *et al.,* 2006) [10] and *Bacillus* spp. (Rajendran *et al.,* 2001; Khan *et al.,* 2001) have been reported. These earlier findings are in line with the mortality and hatching of *M. incognita* observed in the present investigation.

However, among the bio control agents tested the native isolate of *P. lilacinus* showed the highest per cent of parasitism against *M. incognita*. The nematicidal action of culture filtrates might be attributed to fungal metabolites or antibiotics produced by *P. lilacinus*. This fungus has been reported to produce peptidal antibiotics viz., lilacin, leucinostatin and paecila toxin (Arai *et al.,* 1973*;* Mikami *et al.,* 1989). Acetic acid was also identified from culture filtrate of *P. lilacinus*, which affect the movement of nematode (Djiam *et al.,* 1991) [9].

Therefore, nematicidal action of culture filtrates of biocontrol agents may possibly be due to action of antibiotics together with various other metabolic products. It was also observed that isolates of one genus may have differential effect upon larval hatch as seen above. This variation may perhaps be attributed to different levels of toxin production in cultural filtrates of different isolates of antagonistic organisms.

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