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Article

Nematicidal Activity of Some Fluorescent Pseudomonads on Cyst Forming Nematode, *Heterodera cajani* and Growth of *Sesamum indicum* var. RT1

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Among 24 isolates of fluorescent Pseudomonads, 5 isolates named as LPT1, LPT2, LPT3, LPT4 and LPT5 were screened *in vitro* for their nematicidal activity against cyst forming nematode, *Heterodera cajani* causing patchiness, poor and stunting growth besides discoloration in *Sesamum indicum*. Second stage juveniles of *H. cajani* hatched from egg masses were collected from roots of host plant and subjected to fresh and heat-treated culture filtrate of isolates for 24 h. Mortality of *H. cajani* was recorded on the basis of parameters used for test organism bioassay. Among these isolates, *Pseudomonas aeruginosa* LPT5 caused maximum mortality towards second stage juvenile of *H. cajani in vitro*. Five isolates were used as seed coating for the management of cyst forming nematode *H. cajani* on sesame in green house condition. The strains LPT5 was better than the other strains in reducing the population of *H. cajani* both *in vitro* and *in vivo*. The reduction in cyst and juveniles population was found to be 49 and 60%, respectively when seeds were coated with strain LPT5. Among other strains, LPT4 was also found to inhibit the cyst and juveniles population 12 and 36% respectively. Increases in early vegetative plant growth parameters recorded in both *in vitro* and *in vivo* further revealed the significance of indigenous bacteria in comparison to introduced strain.

Key words: *Heterodera cajani, Pseudomonas aeruginosa, Sesamum indicum,* nematicides

The plant growth promoting rhizobacteria such as *P. aeruginosa* has been recognized as a potential biocontrol agent against cyst and root knot nematode.[1)] Many species of *Pseudomonas* reduce the population of root knot nematode and promote plant growth.[2)] Although chemical nematicides are primary means for the control of plant parasitic nematodes including *Heterodera* spp. but their residual effect and health hazardous nature led to a total ban or restricted[3)] and an alternate safe and effective strategy in the form of biological control is required which could provide substantial protection to the crops against plant parasitic nematodes. Consequently, rhizobacteria have been evaluated for their biocontrolling effect on a variety of plant parasitic nematode including root knot nematode *Meloidogyne incognita,*[4-5)] *M. javanica,*[6)] cyst forming nematode *Heterodera glycine*[5)] and *H. schachtii*.[7)] Among the various plant parasitic nematodes associated with sesame, the cyst forming nematode, *Heterodera cajani* is considered as the most serious threat to sesame.[8)]

The present investigation aimed to investigate the nematicidal potential of *P. aeruginosa* against cyst forming nematode, *H. cajani* in *Sesamum indicum*. The vegetative growth and development parameters of *S. indicum* have also been elaborated in a pot trial study.

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Materials and Methods

Nematode culture. Pure inoculum of the nematode culture *Heterodera cajani* was obtained from Department of Plant Protection, Aligarh Muslim University, Aligarh, India and was maintained by allowing it to multiply on pigeon pea (*Cajanus cajan*). A number of culture pots were raised and maintained for further studies.

Bacterial culture. The fluorescent Pseudomonads strains (LPT1, LPT2, LPT3, LPT4 and LPT5) were isolated from the rhizosphere of the mature tomato (*Lycopersicon esculentum*). Appropriate serial dilutions of soil suspension in sterile water were spread on Nutrient agar medium (NAM) supplemented with 100 $\mu g \cdot ml^{-1}$ streptomycin to evaluate the antibiotic resistant strain, and the plates were incubated at 28 ± 1°C for 24-48 h. The fluorescent pigment producing bacterial colonies were carefully picked up and pure culture was kept in NAM slants at 4°C. Morphological and biochemical properties of the bacterial strains were carried out as outlined earlier.[9)] Plant growth promoting activities in fluorescent pseudomonads were determined by growing log phase (24 h old) culture of different strains of pseudomonads. The production of siderophore was estimated qualitatively on Chrom-Azurol S agar medium (CAS), a universal medium for siderophore detection according to Schwyn and Neilands.[10)] For this, the strains were separately spotted on CAS agar medium and plates were incubated at 28 ± 1°C for 48 h. Production of

Hydrocyanic acid production (HCN) was determined by the modified method of Miller and Higgins[11] while Indole acetic acid (IAA) production was observed according to Gupta *et al.*[9]

***In vitro* interaction between juveniles and bacterial culture filtrate.** Healthy cyst along with egg masses of *H. cajani* were kept in petri plates containing sterilized water in the incubator at 29°C for hatching. The active second stage juveniles (J2) were collected and stored in refrigerator at 4°C for further use in the experiment. Bacterial strains were grown in Erlenmeyer flasks containing Nutrient Broth (NB) at 28°C. After 24 h, the bacterial culture was centrifuged at 7000 g for 15 min at 4°C to obtain cell free culture filtrate. The bacterial pellet was used for seed bacterization. To determine the effect of culture filtrate (CF) on juveniles of *H. cajani*, 2 m*l* of bacterial culture filtrate were transferred in cavity glass slide in which 1 m*l* juveniles (40-45 surface sterile Juveniles/m*l*) were added in quintuplicate. The numbers of dead juveniles were counted and mean percentage of the dead larvae was observed after 12 and 24 h.[12] The culture filtrates were heat-treated by boiling in water bath for a period of 5 min and subsequently tested for the nematicidal activity as described by Ali *et al.*[2]

Seed bacterization. The bacterial pellets were washed with sterile distilled water (SDW) and re-suspended in SDW to obtain a population density of 1×10^8 CFU · ml^{-1}. This suspension was mixed with 1% carboxymethylcellulose (CMC) solution. The surface of sterilized seeds (0.5% mercuric chloride) were coated with the slurry and allowed to air-dry overnight in aseptic condition. Bacterized and non-bacterized sesame seeds were sown separately in 21 cm diameter pots containing 2.5 kg steam sterilized soil (77.3% sand, 13.6% silt, 11.7% clay, 0.097% total organic C, pH 6.4 and 36% water holding capacity) in seven sets of treatments: i) *H. cajani* and *Pseudomonas aeruginosa* LPT1, ii) *H. cajani* and *P. aeruginosa* LPT2, iii) *H. cajani* and *P. aeruginosa* LPT3, iv) *H. cajani* and *P. aeruginosa* LPT4, v) *H. cajani* and *P. aeruginosa* LPT5, vi) *H. cajani* and *P. aeruginosa* MTCC 1934, and vii) *H. cajani* alone. After one-week of seedling emergence, each pot was inoculated with 1000 freshly hatched second stage juveniles of *H. cajani*. The inoculation was done with second stage juveniles of *H. cajani* by pouring aqueous suspension of the nematode in four soil cavities (2-3 cm deep) around the collar of the seedling. The holes were then tempted shut and the plants were maintained. The influences of treatments in plant growth characters and nematode population were recorded after 90 days. Pots were arranged in a randomized block design on a bench in a green house. Care of plants was taken as needed. Each treatment was replicated five times.

Root colonization. After 90 days of sowing, count on

Table 1. Comparison of bacterial characteristics of the isolates with the *Pseudomonas aeruginosa* MTCC 1934

Charateristics	Isolates					MTCC 1934
	LPT1	LPT2	LPT3	LPT4	LPT5	
Gram reaction	-	-	-	-	-	-
Growth at 4°C	-	-	-	-	-	-
41°C	+	+	+	+	+	+
Cells short rod	+	+	+	+	+	+
Fluorescent pigment	+	+	+	+	+	+
Motality	+	+	+	+	+	+
Endospore	-	-	-	-	-	-
PHB[a] accumulation	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Urease	+	+	+	+	+	+
MRVP[b] test	-	-	-	-	-	-
H_2S[c] production	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+
Utilization of:						
Glucose	+	+	+	+	+	+
Meso-Inositol	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+
Mannose	-	-	-	-	-	-
Ribose	+	+	+	+	+	+
Maltose	-	-	-	-	-	-

[a]PHB = Poly hydroxy butyrate; [b]MRVP = Methyl red voges prosker; [c]H_2S = Hydrogen sulphide, (-) Negative response, (+) Positive response

bacterial root colonization from 1 g root was recorded by serial dilution plate technique by grinding the root bits. A dilution of the suspension was poured on NAM supplemented with 100 μg · ml^{-1} streptomycin to evaluate the population of *Pseudomonas aeruginosa*$^{strep+}$. After 24 h of incubation at 28 ± 1°C, CFU's per gram root was counted.

Results and Discussion

A total of 24 strains of fluorescent pseudomonads were isolated from the rhizosphere of the mature tomato, out of which 5 strains of pseudomonads LPT1 to LPT5 were screened as the most promising strains for their nemeticidal activity. The strains were motile, Gram-negative, aerobic and non-spore forming rods. The colonies were smooth, translucent, large, low convex and 2-4 mm in diameter with regular spreading edge after 24 h incubation at 28 ± 1°C. The strains were oxidase, catalase, urease and gelatin hydrolysis positive. Greenish blue (Pyocyanin, Hi Media, MU119) and yellow fluorescent pigments were produced on *Pseudomonas* agar (fluorescein, Hi Media, MU120) by the isolates which identified as *Pseudomonas aeruginosa* and confirmed with the evidences, while comparing with known strain of *Pseudomonas aeruginosa* MTCC strain 1934 (Table 1). Strain LPT5 produced more fluorescent pigment, siderophore, HCN and IAA in comparison to that of other isolates and also showed antagonism against *Fusarium oxysporum*, a dreaded pathogen causing wilt of sesame (Table 2).

In this study, we found that the strain LPT5 showed maximum nematicidal effect against the second stage juveniles of *H. cajani*. Strain LPT5 strain showed maximum juvenile mortality of 45 and 65% after 12 and 24 h, respectively, whereas strain LPT4 showed minimum juvenile mortality of 27 and 35% after 12 and 24 h, respectively. The data revealed significant difference in mortality, after an exposure period of 24 h, in all the *Pseudomonas* strains as compared to control. Nematode mortality increased at the increase in the exposure time. Whereas, the nematicidal activity was lost considerably

Table 2. Characteristics of isolated strains of fluorescent pseudomonads from tomato rhizosphere

Strains	CAS blue agar		Fluorescent pigment production[c]	HCN production	IAA production[d]	Antagonism against *F.oxysporum*[e]	% inhibition
	Growth[a]	halo formation[b]					
LPT1	++	+	+	-	+	-	-
LPT2	++	+	+	+c	+	-	-
LPT3	++	++	+	+b	+	-	-
LPT4	++	++	+	-	-	-	-
LPT5	++	+++	+	+a	+	+	56.8
MTCC 1934	++	++	-	-	-	-	-
Control	-	-	-	-	-	-	-

[a]No growth, + minimal growth, ++ normal growth
[b]Absence of halo formation, + small halos <0.5 cm wide surrounding colonies, ++ medium halos >0.5 cm wide surrounding, +++ large halos >1.0 cm wide surrounding
[c]diffusible fluorescent pigment on NAM plates
[d]IAA negative, + IAA positive, +a strong HCN production, +b moderate HCN production, +c low HCN production
[e]Antagonism negative, + antagonism positive
CAS Chrom-Azurol agar medium, HCN hydrocyanic acid, IAA indole acetic acid

Table 3. Effect of culture filtrate and heat-treated culture filtrate of *Pseudomonas aeruginosa* strains and MTCC 1934 strain on mortality of *Heterodera cajani*

Treatment	Fresh culture filtrate		Heat-treated culture filtrate	
	% Mortality after		% Mortality after	
	12 h	24 h	12 h	24 h
Pseudomonas aeruginosa LPT1	30 ± 1.5	38 ± 1.5	12 ± 1.2	18 ± 4.2
Pseudomonas aeruginosa LPT2	35 ± 1.7	45 ± 2.0	15 ± 1.1	20 ± 1.0
Pseudomonas aeruginosa LPT3	40 ± 1.1	52 ± 2.0	12 ± 2.3	19 ± 1.1
Pseudomonas aeruginosa LPT4	27 ± 1.5	35 ± 2.0	10 ± 2.3	15 ± 1.2
Pseudomonas aeruginosa LPT5	45 ± 1.5	65 ± 1.5	15 ± 1.2	25 ± 1.5
Pseudomonas aeruginosa MTCC1934	38 ± 1.5	48 ± 1.1	12 ± 4.4	20 ± 1.5
Control (NB[a])	8 ± 2.3	12 ± 1.2	8 ± 4.8	10 ± 1.0
$LSD_{0.05}$[b]	14.6	9.0	6.5	2.4

[a]NB = Nutrient Broth
[b]$LSD_{0.05}$ means least significant difference at 0.05 probability.

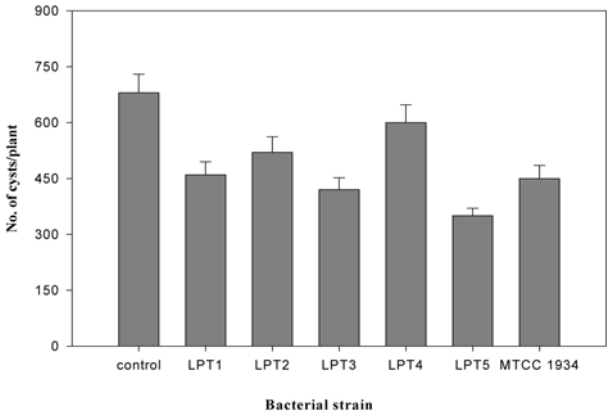

Fig. 1. Influence of *Pseudomonas* strains on the cysts of *H. cajani* on sesame. Lines above bars indicate SE value for three replications.

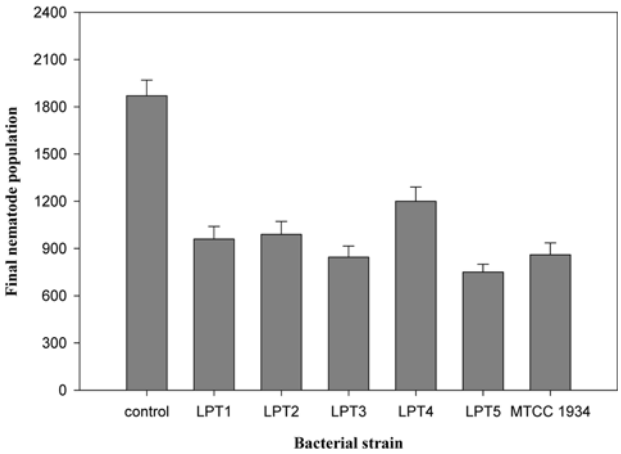

Fig. 2. Influence of *Pseudomonas* strains on second stage juveniles of *H. cajani* on sesame. Lines above bars indicate SE value for three replications.

in heat-treated culture filtrate (Table 3). This indicated the extra-cellular and heat-sensitive nature of nematicidal substance of the *P. aeruginosa*.

Seeds coated with bacterial pellet showed significant increase in plant growth characters in comparison to that of non-bacterized seeds. The maximum increase in shoot and root length, fresh and dry weight of shoot and root was recorded in seedling raised with LPT5 bacterized seeds as compared to control (non bacterized) as shown in Table 4.

The final cyst and J2 population in soil were significantly reduced when the seeds were treated with *Pseudomonas* as compared to control (nematode alone) (Fig. 1 and Fig. 2). The maximum reduction in cyst and J2 population was 49 and 60 % respectively, in treatment that received bacterized seeds with LPT5 in comparison to control. The minimum reduction in cyst and J2 population was 12 and 36%, respectively when the seeds were treated with LPT4. Maximum colonization (3.8×10^5 CFU $\cdot$ g^{-1} root) of rhizobacterium in roots was observed in seeds treated with isolate LPT5 (Table 4).

The strains LPT1 to LPT5 identified as fluorescent pseudomonads belong to *Pseudomonas aeruginosa*. The

Table 4. Effect of *Pseudomonas aeruginosa* strains on *Heterodera cajani* and growth of *Sesamum indicum* after 90 days

Treatment	Plant length (cm)		Growth characters fresh weight (g)		Dry weight (g)		Pod weight/ plant (g)	Bacterial colonization ($\times 10^5$ CFU[a] $\cdot$ g^{-1}) in root
	Shoot	Root	Shoot	Root	Shoot	Root		
LPT1	58.0	17.2	32.0	16.5	7.5	3.5	40	2.9
	(45%)	(63.8%)	(52.3%)	(65%)	(56.2%)	(16.6%)	(60%)	
LPT2	55.0	15.5	29.0	14.0	7.0	3.2	37	2.5
	(37.5%)	(47.6%)	(38%)	(40%)	(45.8%)	(6.6%)	(48%)	
LPT3	60.0	17.5	34.0	17.0	8.0	4.5	42	3.2
	(50%)	(66.6%)	(61.9%)	(70%)	(66.6%)	(50%)	(68%)	
LPT4	51.0	14.0	25.0	12.0	6.0	2.0	32	1.9
	(27.5%)	(33.3%)	(19%)	(20%)	(25%)	(33.3%)	(28%)	
LPT5	68.0	18.0	37.0	18.5	8.6	5.0	43	3.8
	(70%)	(71.4%)	(76.1%)	(85%)	(79.1%)	(66.6%)	(72%)	
MTCC 1934	62.0	17.6	35.0	17.5	8.2	4.6	40	3.0
	(55%)	(67.6%)	(66.6%)	(75%)	(70.8%)	(53.3%)	(60%)	
N[b] alone	40.0	10.5	21.0	10.0	4.8	3.0	25	--
±SEM[e]	1.62	0.736	1.50	0.707	0.158	0.139	3.55	--
CD @ 1%[c]	7.02	0.317	6.50	3.05	0.685	0.600	15.34	--
CD @ 5%[d]	5.01	0.226	4.64	2.18	0.489	0.428	10.95	--

[a]CFU = Colony forming unit; [b]N = Nematode, Each value is an average of three replicates.
Figures in parenthesis represent percentage reduction over control.
[c]CD @ 1% means critical difference at 1% probability.
[d]CD @ 5% means critical difference at 5% probability.
[e]± SEM means standard error of means.

identification of the strains was made on the basis of their morphological and biochemical properties such as siderophore, HCN, and IAA production and these strains showed blue green fluorescent color due to the presence of pyocyanin pigment.[13] The strain LPT5 inhibits the wilt causing *Fusarium oxysporum*. Earlier, Kumar *et al*.[14] reported that the strain PE_{10} reduces the root disease complex due to root knot nematode, *Meloidogyne incognita* and wilt disease causing *F. oxysporum* and promote the growth of tomato. Fluorescent pseudomonads are known to have a significant role in the suppression of fungal pathogen via the production of antifungal metabolites[15] whereas these rhizobacteria reduced the hatching and also invasion due to the production of toxic metabolites,[6] nematicidal components[16] and alteration of specific root exudates that altered nematode behavior.[17] Our results showed that the culture filtrate of the *Pseudomonas aeruginosa* isolates caused significant juveniles mortality due to their nematicidal nature. Earlier, nematicidal activity in some strains of *Pseudomonas* was reported.[2,14] Enhanced larval mortality of *M. incognita* and *H. cajani* were reported when the juveniles exposed to culture filtrate of *Bacillus subtilis* and *P. fluorescens*.[18-19] The plant growth parameters such as length, fresh and dry weight of shoots and roots were also significantly improved in plant treated with *P. aeruginosa* strains. Similar increase in plant growth parameters in *Pseudomonas* treated plants was reported in potato[20] and tomato.[21] Such direct increase in plant growth may be due to production of IAA by *Pseudomonas* mediated IAA activity[22] and indirectly due to production of siderophore.[23] The effectiveness of *P. aeruginosa* as a potential biocontrol agent may involve more than one attribute against *H. cajani*. Oostendorp and Sikora[7] reported that the sugarbeet cyst nematode penetration to sugarbeet was decreased due to *P. aeruginosa* treatment. One of the large varieties of antibiotics produced by fluorescent pseudomonads, 2, 4-diacetyl phloroglucinol[24-26] and chitinases[27] reduced juveniles mobility and delayed egg hatch of the potato cyst nematode, *Globodera rostochiensis*.[28] But other factors such as ability of fluorescent *pseudomonas* to envelop or bind the root surface lectins, thereby interfering with the normal host recognition by the nematode can not be ruled out.[29] Santhi and Sivakumar[30] attributed the best control of plant parasitic nematodes by fluorescent pseudomonads to its best colonizing ability. Root colonization by rhiozospheric bacteria has been reported to reduce nematode invasion[31] thereby protecting the roots during early development stages of the plant.

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