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Isolation and Evaluation of Endophytic Bacteria Against Plant Parasitic Nematodes Infesting Black Pepper (*Piper nigrum* L.)

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ABSTRACT: Abundant and diverse populations of bacterial endophytes have been identified in many plants. In the present study, 80 isolates of endophytic bacteria were isolated from different varieties of black pepper (*Piper nigrum* L.) grown at different locations in India. Another 30, isolates were obtained from tissue cultured black pepper plants. These isolates were tentatively grouped into *Bacillus* spp. (32 strains), pseudomonads (26 strains), *Arthrobacter* spp. (20 strains), *Micrococcus* spp. (10 strains), *Curtobacterium* sp. (one strain), *Serratia* (one strain) and twenty unidentified strains based on morphology and biochemical tests. Their nematicidal properties, when tested in an *in vitro* bioassay using *Meloidogyne incognita* juveniles, varied from 0 -31.03%. Consortia of these endophytic bacteria were made and evaluated in nurseries for their nematode suppression and growth promotion in black pepper rooted cuttings. All the bacterial consortia were able to suppress nematodes, *M. incognita* and *Radopholus sintilis*, significantly. The maximum number of cuttings (243 cuttings / plant) was obtained with phorate treatment followed by treatment with consortia 1 and 4 indicating the potential of these bacteria to be used as nematode biological control agents.

Keywords: Biological control, black pepper, endophytic bacteria, *Meloidogyne incognita, Piper nigrum, Radopholus similis*

Black pepper (*Piper nigrum* L.), the king of spices, is one of the oldest and the most popular spice in the world. Various nematode pests affect this crop of which the burrowing (*Radopholus similis*) and root-knot (*Meloidogyne* spp.) nematodes are the most destructive. However, nematicides being a short-term solution to the problem of nematodes and the time required for the development of resistant cultivars is considerably long, are the impetus behind strong movements in determining the potential of biological management of plant parasitic nematodes. The past two decades investigators are concentrating their efforts on integrating biological control agents in nematode management strategies. Endophytic bacteria are those bacteria that can be isolated from surface-disinfected plant tissue or extract from within the plant, but which do not visibly harm the plant (Hallmann *et al.*, 1997a). Gram-positive and gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species. Abundant and diverse populations of bacterial endophytes were identified in potato (Garbeva *et al.*, 2001; Sturz *et al.*, 1999), maize (Fisher *et al.*, 1992; McInroy & Kloepper, 1995), rice (Stoltzfus *et al.*, 1998), cotton (McInroy & Kloepper, 1995) and cucumber (Mahafee & Kloepper, 1997). Studies have shown that these endophytes entered the plant tissue primarily through the root zone; however, aerial portions of plants, such as flowers, stems, and cotyledons, are also used for entry (Kobayashi & Palumbo, 2000). Endophytes inside a plant are either localized at the point of entry or spread throughout the plant (Hallmann *et al.*, 1997a) and reside within the cells (Jacobs *et al.*, 1985), in the intercellular spaces, or in the vascular system (Bell *et al.*, 1995). Indigenous endophytic bacteria communities cover a broad spectrum of bacterial species like *Pseudomonas fluorescens, Bacillus* spp., *Herbaspirillum* spp., *Serratia marcescens, Streptomyces* spp. etc. (McInroy & Kloepper, 1995). Endophytic bacteria for biocontrol purpose would eliminate the need to select bacterial types with high level of rhizosphere competence that are often considered necessary for successful seed or root bacterization treatment before or at planting. In the present study, attempts were made to isolate endophytic bacteria from healthy black pepper plants and to evaluate their antagonistic potential against plant parasitic nematodes under laboratory and greenhouse conditions.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Runner shoots were collected from different varieties and different geographical regions of black pepper plants grown at Kozhikode, Idukki, and Wayanad district in Kerala State and Kodagu District in Karnataka State of India. Stem, root and leaf samples were taken, rinsed 2-3 times in tap water and weighed. The stem and roots were cut into segments of 2-3 cm long, surface sterilized with 2% sodium hypochlorite for 10 min. and washed with sterile distilled water. Further, they were treated with 70% alcohol for one min. and washed four times in sterile distilled water. For sterility check, the roots, stem and leaves were rolled on nutrient agar and Kings B agar plates as well as 0.1 ml aliquot from the final wash was inoculated to 10 ml nutrient broth (NB) (Stoltzfus *et al.*, 1997; Hallmann *et al.*, 1997a; Gyaneshwar *et al.*, 2001). Samples were discarded if any growth was detected in the sterility check. The endophytic bacteria were isolated by modifying the isolation procedure described by Sturz *et al.* (1999) and Gyaneshwar *et al.*, (2001). Each sample (1g) was homogenized under ascptic conditions with a sterile mortar and pestle in 9 ml of phosphate buffer saline (g/1- NaCl 8, KCl 0.2, Na_2HPO_4 1.44 and KH_2PO_4 0.24, pH 7.4). From this, 1.5 ml of aliquot was transferred to a sterile microfuge tube and centrifuged at 1,300 rpm at 4°C for 10 min. The supernatant was serially diluted up to 10^{-5} and each dilution was pour plated (1 ml) on two different media with three replications. Nutrient agar amended with 2,3,5-triphenyl tetrazolium chloride (30 mg/l) (g/l; peptone 5, beef extract 2, yeast extract 3, sodium chloride 5 and agar 18, pH 7.0) and King's B medium (g/l; protease peptone 20, K_2HPO_4 1.5, $MgSO_4$, $7H_2O$ 1.2, filter sterilized glycerol 20 ml, and agar 18, pH 7.2). To collect the plant sap, a stem pieces (2-3 cm) were transferred to sterile microfuge tubes and were centrifuged at 10000 rpm at 4°C for 20 min. as described by Dong *et al.* (1994). The plant sap (0.1 ml) was serially diluted upto 10^{-5} and each dilution was spread plated (0.1 ml) on media as mentioned above. In order to isolate endophytic bacteria from tissue culture black pepper plants, the exudates from the cut end of the tissue culture plants were touched on nutrient agar and King's B agar medium at the time of subculturing. The plates were incubated at 28°C for 2-3 days. The individual bacterial colonies from each tissue were selected and subcultured on nutrient agar. The representative isolates were cryopreserved at -80°C in 20% glycerol for further studies.

Characterization of endophytic bacteria: Isolates were tentatively grouped and documented based on phenotypic characteristics such as colour, form, elevation, margin, diameter, surface, opacity and texture. Motility, cell morphology, size, Gram reaction, spore formation and production of UV- fluorescent pigments were also recorded using standard procedures. Routine biochemical tests such as Indole, methyl red, Voges-Proskauer, succinic acid and hydrogen cyanide production were assessed for each endophyte as described by Zvyagintsev (1991).

***In vitro* bioassay :** In order to screen the bacteria against nematodes, *in vitro* tests were carried out using bacterial cells suspended in sterile water (Chen *et al.*, 2000; Tian & Riggs, 2000). Bacterial isolates were grown in nutrient broth at 28°C for 24 h and the bacterial cells were separated out by centrifugation at 12,000 rpm at 4°C for 20 min. One hundred and ten endophytic bacteria were tested for their efficacy against *M. incognita.* Two hundred micro litre of bacterial suspension (~ x 10^9 cfu/ml) in sterile distilled water was added to 24 well microtitre plates and each treatment was replicated thrice. Surface sterilized *M. incognita* second stage juveniles hatched out from egg masses of root-knot nematodes cultured on *Coleus* plants were collected, and 50 µl (containing ~ 30 J2) was added to each well. Wells containing sterile distilled water served as control. The plates were incubated at 27°C and the number of live and dead nematodes was counted after 72 h under a stereomicroscope by adding 20 µl of 1N NaOH (Chen & Dickson, 2000). The results were recorded and the percentage mortality of nematodes was calculated. The data were subjected to angular transformation and analyzed using ANOVA. The means were separated by Duncan's Multiple Range Test (DMRT).

***In vivo* screening of endophytic bacterial consortia:** One hundred and ten endophytic bacteria were tested for their efficacy against both *M. incognita* and *R. similis* in an *in vivo* bioassay after grouping them into four different consortia based on their percentage inhibition

of root-knot nematodes (0 to 5%, 6 to 10%, 11 to 20% and 20 to 40%) in the *in vitro* bioassay. The other two groups were formed based on their geographical origin (20 isolates) and nematode antagonism exhibited in previous studies (10 isolates). The *in vivo* bioassay was conducted in two separate systems *viz.*, mother plains trailed on bamboos in a rapid multiplication black pepper many and rooted black pepper cuttings raised in polybags.

Black pepper mother plants trailed on bamboos: Endophytic bacterial consortia were screened in black pepper nurseries at IISR Experimental Farm, Peruvannamuzhi using six consortia of endophytic bacteria. There were eight treatments (6 consortia, chemical control and absolute control in a Randomized Block Design (RBD). The bacteria were multiplied in nutrient broth and applied at monthly intervals to black pepper plants (IISR, Sreekara) in a rapid multiplication shed. Observations on growth of.the plant, nematode and bacterial populations in the soil and root were periodically recorded for 3 months.

Rooted cuttings in polybags: The above mentioned consortia of endophytic bacteria were further evaluated for their role in growth of rooted cuttings and nematode population build up using rooted cuttings of another variety of black pepper (Panniyur 3). For this, the black pepper cuttings were taken from mother plants treated with the consortia for two months and transplanted in polybags containing normal potting mixture (1 kg). Fifty each cuttings were further treated with the above consortia of endophytic bacteria (~ x 10^9 cfu/ml) at monthly intervals. The growth (height of the plant, no. of leaves etc.) and nematode population in various treatments were continuously monitored for 3 months.

RESULTS AND DISCUSSION

Isolation and characterization of endophytic bacteria

A total of 110 endophytic bacteria were isolated from the black pepper samples drawn from different locations. Among the 110 endophytic bacteria, eighty were isolated from different varieties of black pepper namely Panniyur-1, Panniyur-4, Panniyur-5, IISR Panchami, IISR Sreekara, IISR Karimunda and IISR Thevam (Table 1) and another 30 isolates were obtained from tissue culture black pepper plants. These isolates were tentatively grouped into *Bacillus* spp. (32 strains), pseudomonads (26 strains), *Arthrobacter* spp. (20 strains), *Micrococcus* spp. (10 strains), *Curtobacterium* sp. (one strain), *Serratia* (one strain) and twenty unidentified strains based on the keys provided in the Bergey's manual. Among the plant parts the roots contributed for more number of endophytes (49) than stem (28) and leaf (3) and another 30 isolates were obtained from tissue culture black pepper plants.

Table 1. Isolation of endophytic bacteria from different parts of improved varieties of black pepper

Variety	No. of endophytic bacteria			
	Root	Stem	Leaf	Total
Panniyur-I	3	1	-	4
Panniyur- IV	-	1	-	1
Panniyur-V	23	16	-	39
IISR Panchami	-	-	1	1
IISR Sreekara	20	10	2	32
IISR Karimunda	2	-	-	2
IISR Thevam	1	-	-	1
Total	49	28	3	80

In vitro bioassay

In the *in vitro* bioassay, 77 isolates out of the 110 isolates caused significant mortality ($P \leq 0.01$) of the nematode J2s after 72 h of exposure to bacterial cell suspensions (Table 2). Fifty three isolates were either not inhibitory to nematodes or the inhibition was below 5%. Twenty five isolates were categorized as less antagonistic (6-10% mortality) and 22 were moderately antagonistic (11-20%). However, only ten isolates (BP 8, BP 14, BP 16, BP 31, BP 33, BP 61, BP 62, BP 64, BP 66 and BP 65) exhibited more than >20% mortality to the J2. The maximum mortality observed was only 31.03% in the case of isolate BP 62. These ten isolates were obtained from either Sreekara or Panniyur 5.

Table 2. Mortality of root-knot nematode juveniles (J2s) exposed to endophytic bacterial isolates isolated from black pepper

Sl. No	Mean mortality (%) range	No. of isolates	Isolate No.
1	0-5	53	BP 1-7, BP 9, BP 11, BP 21, BP 24, BP 27, BP 29 BP 32, BP 34, BP 36, BP 37, BP 39, BP 43, BP 45, BP 48, BP 49, BP- 50, BP 51, BP 53, BP 54, BP 59, BP 72-77, BP 79, BP 80,TC2,TC6,TC7,TC 12,TC 13,TC 15andTC 19-30
2	5-10	25	BP 12, BP 22, BP 25, BP 30, BP 35, BP 38, BP 40-44, BP 47, BP 52, BP 55-57, BP 60, BP 78, TC 5, TC 8-11 and TC 16-18,
3	10-20	22	BP 10, BP 13, BP 15, BP 17, BP 18, BP 19, BP 20, BP 23, BP 26, BP 28, BP 46, BP 58, BP 63, BP 65, BP 67-71, TC 1, TC 3 andTC4.
4	20-40	10	BP 8, BP 14, BP 16, BP 31, BP 33, BP 61, BP 62, BP 64, BP 66 and BP 65.

The mortality data was subjected to angular transformation and analyzed using ANOV A (SE/Plot = 3.50; CV = 28.97). The means were separated into 34 groups using DMRT. For convenience the data is grouped on the basis of increasing mortality range.

In vivo screening of endophytic bacterial consortia

Black pepper mother plants trailed on bamboos: Growth of black pepper cuttings (IISR Sreekara) and reduction in nematode population after three months of bacterial application (Fig. 1). All the bacterial consortia were able to suppress the nematodes significantly. However, phorate treated plants were more vigorous in growth and yielded more number of cuttings. The maximum number of cuttings (243 cuttings / plant) was obtained with phorate treatment followed by treatment with consortia 1 and 4. All the bacterial consortia reduced the *R. similis* population. However, the total bacterial count in soil was highest in the case of consortium 2 (T4).

Rooted cuttings in polybags: When the same consortia were evaluated using black pepper var. Panniyur 3 in polybags, there was no significant difference among various treatments with regard to height and total number of leaves per plant (Fig. 2). As in the previous experiment, the phorate treatment was the most effective one in suppressing the nematodes. Significant reduction in nematode population was also observed with bacterial consortia 1 (T3), 3 (T5) and 4 (T6). However, maximum nematode population and mortality were observed in plants treated with-consortia 6 (T8). Application of consortium 2 (T4) has significantly increased the total number of bacteria in rhizosphere soil and plant tissues. However, consortium 4 (T4) had a clear edge over other consortia in growth promotion as well as nematode suppression.

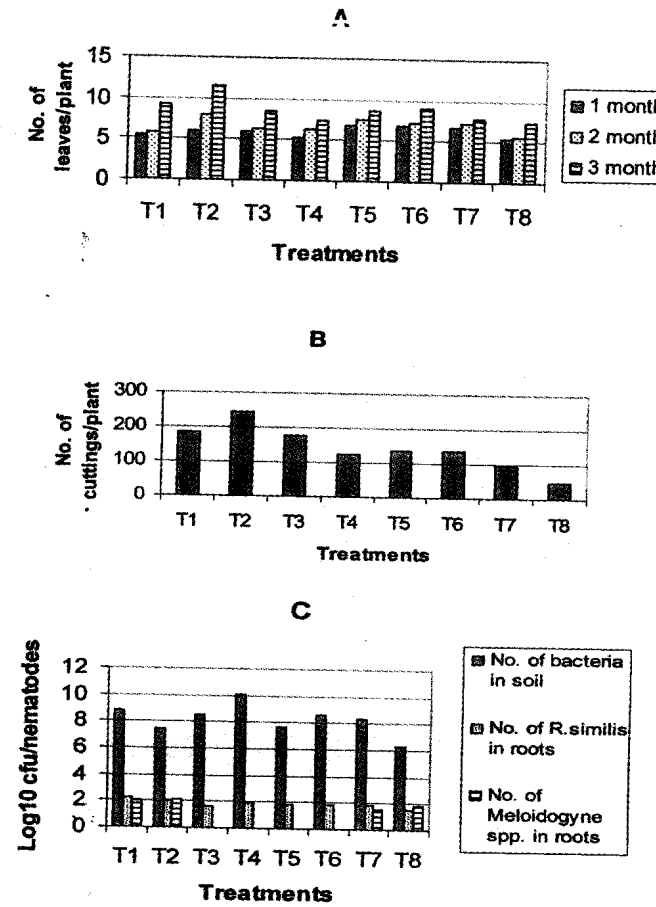

Fig. 1. Effect of different bacterial consortia on growth and nematode infestation in a black pepper nursery. A - No. of leaves per plant; B - Total number of cuttings from treated plants; C - Nematode population in roots of black pepper plants (T1 - control, T2 - phorate treated, T3 to T8 - bacterial consortia 1-6)

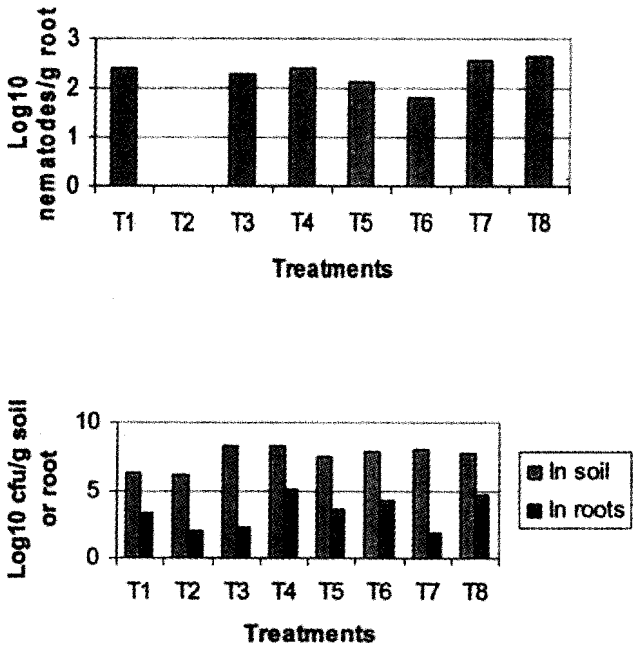

Fig. 2. Effect of different bacterial consortia on growth and nematode infestation in a black pepper rooted cuttings. *Top* - *R. similis* population in roots; *Bottom* - Total number of bacteria in soil and roots (T1 - control, T2 - phorate treated, T3 to T8 - bacterial consortia)

It is interesting to note that majority of endophytic bacteria isolated were obtained from roots of black pepper plants. It has been reported that the highest bacterial densities are usually observed in the roots and decrease progressively from stem to leaves (Quadt-Hallmann & Kloepper, 1996; Lamb *et al.*, 1996), this is because of the fact that the root is the primary site where endophytes gain entry into plants. Since several factors like growth media used, surface sterilization method employed etc. influence the recoverable bacterial population (Lodewyckx *et al.*, 2002), the bacterial isolates obtained need not represent the complete diversity present in the system.

Most of the endophytic bacteria isolated were Gram positive (80%) and rest Gram negative. Among the Gram positive, the dominant ones were *Bacillus* spp., followed by *Arthrobacter* spp, *Micrococcus* spp and *Curtobacterium* spp. Among the Gram negative *Pseudomonas* spp dominated followed by *Serratia* spp. Predominance of endophytic genera such as *Bacillus* spp., pseudomonads *Arthrobacter* spp. and *Micrococcus* spp. Mhafee & Kloepper (1997) have also reported predominant endophytic genera belong to *Bacillus* spp., *Arthrobacter* spp. and pseudomonads.

Comparatively low mortality observed in the *in vitro* bioassay could be because of the low production of toxic metabolites by the endophytes in the aqueous medium used in this study. Toxic metabolites that are lethal to nematodes are produced by most of the rhizobacteria when they are grown in specific nutrient rich media (Tian & Riggs, 2000). Nevertheless the exact reason for the nematode mortality has to be studied critically. Several *in vitro* studies had shown that rhizobacteria able to inhibit nematode viability and egg hatching (Neipp & Becker, 1999; Tian & Riggs, 2000). Generally, in these studies the efficacy was found to be concentration dependent. Although, the *in vitro* bioassays are rapid and space-efficient, these evaluations are not able to detect the effective endophytic bacterial strains. There are reports that organisms that have good antagonistic activity *in vitro* often have no biocontrol activity in soil (Racke & Sikora, 1992). There was no efficient and full proof method for screening of endophytic bacteria or rhizobacteria against plant parasitic nematodes in *in vitro* conditions.

Different mechanisms of action for various PGPR strains may explain why combinations of strains provided more consistency in disease suppression. Increasing the diversity of biological control systems though the use of mixtures of microorganisms may result in treatment that persist longer in the rhizosphere and utilize a wider array of biocontrol mechanisms (e.g. induction of systemic resistance, production of antibiotics and competition of nutrients) under a broader range of environmental conditions. These results are in agreement with studies by Pierson & Weller (1994) and Duffy & Weller (1995), both of which demonstrated that certain mixtures of fluorescent pseudomonads were significantly more suppressive of take-all than either treatment used alone. According to the above report endophytic consortia were made based on the *in vitro* studies. However, when bacteria were grouped based on their in vitro nematicidal activity and used in *in vivo* assay, the results did not tally with their *in vitro* bioassay.

The experiment has failed to clearly indicate the superiority of any single consortium in enhancing the growth of the treated black pepper plants or reducing the population of the target nematode, *R. similis* and *M. incognita*. Since the experiment was conducted in a naturally infested nursery, the distribution of nematodes was not uniform and this would have oreover, mixing large number of bacterial populations their compatibility is not advisable. The consortium 6 which consisted of best performers of *in vitro* bioassay was not superior in any respect in this assay. The maximum reduction in nematode population was observed with T6 (consortium 4), which showed 6-10% nematicidal activity in *in vitro* bioassay. The study has clearly shown the inadequacy of *in vitro* bioassays to identify isolates that really suppress nematodes. However, introducing combinations of biocontrol microorganisms does not always result in a better and more consistent disease suppression, as was demonstrated by Dandurand & Knudsen (1993). To our knowledge this study is the first to employ indigenous bacterial endophytes from native black pepper plants to manage plant parasitic nematodes. The use of specific culturable endophytes is preferable to the use of non-specific chemical fertilizer and pesticides because of cost effectiveness, environment safety and contributions to sustainable agricultural systems.

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