DBT-CP 2 (813): Final Report

Endophytic Bacteria for the Biological System Management of *Radopholus similis*, the Key Nematode Pest of Black Pepper (*Piper nigrum* L.)

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INDIAN INSTITUTE OF SPICES RESEARCH
CALICUT-673 012, KERALA

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PART I: GENERAL INFORMATION

800 Project Code

8001 Institute Project Code No. : DBT CP2 (813)

8002 ICAR Project Code No. : -

801 Name of the Institute and Division

8011 Name and address of Institute: Indian Institute of Spices Research, Calicut – 673012, Kerala

8012 Name of Division / Section : Crop Protection/Nematology

8013 Location of the Project : IISR, Calicut

802 Project Title : **Endophytic Bacteria for Biological System Management of *Radopholus similis*, The Key Nematode Pest of Black Pepper (*Piper nigrum* L.)**

803 Priority Area :

8031 Research Approach :

Applied Research	Basic Research	Process/Technology development	Transfer of Technology
02	01	03	-

804 Specific Area : **BIOLOGICAL CONTROL**

805 Duration of Project :

8051 Date of start : 2003

8052 Date of Completion : 2008

806 Total cost /Expenditure Incurred : Rs. 23.84 lakhs

(Give reasons for variation, if any from original estimated cost)

- Due to escalation in costs
- Due to extension of the project period
- Change in the scope of the project

807 EXECUTIVE SUMMARY

One hundred and seventy three isolates of endophytic bacteria were isolated from12 different varieties/cultivars of black pepper. All the bacterial isolates were characterized and grouped based on colony morphology. Out of these, 81 isolates (from the roots) were characterized using C utilization pattern, antibiotics sensitivity, production of metabolites like DAPG, chitinase, HCN etc. and other biochemical tests. They were identified as fluorescent pseudomonads (12 strains), non fluorescent pseudomonads (9 strains), *Serratia* (one strain), *Bacillus* spp. (26 strains), *Arthrobacter* spp. (20 strains), *Micrococcus* spp. (6 strains), *Enterobacter* sp. (one strain) and seven unidentified strains based on Bergey's manual. The host genotype was found to have profound influence on the diversity and colonization of endophytic bacteria. Molecular techniques like ARDRA and bioinformatics techniques were effectively used for identifying duplicate isolates, confirming species identity and for marker assisted selection of efficient strains. Species identification of short listed endophytic bacterial isolates by cloning and sequencing of rDNA has been initiated.

In vitro screening of 110 bacteria for nematicidal activity has shown that the mortality of nematodes ranged from 0 to 31.03% and eight isolates caused more than 20% mortality of the nematodes. Based on preliminary studies in pots and *in-situ* trials in black pepper nurseries, 14 isolates of endophytic bacteria were short-listed for *R. similis* suppression and colonization in black pepper roots. Among them, BP 17 (*Bacillus* sp.) and TC 10 (*Enterobacter* sp.) followed by BP 10 and BP 35, two *P. fluorescens* isolates, consistently reduced lesion index and *R. similis* population and increased root weight and total biomass of the cuttings, irrespective of the black pepper variety. BP 17, BP 25 and BP 35 are very good inhibitors of *Phytophthora capsici* too. Root inoculation of bacteria (BP 35 and IISR 859) elicited very high activity of oxidative enzymes like peroxidase, polyphenol oxidase and phenyl ammonia lyase in black pepper leaves indicating induced systemic resistance.

Chitin based formulations sustained comparatively higher population of endophytic bacteria (x 10^7) even after three months of storage and has a better shelf-life than talc based formulations. Evaluation of chitin based formulations is

in progress. Additional field trials to evaluate the promising endophytic bacteria for suppression of *R. similis* and promotion of plant growth have been initiated.

808 Key words :
ARDRA, *Bacillus* sp., biological control, black pepper, burrowing nematodes, endophytic bacteria, *Enterobacter* sp., *Piper nigrum*, *Pseudomonas fluorescens*, rhizobacteria, root knot nematodes, *Radopholus similis*

PART II : INVESTIGATORS' PROFILE

810	**Principal Investigator**	**:**	
81011	Name	:	**K.V. Ramana** (2003-05)
81021	Designation	:	Principal Scientist (Nematology) & Project Coordinator (Spices)
81012	Name	:	**Santhosh J. Eapen** (2005-08)
81022	Designation	:	Sr. Scientist (Nematology)
8103	Division/ Section	:	Division of Crop Protection
8104	Location	:	IISR, Calicut
8105	Institute Address	:	P.B. No. 1701, Marikunnu Post Calicut – 673 012, Kerala
811	**Co- Investigators:**		
81111	Name	:	A Kumar (2003 - 08)
81121	Designation	:	Scientist SS (Plant Pathology)
8113	Division/ Section	:	Division of Crop Protection
8114	Location	:	IISR, Calicut
8115	Institute Address	:	P.B. No. 1701, Marikunnu Post Calicut – 673 012, Kerala
812	**Co- Investigators:**		
8121	Name	:	R. Ramakrishnan Nair (2003-07)
81121	Designation	:	Sr. Scientist (Gen. & Cytogen.)
8113	Division/ Section	:	Division of Crop Improvement
8114	Location	:	IISR, Calicut
8115	Institute Address	:	P.B. No. 1701, Marikunnu Post Calicut – 673 012, Kerala

PART III: TECHNICAL DETAILS

820 Introduction and objectives

8201 Immediate objectives

a. To isolate and identify the endophytic bacteria associated with black pepper.
b. To screen for identification of efficient and effective endophytic bacterial strains for suppressing *R. similis* infestation in black pepper.
c. To evaluate the effect of the PGPR under nursery and field conditions and to select the appropriate endophytes for maximum performance in black pepper under high and low nematode pressures.
d. To study the biochemical and molecular interactions in the plant-nematode-bacterial system
e. Scaling up and formulation of promising isolates of endophytic bacteria

8202 Background information and importance of the project

Black pepper, the 'King of Spices' is the most important and widely used spice in the world. The black pepper of commerce is the dried, mature berries of the tropical, perennial climbing plant *Piper nigrum* L., which belongs to the family *Piperaceae*. It is a woody climber, grown in Kerala, Karnataka, Tamil Nadu, and Andhra Pradesh. Black pepper, with its characteristic pungency and flavour, is an ingredient in many food preparations (Ravindran, 2000). It is one of the major export earning crops of India. The production of black pepper is limited by heavy crop losses caused by insect pests, pathogens and nematodes (Sarma et al., 1994).

Various nematode pests affect this crop of which the burrowing nematode (*Radopholus similis*) and root knot nematode (*Meloidogyne* spp.) are more destructive. Slow decline caused by *M. incognita* and *R. similis* are severe threats to pepper cultivation. Etiological studies carried out recently have shown that slow decline is caused due to feeder root damage either by *P. capsici, R. similis* and *M. incognita* or various combinations (Sarma et al., 1994; Anandaraj, 1996). The plant-parasitic nematodes and pathogenic organisms form complex association within the host plant. Nematodes can play an important role in predisposing the host plant to invasion by secondary pathogens. Biochemical changes within the host tissue associated with nematode infestation presumably result in an environment beneficial for pathogenic organisms (Sitaramaiah & Pathak, 1993).

Chemical control of the diseases though effective, is undesirable as they pollute the environment and the residues left in the product are hazardous to human health. To minimize the use of pesticides, biological control becomes imperative in any integrated management of pests and diseases (Sarma et al., 1988). Biological control, being ecologically safer than the chemical pesticides, is preferred and has come to stay as an important crop protection strategy in many crops (Cook & Baker, 1983).

The burrowing nematode (*R. similis*) is the most destructive and highly pathogenic nematode pest of black pepper. It has a wide host range of about 370 plant species and is widely distributed in tropical sub-tropical regions of the world. It is present in almost all black pepper plantations of India. They feed on roots of these plants to produce lesions that lead to root rotting. The main source of nematode spread is through the rooted cuttings used as planting materials. Hence production of healthy and nematode-free planting materials is a major step in this direction. The biocontrol agents developed against root knot and cyst nematodes are less effective on this nematode. So there is a need for identifying candidate organisms that have the potential to suppress these nematodes.

Burrowing nematodes being migratory endoparasites are not accessible to the introduced biocontrol agents unless they are capable of colonizing the root system or inducing some resistance in the host plant through some means. In this context, rhizobacteria and endophytic bacteria need a closer look. Such bacteria are better adapted to the crops and may provide better control of these nematodes and possibly other soil-borne diseases. Bacterial endophytes may also be important in perennial crops like black pepper by effectively increasing phenotypic plasticity of their long-lived hosts under variable or deleterious environmental conditions. Thus organisms acting singly or as communities have been shown to elicit new and unique modification that are often coupled with plants own defence mechanisms like alterations of root exudates, induced resistance, increased plant tolerance and stimulation of allelopathic substances.

Therefore, the aim of the project was to identify suitable endophytic bacteria that improve health of roots by warding off the nematodes. Incorporation of such organisms, right in the nursery, will ensure its distribution and can avoid frequent application of such agents in the field. This novel approach to biologically deal with nematodes in the nurseries by deploying endophytic bacteria isolated from the host

plant itself is environment –friendly and sustainable. In lieu of the conventional integrated disease/pest management, the new approach is called biological system management and can be easily adopted in black pepper nurseries for large scale production of nematode-free planting materials.

821 Project Technical Profile

8211 Technical programme
(Indicate briefly plan of procedure, techniques, instruments and special materials, organisms, special environments etc.)

- Collection of endophytic bacterial isolates colonizing black pepper from different locations
- Characterization and identification of bacterial strains
- Screening for nematicidal activity of bacterial strains isolated
- Evaluation of promising endophytic bacteria in black pepper nurseries
- Studies on colonization of endophytic bacteria
- Studies on formulation of endophytic bacteria
- Molecular characterization of endophytic bacteria

8212 Total man months involvement of component project workers

27 man months by investigators

84 man months by research fellows

822 Final Report on the Project

Detailed report containing all relevant data with a summary of results

8221 Achievements in terms of targets fixed for each activity

Please see Annexure I.

8222 Questions answered

Do black pepper plants harbor endophytic bacteria?

Does the host genotype influence the diversity and population level of endophytic bacteria inside the plant tissues?

What is the role of these bacteria in plant growth?

Does any of the bacterial isolates exhibit nematicidal activity, especially against *R. similis*?

Can they be deployed in black pepper nurseries for management of R. similis?

8223 Process/ Product/ Technology/ Developed

The promising isolates of endophytic bacteria short-listed through this study include one isolate each of *Curtobacterium luteum* and *Bacillus megaterium*.

Two of the promising isolates have been registered with IMTECH, Chandigarh and obtained MTCC numbers viz. IISR BP 17 – MTCC 5409 and IISR BP 35 – MTCC 5410.These can be effectively used on a large scale for control of burrowing nematodes infesting black pepper.

8225 Practical Utility
(not more than 150 words)

R. similis is the most destructive and highly pathogenic nematode pest of black pepper. It is widely distributed in most of the black pepper growing tracts of the country. It is the root cause of 'slow decline' disease in black pepper. Management of these nematodes in black pepper is done mainly through application of nematicides. The nematode biocontrol agents currently available are not effective against *R. similis*. Good resistant source for this nematode also is yet to be identified.

Biological system management is an alternative approach to integrated pest management. It is an approach to improve root health of plants, especially rooted cuttings, using antagonists for biological control and targeted disruption of sensitive developmental stages of a pest. Site specific deployment of the endophytes can bring down the high inputs required for the mass production and release of biocontrol agents. The use of specific endophytic bacteria will provide an inexpensive and environment-friendly procedure of nematode management in nurseries. Their endophytic nature ensures targeted placement of the organism and hence higher levels of activity against nematodes. Many of these isolates possess growth promoting ability which in turn can reduce the fertilizer requirements of black pepper.

The promising isolates can very well be a part of the IPM strategies, which would minimize the costly and toxic inputs like nematicides and chemical fertilizers. This is highly relevant and readily applied in organic agriculture for which there is a global awareness.

8225 Constraints, if any

- Non availability of biosafety data for any of these new bioagents
- Inadequate manpower for in-house mass multiplication of bioagents
- Perennial nature of the crop and therefore lack of field data
- Poor awareness among the growers regarding the need for nematode control

823 PUBLICATIONS AND MATERIAL DEVELOPMENT

8231 Reviews

a. Koshy, P.K., Santhosh J. Eapen and Rakesh Pandey 2005. Nematode Parasites of Spices, Condiments and Medicinal Plants. In: *Plant Parasitic Nematodes in Tropical and Subtropical Agriculture -2 Edition*. Pp. 751-791. Eds. M. Luc, R.S. Sikora and J. Bridge, CAB International, Wallingford, U.K. (CABI Publishing).

b. Santhosh J. Eapen 2005. Nematodes of Spices. In: *Nematode Pests of Crops in Kerala- An Overview*. Pp 63-86. (Eds.) Sheela M.S. et al., Kerala Agricultural University.

8232 Research papers

a. Aravind, R., Dinu Antony, Santhosh J. Eapen, Kumar, A. and Ramana, K.V. 2004. Isolation and in vitro evaluation of endophytic bacteria against root-knot nematodes infesting black pepper (*Piper nigrum* L.). National Nematology Symposium, 17-19 November 2004, University of Agricultural Sciences, Bangalore.

b. Kumar, A., Dinu, A., Aravind, R., Eapen, S.J., Jisha, S., Anila, G., Beena, N., Anandaraj, M. and K.V.Ramana 2004. Evaluation of genetic diversity of rhizobacterial strains obtained from spice crops. *Agri-Informatics 2004*, pp. 49-55, Indian Institute of Spices Research, Calicut.

c. Bhai, R.S., Kishore, V.K., Kumar, A., Anandaraj, M. and Eapen, S.J. 2005. Screening of rhizobacterial isolates against soft rot disease of ginger (*Zingiber officinale* Rosc.). *Journal of Spices and Aromatic Crops* 14: 130-136.

d. Dinu A, Kumar A, Aravind R and Eapen S J (2007) An improved method for selection of antagonistic bactria against Phytophthora capsici Leonian infections in black pepper (Piper nigrum L.). Journal of Spices Aromatic Crops 16: 1-7.

8233 Popular articles : Nil

8234 Reports

a. Britto Cathrin B. 2004. Comparative genomics of biocontrol genes present in five species of *Pseudomonas*. M.Sc. Project Report submitted to Bharatidasan University, Tiruchirappally, Tamil Nadu.

b. Sreesmitha, V. 2005. Designing of species specific primers for plant growth promoting rhizobacteria (PGPR), *Bacillus* and *Pseudomonas* based on 16S rDNA polymorphism. M.Sc. Project Report submitted to Periyar Universit, Tamil Nadu.

c. Suma E. 2006. Induction of defense enzymes in black pepper by *Radopholus similis* and two isolates of rhizobacteria. M.Sc. Project Report. Submitted to Bharathidasan University, Trichy, Tamil Nadu.

d. Swapna C 2008 Comparative studies on two nematicidal strains of *Bacillus megaterium.* Bharathiar University, Coimbatore, Tamil Nadu. 32 pp. (M.Sc. thesis).

8235 Papers under preparation/communicated journals

a. Aravind, R., Eapen, S.J., Kumar, A., Dinu Antony and Ramana, K.V. 2008. Screening of endophytic bacteria against *Radopholus similis* and evaluation of the promising isolates using three different varieties of black pepper (*Piper nigrum* L.). *Communicated to Nematology*

b. Aravind R, Dinu Antony, Eapen, S.J., Kumar A. and Ramana K.V. 2008. Isolation and in vitro evaluation of endophytic bacteria against root knot nematodes infesting black pepper (*Piper nigrum* L.). *Communicated to Indian J. Nematol.*

c. Aravind R, Dinu Antony, Eapen, S.J. and Kumar A. 2008. Diversity and composition of endophytic bacterial community in black pepper (*Piper nigrum* L.) nurseries. (Under preparation).

d. Eapen S.J., Aravind R. and Kumar A. 2008. Mass multiplication of nematicidal bacterial isolates and their greenhouse evaluation using black pepper rooted cuttings. (Under preparation).

8236 Seminars, conferences and workshops (relevant to the project) in which the scientists have participated. (List abstracts forwarded)

e. 6th International PGPR Workshop, 5-10 October 2003, Indian Institute of Spices Research, Calicut.

f. National Nematology Symposium, 17-19 November 2004, University of Agricultural Sciences, Bangalore.

g. DBT Task Force (Biopesticides) Meeting, 02 February 2005, Department of Biotechnology, New Delhi

h. DBT Task Force (Biopesticides & Crop Management) Meeting, 22 August 2005, TERI, New Delhi

i. National Conference on Agro-biodiversity, 12-15 Feb. 2006, National Biodiversity Authority of India, Chennai.

j. DBT Task Force (Biopesticides & Crop Management) Meeting, 23-24 January 2007, India Islamic Centre, New Delhi

824 Infrastructural facilities developed

(Details of field, laboratory, note books and final material and their location)

8241 Infrastructure developed

a. Mastercycler (Eppendorf) – 1 No.

b. Transilluminator (Fotodyne) – 1 No.

c. Centrifuge (Eppendorf) – 1 No.

d. Electrophoresis unit (Atto) – 1 No.

e. Digital Camera (Nikon) – 1 No.

8242 Details of field, laboratory note books

Laboratory/field note books – 10 Nos. (Available with Senior Scientist, Nematology)

Lab registers - 6 Nos. (Available with Senior Scientist, Nematology)

8243 Biocontrol organisms

Four isolates of promising endophytic bacteria were short-listed out of the study and are maintained in the Institute repository. They are

IISR BP 17 – *Bacillus megaterium* – MTCC 5409

IISR TC 10 – *Curtobacterium luteum*

IISR BP 35 – *Pseudomonas aerugenosa* – MTCC 5410

IISR BP 25 – *Pseudomonas putida*

825 Comments / Suggestions of Project Leader regarding possible future line of work that may be taken up arising out of this Project

The potential endophytes identified in the present study have to be tested at multilocations to understand their field efficacy. The present field study has to be continued further to get the final results. These organisms can be scaled up and suitable delivery systems to be identified. The delivery through tissue culture materials is another area which is worth attempting. The leads obtained in their mode of action can be further explored for commercial exploitation.

PART IV: PROJECT EXPENDITURE (Summary)

Year 2003-2008

830 Total Recurring Expenditure

8301 Salaries: (Designation with pay scale)

	Estimated (Rs.)	Actual (Rs.)
i) Scientific	1,80,000	4,50,000
ii) Technical	6,90,000	7,28,000
iii) Supporting	20,000	0
iv) Wages	10,000	0
Sub-Total	8,70,000	11,78,000

8302 Consumables

i) Chemicals & Glasswares	3,50,000	3,39,000
iii) Others	1,00,000	75,000
Sub-Total	4,50,000	4,14,000

8303	Travel	75,000	30,000
8304	Miscellaneous (other costs)	2,60,000	2,30,000
8305	Sub-Total (Recurring)	16,55,000	18,52,000

831 Total Non – Recurring Expenditure
(Equipments and works)

i) Thermocycler - 1 No.	2,50,000	
ii)Transilluminator - 1 No.	50,000	
iii) Centrifuge - 1 No.	60,000	
iv) Electrophoresis - 1 No.	1,00,000	
v) Digital camera - 1 No.	40,000	Total 5,32,000

832	**Total** (830 and 831)	21,55,000	23,84,000

PART V: DECLARATION

This is to certify that the final report of the Project has been submitted in full consultation with the Project workers as per the approved objectives and technical programme and the relevant records, note-books, materials are available for the same.

Signature of the Project Investigator:

Co-Investigators 1.

2.

Signature & Comments of the Head
Of the Division/ Section

Signature & Comments of the
Director

ANNEXURE I

SALIENT ACHIEVEMENTS

ISOLATION OF ENDOPHYTIC BACTERIA

Methodology

Runner shoots were collected from different varieties of black pepper plants grown in different geographical regions (IISR Experimental Farm, Peruvannamuzhi, Idukki, and Wynaad in Kerala State and Kodagu in Karnataka State). 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. Samples were then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 minutes and washed with sterile distilled water. Then samples were treated with 70% alcohol for one minute and washed four times in sterile distilled water. The roots, stem and leaves were checked for the efficacy of sterilization by rolling them 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., 1998; Hallmann et al., 1997; Gyaneshwar et al., 2001). This was then incubated at 28^0 C for 3 days to serve as a sterility check. Samples were discarded if any growth was detected in sterility check.

The endophytic bacteria were isolated using the methods described by Sturz et al. (1999) and Gyaneshwar et al. (2001). Each sample (1g) was homogenized carefully under aseptic conditions with a sterile mortar and pestle in 9 ml of phosphate buffer saline (g/l – 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 / 4^0C / 10 minutes. 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 (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, the stem pieces (2-3 cm) were transferred to sterile microfuge tubes and were centrifuged at 10,000 rpm / 4^0C / 20 minutes as described by Dong et al. (1994). The plant sap (0.1 ml) was serially diluted up to 10^{-5} and each dilution was spread plated (0.1 ml) on two different media with three replications as mentioned above. At the time of subculturing endophytic bacteria from the exudates of tissue culture black pepper plants were isolated by touching the cut end on nutrient agar and King's B agar medium. The plates were incubated at 28^0C for 2-3 days. Colonies with differentiable colony morphologies were selected and were cryo-preserved at -80°C in 20% glycerol and were used for further assays.

Results

A total of 173 isolates of endophytic bacteria were isolated from 12 different varieties/cultivars of black pepper (Table 1) and are cryopreserved at -80°C in 20% glycerol for further assays. Roots of black pepper plants harbour maximum endophytes compared to leaf and stem. However, in P24, leaves had maximum endophytic bacteria. Out of the 11 black pepper improved varieties tested HP 813 possessed the maximum (cfu 8.5 x 10^3) population of endophytic bacteria while the lowest was found in Panniyur 4.

Table 1. Endophytic bacteria isolated from different varieties of black pepper

Variety	No. of Samples	No. of endophytic bacteria			
		Root	Stem	Leaf	Total
Panniyur-I	3	12	8	4	24
Panniyur-II	2	4	1	-	5
Panniyur-IV	1	-	1	-	1
Panniyur-V	4	23	16	-	39
Panchami	1	-	-	1	1
Sreekara	4	20	10	2	32
Karimunda	1	6	9	1	16
Karimundi	1	3	2	6	11
Kalluvally	2	-	1	3	4
Balankotta	1	-	3	3	6
Coll.1041	1	1	-	-	1
Wild (756)	1	3	-	-	3
Total	22	72	51	20	173

CHARACTERIZATION OF ENDOPHYTIC BACTERIA

Methodology

Morphological and biochemical

For studying the colony morphology of bacterial isolates, they were grown on Nutrient Agar medium for 48 h and observations like shape, surface elevation, margin, transparency and pigmentation of each colony were recorded. Further the isolates were smeared on clean glass slides and stained with Grams crystal violet stain. A series of biochemical tests viz. KOH test, Indole test, methyl red test, Voges Proskauer test, catalase test, oxidase test etc. were carried out using standard procedures. Biochemical tests were carried out for 81 isolates (72 root inhabiting endophytes and nine other promising isolates). The data for 81 isolates were compiled and plotted as a tree based on the nearest neighbourhood analysis using NTSYS software package.

Carbon source utilization

Total of 18 carbon sources (mono and disaccharides) viz. Glucose, Dulcitol, D-Mannitol, D-Sorbitol, D-Galactose, D-Xylose, L-Arabinose, Inositol, Raffinose, L(+)Rhamnose, D(-)Fructose, Maltose, Lactose, Sucrose, D-Ribose, D(+)Trehalose, Adonitol and D-Arabinose were used for this screening. Ten per cent solution of these carbon sources was prepared, filter sterilized by membrane filtration (0.22µm) and sufficient quantity was added to the basal medium (Peptone - 1 g l^{-1}, NH4 H2 PO4 – 1 g l^{-1}, KCL - 0.2 g l^{-1}, MgSO4.7H2O - 0.2 g l^{-1}, Agar – 3 g l^{-1}, BTB - 0.08, distilled water - 1000 ml) to give final concentration of 1%. From this, 100µl aliquots were dispensed in the wells of microtitre plates. Bacteria suspension was inoculated in these wells. Each isolate was replicated twice and two wells containing only basal medium without carbon source served as control. The wells were examined after 72 h for any colour change. The colour reaction in comparison with the control wells indicates the organism's utilization of a particular carbon source and to produce acids. The colour change from green to yellow (acid pH < 6) indicates oxidation of the carbohydrates.

Antibiotic sensitivity test

Nine antibiotics viz. streptomycin, spectinomycin, gentamycin, nalidixic acid, ampicillin, chloromphenicol, kanamycin, tetracycline and penicillin were dissolved in

distilled water or in ethyl alcohol and filter sterilized by using membrane filtration (0.22µm) in a Millipore system. Nutrient agar (peptone: 5g l^{-1}, sodium chloride: 5g l^{-1}, Beef extract: 2g l^{-1}, yeast extract: 3g l^{-1}, Agar: 18g l^{-1} and pH: 7.0) (50ml) was taken in 100ml conical flask and autoclaved at 121oC for 20 min at 15 lbs. Required quantity of antibiotics was added to the medium to get the following final concentrations: streptomycin (100µg ml^{-1}), spectinomycin (25µg ml^{-1}), gentamycin (20µg ml^{-1}), nalidixic acid (40µg ml^{-1}), ampicillin (100µg ml^{-1}), chloromphenicol (40µg ml^{-1}), kanamycin (60µg ml^{-1}), tetracycline (30µg ml^{-1}) and penicillin (30µg ml^{-1}) were added. Antibiotic amended medium was poured in Petri plates and inoculated with 2µl of 24 h old bacterial cultures in the marked columns. The plates were incubated for 24 – 48 h at 28^{o}C and observations were taken.

Molecular characterization

Isolation of DNA: Genomic DNA from selected endophytic bacteria was isolated with the following protocol. Bacterial cells were lysed in CTAB (Cetyl Trimethyl Ammonium Bromide) buffer (100mM Tris-Cl, 100mM EDTA, 100mM Na_2HPO4, 1.5M NaCl, 1% CTAB, 20µg proteinase K, 100µg Lysozyme) at 37^{o}C for 30 min and subsequently at 65^{o}C for about 2 h in the presence of Sodium Dodecyl Sulphate (SDS) 15mg/tube. The aqueous phase was extracted with equal volumes of chloroform-isoamyl alcohol (24:1) and the DNA was precipitated with isopropanol. The isolated DNA was dissolved in TE buffer (10mM Tris-Cl, 0.1mM EDTA, pH 8).

Amplified Ribosomal DNA Restriction Analysis (ARDRA): DNA was isolated as described above and amplification was done using 16-23S primer. PCR was done with following conditions (Water 1X, Buffer 1 X, dNTPs 50µm/base, B.S.A 50 µg, MgCl2 3mM, Primers 5Pm each, DNA polymerase 0.5 units, Template DNA 50ng/µl) and with the temperature profile, denaturation at 92°c 1 min 10 sec, annealing at 48°c 1min and elongation at 72 °c 2min 10 sec (35 cycles). The PCR product was resolved in a 1.5% gel with 500 bp ladder as marker. The amplicon was then digested with Msp I restriction enzyme (4 units) and the product was then resolved in a 3% agarose gel and the banding pattern was studied. The banding pattern in the restriction profile was subjected to NTSYS analysis.

DNA from *Pseudomonas* isolates was isolated and amplified using *P. fluorescens* specific primers, 16S-23S rRNA intervening sequence specific primers ITS1F (AAGTCGTAACAAGGTAG) and ITS2R (GACCATATATAACCCCAAG) to confirm their identity (Ramesh Kumar et al., 2002).

Amplification and sequencing of of 16s rRNA gene: Amplification of 16s rRNA gene of endophytic bacteria was performed with a universal primer set pA (Fp) (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (Rp) (5'-AAGGAGGTGATCCAGCCGCA -3') (Woese 1987)(Goebel 1994) in 25 µl of reaction mixture containing 1X buffer (10mM Tris pH 9, 50mM KCl, 0.01% gelatin), 200µm dNTP's mix, 3mM $MgCl_2$, 10µg BSA, 5pM each primer, 0.5 units of Taq DNA polymerase and 100ng template DNA. The thermocycling conditions consisted of an initial denaturation at 94ºC for 2 min, 35 amplification cycles of 94ºC for 1 min 10s, 48ºC for 30s, 72ºC for 2 min 10s and a final polymerization step of 72ºC for 6 min 10s with Eppendorf master thermal cycler. The final PCR product was resolved in 0.8% agarose in Tris Acetate EDTA buffer at 4V/cm. The PCR products were excised and purified with Sigma elution kit.

DNA sequencing was performed with an ABI 377 Prism DNA sequencer at Avastagen in Bangalore. Nucleotide sequence similarities were determined by using BLAST, version 2.0 (National Center for Biotechnology Information databases).

Results

Colony morphology

Colony morphology of all the 173 bacterial isolates was studied and documented. Based on the colony characters the bacterial isolates were divided into 27 groups (Table 2). The details are given in Annexure 2. Among these, 22.5% belonged Circular/Raised/Entire group followed by Irregular/Raised/Entire (15.6%) and Circular/Convex/Entire (13.9%) groups. Colony morphology of selected isolates is given in Plate 1.

Table 2: Grouping of endophytic bacteria based on colony characters

Group	Colony characters	Number of isolates
1	Circular/Flat/Entire	1
2	Circular/Raised/Entire	39
3	Circular/Raised/Erose	2
4	Circular/Raised/Undulate	2
5	Circular/Convex/Entire	24
6	Circular/Convex/Erose	2
7	Irregular/Flat/Entire	6
8	Irregular/Flat/Erose	3
9	Irregular/Flat/Undulate	8
10	Irregular/Flat/Lobate	3
11	Irregular/Raised/Entire	27
12	Irregular/Raised/Erose	15
13	Irregular/Raised/Undulate	18
14	Irregular/Raised/Filamentous	2
15	Irregular/Raised/Lobate	2
16	Irregular/Convex/Entire	3
17	Irregular/Convex/Undulate	2
18	Irregular/Convex/Filamentous	1
19	Punctiform/Raised/Entire	1
20	Punctiform/Raised/Undulate	2
21	Rhizoid/Raised/Filamentous	1
22	Rhizoid/Flat/Undulate	2
23	Filamentous/Raised/Entire	2
24	Filamentous/Raised/Undulate	1
25	Filamentous/Raised/Filamentous	2
26	Filamentous/Flat/Undulate	1
27	Filamentous/Flat/Filamentous	1

Biochemical tests

The 81 isolates were grouped into nine clusters at 80% similarity coefficient. These groups were identified as fluorescent pseudomonads (12 strains), non fluorescent pseudomonads (9 strains), *Serratia* (one strain), *Bacillus* spp. (26 strains), *Arthrobacter* spp. (20 strains), *Micrococcus* spp. (6 strains), *Enterobacter* sp. (one strain) and seven unidentified strains based on Bergey's manual. The cluster diagram depicting the groupings is given in Fig.1. Details are given in Annexure 1.

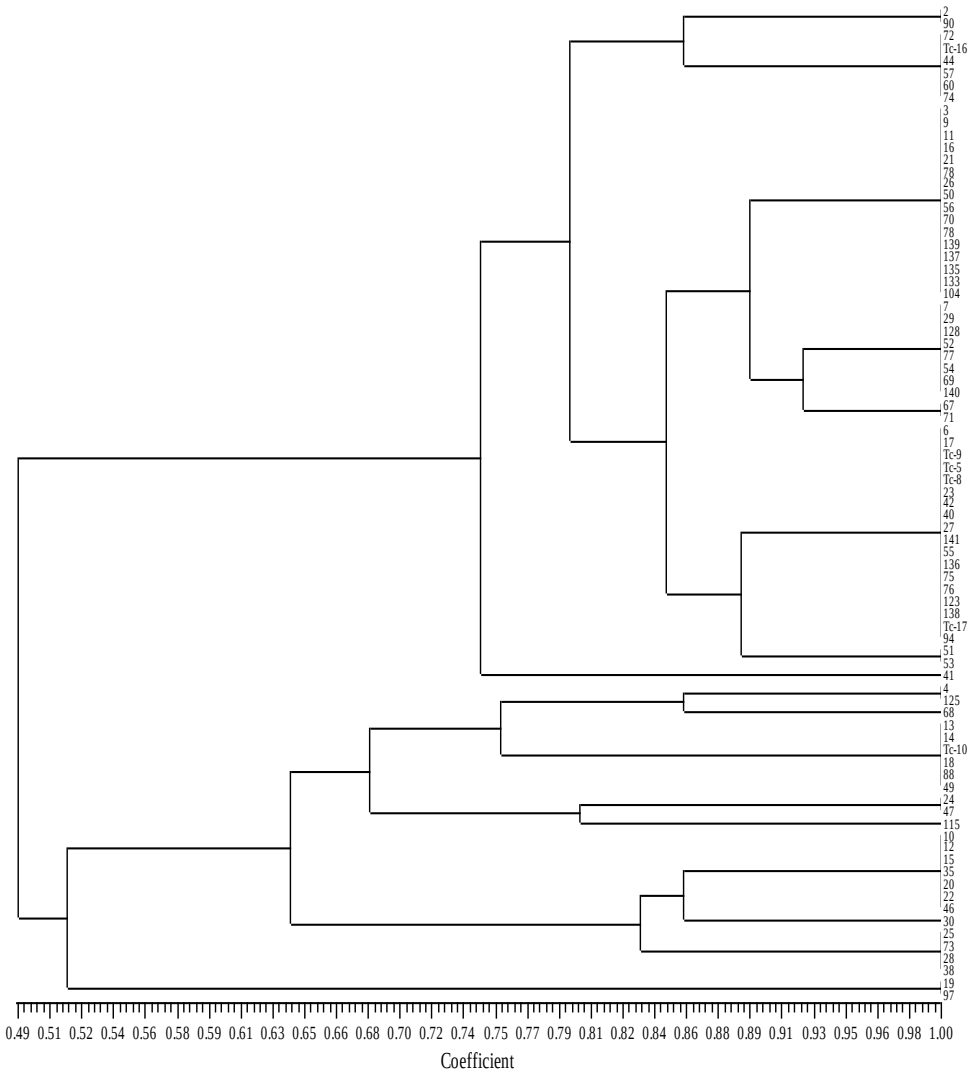


Fig.1. Dendrogram showing the grouping of 81 isolates based on their morphology and other biochemical parameters.

Carbon source utilization and antibiotics sensitivity

Seven of the total endophytic isolates were able to use all the carbon sources. All the isolates were able to utilize a minimum of ten carbon sources. Most of the isolates from tissue culture materials are dulcitol negative. BP 35, BP 72 and BP 137 were

resistant to eight antibiotics while BP 4, BP 55 and BP 88 to seven of them. This information can be utilized for developing specific media for tracking the specific bacteria.

Molecular characterization

ARDRA: The PCR amplification resulted in a product of 1500 bp (Plate 2). The NTSYS analysis of the restriction profile has grouped the endophytes into 18 groups at 78% similarity co-efficient. ARDRA helped to identify duplicates in the bacteria isolated and the results were matching with those of morphological and biochemical analysis. The identity of *P. fluorescens* was confirmed using species specific primers in a PCR assay which produced an amplicon of size 560 bp (Plate 2). This amplicon is reported to be specific for *P. fluorescens* (Ramesh Kumar et al., 2002).

16S rRNA amplification and sequencing: The PCR amplification yielded 1500bp amplicon which was purified and sequenced. Promising endophytic bacteria were identified as *Pseudomonas aeruginosa* (BP35), *Pseudomonas putida* (BP25), *Bacillus megaterium* (BP17) and *Curtobacterium luteum* (TC10) based on 16S rDNA analysis. The percentage nucleotide identity was more than 99 for all the species. Partial sequence data for the 16S rDNA gene have been deposited in the EMBL/GenBank/ DDBJ nucleotide sequence database libraries. Data for endophytic strains have been deposited under the following accession numbers: BP17: *Bacillus megaterium* EU071712 and TC 10: *Curtobacterium luteum* EU071713.

IN VITRO BIOASSAY OF ENDOPHYTIC BACTERIA

Methodology

In vitro screening of bacteria against nematodes was 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 / 4^0C / 20 minutes. Two hundred microlitre of bacterial suspension (~ x 10^9 cfu ml^{-1}) 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* plant 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.

DAPG (2, 4 - diacetylphloroglucinol)

Bacterial cells were suspended in sterile distilled water and the optical density (OD) was adjusted 0.5 in spectrophotometer. PCR amplification was carried out in 10µl reaction mixture contains 2µl of bacterial suspension. 1X PCR buffer, 0.5g l^{-1} bovine serum albumin, 5% dimethyl sulphoxide, 2.5µM of dNTPs, 0.2µM of each primer and 1.4U of Taq DNA polymerase. Amplification was performed with thermal cycler (Eppendorf), using following PCR conditions: initial denaturation for 10 min, 940C for 30 s, 600C for 30 s, 720C for 60 s, followed by 30 PCR cycles and a final extension at 720C for 10 min. PCR product were separated on a 1.2% agarose gel. The expected product size was 745bp DNA fragment (Velusamy & Gnanamanickam, 2003).

Hydrogen cyanide (HCN)

Log phase culture (25µl) of bacterial was inoculated to 10ml of King's B broth supplemented with 4.4g l^{-1} of glycine and added in vials. Filter paper strips soaked in picric acid solution (2.5g picric acid and 12.5g Na2CO3 in one litre) were placed in vials without touching the broth and tightly closing with screw caps. The vials are

incubated in a shaker at 100 rpm for 48h. The production of HCN was indicated by the change in colour of the filter paper strips from yellow to brown to red (Kloepper et al., 1991).

Chitinase

Chitinolytic activity of bacteria was tested on plates by streaking colonies on minimal media supplemented with colloidal chitin (0.2%) and solidified with 1.5% of agar. The plates were incubated at 28^0C for 96 h until the zone of chitin clearing could be seen around the colonies (Chernin et al., 1995).

Protease

To test for proteolytic activity on plates, colonies were streaked on peptone broth supplemented with skim milk powder (10%) and solidified with 1.5% of agar. The plates were incubated at 28^0C for 96h until the zone of chitin clearing could be seen around the colonies.

Results

In the *in vitro* bioassay, 77 isolates out of the 110 isolates caused significant mortality ($P \leq 0.01$) of the nematode juveniles after 72 h of exposure to bacterial cell suspensions (Table 3). Thirty three isolates were not inhibitory to nematodes at all. However, only eight isolates (BP-14, BP-31, BP-33, BP-61, BP-62, BP-64, BP-66 and TC-14) exhibited more than >20% mortality to the J2. The maximum mortality observed was only 31.03% in the case of isolate BP-62. These eight isolates were obtained from either Sreekara or Panniyur 5. 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 (Oka et al.,1993) (Tian & Riggs, 2000). Nevertheless the exact reason for the nematode mortality has to be studied critically. There are reports that organisms that have good antagonistic activity *in vitro* often have no biocontrol activity in soil (Becker et al., 1988; Racke & Sikora, 1992). Under these circumstances it is better to screen the isolates under greenhouse conditions where multiple mechanisms of antagonisms operate.

Table 3. Mortality of root-knot nematode juveniles (J_2) exposed to endophytic bacterial isolates in the in vitro bioassay.

Sl. No.	Isolate No.	Plant part used	Mean mortality (%)	Group[1]
1	BP-1	Stem	0.00 ± 0.00[2]	34
2	BP-2	Root	0.00 ± 0.00	34
3	BP-3	Root	0.00 ± 0.00	34
4	BP-4	Root	0.00 ± 0.00	34
5	BP-5	Stem	0.00 ± 0.00	34
6	BP-6	Root	0.00 ± 0.00	34
7	BP-7	Root	0.00 ± 0.00	34
8	BP-8	Root	16.75 ± 10.52	5-9
9	BP-9	Root	0.00 ± 0.00	34
10	BP-10	Root	10.89 ± 2.00	7-19
11	BP-11	Root	0.00 ± 0.00	34
12	BP-12	Root	7.55 ± 4.78	14 –27
13	BP-13	Root	16.31 ± 14.77	5 – 11
14	BP-14	Root	24.45 ± 10.84	1-4
15	BP-15	Root	15.75 ± 7.95	5-10
16	BP-16	Root	17.03 ± 3.77	4-8
17	BP-17	Root	16.43 ± 4.95	4-8
18	BP-18	Root	13.65 ± 3.38	6-13
19	BP-19	Root	14.28 ± 7.85	6-13
20	BP-20	Root	16.38 ± 1.97	4-8
21	BP-21	Root	4.10 ± 0.55	25-33
22	BP-22	Root	7.80 ± 3.22	14-27
23	BP-23	Root	9.31 ± 1.67	9-22
24	Bp-24	Root	4.31 ± 2.45	24-33
25	BP-25	Root	6.12 ± 2.38	18-28
26	BP-26	Root	9.26 ± 2.31	9-22
27	BP-27	Root	2.71 ± 2.78	30-33
28	BP-28	Root	10.46 ± 0.89	8-21
29	BP-29	Root	2.44 ± 2.38	31-33
30	BP-30	Stem	8.96 ± 2.48	10-24
31	BP-31	Stem	20.58 ± 2.28	3-6
32	BP-32	Stem	2.85 ± 2.48	29-33
33	BP-33	Stem	26.28 ± 5.85	1-3
34	BP-34	Stem	4.64 ± 3.21	23-33
35	BP-35	Stem	5.70 ± 5.06	26-33
36	BP-36	Stem	3.06 ± 3.13	29-33
37	BP-37	Stem	0.00 ± 0.00	34
38	BP-38	Stem	7.26 ± 0.53	14-27
39	BP-39	Stem	0.00 ± 0.00	34
40	BP-40	Stem	7.16 ± 1.00	14-27
41	BP-41	Stem	6.54 ± 0.93	16-27
42	BP-42	Stem	6.69 ± 0.45	16-27
43	BP-43	Stem	5.34 ± 2.12	21-30
44	BP-44	Stem	5.88 ± 2.01	19-28
45	BP-45	Stem	2.14 ± 1.96	32-33
46	BP-46	Root	17.19 ± 3.67	4-8
47	BP-47	Root	5.36 ± 1.42	20-29
48	BP-48	Root	0.00 ± 0.00	34

49	BP-49	Root	0.00 ± 0.00	34
50	BP-50	Root	0.00 ± 0.00	34
51	BP-51	Root	4.96 ± 0.53	22-31
52	BP-52	Root	6.35 ± 0.94	17-27
53	BP-53	Root	0.00 ± 0.00	34
54	BP-54	Root	0.00 ± 0.00	34
55	BP-55	Root	7.00 ± 0.49	14-27
56	Bp-56	Root	5.34 ± 1.9	22-30
57	BP-57	Stem	8.98 ± 1.38	10-23
58	BP-58	Stem	12.27 ± 1.95	7-16
59	BP-59	Stem	2.46 ± 2.23	31-33
60	BP-60	Stem	8.16 ± 2.67	13-26
61	BP-61	Stem	23.61 ± 4.38	1-4
62	BP-62	Stem	31.03 ± 9.68	1
63	BP-63	Stem	14.76 ± 1.96	5-11
64	BP-64	Stem	21.88 ± 5.41	2-5
65	BP-65	Stem	17.70 ± 7.15	4-7
66	BP 66	Stem	27.85 ± 5.56	1-3
67	BP-67	Root	11.43 ± 3.18	7-18
68	BP-68	Root	13.10 ± 5.46	7-14
69	BP-69	Root	12.71 ± 1.89	7-15
70	BP-70	Root	12.00 ± 4.27	7-17
71	BP-71	Root	10.46 ± 0.89	8-21
72	BP-72	Root	4.20 ± 0.49	22-33
73	BP-73	Root	3.97 ± 1.26	25-33
74	BP-74	Root	0.00 ± 0.00	34
75	BP-75	Root	0.00 ± 0.00	34
76	BP-76	Root	4.59 ± 0.22	22-32
77	BP-77	Root	5.09 ± 5.00	27-33
78	BP-78	Root	7.06 ± 0.53	14-27
79	BP-79	Leaf	3.48 ± 3.26	28-33
80	BP-80	Leaf	0.00 ± 0.00	34
81	TC-1	T.C plant	10.57 ± 1.55	8-20
82	TC-2	T.C plant	0.00 ± 0.00	34
83	TC-3	T.C plant	14.68 ± 3.17	5-12
84	TC-4	T.C plant	9.41 ± 3.74	9-22
85	TC-5	T.C plant	6.93 ± 1.32	15- 27
86	TC-6	T.C plant	0.00 ± 0.00	34
87	TC-7	T.C plant	4.22 ± 1.38	24-33
88	TC-8	T.C plant	6.43 ± 3.59	18-28
89	TC-9	T.C plant	5.23 ± 0.29	22-30
90	TC-10	T.C plant	6.64 ± 1.40	16-27
91	TC-11	T.C plant	7.63 ± 2.34	14-27
92	TC-12	T.C plant	4.13 ± 1.42	25-33
93	TC-13	T.C plant	0.00 ± 0.00	34
94	TC-14	T.C plant	29.41± 5.88	1-2
95	TC-15	T.C plant	0.00 ± 0.00	34
96	TC-16	T.C plant	8.49 ± 1.44	12-26
97	TC-17	T.C plant	7.36 ± 2.21	14-17
98	TC-18	T.C plant	8.65 ± 2.44	11-25
99	TC-19	T.C plant	0.00 ± 0.00	34
100	TC-20	T.C plant	1.83 ± 1.58	33
101	TC-21	T.C plant	4.29 ± 1.41	22-33

102	TC-22	T.C plant	0.00 ± 0.00	34
103	TC-23	T.C plant	0.00 ± 0.00	34
104	TC-24	T.C plant	0.00 ± 0.00	34
105	TC-25	T.C plant	0.00 ± 0.00	34
106	TC-26	T.C plant	0.00 ± 0.00	34
107	TC-27	T.C plant	0.00 ± 0.00	34
108	TC-28	T.C plant	0.00 ± 0.00	34
109	TC-29	T.C plant	0.00 ± 0.00	34
110	TC-30	T.C plant	0.00 ± 0.00	34

S.E/ plot 3.50
Gen. Mean 12.10
C.V 28.97
[1]Grouping of means based on DMRT
[2]Mean ± S.D.

Production of toxic metabolites

The endophytes isolated were screened biochemically for the production of 2, 4-diacetyl phloroglucinol (DAPG), hydrogen cyanide (HCN) etc. The results were further confirmed by amplifying the genomic DNA with primers specific for the compounds. None of isolates out of the 111 screened were positive for DAPG.

However, 10 isolates viz. PD-13, PD-16, BP 10, BP 12, BP 15, BP 20, BP 22, BP 30, BP 35 and BP 46 were capable of HCN production, which is detrimental to nematodes. Similarly, 8 isolates (PD-2, PD-3, PD-7, PD-10, PD-12, PD-15, PD-21 and PD-29) out of 53 are chitinase producers and 21 isolates out of 30 are protease producers. Thirteen strains out of 53 are efficient phosphate solubilizers.

Culture filtrates of two strains of *B. megaterium* (BP17 and IISR 522) were toxic to *R. similis* and caused >60% mortality. However, BP17 was slightly more efficient when compared with IISR 522. Similarly, the volatile metabolites produced by both isolates too were toxic to *R. similis* and the mortality was significantly high in the case of IISR 522. Both the isolates had moderately caused repellence of *R. similis*.

Suppression of Phytophthora capsici

When evaluated in the greenhouse for disease management, the isolates BP35, BP25 and BP17 recorded over 70% disease suppression irrespective of the variety. However, the disease suppression was marginally better on Panniyur-1 type (80%) compared to Karimunda type (60-70%). The endophytes could offer protection even with the pathogen population size of 6-7 Log cfu g-1 of soil which showed the role of endogenous population size for protecting the plants.

Another greenhouse trial is in progress to study the performance of endophytic bacteria *P. aeruginosa* (BP35) in 12 varieties of black pepper for the management of *P. capsici* and nematodes. When evaluated in the greenhouse for *Phytophthora* disease management, the isolates endophytic BP35, BP25, BP17 recorded over 70% disease suppression. The disease suppression was marginally better on Panniyur-1 type (80%) when compared to Karimunda type (60-70%). The endophytes could offer protection even with the pathogen population size of 6-7 Log cfu g^{-1} of soil which showed the role of endogenous population size for protecting the plants.

IN VIVO BIOASSAY OF ENDOPHYTIC BACTERIA

Methodology

In vivo screening of 74 endophytic bacterial isolates was done on rooted cuttings of Panniyur-1 variety of black pepper. The black pepper cuttings were dipped in a bacterial suspension and allowed to root in poly bags containing one kg of sterilized potting mixture. After two months the rooted plants were further treated with the bacteria by pouring the bacterial suspension (~ x 10^{10} cfu ml^{-1}) in the root zone. The bacterized plants were challenge inoculated with *R. similis* (150 nematodes / bag) after one month. There were three replications for each treatment. Plants not bacterized but challenged with nematodes served as controls. The nematode suppression, growth parameters (height, no. of leaves, total biomass and root biomass) and bacterial counts were recorded after two months of nematode inoculation and data were statistically analyzed.

Results

In the *in vivo* bioassay, 74 endophytic bacterial isolates were evaluated against the *R. similis* on the rooted cuttings in a greenhouse. The endophytic bacteria were short listed based on the nematode suppression, growth and bacterial colonization in black pepper cuttings. Out of the 74 bacterial isolates, only 11 isolates such as BP 4, BP 9, BP 10, BP 12, BP 17, BP 18, BP 25, BP 35, BP 71, BP 104 and TC 10 significantly reduced *R. similis* population in black pepper roots. The lowest population of nematodes was observed with TC 10 followed by BP 4, BP 10, BP 12, BP 17, BP 9, BP 104, BP 18, BP 71, BP 25 and BP 35. The low nematode population in the case of BP 4, BP 9, BP 10, BP 12 and BP 18 could be due to fewer roots (below 0.43g) available in these plants. BP17, 25, 35, 71, 104 and TC 10 significantly suppressed the nematode population and sufficient root mass was also available. However, the best root system was seen with TC 10 followed BP 17. The growth promotion observed with BP 2, BP 12, BP 25, BP 28, BP 49, BP 53, BP 67 and TC 10 was noteworth**y**. However among them only BP 12, BP 17, BP 25 and TC 10 caused significant reduction in *R. similis* population (Table 4).

Out of the 74 endophytic bacterial isolates evaluated 14 isolates significantly increased the total biomass of black pepper rooted cuttings. The highest biomass (25g) was obtained with two strains viz. BP 2 and BP 49. Several strains increased the height of the cuttings significantly, BP 49 and BP 67 being the best isolates. The

highest number of leaves was observed in plant treated with endophytes BP 53 and BP 49. Out of the 74 endophytic bacteria, only 40 isolates could be reisolated from either roots or shoots of inoculated black pepper plants. From roots only less number of bacteria (24 out of 74) were reisolated as most of the roots were rotten due to nematode infection. Bacterial population was comparatively high in roots than in shoots.

Table 4. Effect of endophytic bacteria on *Radopholus similis*, growth and endophytic bacteria populations colonizing black pepper rooted cuttings in a polybags.

Treatment	*R. similis* per g root (Log_{10})	Growth parameters				No. of inoculated bacteria (Log_{10})	
		Height	No. of. Leaves	Total Biomass	Root weight	Root	Shoot
BP-2	3.40	51.50	10.50	25.00	0.67	4.33	0.00
BP-3	3.43	36.00	3.67	9.67	0.73	2.30	0.00
BP-4	2.00	17.67	2.67	6.33	0.10	IR	2.60
BP-6	3.90	23.00	2.00	6.00	1.10	0.00	2.30
BP-7	3.10	25.00	5.00	11.00	0.70	0.00	2.70
BP-9	2.70	20.00	2.00	5.00	0.30	IR	2.00
BP-10	2.03	32.33	5.33	12.33	0.43	IR	3.25
BP-11	3.33	19.00	1.50	7.00	0.67	0.00	3.70
BP-12	2.10	12.00	4.00	16.00	0.40	IR	2.60
BP-13	3.43	25.00	5.00	12.67	0.60	2.75	0.00
BP-14	3.37	18.00	2.00	7.00	0.60	2.50	3.60
BP-15	3.20	23.33	5.00	14.33	0.87	0.00	0.00
BP-16	3.43	19.00	4.67	10.67	0.63	4.10	2.85
BP-17	2.70	42.67	4.67	14.67	1.97	4.10	0.00
BP-18	2.83	21.67	3.00	10.67	0.43	2.70	0.00
BP-19	3.60	23.00	3.00	10.00	0.30	IR	0.00
BP-21	3.53	22.50	3.00	10.00	0.93	2.80	2.25
BP-23	3.53	28.33	4.67	11.00	0.43	IR	0.00
BP-24	3.63	23.33	3.67	10.00	0.53	0.00	0.00
BP-25	2.90	45.33	5.67	18.67	0.73	3.80	3.20
BP-26	3.57	32.33	4.33	13.33	0.43	IR	0.00
BP-27	3.27	20.67	3.67	10.67	0.40	IR	2.00
BP-28	3.00	59.00	8.00	20.00	1.33	3.50	0.00
BP-29	3.37	25.00	5.67	10.67	0.37	IR	2.00
BP-30	3.10	19.00	2.00	8.00	0.40	IR	3.40
BP-35	2.90	37.00	5.33	17.33	1.03	3.00	0.00
BP-40	3.50	34.00	5.00	14.67	1.10	4.00	3.25
BP-41	2.93	23.33	5.00	8.67	0.43	IR	0.00
BP-42	3.63	25.00	3.33	12.33	0.83	2.30	0.00
BP-44	3.30	13.33	4.00	10.00	0.20	IR	2.25
BP-47	4.50	9.00	3.00	7.00	0.40	IR	0.00
BP-49	3.40	147.00	11.00	25.00	0.90	0.00	0.00
BP-50	3.60	20.00	5.00	7.00	0.50	0.00	0.00
BP-51	3.20	33.00	6.67	14.00	0.80	2.60	0.00
BP-52	D	D	D	D	D	D	D
BP-53	3.40	60.00	16.00	20.00	0.30	IR	0.00

BP-54	3.80	14.00	4.00	6.00	0.80	0.00	0.00
BP-55	D	D	D	D	D	D	D
BP-56	D	D	D	D	D	D	D
BP-60	3.33	24.00	3.67	11.33	0.63	2.95	2.95
BP-67	4.00	115.00	7.00	20.00	1.00	0.00	0.00
BP-68	3.33	29.33	5.00	6.00	0.73	2.70	2.00
BP-69	3.50	30.00	7.00	10.00	0.40	IR	2.00
BP-70	3.38	19.00	3.33	6.00	0.73	4.50	2.30
BP-71	2.87	20.67	3.67	10.67	1.10	IR	2.00
BP-72	3.60	19.33	2.67	4.33	0.30	IR	0.00
BP-73	D	D	D	D	D	D	D
BP-74	3.60	15.00	4.00	5.00	0.20	IR	0.00
BP-75	3.33	24.67	5.00	6.67	0.57	0.00	0.00
BP-76	3.33	22.33	4.67	8.00	0.33	IR	0.00
BP-77	3.67	19.33	2.67	4.33	0.30	IR	0.00
BP-88	3.37	18.50	4.00	5.50	0.47	IR	0.00
BP-90	3.60	27.33	4.33	8.67	0.30	IR	2.30
BP-94	3.37	19.00	1.67	3.00	0.27	IR	0.00
BP-97	3.73	22.33	2.67	7.33	0.53	0.00	0.00
BP-104	2.80	28.67	4.67	8.00	1.17	3.30	0.00
BP-115	3.63	25.50	5.00	7.00	0.53	0.00	3.50
BP-123	3.60	20.33	3.67	4.67	0.50	0.00	0.00
BP-125	D	D	D	D	D	D	D
BP-128	3.50	20.50	3.50	5.50	0.53	3.30	0.00
BP-133	3.57	14.33	3.00	9.67	0.43	IR	0.00
BP-135	3.53	15.00	5.33	11.33	0.67	0.00	0.00
BP-136	D	D	D	D	D	D	D
BP-137	3.37	21.33	4.33	6.67	0.67	0.00	0.00
BP-138	3.40	28.33	4.67	11.00	0.43	IR	0.00
BP-139	3.20	33.00	6.67	14.00	0.80	2.60	0.00
BP-140	3.53	22.33	5.00	9.00	1.10	4.00	3.25
BP-141	3.23	21.33	3.67	10.67	0.40	IR	2.00
TC-5	3.60	19.33	2.67	4.33	0.30	IR	0.00
TC-8	3.30	26.00	3.67	10.00	0.30	IR	2.90
TC-9	2.97	24.00	4.33	10.33	1.20	3.80	3.50
TC-10	1.70	41.00	6.67	19.67	2.80	4.10	0.00
TC-16	3.27	28.67	4.33	13.33	1.53	3.25	0.00
TC-17	3.37	26.50	4.00	9.00	0.30	IR	0.00
Control	3.60	12.00	3.33	7.00	0.33	0.00	0.00
$LSD_{0.05}$	**0.7037**	**13.66**	**1.970**	**5.937**	**0.5379**	**0.1457**	**0.1994**

D - Plants died due to severe infection of *R. similis*; IR – Insufficient roots

GREENHOUSE EVALUATION OF ENDOPHYTIC BACTERIA

Methodology

A series of greenhouse and nursery experiments were conducted using isolates of endophytic bacteria and rooted cuttings of different varieties of black pepper. Nematode-free test plants were treated with endophytic bacterial suspension (Log phase culture) and planted in sterile potting mixture. The bacterial treatment was repeated in regular intervals and challenge inoculated with *R. similis* and the final observations like height of the plant, number of leaves, total biomass, number of bacteria present in various parts of the plant and number of nematodes in root were recorded after three months. The experimental details are given below.

Experiment 1

Black pepper cuttings were dipped in a bacterial suspension and allowed to root in polybags containing one kg of sterilized potting mixture. After two months the rooted plants were further treated with the bacteria by pouring the bacterial suspension (containing ~10^8 cfu ml^{-1}) in the root zone. The bacterized plants were challenge inoculated with *R. similis* (150 nematodes / bag) after one month. The growth, nematode multiplication and bacterial counts were recorded after three months of nematode inoculation.

No. of treatments	-	Main plot: 2 (*R. similis* inoculated and uninoculated) Subplot: 26 (25 bacterial isolates and a control)
No. of replications	-	3
Test plant	_	2 m old black pepper rooted cuttings (Sreekara)
Design	-	Split plot

The experiment was repeated with another 23 isolates of bacteria. The experimental details were same as above.

Experiment 2

Another greenhouse trial was conducted to evaluate 12 short-listed endophytes using three-noded rooted cuttings of Panniyur 1 variety of black pepper. The pepper cuttings (6 leaf stage) were washed free of soil and dipped in a bacterial suspension (bacterial cells were suspended in 100 ml of 10 mM MgSO4 and diluted in 600 ml of water) for one hour. The plants were planted in polybags containing sterilized potting mixture (1 kg). After 15 days the plants were drenched with the bacterial suspension. These plants were challenged with *R. similis* @ 200 nematodes / bag one month after planting in polybags. The experiment was concluded after three months and

final observations viz. height of the plant, number of leaves per plant, total biomass, root biomass, final nematode population in roots and bacterial population in plant were recorded.

No. of treatments	-	Main plot: 2 (*R. similis* inoculated and uninoculated) Subplot: 14 (12 short-listed bacterial isolates and two controls)
No. of replications	-	6
Test plant	_	Three-noded cuttings (Panniyur 1)
Design	-	Split plot

Experiment 3

In the light of the above two experiments, a few isolates were short-listed and further tested in another greenhouse trial using three varieties of black pepper. This was to find out the influence of the host genotype on the establishment and efficacy of introduced bacteria. For this, three one month-old rooted cuttings of black pepper of each variety (viz. Panniyur 2, Panniyur 4 and Sreekara) were planted in earthen pots (30 cm dia) containing sterilized potting mixture.

No. of treatments	-	Main plot: 2 (*R. similis* inoculated and uninoculated) Subplot: 3 (three black pepper varieties) Sub subplot: 9 (eight short-listed bacterial isolates and a control)
No. of replications	-	3
Test plant	-	Rooted cuttings of black pepper (Panniyur 2, Panniyur 4 & Sreekara)
Design	-	Split plot

Experiment 4

The endophytic bacteria were grouped into different consortia based on their in vitro efficacy as well as geographical origin. Preliminary trials were laid out in black pepper nurseries at IISR Experimental Farm, Peruvannamuzhi using six consortia of endophytic bacteria. The bacteria were multiplied in nutrient broth and applied at monthly intervals to black pepper plants in a rapid multiplication shed. Observations on growth of the plant, nematode and bacterial populations in the soil and root etc. are periodically recorded.

No. of treatments	-	8 (six consortia of endophytic bacteria, chemical control and an absolute control)
Consortia 1	:	10 promising rhizobacteria identified in earlier studies

Consortia 2	:	20 endophytes collected from different geographical regions
Consortia 3	:	53 endophytes having very low (0-5%) nematicidal activity under *in vitro* conditions
Consortia 4	:	25 endophytes that showed moderate (5-10%) nematicidal activity
Consortia 5	:	22 isolates having high (10-20%) nematicidal activity
Consortia 6	:	10 endophytes that showed maximum (20-40%) nematicidal activity in *in vitro* bioassay
No. of replications	-	14
Test plant	_	Black pepper rooted mother plants (cv. Sreekara)
Design	-	RBD

Further, the growth and nematode population build up were monitored in another variety of black pepper (Panniyur 3). For this, the black pepper cuttings were taken from mother plants treated with different 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 at monthly intervals. The growth (height of the plant, no. of leaves etc.) and nematode population in various treatments were continuously monitored.

No. of treatments	-	8 (six consortia of endophytic bacteria, chemical control and an absolute control)
No. of replications	-	50
Test plant	_	Black pepper rooted cuttings (cv. Panniyur 3)
Design	-	RBD

Results

Experiment 1

In the first batch of isolates, there was no significant difference in any of the growth characters of plants treated with either bacteria alone or bacteria with nematodes. However, there was significant reduction in the nematode population in response to treatments with Bp 9, Bp12, Bp 15 and TC 9. The results are given in table 5 and Plate 4. However, in the second batch wherein 23 bacterial isolates were evaluated significant reduction in nematode population was observed with three endophytic

Table 5. Changes in *Radopholus similis* and endophytic bacteria populations colonizing black pepper seedlings in a greenhouse (Mean of three replications).

Treatment	*R. similis* per g root	No of total bacteria (x 10^5)				No. of inoculated bacteria (x 10^4)			
		Shoot		Root		Shoot		Root	
		N+	N-	N+	N-	N+	N-	N+	N-
Control	1886.99	0.10[a]	4.00	0.20[a, b]	0.02 [a]	-	-	-	-
Bp 3	2678.17	0.00[a]	0.00[a]	0.04[a]	0.07 [a, b]	0.00[a]	0.00[a]	0.02[a]	0.03[a]
Bp 4	1022.29	0.05[a]	0.04[a, b]	-	0.67 [b, c]	0.04[a, b]	0.03[a]	-	0.48[a]
Bp 7	1278.38	0.19[a, b]	0.09[b]	-	0.90 [c]	0.20 [b, c]	0.05[a, b]	-	0.67[a]
Bp 9	505.82*	2.00[d, e]	-	-	-	0.01[a]	-	-	-
Bp 10	4445.31	0.67[b, c]	-	-	3.94 [d]	0.19 [b, c]	-	-	-
Bp 11	1171.20	0.04[a]	-	-	-	0.48[c, d]	-	-	-
Bp 12	114.88*	-	-	-	-	-	-	-	-
Bp 13	2659.73	1.87[d, e]	0.60[c]	-	-	-	-	-	-
Bp 14	2284.60	0.06[a]	0.06[a, b]	-	0.80 [c]	0.39[c]	0.10 [b]	-	0.49[a]
Bp 15	1481.52	0.03[a]	0.08[b]	-	0.05[a, b]	-	-	-	-
Bp 25	1388.95	-	-	-	-		-	-	-
Bp 30	1388.95	0.06[a]	0.00[a]	-	0.04[a]	0.25[c]	0.01[a]	-	0.10[a]
Bp 35	2896.34	0.90[c, d]	1.97[d]	0.27[a, b]	0.69 [b, c]	-	0.05[a, b]	0.10[a, b]	0.40[a]
Bp 40	1269.57	3.94[f]	0.49[c]	0.80[c]	2.00 [c, d]	0.19[b, c]	0.58[c]	0.99[c]	2.91[b]
Bp 44	2003.47	2.00[d, e]	0.03[a, b]		0.99 [c]	0.02[a]	0.02[a]	0.02[a]	0.03[a]
Bp 47	28972.44	-	-	0.37[b, c]	-	-	-	-	-
Bp 52	1580.25	-	-	-	-	-	-	-	-
Bp 55	404.51*	-	-	-	-	-	-	-	-
Bp 56	1341.76	-	-	-	-	-	-	-	-
Bp 60	2097.94	2.91[e, f]	1.50[d]	2.00[d]	0.80 [c]	0.87[d]	0.67[c]	0.90[c]	0.02[a]
TC 8	2453.71	6.00[g]	0.01[a, b]	-	0.08 [a, b]	7.00[e]	0.02[a]	-	0.05[a]
TC 9	890.25*	0.07[a]	0.02[a, b]	0.39[b, c]	3.00 [d]	0.30[c]	0.06[a, b]	0.60[c]	6.66[a]
TC 16	1930.97	0.00[a]	0.00[a]	0.10 [a, b]	2.62 [d]	0.00[a]	0.00[a]	0.19[b]	0.10[a]
TC 17	2370.37	-	-	-	-	-	-	-	-

- Not processed due to complete decay of roots/shoot; * significant difference from the control. Figures followed by the same letter are not significantly different based on DMRT. N+ *R.similis* inoculated; N- uninoculated

bacteria viz. Bp 17, Bp 51 and Bp 53. A few of these isolates significantly improved the growth of both nematode infested and nematode uninoculated black pepper cuttings (Table 6).

Experiment 2

In this experiment, out of the 12 endophytes none of the isolates could give a statistically significant reduction in the nematode population (Table 7; Plate 5). However, appreciable level of control could be seen in the case of BP 10 and BP 46. Phorate application gave excellent control of nematodes and growth promotion. None of the isolates failed to give any appreciable level of growth promotion too. More than 50% reduction in the *R. similis* population was noticed with isolates BP 25, BP 35, BP 70, BP 76 and TC 10.

Experiment 3

In this exhaustive experiment, except IISR 859, all the other three bacteria were good in promoting growth of all the varieties used (Table 8; Plate 6-9). However, the performance of other isolates was strongly influenced by the host genotype. BP 35, TC 10 and IISR 6 induced root proliferation in all the varieties irrespective of *R. similis* infestation. It was conclusively proved that BP 17, TC 10, IISR 859 and IISR 6 were very effective in suppressing *R. similis* population in black pepper, irrespective of the variety (Table 9). Maximum population of the inoculated bacteria was retrieved from the roots and shoots of inoculated black pepper plants in the case of BP 25 followed by BP 17. IISR 859 could not be located in either roots or shoots of inoculated plants as it is not an endophyte. Similarly, the population level of IISR 6 too was very low in black pepper tissues. TC 10 was present in high numbers only inside roots of inoculated plants. Among the varieties, Sreekara was inhabited by maximum number of introduced bacteria showing the congenial physiology of this variety for supporting endophytes.

Experiment 4

Growth of black pepper cuttings (cv. Sreekara) and reduction in nematode population after three months of bacterial application is depicted in Fig.2 and Plate 3A & B. 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

Table 6. Effect of endophytic bacteria on *Radopholus similis* and growth of black pepper rooted cuttings in a greenhouse (Mean of three replications).

Treatment	*R.similis* / g root	Height (cm)			Total biomass (g)			Root wt. (g)		
		N+	N-	Mean	N+	N-	Mean	N+	N-	Mean
Control	632.87a	17.67	23.00	20.33	7.00	18.00	12.50	0.61	2.36	1.49
Bp 2	141.56ab	112.5	20.00	66.25	25.00	8.00	16.50	1.35	0.72	1.04
Bp 16	166.49ab	0	30.00	24.50	10.67	15.00	12.83	0.59	1.21	0.90
Bp 17	4.71cd	19.00	-	-	-	-	-	-	-	-
Bp 18	370.53ab	-	-	-	-	-	-	-	-	-
Bp 19	600.17ab	-	30.00	26.50	10.00	15.00	12.50	0.29	1.38	0.84
Bp 20	202.70ab	23.00	56.67	38.67	10.67	26.00	18.33	1.71	2.92	2.31
Bp 21	238.33ab	20.67	-	-	-	-	-	-	-	-
Bp 23	305.20ab	-	62.00	45.17	11.00	25.83	18.42	0.44	2.48	1.50
Bp 24	276.33ab	28.33	79.00	51.17	10.00	25.00	17.50	0.55	2.83	1.69
Bp 26	307.32ab	23.33	45.00	38.67	13.33	20.00	16.67	1.08	1.61	1.34
Bp 28	388.05ab	32.33	75.00	67.00	20.00	25.00	22.50	1.30	3.64	2.47
Bp 29	562.64ab	59.00	65.00	45.00	10.67	16.00	13.33	0.77	1.85	1.31
Bp 46	301.69ab	25.00	-	-	-	-	-	-	-	-
Bp 49	227.03ab	-	-	-	-	-	-	-	-	-
Bp 50	199.91ab	-	132.33	76.17	7.00	22.00	14.50	0.54	2.41	1.48
Bp 51	29.62bc	20.00	35.00	34.00	14.00	15.00	14.50	0.82	1.70	1.26
Bp 53	0.00d	33.00	-	-	-	-	-	-	-	-
Bp 54	232.88ab	-	-	-	-	-	-	-	-	-
Bp 67	201.77ab	-	42.00	59.75	20.00	13.00	16.50	2.75	2.80	2.78
Bp 68	57.62abc	77.50	55.00	42.17	6.00	17.50	11.75	0.74	3.92	2.33
Bp 69	94.06ab	29.33	60.00	45.00	10.00	20.00	15.00	0.40	2.15	1.27
Bp 70	71.28abc	30.00	45.00	32.00	6.00	15.00	10.50	0.74	4.75	2.74
Bp 74	156.04ab	19.00 15.00	26.17	20.58	5.00	11.50	8.25	0.24	2.00	1.12
Mean	-	34.39	51.83*	-	11.55	18.11*	-	0.88	2.40*	-
$LSD_{0.05}$	-	48.49		26.99	8.77		5.92	1.23		0.75

N+ *R.similis* inoculated; N- uninoculated; * significant difference between N+ and N-. Figures followed by the same letter are not significantly different based on DMRT.

Table 7. Effect of endophytic bacteria on *Radopholus similis* and growth of black pepper rooted cuttings in a greenhouse (Mean of six replications).

Treatment	R. similis / g root	Height (cm)			No. of leaves			Total biomass (g)			Root biomass (g)		
		N+	N-	Mean	N+	N-	Mean	N+	N-	Mean	N+	N-	Mean
R. similis	125.18abc	74.83	97.50	86.17	13.67	15.00	14.33	55.00	59.83	57.42	8.20	15.33	11.77
Phorate	0.00d	91.83	145.75	118.79	12.00	18.00	15.00	54.50	78.75	66.63	8.12	17.00	12.56
BP 3	107.39abc	60.50	112.67	86.58	9.83	13.50	11.67	48.00	49.33	48.67	8.84	8.00	8.42
BP 10	11.27c	104.83	98.50	101.67	12.33	14.33	13.33	43.50	53.33	48.42	6.01	10.83	8.42
BP 25	52.95abc	59.60	88.60	74.10	11.80	15.60	13.70	43.40	53.60	48.50	7.05	8.20	7.63
BP 28	437.53a	53.17	100.00	76.58	9.33	11.50	10.42	35.33	38.50	36.92	7.00	7.00	7.00
BP 35	61.95abc	58.17	68.00	76.58	11.67	15.40	13.53	43.67	49.00	46.33	4.07	7.00	5.53
BP 40	130.22abc	77.50	86.17	81.83	11.67	15.17	13.42	42.00	56.50	49.25	5.38	12.17	8.77
BP 42	188.23ab	89.00	48.00	68.50	12.67	11.40	12.03	46.83	42.80	44.82	7.15	12.40	9.77
BP 46	27.12bc	82.67	87.17	84.92	12.00	17.17	14.58	47.50	64.33	55.92	5.18	14.17	9.68
BP 70	59.26abc	65.17	85.00	75.08	10.83	15.83	13.33	40.00	51.00	45.50	6.21	10.00	8.10
BP 76	54.59abc	65.00	84.67	74.83	12.50	13.83	13.17	38.00	53.67	45.83	6.48	11.83	9.16
BP 97	426.56a	51.67	94.20	72.93	8.67	12.60	10.63	37.00	55.80	46.40	9.39	13.80	11.60
TC 10	54.08abc	65.17	63.33	64.25	12.50	12.33	12.42	44.50	43.33	43.92	7.98	11.60	9.79
Mean	-	73.29	89.97*	-	11.53	14.40	-	44.23	53.56*	-	6.93	11.38*	-
$LSD_{0.05}$		31.75		22.45	NS		2.88	NS		9.88	3.91		2.76

N+ *R.similis* inoculated; N- uninoculated; NS Not significant, * significant difference between N+ and N-. Figures followed by the same letter are not significantly different based on DMRT.

Table 8. Effect of short-listed endophytic bacteria on the growth of nematode-free and *Radopholus similis* infested black pepper plants belonging to three varieties (Mean of 9 replications).

Variety	Isolates	Height (cm)			Total biomass (g)			Root Biomass (g)		
		R.s +	R.s -	Mean	R.s +	R.s -	Mean	R.s +	R.s -	Mean
Panniyur-2	BP-17	18.22	41.56	29.89	10.67	25.89	18.28	1.52	2.93	2.23
	BP-25	36.57	77.78	57.18	25.11	37.11	31.11	2.95	2.96	2.96
	BP-35	23.78	70.44	47.11	14.67	43.44	29.06	1.82	4.87	3.35
	BP-71	11.68	68.11	39.90	7.88	33.11	20.50	1.55	3.73	2.64
	BP-104	17.57	71.44	44.51	10.89	43.78	27.34	1.24	4.87	3.06
	TC-10	26.89	52.44	39.67	17.00	29.44	23.22	2.13	5.58	3.86
	IISR-6	21.69	70.56	46.13	13.44	46.11	29.78	1.72	5.07	3.40
	IISR-859	17.22	46.33	31.78	12.56	24.56	18.56	1.74	2.27	2.01
	Control	14.11	45.78	29.95	10.67	24.56	17.62	1.52	2.05	1.79
	Mean	20.86	60.49	40.68	13.65	34.22	23.94	1.80	3.81	2.81
	$LSD_{0.05}$	20.94		23.57	9.00		10.13	0.97		1.09
Panniyur-4	BP-17	14.89	80.78	47.83	9.67	43.33	26.50	0.79	4.08	2.44
	BP-25	21.22	46.11	33.67	16.33	22.89	19.61	1.65	2.46	2.06
	BP-35	21.00	81.22	51.11	16.78	41.89	29.33	1.15	3.62	2.39
	BP-71	20.57	54.22	37.38	15.89	27.67	21.78	1.58	2.34	1.96
	BP-104	21.45	49.11	35.28	13.56	27.11	20.33	1.02	1.82	1.42
	TC-10	22.00	74.78	48.39	21.22	30.89	26.06	1.83	2.45	2.14
	IISR-6	20.57	75.56	48.06	13.33	30.56	21.94	1.53	2.00	1.77
	IISR-859	23.89	50.44	37.17	17.22	23.00	20.11	1.83	2.22	2.03
	Control	13.78	37.67	25.72	9.11	17.00	13.06	0.63	1.84	1.24
	Mean	19.93	61.10	40.51	14.79	29.37	22.08	1.33	2.54	1.94
	$LSD_{0.05}$	20.94		23.57	9.00		10.13	0.97		1.09
IISR Sreekara	BP-17	27.89	85.89	56.89	13.44	44.44	28.94	1.29	4.10	2.70
	BP-25	38.00	59.67	48.83	16.00	27.11	21.56	1.27	2.07	1.67
	BP-35	19.22	120.78	70.00	9.00	57.44	33.22	1.29	5.22	3.26
	BP-71	41.22	121.33	81.28	16.78	39.33	28.06	1.75	3.31	2.53
	BP-104	32.00	162.89	97.45	16.22	71.78	44.00	1.10	6.75	3.93
	TC-10	42.33	127.22	84.78	25.00	51.67	38.33	3.45	4.15	3.80
	IISR-6	47.56	113.67	80.61	21.67	54.89	38.28	2.45	5.61	4.03
	IISR-859	61.86	90.44	76.17	28.00	37.78	32.89	2.20	4.28	3.24
	Control	75.22	72.11	73.67	27.44	35.22	31.33	3.12	3.17	3.15
	Mean	42.81	106.00	74.41	19.28	46.63	32.96	1.99	4.30	3.14
	$LSD_{0.05}$	20.94		23.57	9.00		10.13	0.97		1.09
Gen Mean		27.86	75.86*	-	15.91	36.74*	-	1.71	3.55	-

* Significant difference between the pair of means; R.s. – *Radopholus similis*

Table 9. Effect of short-listed endophytic bacteria on *R. similis* and bacterial populations in three varieties of black pepper (Mean of 9 replications).

Isolate	*R. similis* in roots (log N / g)	No.of inoculated bacteria in roots (log cfu / g)	Total bacteria in roots (log cfu / g)	No.of inoculated bacteria in shoot (log cfu / g)	Total bacteria in shoots (log cfu / g)
Panniyur 2					
BP-17	0.00	0.67	3.52	0.77	2.30
BP-25	1.65	1.69	3.49	1.54	2.38
BP-35	1.90	0.00	3.26	0.00	2.60
BP-71	0.73	2.16	3.13	0.00	0.67
BP-104	1.25	0.00	3.60	0.00	2.73
TC-10	1.42	0.83	3.44	0.00	2.83
IISR-6	1.03	0.00	3.18	0.00	2.30
IISR-859	1.08	0.00	3.13	0.00	2.55
Rs alone	1.85	0.00	1.59	0.00	1.59
Mean	1.21	0.59	3.15	0.26	2.22
Panniyur-4					
BP-17	1.58	0.67	3.54	0.77	2.83
BP-25	0.51	1.69	3.39	1.54	2.54
BP-35	2.35	0.00	3.21	0.00	2.90
BP-71	2.82	2.16	3.05	0.00	1.85
BP-104	1.86	0.00	3.32	0.00	2.72
TC-10	0.00	0.83	2.70	0.00	2.50
IISR-6	1.18	0.00	2.49	0.00	2.46
IISR-859	0.71	0.00	2.54	0.00	2.49
Rs alone	2.30	0.00	2.59	0.00	2.20
Mean	1.48	0.60	2.98	0.26	2.50
IISR Sreekara					
BP-17	1.43	2.79	3.80	1.49	2.76
BP-25	2.06	3.35	4.10	2.36	2.83
BP-35	1.69	2.32	3.72	0.67	2.73
BP-71	0.67	0.83	3.62	0.77	2.76
BP-104	1.80	0.00	3.53	1.85	2.60
TC-10	0.91	1.64	3.26	0.00	2.62
IISR-6	0.82	0.83	3.98	0.67	2.20
IISR-859	0.68	0.00	3.74	0.00	2.75
Rs alone	1.89	0.00	3.48	0.00	2.52
Mean	1.33	1.31	3.69	0.87	2.64
$LSD_{0.05}$ Var. x Is.	1.41	1.29	0.68	0.84	0.84
General Mean					
BP-17	1.00	1.38	3.62	1.01	2.63
BP-25	1.41	2.24	3.66	1.81	2.59
BP-35	1.98	0.77	3.40	0.22	2.74
BP-71	1.41	1.72	3.27	0.26	1.76
BP-104	1.64	0.00	3.48	0.62	2.68
TC-10	0.78	1.10	3.13	0.00	2.65
IISR-6	1.01	0.28	3.22	0.22	2.32
IISR-859	0.83	0.00	3.14	0.00	2.60
Rs alone	2.01	0.00	2.55	0.00	2.11
$LSD_{0.05}$	**0.89**	**0.81**	**0.43**	**0.84**	**0.53**

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). The consortium 6 which consisted of best performers of in vitro bioassay was not superior in any respect. The study has clearly shown the inadequacy of in vitro bioassays to identify isolates that really suppress nematodes.

When the same consortia were evaluated using another variety of black pepper (Panniyur 3) in polybags, there was no significant difference among various treatments with regard to height and total number of leaves per plant (Fig. 3; Plate 3C & D). 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. 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*. Since the experiment was conducted in a naturally infested nursery, the distribution of nematodes was not uniform and this would have affected the outcome of the trial. Moreover, mixing large number of bacterial populations without a clear understanding about their compatibility is not advisable. However, consortium 4 (T4) had a clear edge over other consortia in growth promotion as well as nematode suppression.

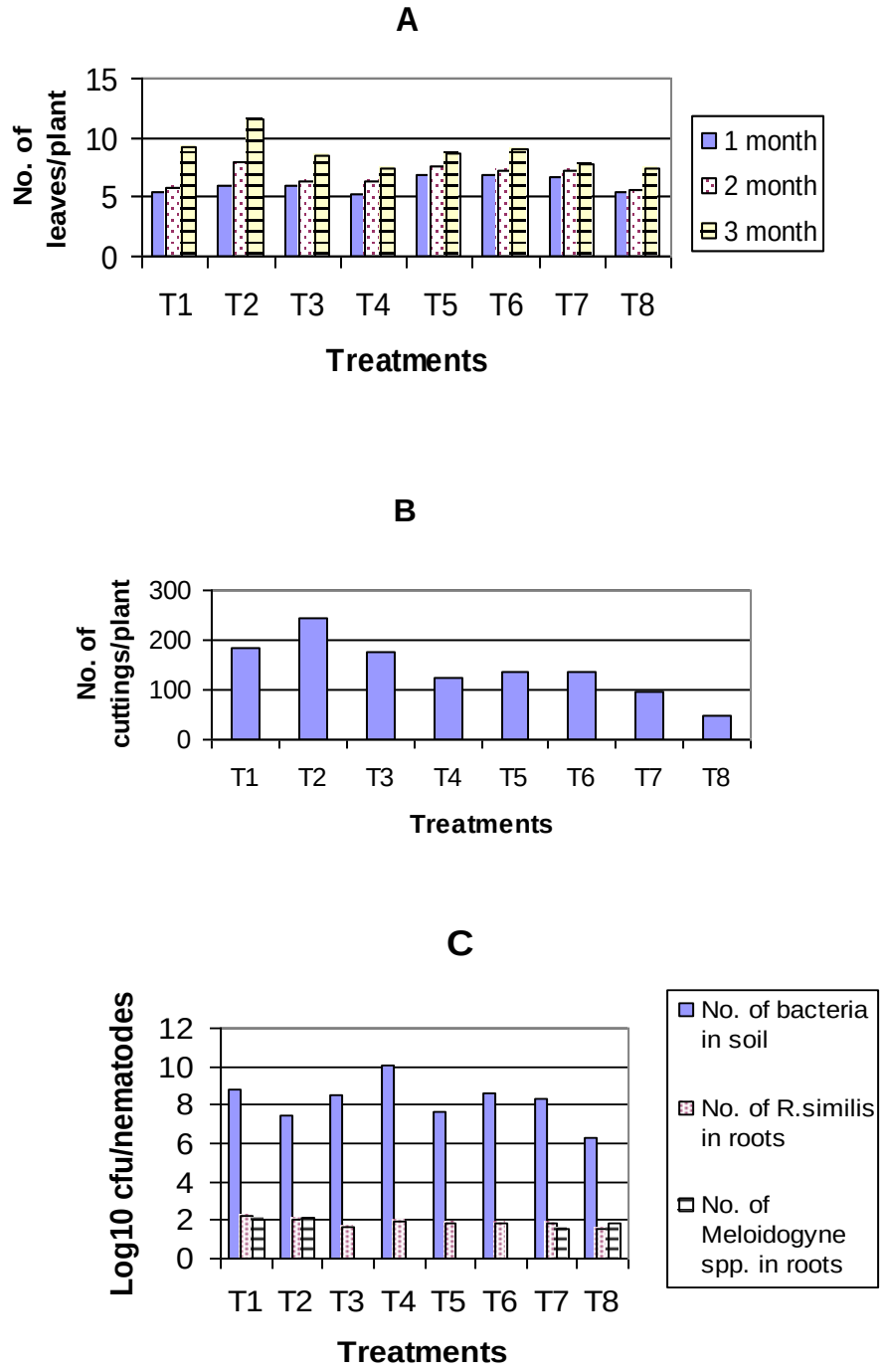


Fig. 2. Effect of different bacterial consortia on growth and nematode infestation in a black pepper nursery. **A** – No. of leaves per plant; **B** – Total number of cuttings from treated plants; **C** – Nematode population in roots of black pepper plants (T1 – control, T2 – phorate treated, T3 to T8 – bacterial consortia 1-6).

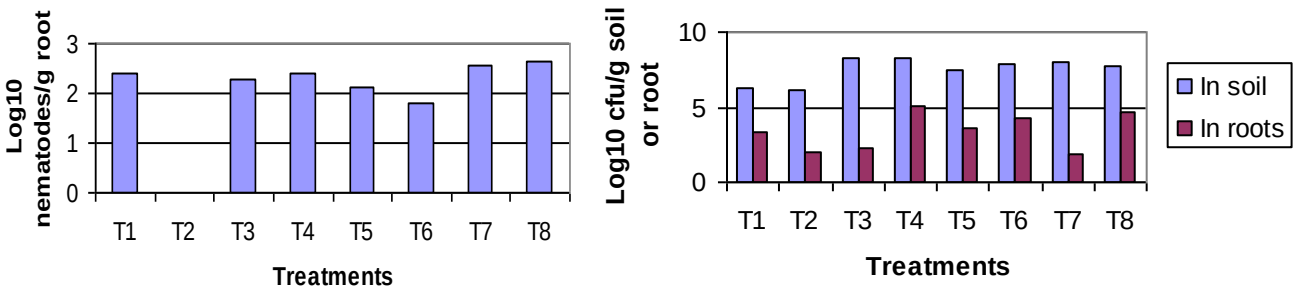


Fig. 3. Effect of different bacterial consortia on growth and nematode infestation in black pepper rooted cuttings **Left** — *R. similis* population in roots; **Right** – Total number of bacteria in soil and roots (T1 – control, T2 – phorate treated, T3 to T8 – bacterial consortia).

STUDIES ON PLANT- NEMATODE - BACTERIA INTERACTION

Methodology

To study the interaction between endophytes in black pepper, both in presence of and absence of *R. similis*, a separate lab experiment was set up using TC black pepper plants (3-4 leaves). The plants were dipped in a suspension of BP 35 and IISR 859 (x 10^{10} cells) for 15 min and planted in sterile sand under controlled conditions. One set of bacterized plants were inoculated with *R. similis* (150 nematodes / cup) after three days of inoculation with bacteria. The treatments (2 bacteria and a control) were replicated thrice in a RBD design. Two plants from each set were sampled at 0, 3, 6, 9 and 12 days intervals. The plants were washed and cut into three portions viz. roots, stem and leaves. From each sample 1 g tissue was homogenized in sodium phosphate buffer containing poly vinyl poly pyrrolidone (PVPP) and 50 mg sodium sulphite. The homogenized tissue was filtered, centrifuged at 12,000 rpm for 20 min and stored at -80^0C. The protein was estimated using the Lowry's method (Lowry et al., 1951). The activities of peroxidase (Hammerschmidt et al., 1982), polyphenol oxidase (Mayer et al., 1965) and phenyl ammonia lyase (PAL) (Baudoin-Eagan & Thorpe 1985) were estimated using standard procedures.

Results

The activities on of all the enzymes in black pepper in response to bacterization with BP 35 and IISR 859 and *R. similis* inoculation are depicted in Fig. 4 & 5 and Plate 10. The activity of PPO was very low in *R. similis* inoculated plants. The activities of all the enzymes were significantly high in leaves compared to other tissues indicating the induced systemic resistance (ISR) operating as a result of bacterial inoculation. PAL activity was very much pronounced with IISR 859 than BP 35. *R. similis* inoculation reduced the activity of PO. But BP 35 inoculation significantly enhanced its activity on the 6^{th} day of inoculation. Similarly PPO activity too was enhanced on inoculation with *R. similis*. However there was a delayed response in bacterized plants when they are challenged with nematodes. PAL being a key enzyme in the phenyl propanoid and flavanoid pathways and are responsible for the biosynthesis of phenolics that are effective chemical barriers to pathogens. In the case of PO activity also there is an indication of hypersensitive resistance (HR) reaction and is observed in plants inoculated with BP 35. However, PPO activity is a general wound response indicating the massive root damage caused by *R. similis*. The study clearly

revealed the potential of these bacteria to activate the defense genes of black pepper plants to contain the nematode infestation.

Colonization of endophytic bacteria in black pepper

The endogenous population and spatiotemporal colonization of the bacterium in bacterized shoot was analysed. Minimal concentration of bacterium required for suppression of P. capsici was found to be 1012-1013 cells ml-1. At this concentration the endogenous population of the bacterium was 105-106 cells g-1 of tissue. Duration of bacterization vis-à-vis the endogenous population of bacteria and suppression of *P. capsici* was optimized. The minimum duration of bacterization for suppression of lesion on shoot was found to be 15-20 minutes. At this duration endogenous population of *Pseudomonas aeruginosa* (BP 35) was106 cells g-1 of tissue with significant lesion inhibition (96-100%) on the excised shoots.

Similarly *B. megaterium* (BP 17) was inoculated onto black pepper rooted cuttings and their colonization level was monitored at different intervals and was confirmed by PCR assay using species-specific primers. The effect of dipping black pepper rooted cuttings in the bacterial suspension for different durations was studied. The results have shown that dipping the cuttings for different time intervals does not have a profound influence on the entry of the bacteria into the root tissues. The cuttings when dipped in the suspension for 15 minutes itself had resulted in ~ x 10^5 cells per g of the root tissue (Table 10). The bacterial entry in to the tissues was on par when dipped for 15 or 30 minutes. However, dipping for 60 minutes was not favorable for the bacterial colonization. The retrieval of bacteria from these tissues also did not show much variation in relation to the sampling intervals. The bacterial entry in to the tissues was further confirmed by obtaining 780 bp size products in the direct cell PCR using the species specific primers.

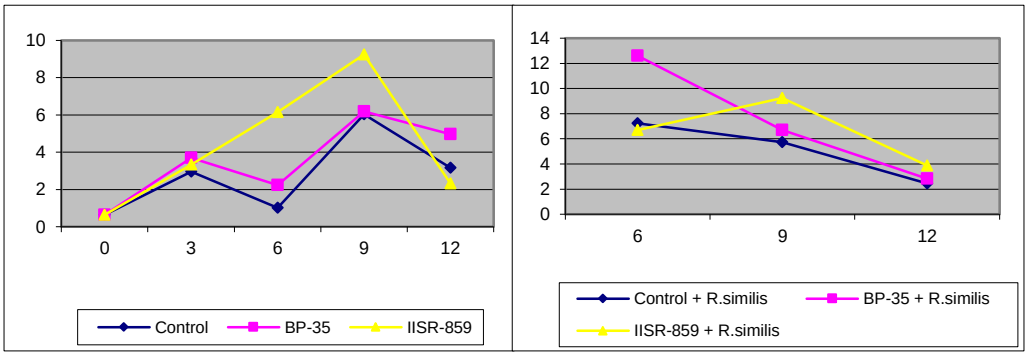
BP17 and IISR 522 have the ability to decrease the length of primary roots of tomato plants. There was significant difference in the root hair production and root architecture of tomato plants indicating the crucial role of endophytes in growth promotion.

Table 10. Colonization of BP17 in black pepper roots dipped for different durations (Mean of six relications).

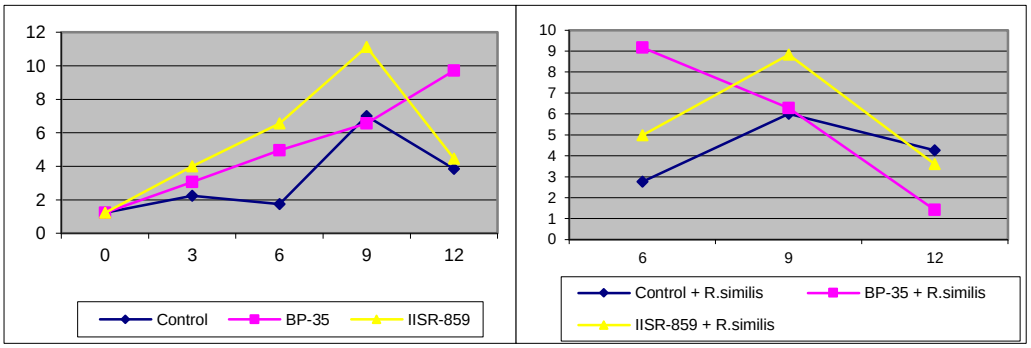
Dip duration (min)	Colonization of bacteria in roots (log cfu/g)			
	24h	**48h**	**72h**	**Mean**
15	4.82 a	4.63 ab	4.75 a	4.73 a
30	4.73 a	4.86 a	4.44 bc	4.68 ab
60	4.31 c	4.64 ab	4.81 a	4.59 b
Mean	4.62	4.71	4.67	-

Means followed by the same alphabet are not statistically different.

Root



Stem



Leaf

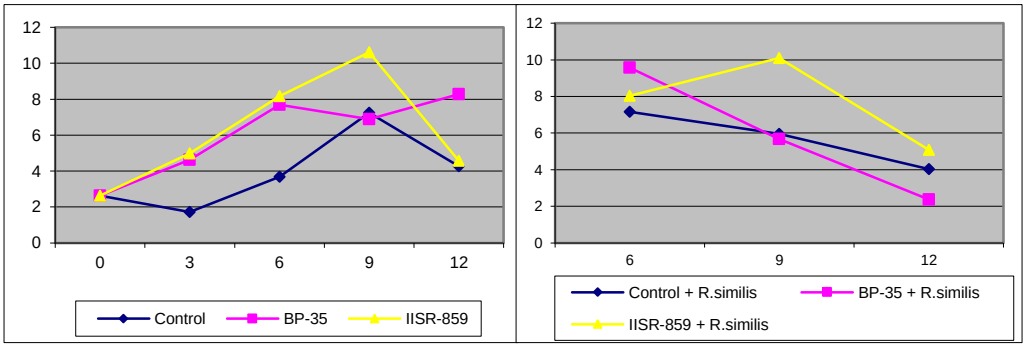
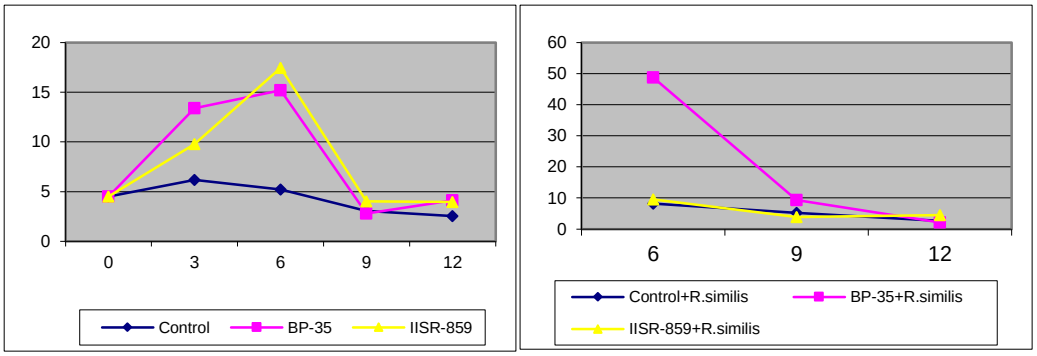
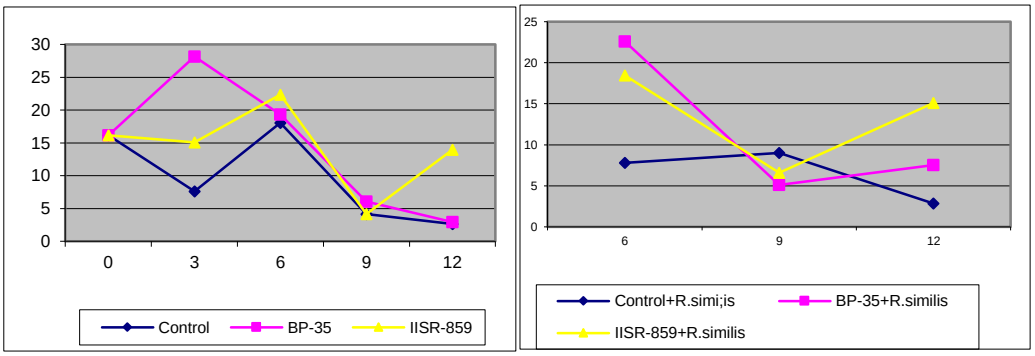


Fig. 4. Activity of phenyl ammonia lyase (PAL) in black pepper plants in response to bacterial and *R. similis* inoculation

Root



Stem



Leaf

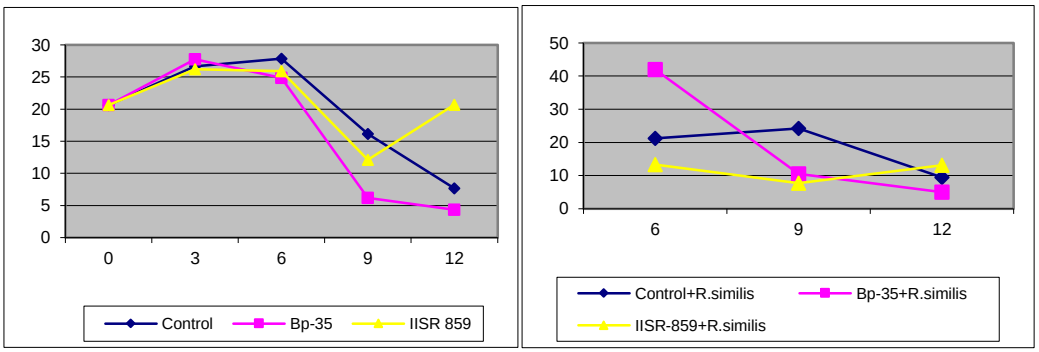


Fig. 5. Activity of peroxidase (PO) in black pepper plants in response to bacterial and *R. similis* inoculation.

STUDIES ON BACTERIAL FORMULATIONS AND THEIR SHELF LIFE

Methodology

Three promising endophytes (BP 17, BP 35 and TC 10) and two recommended rhizobacteria (IISR 6 and IISR 859) were multiplied on nutrient broth and mixed with two carriers viz. talc and chitin powder. For this 50 g each of carrier was weighed, added to tissue culture bottles and autoclaved. The log phase culture (after 24 hours) (50 ml) was added to each bottle and mixed with the carriers thoroughly under aseptic conditions. The sampling was done in fixed intervals and colony count was recorded after plating in nutrient agar medium amended with TTC. The data on number of colonies obtained at each sampling was log transformed and ANOVA test was carried out. The means were separated using Duncan's Multiple Range Test.

A greenhouse trial has been initiated to evaluate the efficacy of new formulations in controlling R. similis infesting black pepper plants. For this two each rooted cuttings of black pepper (Sreekara) were planted in earthen pots (30 cm dia) containing solarized potting mixture. At the time of planting the bacterial formulations were applied @ 10 g /pot and was repeated after one month. For comparison, a liquid suspension of bacteria was also tried. *R. similis* was applied two months after planting @ 200 nematodes / plant. The details are given below.

No. of treatments	-	Main plot: 3 (Chitin, talc and nutrient broth based formulations) Subplot: 7 (5 bacterial isolates – BP 17, BP 35, TC 10, IISR 6 and IISR 859 and two controls)
No. of replications	-	5
Test plant	_	Rooted cuttings (Sreekara)
Design	-	Split plot

Results

Two strains of *B. megaterium* (BP17 and IISR 522) having nematicidal properties were compared for the pH and temperature requirements for their optimum growth. It was found that the optimum pH ranged between 5 to 9 for both BP17 and IISR 522. BP 17 had a better multiplication rate across a pH range of 5 to 9. Highly acidic or alkaline pH was detrimental to both bacterial strains. Similarly, the optimum temperature for both strains was 25° C. In general BP 17 has a better multiplication rate than IISR 522.

Three isolates of endophytic bacteria (Bp-17, Bp-35 and Tc-10) were used for developing a suitable formulation for field level use. IISR 6 and IISR 859, two recommended rhizobacteria were also included in this study as controls.

Shelf life of endophytic bacterial isolates

The results on number of colony forming units recorded at each sampling day are depicted in Fig. 7. Chitin was a superior medium than talc for all the bacterial isolates (Plate 11 A & B). IISR 6, IISR 859 and BP 35 have better multiplication rate than the remaining two isolates, irrespective of the carriers. The highest cfu in chitin was noticed for IISR 6 while in talc it was BP 35. The multiplication rate of TC 10 was the lowest in both the carriers. The cfu of all the isolates declined as the storage time increased. There was a steep decline of bacterial population in talc from 3.06×10^9 to 9×10^5. In the case of chitin, all the isolates excluding TC 10 maintained x 10^7 cfu even after 90 days of storage. On the contrary, talc based formulations contained only 10^4 to 10^6 cfu after 90 days. This is quite advantageous for a solid carrier.

Moreover, there are several reports on nematode control through chitin amendments. Other advantages of chitin amendments include its light weight and higher bulk density which makes it ideal in handling.

Greenhouse evaluation

The above two formulations were compared for their efficacy in a greenhouse trial. The performance of TC 10 was superior to other isolates irrespective of the formulation in improving the growth parameters of black pepper as well as reducing the nematode damage (Table 11; Plate 11 C & D). However, chitin formulation was found more ideal for TC 10 while talc formulation was found more suitable for BP 17.

Table 11. Effect of different formulations of endophytic bacteria on growth of black pepper rooted cuttings.

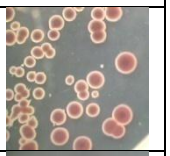
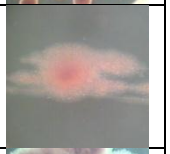
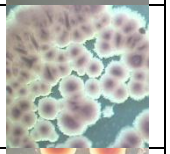
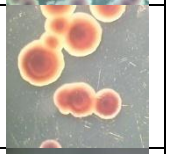
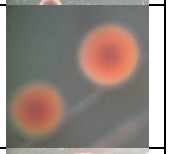
Treatment	Biomass (g)				Root rot index			
	Chitin	Talc	NB	Mean	Chitin	Talc	NB	Mean
BP17	34.0	67.0	19.4	40.1bc	2.8	1.2	4.7	2.9a
BP35	27.9	32.0	43.7	34.6c	3.0	3.2	4.0	3.4a
TC10	87.5	72.5	35.0	65.0a	0.0	2.2	2.4	1.5b
IISR6	62.5	51.2	46.0	53.2abc	2.2	2.7	3.6	2.9a
859	52.1	40.8	35.8	42.9bc	2.7	3.5	4.7	3.6a
Carrier	64.0	75.0	33.3	57.4ab	2.2	2.6	4.8	3.2a
Control	58.3	55.6	64.6	59.5ab	1.5	2.8	2.9	2.4ab
Mean	55.2a	56.3a	39.7b	LSD c x t =31.6	2.1b	2.6b	3.9a	LSD c x t =1.9

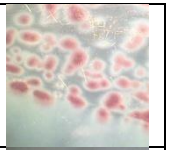
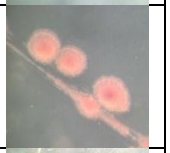
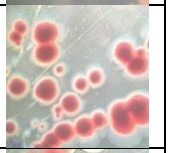
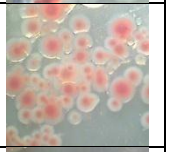
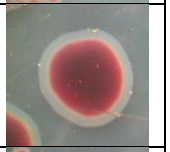
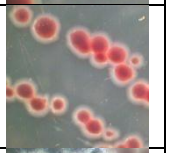
FIELD EVALUATION OF ENDOPHYTIC BACTERIA

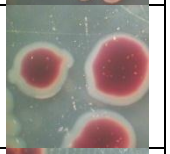
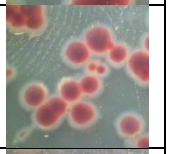
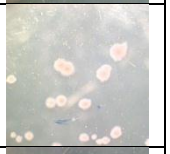
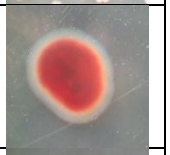
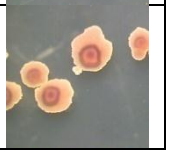
A field trial is in progress at IISR Experimental Farm, Peruvannamuzhi on the effect of bacterial endophytes on *R.similis* as well as *P.capsici* (Plate 12). Three varieties (IISR-Thevam, OPKM and IISR-Sreekara) are being evaluated. The bacterized rooted cuttings were planted and observations on establishment and disease incidence are being monitored at bimonthly intervals. The initial data revealed that the bacterial endophytes could enhance the establishment of the vines. The percentage establishment recorded for the endophytic bacteria are *Bacillus megaterium* IISR BP17 -82%, *Pseudomonas putida* IISR BP25- 72%, *Pseudomonas aeruginosa* IISRBP35 - 82% and *Curtobacterium luteum* IISRTC10- 88%. The chemical check recorded 85% and the untreated check recorded 78% of establishment. The trial is in progress for other observations on disease incidence and yield.

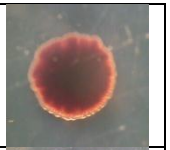
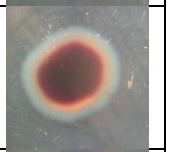
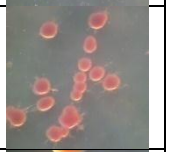
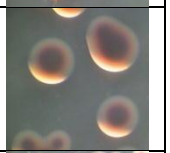
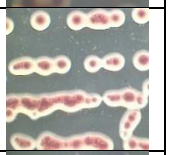
ANNEXURE II

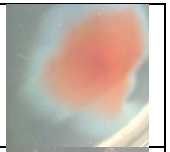
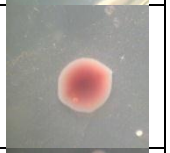
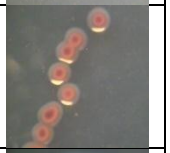
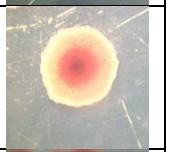
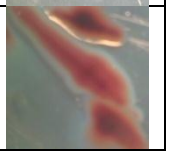
LIST OF ENDOPHYTES

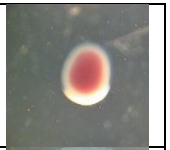
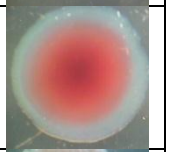
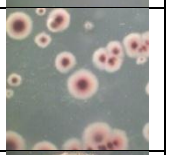
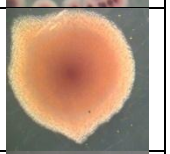
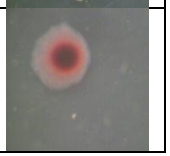
Identity	Plant parts	Varieties	Location	Colony Morphology					Tentatively Identity of Bacteria	Photo (TTC amended medium)
				Form	**Surface/ Elevation**	**Margin**	***Transparency***	**Colour**		
BP-2	Root	P51	Peruvanamuzhi	Irregular	Raised	Entire	Semi-Transparent	Pale yellow	*Micrococcus* spp.	
BP-3	Root	1041	Peruvanamuzhi	Filamentous	Raised	Entire	Opaque	Pale yellow	*Bacillus* spp.	
BP-4	Root	P51	Peruvanamuzhi	Irregular	Raised	Erose	Opaque	White	unidentified strains	
BP-6	Root	Panniyur - I	Peruvanamuzhi	Circular	Convex	Entire	Opaque	Milky white	*Arthrobacter* spp.	
BP-7	Root	Panniyur - I	Peruvanamuzhi	Irregular	Raised	Undulate	Opaque	White	*Bacillus* spp.	
BP-9	Root	Panniyur - I	Peruvanamuzhi	Irregular	Raised	Undulate	Opaque	White	*Bacillus* spp.	

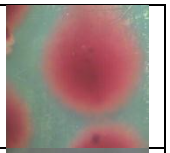
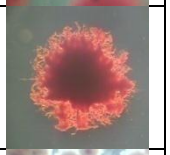
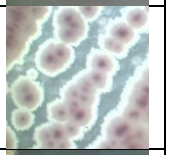
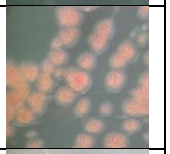
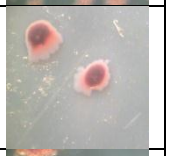
BP-10	Root	Karimunda	Peruvanamuzhi	Circular	Flat	Entire	Semi-Transparent	Green	fluorescent pseudomonads	
BP-11	Root	Karimunda	Peruvanamuzhi	Irregular	Flat	Entire	Opaque	White	*Bacillus* spp.	
BP-12	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Entire	Semi-Transparent	Pale yellow	fluorescent pseudomonads	
BP-13	Root	Panniyur - V	Peruvanamuzhi	Irregular	Flat	Entire	Semi-Transparent	Pale yellow	non fluorescent pseudomonads	
BP-14	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Entire	Semi-Transparent	Yellow	non fluorescent pseudomonads	
BP-15	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Erose	Opaque	Pale white	fluorescent pseudomonads	
BP-16	Root	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Opaque	Yellow	*Bacillus* spp.	

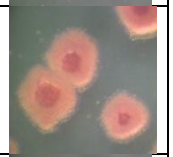
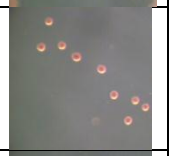
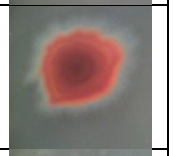
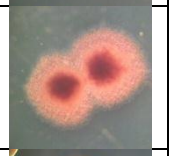
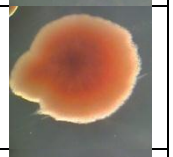
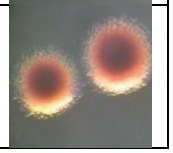
BP-17	Root	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Opaque	Creamy	*Bacillus megaterium*	
BP-18	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Erose	Opaque	Creamy	non fluorescent pseudomonads	
BP-19	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Entire	Transparent	Creamy	unidentified strains	
BP-20	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Filamentous	Semi-Transparent	Creamy	fluorescent pseudomonads	
BP-21	Root	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Semi-Transparent	Yellow	*Bacillus* spp.	
BP-22	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Filamentous	Semi-Transparent	Pale yellow	fluorescent pseudomonads	
BP-23	Root	Panniyur - V	Peruvanamuzhi	Filamentous	Raised	Entire	Opaque	White	*Arthrobacter* spp.	

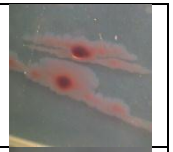
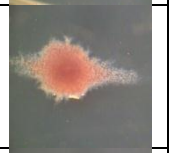
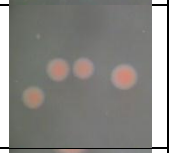
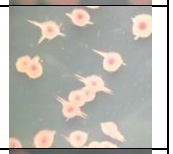
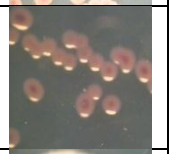
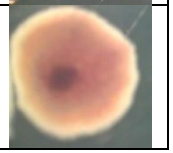
BP-24	Root	Panniyur - V	Peruvanamuzhi	Circular	Raised	Erose	Opaque	Yellow	non fluorescent pseudomonads	
BP-25	Root	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Opaque	Yellow	*P. aerugenosa*	
BP-26	Root	Panniyur - V	Peruvanamuzhi	Irregular	Flat	Entire	Semi-Transparent	Pale yellow	*Bacillus* spp.	
BP-27	Root	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Semi-Transparent	Yellow	*Arthrobacter* spp.	
BP-28	Root	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Semi-Transparent	Creamy	non fluorescent pseudomonads	
BP-29	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Entire	Semi-Transparent	Creamy	*Bacillus* spp.	
BP-30	Stem	Panniyur - V	Peruvanamuzhi	Circular	Convex	Erose	Opaque	Black (outer) Orange (inner)	non fluorescent pseudomonads	

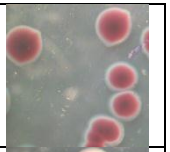
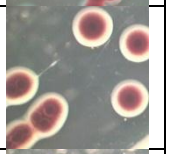
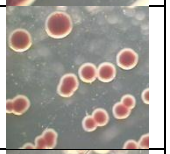
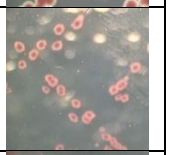
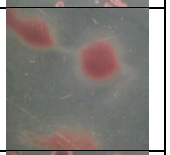
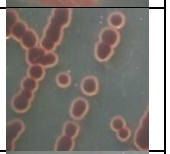
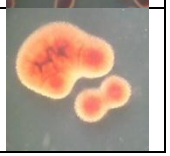
BP-35	Stem	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Erose	Opaque	Creamy	fluorescent pseudomonads	
BP-38	Stem	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Opaque	Creamy	non fluorescent pseudomonads	
BP-40	Stem	Panniyur - V	Peruvanamuzhi	Circular	Convex	Entire	Semi-Transparent	Yellow	*Arthrobacter* spp.	
BP-41	Stem	Panniyur - V	Peruvanamuzhi	Circular	Raised	Entire	Opaque	Fluorescence	*Arthrobacter* spp.	
BP-42	Stem	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Undulate	Opaque	Orange	*Arthrobacter* spp.	
BP-44	Stem	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Erose	Opaque	Creamy	*Micrococcus* spp.	
BP-46	Root	Panniyur - V	Peruvanamuzhi	Circular	Convex	Erose	Opaque	Black (outer) Orange (inner)	fluorescent pseudomonads	

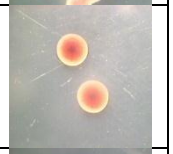
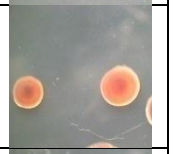
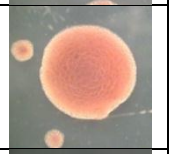
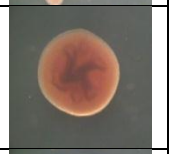
BP-47	Root	Panniyur - V	Peruvanamuzhi	Irregular	Raised	Erose	Opaque	Light yellow	non fluorescent pseudomonads	
BP-49	Root	Sreekara	Peruvanamuzhi	Irregular	Convex	Entire	Opaque	Pale yellow	non fluorescent pseudomonads	
BP-50	Root	Sreekara	Peruvanamuzhi	Irregular	Flat	Erose	Opaque	Creamy	*Bacillus* spp.	
BP-51	Root	Sreekara	Peruvanamuzhi	Filamentous	Raised	Filamentous	Opaque	Creamy	*Arthrobacter* spp.	
BP-52	Root	Sreekara	Peruvanamuzhi	Irregular	Raised	Filamentous	Transparent	Creamy	*Bacillus* spp.	
BP-53	Root	Sreekara	Peruvanamuzhi	Irregular	Flat	Entire	Opaque	Creamy	*Arthrobacter* spp.	
BP-54	Root	Sreekara	Peruvanamuzhi	Irregular	Raised	Undulate	Transparent	Creamy	*Bacillus* spp.	

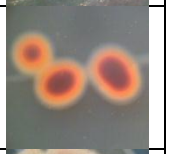
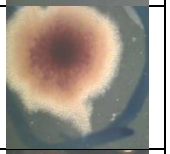
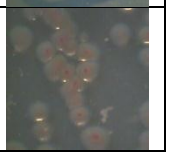
BP-55	Root	Sreekara	Peruvanamuzhi	Irregular	Submerged	Erose	Semi-Transparent	Creamy	*Arthrobacter* spp.	
BP-56	Root	Sreekara	Peruvanamuzhi	Filamentous	Raised	Undulate	Opaque	Creamy	*Bacillus* spp.	
BP-57	Stem	Sreekara	Peruvanamuzhi	Circular	Raised	Entire	Opaque	Creamy	*Micrococcus* spp.	
BP-60	Stem	Sreekara	Peruvanamuzhi	Irregular	Raised	Entire	Opaque	Creamy	*Micrococcus* spp.	
BP-67	Root	Sreekara	Peruvanamuzhi	Irregular	Flat	Undulate	Opaque	White	unidentified strains	
BP-68	Root	Sreekara	Peruvanamuzhi	Filamentous	Flat	Filamentous	Opaque	Dry white	*Serratia* spp.	
BP-69	Root	Sreekara	Peruvanamuzhi	Irregular	Raised	Lobate	Semi-Transparent	White	*Bacillus* spp.	

BP-70	Root	Sreekara	Peruvanamuzhi	Irregular	Raised	Undulate	Opaque	White	*Bacillus* spp.	
BP-71	Root	Sreekara	Peruvanamuzhi	Rhizoid	Flat	Lobate	Opaque	Dry white	unidentified strains	
BP-72	Root	Sreekara	Peruvanamuzhi	Irregular	Flat	Lobate	Transparent	White	*Micrococcus* spp.	
BP-73	Root	Sreekara	Peruvanamuzhi	Puntciform	Convex	Undulate	Opaque	White	*Bacillus* spp.	
BP-74	Root	Sreekara	Peruvanamuzhi	Irregular	Raised	Lobate	Opaque	White	*Micrococcus* spp.	
BP-75	Root	Sreekara	Peruvanamuzhi	Irregular	Convex	Undulate	Opaque	Light brown	*Arthrobacter* spp.	
BP-76	Root	Sreekara	Peruvanamuzhi	Irregular	Raised	Undulate	Opaque	Orangish yellow	*Arthrobacter* spp.	

BP-77	Root	Sreekara	Peruvanamuzhi	Irregular	Convex	Undulate	Opaque	Brown	*Bacillus* spp.	
BP-78	Root	Sreekara	Peruvanamuzhi	Irregular	Raised	Rhizoid	Opaque	White	*Bacillus* spp.	
BP-88	Root	Karimunda	Idukki	Circular	Raised	Entire	Opaque	Orangish yellow	non fluorescent pseudomonads	
BP-90	Root	Karimunda	Idukki	Irregular	Raised	Entire	Opaque	White	*Micrococcus* spp.	
BP-94	Root	Panniyur - I	Idukki	Irregular	Raised	Undulate	Opaque	Dry white	*Arthrobacter* spp.	
BP-97	Root	Panniyur - I	Idukki	Irregular	Raised	Entire	Opaque	White	unidentified strains	
BP-104	Root	Karimundi	Kodagu	Irregular	Raised	Undulate	Opaque	White	*Bacillus* spp.	

BP-115	Root	Panniyur - I	Kodagu	Irregular	Raised	Entire	Opaque	Creamy	non fluorescent pseudomonads	
BP-123	Root	Panniyur - I	Kodagu	Circular	Convex	Entire	Opaque	Yellow	*Arthrobacter* spp.	
BP-125	Root	Panniyur - I	Kodagu	Irregular	Raised	Undulate	Opaque	Creamy	unidentified strains	
BP-128	Root	Karimundi	Kodagu	Irregular	Raised	Undulate	Opaque	White	*Bacillus* spp.	
BP-133	Root	Panniyur – I	Kodagu	Irregular	Flat	Undulate	Opaque	White	*Bacillus* spp.	
BP-135	Root	Panniyur - I	Kodagu	Irregular	Raised	Entire	Opaque	White	*Bacillus* spp.	
BP-136	Root	Wild 756	Kodagu	Irregular	Raised	Erose	Opaque	White	*Arthrobacter* spp.	

BP-137	Root	Wild 756	Kodagu	Circular	Raised	Entire	Opaque	Milky white	*Bacillus* spp.	
BP-138	Root	Wild 756	Kodagu	Circular	Raised	Entire	Opaque	White	*Arthrobacter* spp.	
BP-139	Root	Panniyur - II	Kodagu	Circular	Raised	Entire	Opaque	Dry white	*Bacillus* spp.	
BP-140	Root	Panniyur - II	Kodagu	Circular	Raised	Entire	Opaque	White	*Bacillus* spp.	
BP-141	Root	Panniyur - II	Kodagu	Circular	Raised	Entire	Opaque	White	*Arthrobacter* spp.	
TC-5	TC plant			Irregular	Raised	Undulate	Opaque	White	*Arthrobacter* spp.	
TC-8	TC plant			Irregular	Raised	Entire	Opaque	Light white	*Arthrobacter* spp.	

TC-9	TC plant			Irregular	Raised	Serrate	Semi-Transparent	Creamy	*Arthrobacter* spp.	
TC-10	TC plant			Circular	Raised	Entire	Semi-Transparent	Yellow	*Curtobacterium luteum*	
TC-16	TC plant			Irregular	Raised	Erose	Opaque	Pale yellow	*Micrococcus* spp.	
TC-17	TC plant			Irregular	Raised	Entire	Transparent	Dark cream	*Arthrobacter* spp.	

ANNEXURE III

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LITERATURE CITED

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ANNEXURE IV

COPIES OF RESEARCH PUBLICATIONS