

**STUDIES ON BACTERIAL ANTAGONISTS OF
PLANT PARASITIC NEMATODES OF BLACK PEPPER
(*Piper nigrum* L.)**

Thesis submitted to
The Faculty of Science, University of Calicut

In partial fulfillment of the award of

Doctor of Philosophy
(Microbiology)

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CERTIFICATE

This is to certify that the thesis entitled “**STUDIES ON BACTERIAL ANTAGONISTS OF PLANT PARASITIC NEMATODES OF BLACK PEPPER (*Piper nigrum* L.)**” is a bonafide research work carried out by **Ms. B. Beena** in the Division of Crop Protection, Indian Institute of Spices Research, Calicut, Kerala under my supervision and also under the co supervision of **Dr. M. Anandaraj**. It has not been previously formed the basis for the award of any other degree or diploma. All sources of help received by her during the course of this investigation have been duly acknowledged.

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Calicut

Dated 12 Feb 2008


M. ANANDARAJ

DECLARATION

I, B. Beena hereby declare that the thesis entitled “**STUDIES ON BACTERIAL ANTAGONISTS OF PLANT PARASITIC NEMATODES OF BLACK PEPPER (*Piper nigrum* L.)**” submitted by me for the award of the degree of Doctor of Philosophy in Microbiology to the University of Calicut is bonafide research work carried out by me in the Crop Protection Division of Indian Institute of Spices Research, Calicut, Kerala under the supervision of **Dr. K V Ramana**, Former Assistant Director General (Horticulture), Indian Council of Agricultural Research, New Delhi and co-supervision of **Dr. M Anandaraj**, Project Coordinator, All India Coordinated Research Project on Spices (AICRPS), Indian Institute of Spices Research. This thesis or part of it has not been submitted to any University for the award of any degree or diploma.

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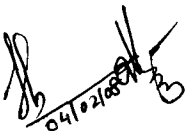
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ABBREVIATIONS

%	Percent
µg	Micro gram
@	At the rate of
Abc	Absolute control
ACC	Amino cyclopropane carboxylate
Amp	Amplicilin
ANOVA	Analysis of Variance
Ap	Antagonistic plant
ATCC	American Type Culture Collection
Bac	Bacitracin
BCA	Biocontrol Agent
C	Celcious
c.f.u	Colony forming unit
CA	Carrot Agar
CC	Coir Compost
CFCF	Cell free culture filtrate
Chl	Chloramphenicol
CMA	Corn meal agar
CMC	Carboxy Methyl Cellulose
CTAB	Cetyl trimethylammonium bromide
DAPG	Diacetyl Phloroglucinol
DMRT	Duncan's Multiple Range Test
Dul	Dulcitol
g	gram
GA	Gum Arrabica
Gal	Galactose
Gen	Gentamycin
Glu	Glucose
H ₂ S	Hydrogen Sulphide
HCN	Hydrogen Cyanide
ICAR	Indian Council of agricultural research
IISR	Indian Institute of Spices Research
IOC	Increase Over Control
IPM	Integrated Pest management
ISR	Induced Systemic Resistance
J ₂	Second stage juvenile
JA	Jasmonic acid
Kan	Kanamycin
KBM	King's B Medium
kDa	Kil o dalton
Lac	Lactose
LPS	Lipopoly saccharide
M	Molar
Mal	Maltose
Man	Mannitol
M.i	<i>Meloidogyne incognita</i>
MHA	Mueller Hinton Agar
MM	Minimal Medium

mM	Milli molar
NA	Nutrient Agar
Nal	Nalidixic Acid
NaRPh	National Repository of <i>Phytophthora</i>
NB	Nutrient broth
NCBI	National Centre for Biotechnology information
ng	Nano gram
NHB	Nematophagous fungus Helper Bacteria
nm	Nanometer
O/F	Oxidation /Fermentation
P. a	<i>Pseudomonas aureginosa</i>
P. c	<i>Pochonia chlamydosporia</i>
P.l	<i>Paecilomyces lilacinus</i>
PAF	Pseudomonas agar for fluoresin
PAGE	Polyacrylamide gel electrophoresis
PAP	Pseudomonas agar for pyocyanin
PCNB	Pentachloronitrobenzene
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
Pen	Penicillin
PGPR	Plant Growth Promoting Rhizobacteria
PHPR	Plant health promoting rhizobacteria
Pol	Polymixin
Pp	Polypropylene
ppm	Parts per million
R.s	<i>Radopholus similis</i>
RAPD	Random amplified Polymorphic DNA
RB	Rice Bran
Rif	Rifampicin
RKN	Root knot nematode
SA	Sodium Alginate
SAR	Systemic Acquired Resistance
SDS	Sodium Dodecyl Sulfate
SDW	Sterile Distilled Water
Sor	sorbitol
spec	Spectinomycin
str	Streptomycin
T.h	<i>Trichoderma harzianum</i>
TAE	Tris Acetate EDTA buffer
tet	tetracyclin
TTC	Trichloro Triphenyl Tetazolium chloride
UPGMA	Unweighed Pair Group Arithmetic ^{linked with} mean

INTRODUCTION

B. Beena “Studies on bacterial antagonists of plant parasitic nematodes of black pepper (*Piper nigrum* L.) ” Thesis. Indian Institute of Spices Research Calicut , University of Calicut, 2008

Chapter 1

INTRODUCTION

India, the spice bowl of the world, with the amazing richness and diversity of spices, attracted foreign traders in ancient times. Spices are mostly grown in tropical and warm temperate regions for their characteristic aroma and pungent principles that add flavour to food and beverages. In India, spices are grown in 2.5 million ha with a production of 2.7 million tones annually. Among the spices, black pepper (*Piper nigrum* L.), the 'King of Spices' or 'Black Gold' is an important foreign exchange earner for the country. Vietnam (85000 tons), Indonesia (67000 tons), India (65000 tons), Brazil (35000 tons), Malaysia (22000 tons), Sri Lanka (12750 tons), Thailand and China are the major producers of pepper in the World (Indiamart 2007). It is a well known culinary spice and condiment with several medicinal properties and is used widely in indigenous system of medicine. India is one of the largest producers and consumers of black pepper in the world. Kerala and Karnataka are the two major states in cultivation and production of black pepper in the country. This crop is rapidly spreading to non-traditional areas like Andhra Pradesh and North Eastern regions. Indian black pepper has a prime place in the international market because of its intrinsic quality. India exported 3,73,750 tonnes of spices and spice products during 2006-2007 valued Rs. 3,575.75 cores. During 2005-06 it was 3,50,363 tonnes valued Rs. 2,627.62 cores (Spices Board 2007a). Black pepper accounts for 8.6% of export earnings among all the spices during 2006-2007 compared to 5.7 % in 2005-2006 and 5.2% in 2004-2005 (Spices Board 2007b). Its export increased from 17,363 tonnes during 2005-06 to 28,750 tonnes during 2006-07 and earnings increased from Rs. 150.95 cores to Rs. 306.20 cores (International Pepper Community

2007). Major portion of black pepper is exported to America (13,886 tonnes). This crop is the major source of income and employment for rural households in the predominantly black pepper growing regions of Kerala (Sarma 2000). World production of black pepper has come down from 3,14,270 tons in 2005 to 2,69,000 tons in 2006.

Plant parasitic nematodes are important pathogens of all agricultural and horticultural crops and cause severe losses in yield and quality, if appropriate control measures are not taken at a proper time. It is estimated that crops worth of \$ 100 billion is lost world wide due to plant parasitic nematodes annually and spices are not an exception. Almost all spice crops are attacked by plant parasitic nematodes and the extent of damage to a particular crop is influenced by several factors, of which host plant, crop variety, nematode species and their population density and environmental factors are important (Ramana & Eapen 1999).

Plant parasitic nematodes attack both aerial and underground plant parts, but most of the nematodes parasitize the underground plant parts, particularly the roots. The symptoms produced on roots are more specific and diagnostic, while the symptoms on aerial plant parts are of general nature. Lesions, galls, abnormal branching, cessation of growth, rotting and decay are common symptoms produced on the root system due to nematode infestation (Plate 1) Due to the damage, the roots do not carry the normal functions of absorbing moisture and nutrients from the soil medium and as such the plants express symptoms on the aerial parts such as yellowing of foliage, stunted growth, wilting etc. Nematodes rarely kill the plant but cause a gradual decline in the health of the plant and production. Nematodes interact with other organisms and cause diseases of complex nature and sometimes breakdown the resistance to fungal and bacterial pathogens in several crops (Raman & Eapen 2000).

Black pepper, a perennial climbing shrub, is cultivated as a mono-crop in most of the pepper growing countries like Indonesia, Malaysia and Brazil. In Kerala, it is mostly grown as a mixed crop trailed either on arecanut or coconut palm or other trees in the homesteads or as a companion crop in coffee or tea plantations. The average production of black pepper in India is 306 kg/vine, which is far below the yield realized in other pepper growing countries like Malaysia (2600 kg/vine) and Brazil (1500 kg/vine). Several biotic and abiotic factors affect the production and productivity of black pepper in India. Among the various diseases and pests causing enormous loss in quality and quantity of

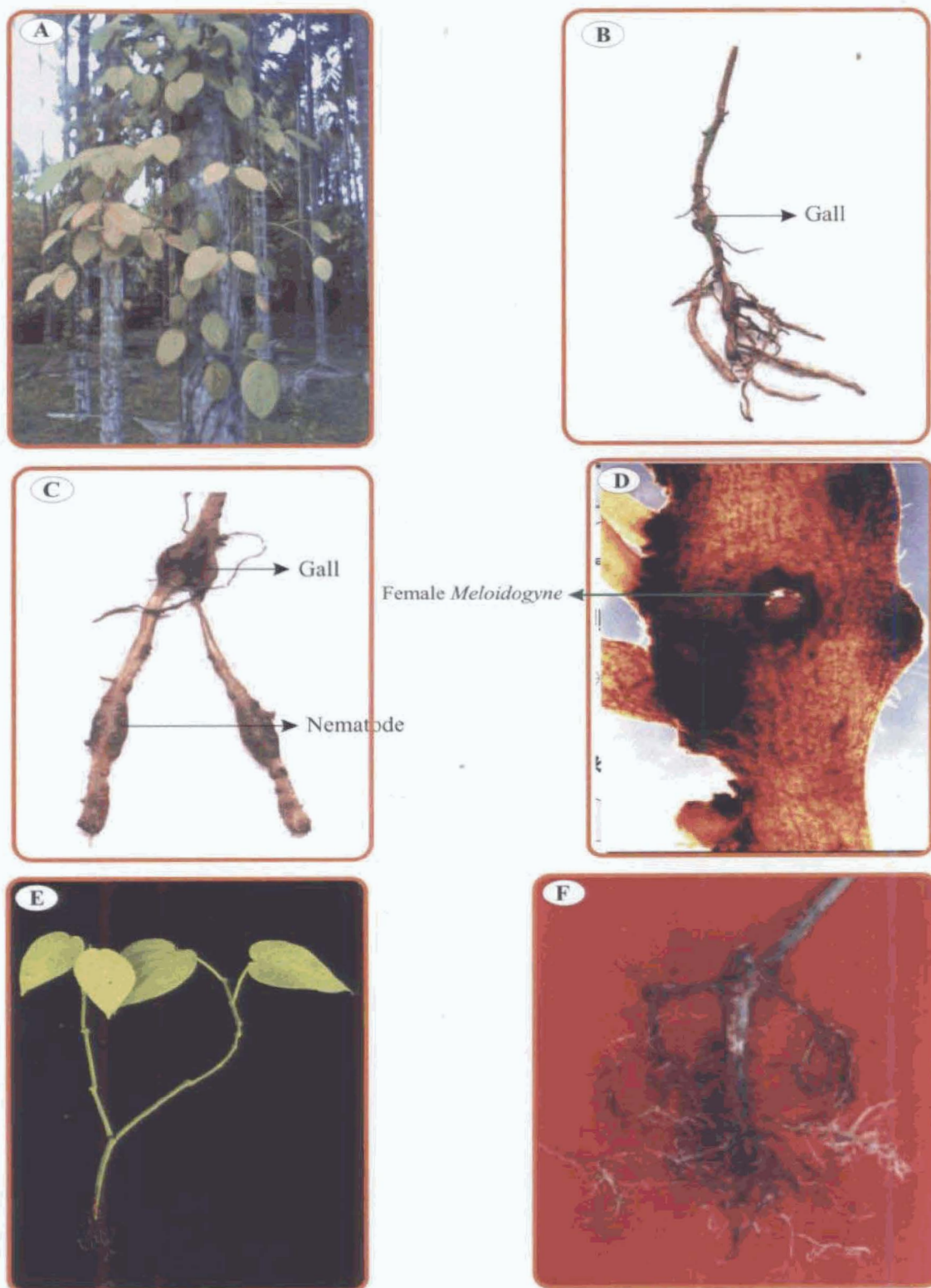


Plate 1. Nematode problems in black pepper

- A. Symptoms of yellowing in a black pepper vine due to the attack of nematodes
- B. Root system of a *Meloidogyne* infected black pepper cutting.
- C. *Meloidogyne* infected root split opened.
- D. Galls due to *Meloidogyne* in black pepper root
- E. *R. similis* infected black pepper cutting.
- F. Lesions, rotting and loss of feeder roots due to *R. Similis* infestation

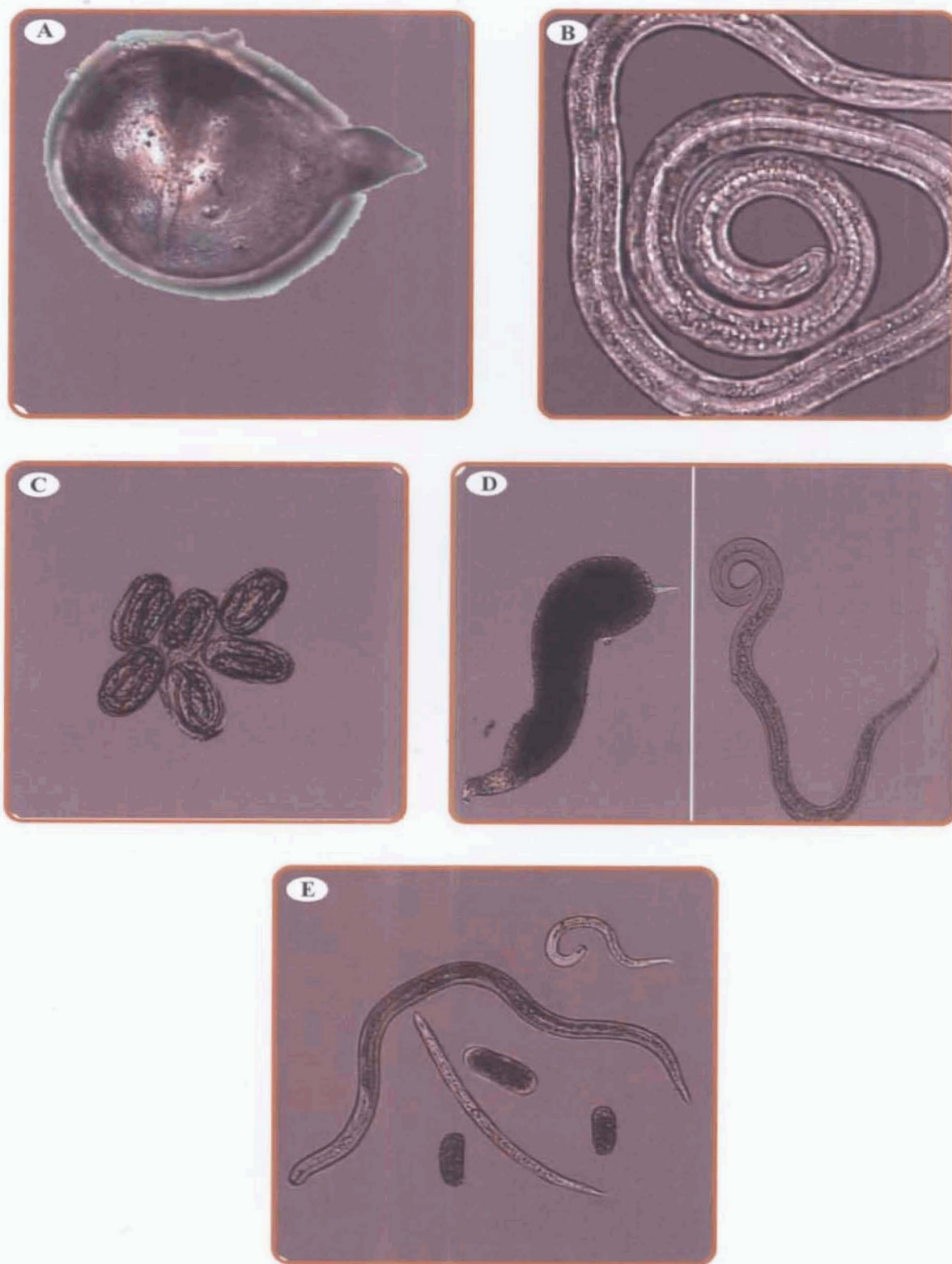


Plate 2. Major nematodes infecting black pepper

- | | |
|---|--------------------------------|
| A. Female <i>Meloidogyne incognita</i> | B. Male <i>Meloidogyne</i> |
| C. Eggs of <i>Meloidogyne</i> | D. Juvenile <i>Meloidogyne</i> |
| E. Eggs, juveniles, and female of <i>Radopholus similis</i> | |

black pepper, diseases due to plant parasitic nematodes have been recognized as a major production constraint. It is estimated that the losses due to nematode infestation in black pepper may go up to 50 to 60%. Soil borne diseases like *Phytophthora* foot rot caused by *Phytophthora capsici* and slow decline, primarily due to infestation by root knot (*Meloidogyne* spp.) and burrowing (*Radopholus similis*) nematodes are very serious and cause substantial loss in the black pepper production (Anandaraj *et al.* 1996).

Though, plant parasitic nematodes belonging to 30 genera and 54 species were reported on black pepper all over the world, only 14 genera were found in association with the crop in the major pepper growing areas in Kerala and Karnataka. Among these, only a few species like *M. incognita*, *R. similis*, *Trophotylenchulus piperis*, *Rotylenchulus reniformis* and *Helicotylenchus* spp. are predominantly associated with the crop in India (Ramana & Mohandas 1987; 1989). However, in all black pepper growing countries (India, Malaysia, Indonesia, Sri Lanka and Brazil) *Meloidogyne* spp. and *R. similis* assumed greater economic importance since these nematodes are responsible for slow decline or yellows disease, a major threat to black pepper cultivation.

Root knot nematodes (*Meloidogyne* spp.) are important group as they infest several crops and almost all spice crops. These are obligate endoparasites and are prevalent throughout the tropical and subtropical countries. Among several species of root knot nematodes, *M. incognita*, *M. javanica* and *M. arenaria*, *M. hapla*, *M. piperi* are reported on black pepper (Jacob & Kuriyan 1979; Ramana & Mohandas 1987; 1989; Sahoo *et al.* 2000). *M. incognita* is the most commonly and widely distributed nematode in black pepper gardens in all pepper growing countries as well as in Kerala and Karnataka (Kumar *et al.* 1971; Venkitesan 1972). *M. incognita* is a sedentary endoparasite that feed on vascular tissues and cause hypertrophy and hyperplasia resulting in series of tumors (galls) on the roots and these tumors decay due to secondary infection by microorganisms, resulting in the total loss of root system. In severely infested vines the leaves show dense yellowing of interveinal areas with deep green veins. Population of this nematode in black pepper gardens reaches maximum during December/January (Ramana 1992).

The burrowing nematode (*R. similis*), a migratory endoparasite, is another important nematode pest of black pepper. It is the most destructive and highly pathogenic root parasite of several plantation and spice crops with a wide host range of about 370

plant species. The nematode is prevalent throughout tropical and warm temperate regions. The nematode causes extensive damage to root cortical tissues and in association with other microorganisms results in root decay. The nematode prefers succulent root tips for invasion. Dark brown lesions are produced at the sites of infection and the nematodes move inter and intracellularly in the cortical tissues, producing large necrotic areas throughout the length of the root. All stages of the nematode (egg to adult) are found in the root lesions. Due to severe infestation, the plants lose almost all vital roots and are left with a bunch of decayed root mass (Venkitesan & Setty 1977; Mohandas & Ramana 1987). As a result, the roots lose their function and will not be able to absorb moisture and nutrients from the soil medium and plants express above ground symptoms such as foliar yellowing, defoliation and retarded growth (Venkitesan & Setty 1977; Mohandas & Ramana 1987). About 20 million pepper vines were lost due to the burrowing nematode in Bangka Islands (Indonesia) during 1950s (Van der Vecht 1950). *R. similis* population is maximum during September-October and minimum during April-June in black pepper roots. Rainfall and number of rainy days had significant influence on the population build up of the nematode (Mohandas & Ramana 1988).

Black pepper being a perennial crop, nematodes can survive well in the agro ecosystem. Other intercrops and common weeds in black pepper gardens are also good hosts of root knot and burrowing nematodes. Crops like banana, ginger, turmeric and a variety of vegetables are also grown along with black pepper in the homesteads in Kerala and such crop combinations result in build up the populations of nematodes and other pests and pathogens.

Nematodes interact with other microorganisms and cause complex diseases such as slow decline in black pepper. Studies in India showed that feeder root loss caused by *R. similis*, *M. incognita* and *P. capsici* either alone or in combinations leads to slow decline in black pepper (Anandaraj *et al.* 1996). The plant-nematode biochemical interactions that impair the overall physiology of the plant, as well as the role nematodes play in providing courts for entry of other pathogens are primarily responsible for plant injury that lead to the overall decline of plant health. Although nematodes can cause diseases in plants by themselves, most of them live and operate in the soil, where they are constantly surrounded by fungi and bacteria, many of which can also cause plant diseases. In many cases, an association develops between nematodes and other pathogens.

Nematodes then become a part of an etiological complex resulting in a compounded pathogenic potential that sometimes appears to be far greater than the sum of the damages, either of the pathogens can produce individually.

Nematode problems often go unnoticed or considered insignificant as nematodes are invisible and as they do not produce specific external symptoms in many cases. As a result, timely remedial measures are not taken to manage these pests. This complacency can lead to serious consequences, especially in a perennial crop like black pepper. So it is obvious to have a clear strategy to manage these hidden enemies.

Black pepper is propagated vegetatively and the planting materials (rooted cuttings) are produced in the nurseries. The major means of dissemination of nematodes is through infested planting materials. Most of the nurseries are rich in endemic populations of *R. similis* and *M. incognita* (plate 2). Production of healthy, nematode free planting materials is still a challenging task and is a major step in the management of nematodes in black pepper. Though fumigation of nursery mixture with fumigants like methyl bromide helps in elimination of the nematodes in the nurseries to some extent, in plantations it is practically impossible to use the fumigants. Soil solarization, a method of pasteurization, is an excellent means for reducing the initial nematode load (Eapen 1995; Sukla *et al.* 2000). Supplementing soil solarization with organic amendments or biocontrol agents also gives better protection (Gamliel & Stapleton 1993).

Several chemical pesticides like phorate, carbofuran etc. are highly effective in reducing the nematode populations in the plantations (Ramana & Eapen 1999). However, the usage of these chemicals in black pepper is very much limited since they are highly toxic, hazardous and can lead to environmental pollution, particularly the underground water. They are also not cost effective in many situations. The usage of these chemicals in export oriented crops like black pepper is further restricted because of the strict standards imposed by the importing countries.

Attempts made to identify the source of resistance/tolerance to the root knot or burrowing nematode in black pepper were not highly successful. *In vitro* propagation in black pepper has been standardized but the process involves lengthy spells of time for rooting and hardening. Hence, focus is given for the development of biological control, an environment friendly strategy, as an alternative for the protection of agricultural and

horticultural crops against phytopathogens. There is a need to develop integrated nematode management strategies with more emphasis on biocontrol agents.

In India, biological control of plant parasitic nematodes is still in its infancy. Much of the research was mainly focused on root knot and cyst nematodes using fungal antagonists for nematode management. Further, very few attempts were made for the management of *R. similis* using biocontrol agents. The proposed line of approach in the present study is seldom used in any of the perennial crops. Though a biocontrol agent as the sole source of nematode management is still debatable, it has the potential to form a component of integrated nematode management programme. Plant associated microorganisms have been extensively examined for their role in natural and induced suppressive abilities for soil borne diseases. Among the many groups of such organisms are root associated bacteria, which generally represent a subset of soil.

Plant growth promoting rhizobacteria (PGPR), besides offering protection against phytopathogens, are also good growth promoters, an added advantage for any practical agriculture system. These bacterial strains may be useful in multi-crop, multi-pathogen management programmes to augment genetic resistance to plant diseases. It is evident that rhizobacteria could possibly serve as an ecofriendly and sustainable alternative to the hazardous chemicals used for growth promotion and management of crop diseases. These bacteria can be exploited individually or in compatible combinations in a formulation. Development of a suitable microbial pesticide for disease management as well as plant growth promotion is an urgent need that would go a long way as a new dimension towards the philosophy of biological control that leads to the increased production with quality improvement of various crops. Rhizobacteria and *Trichoderma* have been used to get healthy cuttings of black pepper (Sarma 2000; Anandaraj *et al.* 2001 and Thankamani *et al.* 2003).

The use of suitable bacteria for suppression of plant parasitic nematodes of black pepper, provide an inexpensive, non-polluting strategy for plant protection. The accelerated growth obtained by bacterial inoculation of rooted cuttings of black pepper is another advantage in the production of healthy, nematode free planting materials.

Therefore, the present study was undertaken with the following objectives

- To isolate, identify and characterize bacteria suppressive to nematodes of black pepper.
- To study the ability of bacterial isolates to induce systemic resistance in black pepper.

REVIEW OF LITERATURE

B. Beena “Studies on bacterial antagonists of plant parasitic nematodes of black pepper (*Piper nigrum* L.) ” Thesis. Indian Institute of Spices Research Calicut , University of Calicut, 2008

Chapter 2

REVIEW OF LITERATURE

Biological control of plant parasitic nematodes in spice crops assumes significance as many of these crops are export oriented and the produce should be free from pesticide residues to meet the quality standards of importing countries in the world market. Biological control of nematodes of spice crops is in the initial stages of development. Though several fungi antagonistic to plant parasitic nematodes of black pepper have been identified, bacterial antagonists, particularly, rhizobacteria have not received due attention as potential biocontrol agents in spice crops.

2.1. BIOLOGICAL CONTROL

‘Antagonist’ is an umbrella term for parasites, predators, competitors, and other organisms that repel or kill a pathogen. A large number of biocontrol agents having very good potential for suppressing nematode populations have been identified in recent years. These rhizosphere microorganisms encounter the pathogens during primary infection and also during secondary spread on roots. Although an encounter between a nematode and its antagonist may occur anywhere in the soil, certain developmental stages of most plant parasitic nematodes, which commonly occur in the root zone are the primary targets of the antagonists. Potential targets are nematode eggs, juveniles in the soil and sedentary adults on the host roots. The antagonists also affect egg hatching, movement of the juveniles through the soil and their attraction and orientation to the host roots, recognition of host tissue and feeding sites, penetration of root tissues, attraction to opposite gender

etc. These critical stages of the nematodes are exposed to countless interactions among roots of host or non-host plants, soil biota, and the physical and chemical environment of the rhizosphere (Neipp & Becker 1999).

In India, research on biological control is mainly focused on root knot and cyst nematodes and several potential biocontrol agents suppressive to these nematodes have been identified and are effectively used for their management. However, biological management of the burrowing nematode, *R. similis*, a serious pest of several horticultural crops has not made much headway. There are very few reports on biological suppression of *R. similis* either by fungi or bacteria.

2.2. BIOLOGICAL CONTROL OF NEMATODES USING BACTERIA

Both rhizobacteria and endophytic bacteria have the potential to reduce the damage caused by plant parasitic nematodes and to be employed as biocontrol agents for nematode management. They have been shown to promote plant growth and to inhibit disease development and nematode pests (Sturz & Matheson 1996; Akhtar 1997; Hallmann *et al.* 1998a, 1998b; Azevedo *et al.* 2000; Munif *et al.* 2000; Shaukat *et al.* 2002; Sturz & Kimpinski 2004; Compant *et al.* 2005a, 2005b).

According to Kerry (1990), the advantages of bacterial biocontrol agents are (i) easy to culture *in vitro*, (ii) can be applied as seed treatment, (iii) reduce plant damage and the limitations are (i) they are effective for a relatively short period, (ii) activity affected by crop cultivar and nematode species and (iii) little effect on nematode multiplication. Endophytic bacteria have the capability of colonizing internal host tissues and this makes them a valuable tool to improve crop performance. By systemically colonizing roots they have potential to develop into biological control agents of plant parasitic nematodes. This is because (i) they are easy to culture *in vitro*, (ii) can be applied as seed treatments, (iii) reduce initial root damage, (iv) escape microbial competition and also capable of influencing host's response to pathogen attack, (v) do not produce any phytotoxic symptoms instead, promote plant growth and (vi) depend on root exudates for multiplication (Siddiqui & Shaukat 2003f).

Nematode species like cyst nematodes that have a single generation per year, react strongly to hatching factors and cause damage mainly in the early stages of root infection

process, were considered easy targets for control with antagonist rhizobacteria, while nematodes with multiple generations and cause damage throughout the crop growth are more difficult targets (Sikora 1992). Most plant parasitic nematodes remain mobile throughout their life cycle and microbial parasites must produce traps or adhesive spores to infect them. Nematodes with sedentary stages such as cyst and root-knot nematodes may be parasitised from vegetative hyphae in the rhizosphere without the production of specialized infective structures like that of fungi (Kerry 1990). Usually only the eggs and not the females of root-knot nematodes are exposed in the rhizosphere and these complete their embryonic development and hatch within about ten days at 25 °C. Non-parasitic micro-organisms that degrade soil amendments and release nematicidal compounds, such as the bacterium which degrades chitin to produce ammonia (Spiegel *et al.* 1991), are likely to kill most nematodes in soil.

The host plant influences the rate of nematode development and fecundity and the establishment of some natural enemies in the rhizosphere. Despite these effects the role of the host plant has rarely been considered in research on the biological control of nematodes (Kerry 1990). Bacterial control of nematodes was successful in variety of crops like lettuce (Hoffmann Hergarten *et al.* 1998), table grape (Ali & Kamal 1998; Shanthi *et al.* 2000), tomato (Hoffmann Hergarten *et al.* 1998; Khan & Tarannum 1999; Salh *et al.* 1999; Khan *et al.* 2002a; Jyothy & Sivakumar 2003; Leontopoulos *et al.* 2003), blackgram (Latha & Sivakumar 1998; Latha & Narasimhan 2001), banana (Mena *et al.* 1997; Vidya & Reddy 1998; Aalten *et al.* 1998; Aalten & Gowen 1998), pegion pea (Siddiqui *et al.* 1998), mustard (Neipp & Becker 1999), (Sivakumar & Mehta 1998, 1999), rice (Ramakrishnan *et al.* 1998b, 2003; Sivaraman & Lakshmanan 2003, Seenivasan & Devrajan 2002) black pepper (Eapen *et al.* 1997; Beena *et al.* 2001; Ramana 2002) cotton (Jayakumar *et al.* 2003), mulberry (Sarma 1998), mungbean (Ali *et al.* 2001, Siddiqui & Shaukat 2002d) peach (Sterling *et al.* 1979, Kluepfel *et al.* 1993, 2002) etc.

2.2.1.1. Bacterial biocontrol agents of nematodes

The antagonistic effects of naturally occurring rhizobacteria on plant parasitic nematodes can be significant (Yepsen & Robert 1984; Sikora 1992; Whipps 1997 Compant *et al.* 2005a, 2005b).

Meloidogyne incognita could be effectively controlled by rhizobacteria like *Bacillus cereus* (Jonathan *et al.* 2000; Cannayane *et al.* 2001), *B. subtilis* (Sharma 1998;

Kokalis-Burelle *et al.* 2002; Rajendran *et al.* 2001), *B. sphaericus* (Vyalykh *et al.* 2001), *B. thuringiensis* (Khan 1999; Chahal *et al.* 2003; El-Nagdi & Youssef 2004), *B. licheniformis* (Marquez *et al.* 1998; Siddiqui & Husain 1991), *B. firmus* (Giannakou *et al.* 2004; Keren *et al.* 2000), *B. amyloliquefaciens*, *B. pumilus* (Kokalis Burelle *et al.* 2003), *B. mojavensis* (Bacon & Hinton 2002), *Rhizobium etli* (Mahdy *et al.* 2001; Reimann & Sikora 2003), *Rhizobium meliloti* (Abdul Ghaffar *et al.* 2000), *Pseudomonas fluorescens* (Jonathan *et al.* 2000; Ali *et al.* 2002; Mahapatra *et al.* 2003; Jothi *et al.* 2003; Siddiqui *et al.* 2003; Senthilkumar & Ramakrishnan 2004; Siddiqui & Shaukat 2004), *P. chlororaphis* (Jonathan *et al.* 2000; Jyothi *et al.* 2003), *P. stutzeri* (Khan 1999), *P. mindocina* (Siddiqui & Husain 1991), *P. aeruginosa* (Siddiqui *et al.* 2002b), *Enterobacter cloacae* (Duponnois *et al.* 1999), *Serratia marcescens* (Ali & Kamal 1998; Kokalis-Burelle *et al.* 2002), *Burkholderia tropicalis* (Guyon *et al.* 2003), *B. ambifaria* (Li *et al.* 2002), *B. cepacia* (Jonathan *et al.* 2000; Meyer *et al.* 2001; Guyon *et al.* 2003), *B. gladioli* (Guyon *et al.* 2003), *B. caribensis*, *B. fungorum* (Vogel *et al.* 2002), *Xenorhabdus nematophilus* (Samaliev *et al.* 2000), *Azotobacter chroococcum* (Khan *et al.* 2002b; Chahal *et al.* 2003), *Agrobacterium radiobacter*, *Azospirillum brasilense* (Siddiqui *et al.* 2002a), *A. lipoferum*, *Beijerinckia indica* (Khan *et al.* 2002b), *Bradyrhizobium japonicum*, *Pantoea agglomerans*, *Cedeca davisae*, *Enterobacter* spp. (Munif *et al.* 2001).

Burkholderia cepacia, *A. radiobacter* (Chen *et al.* 1999), *B. cereus* (Sela *et al.* 1998), *B. subtilis* (Siddiqui 2002; Li *et al.* 2005), *P. aeruginosa* (Ehteshamul-Haque *et al.* 1997; Siddiqui 2001; Siddiqui 2002; Siddiqui & Shaukat 2003; Siddiqui *et al.* 2003c), *P. chitinolytica* (Spiegel *et al.* 1991), *B. japonicum* (Siddiqui *et al.* 2000), *P. fluorescence* (Subbotin 1992; Ali *et al.* 2002; Siddiqui & Shaukat 2003e; Siddiqui *et al.* 2004), *P. oryzihabitans* (Samaliev *et al.* 2000; Leontopoulos *et al.* 2003), *Pseudomonas striata* (Ansari *et al.* 2002), *Rhizobium meliloti* (Khan *et al.* 2002), *Brevibacillus brevis* (Li *et al.* 2005) are known to significantly reduce the populations of *M. javanica*.

The other major species of root knot nematodes which could be suppressed by bacteria are *M. arenaria* by *B. cereus* (Mahdy *et al.* 2000), *M. hapla* by *B. thuringiensis* (Chen *et al.* 1999), *M. chitwoodi* by *B. thuringiensis* and *B. megaterium* (Al-Rehiyani *et al.* 1999), *M. arabicida* by *P. fluorescens* (Rajas Miranda *et al.* 1998), *M. graminicola* by

B. megaterium (Padgham *et al.* 2005) and *M. exigua* by *P. fluorescens* (Maximiniano *et al.* 2001).

Rhizobacteria have been also been found promising against other nematodes like *R. similis* by *B. thuringiensis* var. *kurstaki* (Mena *et al.* 1997) *P. fluorescens* and *P. putida* strains (Aalten *et al.* 1998, Aalten & Gowen, 1998), *Heterodera glycines* by *B. cereus*, *B. pumilus* and *B. thuringiensis* (Kloepper 1992), *P. fluorescens*, *P. putida*, *P. chlororaphis*, *B. lentimorbus*, *Pantoea citrea*, *Alcaligenes piechaudii*, *Variovorax paradoxus*, *Acinetobacter* *genospecies*, *Cellulomonas cartae*, *Hydrogenophaga pseudoflava*, *Burkholderia galthei* and *Streptomyces violaceusniger* (Tian & Riggs 2000; Sarah *et al.* 2003); Reports are available on the biological control of other species *Heterodera* like *H. cajani* by *P. fluorescens* (Siddiqui *et al.* 1998; Latha *et al.* 2001; Subramanian *et al.* 2003), *B. subtilis* (Latha & Sivakumar 1998); *H. schachtii* by *B. cereus* (Wu *et al.* 1992; Siddiqui *et al.* 1998); *H. glycines* by *B. subtilis* (Araujo *et al.* 2002); *H. trifolii* by *B. cereus* (Kempster *et al.* 2001); *H. avenae* by *P. fluorescens* (Anju-Kamra *et al.* 1999) etc.

Rhizobacteria can be effectively used for the control of other nematodes like *Pratylenchus penetrans* by *Pseudomonas chlororaphis* (Hackenberg *et al.* 1997; Hackenberg *et al.* 2000), *Streptomyces* (Samac & Kinkel 2001); *Rotylenchus reniformis* by *Bacillus thuringiensis* (Zuckerman *et al.* 1993), *Arthrobacterium* sp., *Bacillus* sp., *Corynebacterium* sp, *Serratia* sp and *Streptomyces* sp. (Ali 1996), *B. subtilis* (Anver & Alam 2000; Niknam & Dhawan 2002b, 2003a; Visalakshi *et al.* 2003), *B. cereus* (Visalakshi *et al.* 2003), *Pseudomonas* (Niknam & Dhawan 2002a, 2002c, 2003a; Jayakumar *et al.* 2002, 2003 a, 2003b; Senthamizh *et al.* 2003); *Globodera rostochiensis* by *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia*, *Chromobacterium* (Dunne *et al.* 1997; Wronkowska 1988), *Achromobacter* (Zeiruk *et al.* 2003); *Bacillus* (Niknam & Dhawan 2002b, 2003a); *Globodera pallida* by *Rhizobium etli* (Reitz *et al.* 2000); *Hirschmanniella gracilis* by *P. fluorescens* (Ramakrishnan *et al.* 1998b; 2003; Seenivasan & Devrajan 2002, Seenivasan & Lakshmanan 2003; Andreoglou *et al.* 2003).

2.1.2. *Pasteuria penetrans*

Among the bacterial antagonists of nematodes, *Pasteuria* the only obligate parasite of nematodes (Mankau & Prasad 1977; Poiner & Hansen 1986; Davies *et al.* 1991; Esnard *et al.* 1997) and is the most affective in suppressing several nematode

species. *P. penetrans* has several characteristics which makes it a potential biocontrol agent for root knot and other nematodes. However, due to its obligate nature, the bacteria could not be cultured on the artificial media in the laboratory and is a limiting factor for its usage in the biocontrol (Sterling *et al.* 1986; Sterling 1991). The spores of *P. penetrans* have the morphology and biochemical characters of bacterial endospores (Williams *et al.* 1989), tolerate extremities of environment and can be stored in air dried soil and roots for longer period without apparent loss of infectivity (Sterling & Wachtel 1980). The spores of *P. penetrans* attach to free living and parasitic second stage juveniles, but not to males or females of root knot nematodes. *P. penetrans* infests different species of *Meloidogyne* namely, *M. incognita*, *M. arabicida* (Miranda *et al.* 1997), *M. arenaria* (Freitas *et al.* 1986), *M. javanica*, *M. camelliae*, *M. hapla*, and *M. mali*, *M. suginamiensis* (Orui 1997). Geetha *et al.* (1992) reported that *P. penetrans* reduced the damage due to *R. similis* in arecanut to some extent. Beneficial association of other bacteria with *P. penetrans* was reported by Duponnois *et al.* (1999). *P. penetrans* might have evolved from an ancient symbiotic bacteria associate of nematodes, possibly when the root-knot nematode evolved to a highly specialized parasite of plants (Charles 2005; Charles *et al.* 2005). Recently, a project was initiated to sequence the *P. penetrans* genome (Baker *et al.* 2001; Bird *et al.* 2003; Davies *et al.* 2003; Davies 2005). Genomic DNA of bacteria was then extracted (Waterman *et al.* 2006) and four genomic libraries from the broad-host-range *P. penetrans* strain RES147 have been constructed (Anderson *et al.* 1999; Atibalentia *et al.* 2000; Bird *et al.* 2003; Davies *et al.* 2003; Davies 2005).

2.2.2. Fungal biocontrol agents of nematodes

Fungi have been exclusively used for the control of nematodes. Fungi belonging to widely divergent orders and families from Phycomycetes to Basidiomycetes were reported as antagonists of nematodes (Eapen 2003). Organisms that showed some promise against nematodes are endomycorrhizal fungi like *Gigaspora* (Saleh & Sikora 1984; Sharma & Swarup 1988), *Glomus esculentus* (Hussey & Rancadori 2000) and *G. fasciculatum* (Sivaprasad *et al.* 1990; Anandaraj & Sarma 1994a; Anandaraj *et al.* 1991; 1996; Reddy *et al.* 1997; Koshy *et al.* 1998; Sosamma *et al.* 1998; 1999). A plant with well established symbiont is better off because of increased resistance to fungal pathogens and nematodes (Reddy *et al.* 1997). Use of predacious or nematode trapping fungi like *Arthrobotrys dactyloides*, *Dactylaria*, *Dactylella*, *Monacosporium ellisporum*, *M. cinopagum* etc. have been reported to be suppressive to various plant parasitic nematodes

(Barron 1977; Sterling *et al.* 1979; Manaku 1980; Esser 1983; Duckman *et al.* 1987). Obligate and facultative parasites of nematodes include, *Pochonia chlamydosporia*, (Morgan-Ajones & Kabana 1988; de Leij & Kerry 1991; de Leij *et al.* 1992; Sreeja *et al.* 1996; Nitao *et al.* 1999), *Verticillium suchlasporium* (Gams 1988), *V. lecanii* (Hussey 1984; Meyer & Meyer 1995), *V. lamellicola*, *V. leptobactrum* (Godoy *et al.* 1982), *Paecilomyces lilacinus* (Dunn *et al.* 1982; Friere & Bridge 1985; Sharma & Trivedi 1989; Ramana 1994; Sosamma & Koshy 1995; Hollond 1999; Kiewnick *et al.* 2005; Fiedler *et al.* 2007), *P. marquandii* (Marban Mendoza *et al.* 1992), *P. fumosoroseus* *P. nostocoides* (Dun 1983), *Trichoderma harzianum* (Hewlett *et al.* 1988; Eapen & Venugopal 1995), *T. viride* (Goswami *et al.* 1998), *T. koningi* (Marban Mendoza *et al.* 1992), *Trichoderma* spp. (Eapen *et al.* 2000), *Fusarium oxysporum*, *F. dimerum*, *F. solani*, *F. moniliforme* (Mustica 1990; Goswami *et al.* 1998), *Aspergillus niger*, *A. terreus* (Gupta *et al.* 1993). The common fungal egg parasites like *P. lilacinus*, *P. chlamydosporia*, *Trichoderma* etc. are not effective against *R. similis* due to the feeding habit and biology of the nematode. Recently some endophytic fungi have been reported from Latin America to be effective in suppressing *R. similis* (Pocasangre *et al.* 2000, Sikora *et al.* 2000).

2.2.3. Bacterial control of *Phytophthora*

The effective utilization of rhizobacteria for the control of soil borne fungal pathogens have been reviewed by Glick and Bashan (1997) and Kloepper (1993). Inhibition of *Phytophthora* by rhizobacteria in black pepper is reported by Lisha, (2002); Anith *et al.* (2003); Susheela Bhai and Sarma (2003); Saju (2004) Diby Paul and Sarma (2005), Rajkumar *et al.* (2005), Diby Paul *et al.* (2005) etc. Biological control of fungal pathogens results from production of metabolites, which directly inhibit the pathogen, such as antibiotics, hydrogen cyanide, iron chelating siderophores and cell wall degrading enzymes (Defago *et al.* 1990). Variety of bacterial species including *Agrobacterium*, *Bacillus*, fluorescent and non fluorescent *Pseudomonads*, *Xanthomonas*, *Erwinia* are proven to be effective biocontrol agents for *Phytophthora* (Burr *et al.* 1978; Chanway *et al.* 1991; Wei *et al.* 1991). Bacterial antagonism of fungal pathogens like *Phytophthora*, *Fusarium*, *Pythium*, *Sclerotia* etc. are reported (Cook & Weller 1986).

2.3. ISOLATION METHODS

It is well established that the genetic diversity in soil bacteria is high (Torsvik *et al.* 1990; Clegg *et al.* 1998) and that soils contain many bacterial species of lineages for

which no known cultivated isolates are available (Wollum 1982; Liesack *et al.* 1997). Many soil bacteria are referred to as uncultured or even non-culturable. A variety of methods have been developed to study these organisms directly in their habitats (Amann & Ludwig 2000, Gray & Head 2001). These methods are extremely useful for studying the ecology of microorganisms as parts of communities, but initial physiological and genetic studies of pure cultures should greatly facilitate such synecological studies. But many of these bacteria are in fact culturable using relatively simple technologies. Peter *et al.* (2002) reported simple growth medium to investigate the culturability of soil bacteria.

The serial dilution liquid culture techniques are found to be effective for isolating numerically significant members of the community in paddy soil (Grokopf *et al.* 1998; Chin *et al.* 1999; Rosencrantz *et al.* 1999).

Silva *et al.* (2003) developed a root colonization bioassay technique for rapid identifying rhizobacteria for potential biocontrol agents. Methods for isolation and phenotypic characterization of plant growth promoting rhizobacteria were reported by Kravchenko *et al.* (2002). Several workers developed specific culture media for the selective isolation of bacterial biocontrol agents (Guyon *et al.* 2003).

2.3.1. Bacteria isolated from nematode antagonistic plants

Population structure of microbial communities in the rhizosphere is important to the plant (Randall *et al.* 2001). The plant genome determines root exudates and enriches for specific microbial colonists of root (Lugtenberg *et al.* 1999). Seeds of different spices, for example, exude different exudates, a factor that may determine the effectiveness of seed treatment with PGPR. Therefore, plant spices as well as the different cultivars can directly affect the overall ability of naturally occurring or introduced PGPR to establish and persist in the rhizosphere (Abdul Ghaffar *et al.* 2000). Rhizobacteria isolates react differently to different spices of plant and different genotypes within the spices (Aström & Gerhardson 1988; Aström 1991). Many rhizobacteria isolated from the root system of a plant have the ability to limit nematode penetration indicating the potential of the naturally occurring microbial community to provide biological control in the rhizosphere (Sikora 1988).

Insunza *et al.* (2002) reported that root bacteria of nematocidal plants possessed biocontrol potential against nematodes in potato. Sixteen bacteria isolates from the roots

of 12 nematicidal plants and potato out of 44 reduced nematode *Paratrichodorus pachydermus* and *Trichodorus primitivus* densities by 50-100%. These isolates are *Stenotrophomonas maltophilia*, *Bacillus mycoides*, *Pseudomonas* sp. and one unidentified bacterium, isolated from potato, *Plantago major*, *Thymus vulgaris* and *Asparagus officinalis*, respectively. Plants releasing nematicidal compounds may harbour nematode antagonistic bacteria as well. Plant species, *Secale cereale* cv. Raitaro, *Avena sterilis* cv. Negusare-Taiji, *Crotalaria breviflora* cv. Nekobu Killer II, *Panicum maximum* cultivars Natsukaze and Soil Clean, *Sorghum sudanense* cv. Berusudan, *S. bicolor* cultivars Lucky Sorghum II, SS701 and Suddakkus, and *Tagetes erecta* cultivars African Marigold, African Tall and Tall Kongou are reported to be antagonistic to root-knot nematode, *M. incognita* (Alahm *et al.* 1990).

Chromola odorata, *Pimenta dioica*, *Brassica campestris*, *Tagetes zipzquirensis*, *T. erecta*, *T. patula*, *T. minuta* (Hackney & Dickerson 1975; Sikora 1992), *Gaillardia picta* (Yen *et al.* 1998), *Bidens pilosa*, *Ruta graveolens*, *Taraxacum officinale*, *Ricinus communis*, *Piper colubrinum*, (Alvarez *et al.* 1998), *Crotalaria* sp. (Chodhury *et al.* 1998), *Nasturtium officinale* (Watercress), *A Armoracia rusticana* (horseradish) (Kotova *et al.* 1999), *Datura stramonium* (Owino & Mousa 1994), Velvetbean (*Mucuna* sp.) (Santos *et al.* 1994 Vargas *et al.* 1994), Switchgrass (*Panicum virgatum*) (Kokalis Burelle *et al.* 1995) (reported to possess nematode suppressive ability).

It is supposed that the antagonistic plants reduce the damage due to nematodes by directly affecting the nematodes through production of toxic compounds (Alam *et al.* 1990; Gyaneshwar *et al.* 2002), or these plants may indirectly affect the nematodes by encouraging the rhizosphere microorganisms suppressive to nematodes, or by supporting a distinct microflora suppressive to nematodes from non-antagonistic plants (Kloepper *et al.* 1991). Microorganisms from rhizosphere of antagonistic plants should have a higher frequency of inhibition to nematodes than those from non antagonistic plants. Kloepper *et al.* (1991) reported that rhizosphere bacteria from nematode antagonistic plants were physiologically distinct from those of nematode susceptible plants, suggesting that specific groups of microorganisms have adapted to rhizospheres of antagonistic plants.

There are several reports on the root exudates of the plants supporting the enhanced proliferation of rhizosphere microflora. The exudates contain mainly low molecular organic compounds such as sugars, amino acids and organic acids (Chanway *et*

al. 1991). In addition, organic substances are also made available to soil microbes through constant root and root hair turn over (Leinweker *et al.* 1995). This rhizodeposition enhances microbial activity and therefore increases the rate of nitrogen mineralization in the soil (Bradley & Fyles 1996). Still many aspects of the interactions between plants and soil microorganisms are poorly understood due to the high complexity of the soil ecosystem (Kapulnik 1996).

2.4. MOLECULAR METHODS FOR IDENTIFICATION OF BACTERIA

Accurate and definitive microorganism identification is essential for biological control. Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using gram staining, culture and biochemical methods. However, these methods of bacterial identification suffer from major drawbacks. The wide spread application of molecular method for the identification of microorganisms including bacteria in recent years have been a fundamental advance that has dramatically improved the speed, sensitivity and accuracy of the identification procedure of bacteria. In the past decade or so, molecular techniques have proven beneficial in overcoming some limitations of traditional phenotypic procedures for the detection and characterization of bacterial phenotypes. Several non-culture based methods have emerged in the past 15 years. Since the discovery of PCR and DNA sequencing, comparison of the gene sequences of bacterial species showed that the 16S rRNA gene is highly conserved within a species and among species of the same genus, and hence can be used as the new "gold standard" for identification of bacteria to the species level (Tannock 1989; Spilker *et al.* 2004). Using this new standard, phylogenetic trees, based on base differences between species, are constructed, and bacteria are classified and reclassified into new genera (Lau *et al.* 2002 a, 2002 b; Woo *et al.* 2002 a, 2002 b).

Biotin-labeled plasmid DNA probes for detection of bacteria by Tannock (1989); DNA amplification to enhance detection of genetically engineered bacteria in environmental samples by Steffan & Atlas (1988); rapid and correct identification of bacteria with chromosomal DNA probes by whole-cell dot blot hybridization by Morotomi *et al.* (1988); DNA probe method for the detection of specific microorganisms in the soil bacterial community by Holben *et al.* (1988); reverse sample genome probing, by DNA hybridization for the identification of bacteria by Voordouw *et al.* (1991) and

real-time PCR by McDowell & Patrick (2005) etc. are some examples in which the molecular method are being adopted by identification of bacteria.

2.5. *IN VITRO* BIOASSAYS

Nematode antagonism of various bacterial biocontrol agents are studied intensively simple systems in laboratory bioassays.

Various laboratory systems are developed by different workers for screening bacterial antagonists against nematodes. *In vitro* bioassays enabled numerous bacterial strains to be screened within minimum requirements and space (Becker *et al.* 1999). The effect of bacterial cell suspension (Tian & Riggs 2000) the effect of metabolites released into buffer suspension of bacterial isolates (Cronin *et al.* (1997b) culture filtrates (Neipp & Becker (1999); non volatile & volatile metabolites (Ying *et al.* 2007) etc. are some examples. Zavaleta Mejia (1985); Mishra *et al* (1987); Jayakumar *et al.* (2002b) Guyon *et al.* (2003); Niknam & Dhawan (2003); Siddiqui & Shaukat (2003e) and Siddiqui & Shaukat (2004b) used various *in vitro* bioassays for evaluating the efficacy of bacterial isolates against nematodes. In addition, a number of *in vitro* studies have reported direct antagonism by bacterial isolates towards plant-parasitic nematode species belonging to the genera *Meloidogyne*, *Heterodera* and *Rotylenchulus* (Gokta & Swarup 1988; Ooestendorp & Sikora 1990; Kloepper *et al.* 1992b; Sheela & Venkitesan 1997; Madamba *et al.* 1999; Siddiqui & Mahmood 1999; Insunza *et al.* 2002; Kokalis-Burelle *et al.* 2002a; Shaukat *et al.* 2002; Meyer 2003; Siddiqui & Shaukat 2003 a, 2003b, 2003c; Giannakou & Prophetou-Athanasiadou 2004; Li *et al.* 2005). Hatching assays were designed by Neipp and Becker (1999) for evaluating the potential of bacterial strains in suppressing nematode egg hatching. *In vitro* bioassays for detecting the efficacy of *Bacillus subtilis* strain Bst cell concentrations and cell-free filtrates on hatching and mobility of *Rotylenchulus reniformis* were conducted by Niknam and Dhawan (2002b). In an experiment to determine the effect of size fractionated culture filtrates of *B. cepacia* on egg hatch and J2 mobility, culture filtrates grown on Potato Dextrose Broth were fractionated by centrifugation and *in vitro* bioassays were performed with the filtrate (<3KDa fraction), retentate (>3KDa fraction) and the non-fractionated culture filtrate (Meyer *et al.* 2000). Only exposure to non fractionated filtrate resulted in significantly lower egg hatch and there was an apparent effect on the J2 mobility. The *in vitro* plate screening designed by Becker *et al.* (1999) involved streak culturing candidate bacterial

isolate on TSA agar plates in a cross like manner and evaluating them by transferring agar plugs to microtitre plates containing nematode eggs. Agar plugs were transferred Becker *et al.* (1999) also used pouch test, the modified soil-free system for assessing nematicidal activity of bacterial isolates.

2.6. INPLANT BIOASSAYS

In plant bioassays for the evaluation of biocontrol agents were reported by many workers. Some examples are biocontrol of *M. incognita* in tomato and cucumber plants by *Serratia marcescens* (Zavaleta Mejia 1985) endophytic bacteria against *M. incognita* (Munif *et al.* 2000), bacterial control of *Heterodera* in sugar beet by Oostendorp & Sikora (1989); *Bacillus*, *Pseudomonas*, *Variovorax* and *Arthrobacter* against *H. schachtii* (Neipp & Becker 1999). Biocontrol potential of various bacterial isolates were evaluated in green house by Wu *et al.* (1992); Sturtz *et al.* (2000); Vyalykh *et al.* (2001) etc.

The importance of greenhouse trials for the selection and short listing of biocontrol agents are exclusively reviewed. Greenhouse screening though, consumes time and space, would allow understanding multiple mechanisms of antagonism such as antibiosis, competition and parasitism and to study the adaptability of potential biocontrol agents in soil. The greenhouse tests are also useful in detecting strains that have a negative effect on plant growth (Neipp & Becker 1999) and helps to study the adaptability of potential biocontrol agents in soil (Racke & Sikora 1992a). Results from controlled trials, conducted in greenhouses will help to design the field level application of the biocontrol agents.

2.7. FIELD EVALUATION

Scanning of literature revealed the successful use of bacteria to suppress nematodes under field conditions. Biocontrol potential of *Bacillus subtilis*, *B. thuringiensis*, *Pseudomonas stutze* and *Paecilomyces lilacinus* against *M. incognita* on tomato by Khan and Tarannum (1999), *P. fluorescens* against rice root nematode, *Hirschmanniella gracilis* by Ramakrishnan *et al.* (1998 b), *Serratia marcescens* and *Streptomyces avermitilis* against *M. incognita* on grape by Ali & Kamal (1998), *B. thuringiensis* var. kurstaki H-3 against high populations of *R. similis* in banana plantations by Mena *et al.* (1997a) etc. are some examples. Integrated management of *M. incognita* on tomato in nursery and main field was reported by Reddy *et al.* (2000). Field level

experiments with biocontrol agents against various nematodes were conducted by Kloepper *et al.* (2004), Shanthi *et al.* (2003), Ramakrishnan *et al.* (2003), Deepak Kumar *et al.* (2003), Reimann & Sikora (2004) Vogel *et al.* (2002) and Khan & Tarannum (1999). Bio control of *R. similis* on black pepper, under field conditions by Koshy (2003) revealed that the biological control agents individually or in combinations were highly effective in enhancing plant growth and suppressing nematode multiplication.

2.8. MODE OF ACTION

Different mechanisms on the suppressive nature of rhizobacteria on the nematode populations of have been postulated. These mechanisms are generally classified as competition, parasitism/predation and antibiosis. Antibiosis is the antagonism mediated by specific or non-specific metabolites of microbial origin, by lytic enzymes, volatile compounds or other substances (Fravel 1988; Ryu *et al.* 2003b). They may interfere with the host finding process of nematodes by receptor blockage of the lectin carbohydrate recognition sites (Sikora & Hoffman-Hergarten 1993). Gram negative rhizobacteria may have lectin binding structures in the lipopolysaccharide layer of the cell wall membrane. The ability of plant health promoting rhizobacteria (PHPR) to envelop or bind to root-surface and the lectins have the potential to interfere with nematode host recognition and thus affecting the root penetration (Oostendorp & Sikora 1990). Some bacteria alter the root exudates that influence nematode hatch, attachment and penetration in to the host tissues (Oostendorp & Sikora 1990; van Loon *et al.* 1998).

The pathogenic versatility of a bacterium is reflected in its large arsenal of secreted and surface-associated virulence factors and in the complexity of the regulatory circuitry with which it controls these factors. Among the specific virulence factors produced are adhesins such as pili and filamentous hemagglutinin (Dörr *et al.* 1998), protein toxins such as phospholipase, proteases and ADP-ribosylating enzymes and small-molecule poisons such as phenazines, rhamnolipid biosurfactant and cyanide (Temple *et al.* 1998).

2.8.1. Metabolites

Metabolites like avermectins (Stretton *et al.* 1987), 2, 4-diacetyl phloroglucinol (Cronin *et al.* 1997b; Bangera & Thomashow 1999; Rubio *et al.* 2000; Musarrat *et al.* 2000; Webster 2002), valinomycin (Mishra *et al.* 1987; van Wees *et al.* 2000; Siddiqui &

Shaukat 2003d), cyanamide (Walsh *et al.* 2001; Sidique *et al.* 2006), organic acids such as butyric acid (Hollis & Rodriguez-Kabana 1966; 1967; Striling 1991; Burdman *et al.* 2000) and volatile metabolites like hydrogen cyanide (Ahl *et al.* 1986; Blumer and Has 2000), hydrogen sulphide (Rodriguez-Kabana 1965; Lopper *et al.* 1991), ammonia (Rodriguez-Kabana 1986; Zavalecta-Mejia 1985, 1989; Carnerio *et al.* 1998; Velusamy *et al.* 2003) etc. are important in nematode control. According to Oka *et al.* (1993) the active ingredient with nematicidal property in the bacterial culture filtrate is ammonia, released during the breakdown process of peptides in the media by bacterial activity. Milbemycins and dianemycin type polyethers from a strain of *S. hygroscopicus* (Whaley *et al.* 1990), aromatic nitrocompounds and griseulin from *Streptomyces* spp. (Nair *et al.* 1995) are also reported to have nematicidal properties. Hu *et al.* (1999) have reported that indole derivative from the culture filtrate of an isolate of *Photorhabdus luminescens* has nematicidal properties against the root knot nematode, *M. incognita*. The detected volatile compounds from bacterial isolates included alcohols, aldehydes, ketones, alkenes and ethers. Volatiles with strong nematicidal activity include phenol, 2-octanol, benzaldehyde, benzeneacetaldehyde, decanal, 2-nonanone, 2-undecanone, cyclohexene and dimethyl disulfide terpineol, benzeneethanol, propanone, phenyl ethanone and nonane (Ying *et al.* 2007).

The antimicrobial metabolites 2,4-diacetylphloroglucinol (2,4-DAPG) (Notz 2002) and pyoluteorin contribute to the ability of *P. fluorescence* strain CHA0 to control plant diseases caused by soil-borne pathogens including *M. javanica*. Exposure of root-knot nematode to culture filtrates of *P. fluorescens* under *in vitro* conditions significantly reduced egg hatch and substantial mortality of *M. javanica* juveniles.

2.8.2. Toxins

Exotoxins produced by *B. thuringiensis* var. *thuringiensis* (Btt) and *B. thuringiensis* var. *israelensis* (Bti) (Prasad & Thilak 1972; Zuckerman *et al.* 1993; Sharma 1994), delta-endotoxins by *Bacillus sphaericus* (Sharma & Gomes 2000) etc. are nematicidal or reduce egg hatch. Preliminary work conducted in different laboratories have suggested the possibilities for commercial utilization of these toxins for nematode control. Prasad *et al.* (1972) reported that a nematicidal toxin produced by *B. thuringiensis* var. *thuringiensis* was capable of killing root-knot nematode juveniles. The toxin produced in the culture medium was thermo stable and highly potential, even at a 10

fold dilution. These toxins also inhibited hatching of root-knot nematode eggs. Devidas & Rehberger (1992) reported that thuringiensin was capable of protecting tomato plants from infection by nematodes. In addition, there are several reports on the effect of *B. thuringiensis* toxins on animal parasitic nematodes (Bone *et al.* 1985). As thuricide, a biological product of *B. thuringiensis*, it is likely that exotoxin produced by bacteria might have prevented the hatching of eggs (Chahal & Chahal 1990; Bradfish *et al.* 1991; Dhaliwal *et al.* 2001; Wei *et al.* 2003).

B. thuringiensis toxins were used to control nematodes belonging to the genera *Criconebella*, *Ditylenchus*, *Globodera*, *Helicotylenchus*, *Heterodera*, *Longidorus*, *Meloidogyne*, *Paratrichodorus*, *Pratylenchus*, *Radopholus*, *Rotelynchus*, *Rotylenchulus*, *Tylenchulus* or *Xiphinema*. Particularly, the nematode species susceptible to the toxins are *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *N. aberrans*, *P. penetrans*, *P. brachyurus*, *P. scribneri*, *P. zaeae*, *D. dipsaci*, *T. semipenetrans*, *R. reniformis*, *R. similis* and *H. glycines*, and more particularly *M. incognita*, *P. penetrans*, *R. reniformis*, *R. similis* and *H. glycines*. (Crickmore *et al.* 1998; Marroquin *et al.* 2000; Wei *et al.* 2003; Kotze *et al.* 2005).

The toxic principles of *P. aeruginosa* were partially characterized by Nasima *et al.* (2002). The nematode toxins produced by bacteria such as *Agrobacterium radiobacter*, *B. sphaericus* and *B. thuringiensis*, etc. were briefly discussed by Walia *et al.* (2000).

2.8.3. Antibiotics

Antibiotics, the organic compounds produced by microbes, are deleterious to growth or metabolic activities of other microorganisms, even at low concentrations. *In vivo* study by Bone *et al.* (1985) correlating antibiotic production with pathogen inhibition and disease suppression supported the role of bacterial antibiotics in biological control. Further it is well known that the antibiotic production by some bacteria had a major role in disease suppression (Cook *et al.* 1995; Das *et al.* 2000).

The secondary metabolites such as phenazines, pyrrolnitrin, tropolone, pyocyanin and 2,4-diacetylphloroglucinol produced by *P. fluorescens* exhibited antibiotic activity lethal to lesion nematodes in the rhizosphere plants (Cronin *et al.* 1997; Landa *et al.* 2002; Maurhofer *et al.* 2004; Ahmadzadeh *et al.* 2004). Apart from possessing high specific activity and target selectivity, biologically active secondary metabolites are

generally biodegradable (Mondal *et al.* 2002). Regulation of antibiotic production in root colonizing *Pseudomonas* spp. and relevance for biological control of plant diseases was reported by Has & Keel (2003). 2, 4-diacetylphloroglucinol act as a signaling compound inducing the expression of its own biosynthetic genes.

2.8.4. Siderophores

Siderophores are low molecular weight, ferric specific ligands designed for solubilization, transport and storage of iron in microorganisms (Klopper *et al.* 1987; De Meyer & Hofte 1997; Yeole & Dube 2002). The biosynthesis of siderophores and their corresponding membrane located transport systems are carefully regulated by iron (Schroth & Hancock 1982). Since siderophores sequester the limited supply of iron (III) in the rhizosphere, its availability to pathogens is affected, which ultimately suppress their growth (Schroth and Hancock 1982; De Meyer & Hofte 1997). The availability of iron (III) in the soil declines logarithmically with the increase in soil pH. Thus, siderophore mediated suppression should be greater in neutral and alkaline soil than in acid soil (Yeole & Dube 2000).

Siderophores producing strains of *Pseudomonas*, *Alkaligenes*, *Bacillus*, *Enterobacter* etc. have been used as biocontrol agents against damping off in cotton and root rot of wheat caused by *Pythium* and potato seed decay by *Erwinia* (Yeol & Dube 2000). Enterobactin produced by the members of Enterobacteriaceae family namely, *Escherichia coli*, *Salmonella* sp. (Press *et al.* 2001); agrobactin produced by *Agrobacterium tumefaciens*; acetobactin by *Acetobacter* and different species of *Pseudomonas* (Mondal *et al.* 2002); pyochelin by *P. aeruginosa*; pseudobactin by *P. fluorescens* and *P. putida*; pyoverdins by *P. fluorescens* (Mayer & Abdallah 1978); arthrobactin by *B. megaterium* (Verma *et al.* 2001) are important in biocontrol. The siderophore deficient mutants are less suppressive to pathogens than the parent strains (Loper 1988). Apart from the role in iron chelation, siderophores may also act as growth promoting factors (Kloepper *et al.* 1980a) and some are potential antibiotics under low iron concentration (Wei *et al.* 1996).

P. fluorescens produced extracellular siderophores when grown in casamino acid medium under iron deficiency, antagonistic to fungal pathogens like *Fusarium oxysporum*, *Alternaria* sp. and *Colletotrichum capsici*. Isolates of *P. fluorescens* showed increased growth and siderophore production with amino acids. Citric acid and malic acid

prevented the fluorescence and siderophore production. Lactic acid was found to be the best nutrient for growth and siderophore production. Sugars completely suppressed siderophore production (Dave & Dube 1999).

2.8.5. Enzymes

During bacterial infection, the degradation of all the nematode cuticle components around the holes suggests the involvement of hydrolytic enzymes (Cox *et al.* 1981; Cherin & Chet 2002; Decraemer *et al.* 2003; Huang *et al.* 2005). Bacteria produced specific enzymes such as chitinase (Chen *et al.* 1999; Li *et al.* 2002; Zhen-Xiang Lu *et al.* 2005), protease (Miller & Sands 1977) and collagenase (Robertson & Miller 1972) are effective in suppressing nematodes.

2.8.5.1. Protease

The inhibitory action of protease on nematodes has been reported (Sacherer *et al.* 1994; Ahmadzadeh *et al.* 2004). Sela *et al.* (1998) purified and characterized the *B. cereus* collagenolytic/proteolytic enzyme and studied its effect on *M. javanica* cuticular proteins. Alkaline protease from alkalophilic *Bacillus* (Purva *et al.* 1998) and protease from *Lactobacillus* sp. (Kawai *et al.* 1999); *P. fluorescens* (Kumura *et al.* 1999); *B. sphaericus* (Jasvir-Singh *et al.* 1999) etc. are proven to be efficient in biocontrol. Histopathological observations and molecular biological analyses have demonstrated that major pathogenic activity on nematodes could be attributed to an extracellular alkaline serine protease (Huang *et al.* 2005; Tian *et al.* 2006). The most compelling evidence to support the role of protease as virulence factor was derived from studying protease-deficient mutants (Tian *et al.* 2006).

The condition and abundance of protein component in the eggshell would determine its degradability by protease. In nematode eggshells, the protein content is variable and it is 59% in *G. rostochiensis* (Clarke *et al.* 1967), while it is 40% in *M. incognita* (Bird & McClure 1976). When protein content in the eggshell is higher than chitin, proteases may be more important than chitinases. Kumura *et al.* (1999) characterized the proteases from *P. fluorescens* and reported loss of 90% proteolytic activity on heating to 50° C for 2 minutes. Incubation of the protease at 50° C also led to the loss of the intact enzyme, whereas it was stable in the presence of the protease inhibitor o-phenanthroline. The protease was also active over a wide range of pH (5.5-

10.0) and had optimum activity at pH 7.5. Siddiqui *et al.* (2005) identified extracellular protease of *P. fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode, *M. incognita*.

2.8.5.2. Chitinase

Several bacteria like *Stenotrophomonas maltophili*, *Chromobacterium* sp., *S. marcescens* (Cronin 1997a), *Chromobacterium violaceum*, *Aeromonas hydrophila*, *A. caviae* and *Serratia marcescens* (Park *et al.* 1995; Watanabe *et al.* 1997), *B. polymyxa* (Tian *et al.* 2000), *B. pabuli* (Frandsen & Schnurer 1994), *B. circulans* (Chanpen *et al.* 1999; Wiwat *et al.* 2002), *Bacillus* sp., *Streptomyces* sp. (Singh *et al.* 1999), *Eubacterium* sp., *Streptococcus* sp., *Clostridium* sp. and *Bacteroides* sp. (Olsen *et al.* 2000), *S. saraceticus*, proteo-chitinolytic strain of *B. thuringiensis* kurstak (Rojas Avelizapa *et al.* 1999) etc. were reported to be producing chitinase enzyme having inhibitory effect on nematodes.

The chitinase from *S. marcescens* exhibited both endo and exo chitinase activity (Watanabe *et al.* 1997) and consisted of several enzymes. Together they bring about the degradation of chitin ultimately to the dimer; chitobiose and the monomer, N-acetyl-D-glucosamine. The *streptomycete* chitinase similarly consists of a number of enzymes (Watanabe *et al.* 1997).

Purification and characterization of chitinase from *Bacillus circulans* active against nematodes was done by Chanpen *et al.* (1999). The purified enzyme has a single band on SDS-PAGE, estimated to be 45 kDa. This enzyme could hydrolyze colloidal chitin, purified chitin, glycol chitin, carboxymethyl chitin and 4-methylumbelliferyl-beta-D-N, N'-diacetylchitobioside. The crude enzyme extracts from five *Streptomyces* spp. showed high endochitinolytic activity, compared to a commercial chitinase. Exochitinase and chitobiase activities were also detected suggesting that these strains are potential biocontrol agents (Gomes *et al.* 2000).

Bacteria that are able to degrade colloidal chitin in water agar comprised of 0.2-5.7% of the total amount of culturable bacteria (Robbins *et al.* 1988). *Pseudomonas* spp. are the most abundant culturable, chitin-degrading bacteria at the lime-poor sites, whereas *Xanthomonas* spp. and *Cytophaga* spp. are important at the lime-rich sites. Chitinolytic actinomycetes are relatively abundant at all three sites (Boer *et al.* 1980). Increased

hatching of *M. incognita* eggs was observed on incubation with chitinase from *Phaseolus vulgaris*, *Serratia marcescens* and *Streptomyces griseus*. After treatment with chitinases from *S. marcescens* and *S. griseus*, many of the eggs became spherical with a simultaneous increase in the number of dead juveniles. Egg mortality was observed with purified *P. vulgaris* chitinase at concentrations of 17 µg protein/ml and above, suggesting that the exogenous chitinases cause premature hatch of nematode eggs and could be used as an aid in the control of nematodes (Mercer *et al.* 1992). Both proteases and collagenase showed adverse effect on egg hatching.

2.8.5.3. Collagenase

Wronkowska (1988) detected the keratinolytic properties of microorganisms isolated from cyst-nematodes like *H. avenae* and *G. rostochiensis*. Collagenase has stronger lethal effect on the nematode than protease. Both protease and collagenase showed positive effect on larval motility *in vitro*, the former being stronger than the later.

2.8.5.4. Molecular basis of enzyme production

gacA controlled *aprA* gene encodes the major extracellular protease and the mutation of the *gacA*-controlled *aprA* gene (encoding the major extracellular protease) or the *gacA* regulatory gene resulted in reduced biocontrol activity against *M. incognita*. Culture supernatants of strain CHA0 inhibited egg hatching and induced mortality of *M. incognita* juveniles more strongly than did supernatants of *aprA* and *gacA* mutants, suggesting that *aprA* protease contributes to biocontrol (Siddiqui *et al.* 2005). Many genes coding for chitinase enzymes have been cloned and in few cases sequenced (Colwell *et al.* 1988).

2.8.6. Molecular basis of biocontrol

Using latest techniques like southern blot, sequence analysis, open reading frame analysis and amino acid comparative database searches, Wechter *et al.* (2002) identified the genetic loci in a rhizosphere inhabiting species of *Pseudomonas* involved in the expression of a phytoparasitic nematode ovicidal factor. High degree of homology to the *vgrG* gene, the two component sensor kinase protein, *rtpA* (*gacS*) analogue, a UDP-glucose 4-epimerase gene, a siderophore receptor protein, and the 50S ribosomal protein, L21 was found. These genes are involved in the production of the egg kill factor and the loss of these gene results in lack of ovicidal activity and also exhibited altered fluorescent

siderophore and protease production. The molecular basis of biocontrol by rhizobacteria was explained by Lugtenberg (2001).

2.8.7. Mechanisms of suppression of nematode egg hatching

The embryonic development of eggs ceases soon after they are treatment with bacterial, suggesting a rapid injury to the treated eggs. Among different stages of the nematode, the stages before the formation of second stage juvenile are reported to be only sensitive to bacteria. Since adults and second stage juveniles were apparently insensitive, it appears that the nematode cuticle and not the eggshell is a selective barrier (Wharton 1980). First stage juveniles appeared to be sensitive indicating that the cuticle of this stage may not be an effective barrier. Alternatively, first stage juveniles may be sensitive to the bacteria during the first molt (Oostendrop & Sikora 1989; Khan & Javed 2002). Some plant parasitic nematodes require a hatching factor from the host plant, and some bacteria prevent this signal from being received by the nematode (Oostendrop & Sikora 1989).

The gelatinous matrix into which the eggs of *Meloidogyne* spp. are deposited is having several functions in the life cycle of the nematode and it is synthesized in the rectal glands and excreted through the anus just before and during egg laying. It is an irregular mesh containing glycoproteins, polysaccharides and N-acetyl glucosamine containing compounds (Sharon & Spiegel 1993) but no chitin (Bird & Self 1995). Within the roots, the gelatinous matrix is involved in lysing the tissues in which the female nematode is embedded, to form a channel through which the eggs can reach the surface (Orion *et al.* 1987). On the roots and in the soil the gelatinous matrix is thought to protect the eggs from desiccation and from other soil microorganisms (Bird & Bird 1991). Gelatinous matrix compounds affect a number of rhizospheric bacteria where as other bacteria are not inhibited and can use gelatinous matrix components as nutrient sources (Orion & Kirtzman 1991). Papert & Kok (2000) reported that the egg masses support a greater bacterial population even larger than that of the rhizospheric soil, which is generally considered as a densely populated microbial niche. Hence, description of the bacterial population of egg mass is of importance as the resident bacterial population could affect the eggs and/or egg parasitic microorganisms.

Collagen is the major component of the animal extra cellular tissue. It consists of fibrils composed of laterally arranged polarizes tropocollagen molecules (Robertson &

Miller 1972). Collagen is the main component of nematode cuticle (Reddigari *et al.* 1986). Sela *et al.* (1998) showed that collagen amendments to soil drastically reduced galling in tomato compared with other protein and chitin amendments. Ammonia released in the decomposition of collagen was inadequately lethal to *M. javanica* larvae. However, collagenase had a grater effect on egg hatch. Effect of culture filtrate of *B. subtilis* on the hatching suppression of nematodes was reported by Sharma (1998).

Bacteria that produce various lipolytic, proteolytic and chitinolytic enzymes may lyse the eggs. When living eggs were treated with chitinase, they become spherical and nematodes hatch prematurely (Mercer *et al.* 1990; Chen *et al.* 2000). Though, clear evidence of egg shell rupture or change in the shape of eggs is not observed in some cases, change in the permeability could occur without drastic change in egg shape (Khan and Goswami 2000). In eggshells of tylenchid nematodes, chitin is located between the outer vitelline and the inner lipid layer and may occur in association with proteins (Bird & Bird 1991; Decraemer *et al.* 2003). The breakdown of the polymer by chitinase can cause premature hatch, resulting in few viable juveniles (Mercer *et al.* 1992). In soil, chitinases are produced by some actinomycetes, fungi (Mian *et al.* 1982) and bacteria (Ordentlich *et al.* 1988). Besides, they are also released by many plants as part of the defense mechanism against various pathogens (Punja & Zhang 1993) and plant parasitic nematodes (Roberts *et al.* 1992). Chitinases depolymerize the chitin polymer in to N-acetylglucosamine and chitobios. Further microbial activity results in the deamination of the sugars and accumulation of ammonia ions and nitrates (Rodrigues Kabana *et al.* 1983) and these in association with a newly formed chitolytic microflora are believed to be responsible for nematode suppression (Mian *et al.* 1982; Godoy *et al.* 1983). The role of exogenous cyanide prodeuced by *Pseudomonas* inhibited egg hatch of *Meloidogyne* (Way 1984; Sidique *et al.* 2006).

2.8.8. Molecular genetic techniques used in studying bacterial pathogenesis in nematodes

Over the past few years, a number of molecular genetic methods in bacterial pathogenicity have been developed, and it is now possible to introduce these successful techniques to the study of bacterial pathogenesis in plant-parasitic nematodes (Hensel & Holden 1996; Ciancio *et al.* 2000; Abbas *et al.* 2002; Barker 2003).

Reverse genetics is a common approach in identifying and determining functions of virulence determinants. This method involves the isolation of virulence proteins involved in pathogenicity, and cloning of the corresponding genes. The functions of virulence proteins are further confirmed by their expression in other organisms, by the inactivation of the gene in a wild-type strain, or by immunological techniques (Huang *et al.* 2005; Tian *et al.* 2006).

Mutational analysis both directed and random mutagenesis is another popular technique for identifying pathogenic determinants. In directed mutagenesis, a putative virulence determinant encoding a gene postulated to be responsible for a certain pathogenic trait is disrupted or replaced to construct a mutant strain. The mutant and the wild-type strain are then compared to determine the importance of the suspected virulence determinant (Hensel *et al.* 1995; Wechter *et al.* 2001, 2002; Siddiqui *et al.* 2005).

Comparative genomics can identify pathogenic genes by comparing genomic sequences of pathogenic and nonpathogenic strains, or other sequences from strains of interest of the same genus. Similarly, a genomic-bioinformatic approach might further define the evolutionary relationships among the various pathogenic and nonpathogenic bacteria (Hensel & Holden 1996; Bird *et al.* 2003; Preston *et al.* 2003; Charles 2005; Charles *et al.* 2005; Davies 2005).

Some techniques have not yet been employed in the study of bacterial infection against plant-pathogenic nematodes but may prove useful, for example in vivo expression technology (IVET), differential fluorescence induction (DFI), subtractive hybridization and differential display etc (Hensel & Holden 1996; Valdivia & Ramakrishnan 2000; Ogawa *et al.* 2000; Harakava & Gabriel 2003).

2.9. ROOT COLONIZATION

According to Scher *et al.* (2004) while root colonization alone is not a predictive of a beneficial plant response, it is a logical starting point for assays designed to select either growth promoting bacteria or biological control agents.

Root colonization occurs in two phases. In the first phase, the bacteria attach to, and are then transported on the elongating root tip, and in second phase, the bacteria spread locally and proliferate to the limits of the niche in competition with indigenous

organisms and survive (Howie *et al.* 1987). The distribution of introduced bacteria along the root during first phase, and their propagation and survival during the second phase are profoundly affected by abiotic and biotic factors (James *et al.* 1985; De Weger *et al.* 1995). Mechanisms of rhizosphere colonization may involve active bacterial movement or passive transport by percolating water or plant roots. Nematodes can have an important role in rhizosphere colonization by bacteria in soil (Knox *et al.* 2004).

Root colonization is typically examined by quantifying bacterial populations on root surfaces, however, some rhizobacteria can also enter roots and establish at least a limited endophytic phase (Daluz 2001). Hence, root colonization may be viewed as a continuum from the rhizosphere to the rhizoplane to internal tissues of roots. Some endophytic bacteria transcend the endodermis, crossing from the root cortex to the vascular system and subsequently can be located in stem tissues above the ground. Because rhizobacteria and endophytes are part of the natural microflora of healthy plants, they may be considered to be important contributors to plant health and general soil suppressiveness (Zalpel *et al.* 2001; Nanda Kumar *et al.* 2001; Louws *et al.* 2001). Nematodes are important vectors for bacteria rhizosphere colonization in the absence of percolating water (Knox *et al.* 2003, 2004). While root colonization alone not a predictive for beneficial plant response, it is a logical starting point for assays designed to select the strains for growth promotion or disease suppression (Scher *et al.* 2004).

Soil edaphic factors such as temperature (Weller & Cook 1983), soil moisture (Dupler & Baker 1984; Turnbull 2001 a, 2001 b), pH (Berg 1996) and clay content (Campbell & Ephgrave 1983) were reported to influence the root colonization potential of an organism. Gram reaction is also correlated well with the colonizing capacity and Scher *et al.* (2004) reported that all gram positive bacteria are non colonizers, whereas all gram negative bacteria, except four species, are root colonizers of maize roots.

The copper resistant gene present in a copper resistant strain of *Pseudomonas* (09906) plays important role in the competitive survival of *Pseudomonas* (Yang *et al.* 1993). Many of these bacterial root endophytes can also influence plant growth significantly, and some possess PGPR activity (Wilson 1995; Bashan & Hoglin 1998; Chanway 1998; Chanway *et al.* 2000; Surette *et al.* 2003). Because microorganisms that colonize the root interior may be buffered against environmental extremes compared with those that associate with the exterior regions of the root (Law & Lewis 1983),

establishment of PGPR within root tissues may result in greater inoculum persistence compared with those that are restricted to colonizing the rhizosphere. In addition, the comparatively intimate association of root-endophytic bacteria with host plant cells may facilitate greater PGPR efficacy compared with rhizosphere associations, where microorganisms may be physically separated from root systems (van Peer & Schippers 1989; van Peer *et al.* 1990). Van Peer *et al.* (1990) found that *Pseudomonas* strains located on the surface of hydroponically grown tomato (*Lycopersicon esculentum* Mill) roots were biochemically distinct from those found inside root tissues, and observed that, in general, those isolate originating from within roots tissues were more effective colonizers of internal root tissues than where isolates derived from the root surface.

For biocontrol agents that colonize both the rhizosphere and endorhiza, it is difficult to differentiate whether biological control depends on bacterial colonization of the rhizosphere, the endorhiza or both (Hallmann *et al.* 2001). Using the green fluorescent protein (GFP) as a marker, Hallmann *et al.* (2001) studied the root colonization process in relation to the infection of *M. incognita* and found that although the bacteria were found over the entire rhizoplane, they preferentially colonized root tips and galled tissue caused by *Meloidogyne* infection. In the presence of nematode the bacteria colonized in the interior nematode galls in high numbers. A heterogeneous colonization of the bacteria may indicate induced systemic resistance as second mode of action other than nutrient competition in biological control.

To facilitate root colonization, root endophytic bacteria also possess the ability to metabolize structural components of plant cells. In particular, the ability to metabolize pectin (poly-galacturonic acid), the primary component of the middle lamellae of plant wall cells, has been proposed to at least partially explain why bacterial root endophytes are often found in the root cortex intercellularly (Ben *et al.* 2001). Knox *et al.* (2004) reported that internal root colonization by *Bacillus* strains is a root random event and that root-endophytic *Bacillus* strains possess specific physiological and (or) biochemical characteristics that facilitate colonization of internal root tissues.

2.10. INFLUENCE OF ECOLOGICAL AND OTHER FACTORS

Nematode population dynamics and associated crop responses are often related to environmental parameters (Oostenbrink 1972; Barker *et al.* 1985). Thus, such information

is critical for effective nematode control programmes. In addition to enhancing the predictability of crop responses to a given nematode species, environmental data are also helpful in achieving optimum efficacy of a number of management tactics.

Soil conditions, nematode species, their rate of development and density and host plant all have a considerable effect on the establishment and activity of biological control agents of nematodes. It is essential to understand these interactions for effective control of nematodes (Kerry 1990). Too often, biological control agents have failed because they have been used before a basic knowledge of their ecology and biology have been established (Stirling 1991). Nematode population dynamics and associated crop responses are often related to environmental parameters (Oostenbrink 1972; Barker *et al.* 1985). Understanding the environmental factors that regulate the biosynthesis of antimicrobial compounds by disease suppressive strains is an essential step toward improving the level and reliability of their biocontrol activity (Duffy & Defago 1999). Liquid culture assays were used to identify several minerals and carbon sources which had a differential influence on the production of the antibiotics indicating that the ratio of carbon source to nutrient concentration played a key role in the metabolic flow (Das & Mukherjee 2000). These tests provide insight into the biosynthetic regulation of antimicrobial compounds, limit the number of factors for intensive study *in situ*, and indicate factors that can be manipulated to improve bacterial inoculants.

Biological control depend upon the establishment and maintenance of a threshold population of bacteria on planting material or in soil, and a drop in viability below that level may limit the possibility of biological control (Burr *et al.* 1987). The method the bacteria are cultured and processed will affect their viability and tolerance to adverse conditions once applied (Esnard *et al.* 1998b). Repeated culturing of fluorescent pseudomonads *in vitro* can result in a loss of field efficacy, possibly related to changes in cell and colony morphology, loss cell surface structures, or reduction in antibiotic and siderophore production (Schroth and Becker 1999). The efficacy of a biological control agents is affected by the cultural conditions. Yan & Riley (2003) reported how the efficacy of *Dilophospora alopecuri* inoculum was affected by various conditions, including length of fermentation, moisture content, and growth substrate. Many soil edaphic factors like temperature, soil moisture and clay content, influence the survival and establishment of bacteria and their interaction with the pathogen. Many soil edaphic

factors including temperature (Weller & Cook 1983), soil moisture (Siddiqui and Ehteshamul Haque 2001b), pH (Berg 1996), clay content (Campbell & Ephgrave 1983) etc. are reported to be influencing the root colonization potential and thereby the biocontrol potential of an organism. Use of ecologically competent rhizobacteria was highly efficient in plant growth promotion and disease management.

2.10.1. Temperature

Temperature has a direct effect on the growth and sporulation of the biocontrol agent and on the rate of development of the nematode target. Soil texture and structure influence nematode activity and the growth and spread of microorganisms. In temperate zones, the soil temperature ranges between 10° and 15°C for much of the growing season, whereas in warmer climate the temperature vary from 20° to 25°C at 10 cm depth. Often, research workers in temperate regions have tested the effects of biological control agents in pot experiments in greenhouses at temperatures considerably above those which normally prevalent in the field; and data from such tests are of limited value (Andreoglou *et al.* 2003).

Influence of temperature on plant rhizobacteria interactions related to biocontrol potential was studied by Landa *et al.* (2004). Salt and temperature tolerant strains of *P. denitrificans*, *P. rathonis*, *B. laevolacticus*, *B. amyloliquefaciens* and *Arthrobacter simplex* were found to be effective in biocontrol (Egamberdiyeva 2005). The motility and efficacy of *P. oryzihabitans*, a biocontrol agent against the potato cyst nematode *Globodera rostochiensis*, were severally affected by temperature (Andreoglou *et al.* 2003).

Effect of temperature on *in vitro* interactions between *P. chlamydosporia* and other *Meloidogyne* associated microorganisms was reported by Kok & Papert (2002). The antagonism of bacteria and fungi isolated from egg masses of *Meloidogyne* spp. was tested *in vitro* against the egg parasitic fungus, *V. chlamydosporium*, at two temperatures (15 and 20°C). Overall, the bacteria were less active against *V. chlamydosporium* at 15°C than at 20°C. However, this overall temperature effect was not observed with the fungi due to the small sample size tested. The effect of temperature on antagonism of individual strains was more striking than the overall temperature effect. Antagonism was affected by temperature for 33% of the bacteria and 47% of the fungi.

2.10.2. pH

Application of fertilizers and other agrochemicals can affect soil pH, which influence the growth and reproduction of soil organisms (Berg *et al.* 1996). Nitrifying bacteria were also reported to be inhibited, leading to ammonium concentrations toxic to the pathogens. The population of antagonistic species of *Trichoderma* were also reported to increase (Bitton *et al.* 1974).

2.10.3. Moisture

The movement of bacteria through soil by means of water is affected by bacterial characteristics such as cell shape, size, buoyancy, motility (Hazelbauer & Berg 1993; Berg 1990) and electrostatic charge (Marshall 1971), as well as by soil type, pore-size distribution, water content and pH (Hazelbauer & Berg 1993; Berg 1991). Channels left by old roots or worms (Madsen & Alexander 1982) and possible gaps at the root-soil interface created by the diurnal shrinking and swelling of roots might allow more rapid downward movement of bacteria in water than occurs along roots in a more uniform soil matrix (Parke 1991). Moisture in soil is rarely limiting for the growth of most fungi but affects spore dispersal, particularly the zoospores. Bacteria are more sensitive to soil moisture levels than fungi, but are unlikely to be affected at moisture levels that enable nematodes to remain active.

2.10.4. Nutrient flux

Carbon, nitrogen and inorganic phosphate source in liquid culture is reported to regulate the nematicidal activity of fluorescent pseudomonads *in vitro* (Siddiqui & Shaukat 2004). In general, nitrogen tends to increase disease, potassium tends to increase disease resistance, and phosphorus may elicit either response (Huber & Watson 1974). Hamid *et al.* (2003) mentioned that addition of ammonium molybdate enhances the production of nematicidal compounds by *P. fluorescens in vitro* and improves its efficacy as biocontrol agent against root-knot nematode under glasshouse conditions. Role of iron in rhizobacteria mediated suppression of root infecting fungi and root knot nematode in tomato was reported by Siddiqui & Shaukat (2003c). Appropriate quantities of glycerol and zinc alone or in combination enhanced the nematicidal activity of *P. aeruginosa* and *P. fluorescens* and glucose reduced the activity of these bacteria (Siddiqui & Shaukat 2002).

Developing bacterial strains more responsive to certain environmental signals can be exploited for increased secondary metabolite production in pharmaceutical fermentations and this offers new avenues to improve their biocontrol efficacy. Liquid culture amendments with various C, N or P sources modulate the biosynthesis of nematicidal factors by biocontrol agents (Siddiqui & Shaukat 2004a).

Role of micronutrients in the suppression of root knot nematode, *M. javanica* by *P. fluorescens* strain CHA0 and its GM derivatives was studied by Siddiqui *et al.* (2004a). Response to carbon starvation in *Pseudomonas aeruginosa* strain IE-6S+ on general cross protection, production of some nematicidal compounds *in vitro*, and the biological control of *M. javanica* in tomato were reported by Siddiqui & Shaukat (2003). Role of zinc in rhizobacteria mediated suppression of root infecting fungi and root knot nematode was reported by Siddiqui *et al.* (2002a). Regardless of the nematode densities, Zn applied alone or in combination with *P. aeruginosa* caused marked suppression of *M. javanica*.

2.10.5. Cultural conditions

The efficacy of biological control agents is affected by several cultural conditions. Fermentation period, moisture content and substrate effects on the effectiveness of biocontrol agents inoculation were studied by Yan & Riley (2003).

Suppression of *M. javanica* by *P. aeruginosa* in tomato was reported to be influenced by NaCl, oxygen and iron levels. Culture filtrate of the bacterium resulting from low oxygen level caused greater mortality of *M. javanica* juveniles *in vitro*, compared with the filtrates obtained from ambient oxygen conditions. Culture filtrate from low oxygen conditions also showed the presence of hydrogen cyanide while those from ambient oxygen condition did not have hydrogen cyanide. Under *in vitro* conditions, with an increased iron concentration in the form of FeEDDHA, growth of *P. aeruginosa* and its nematicidal activity increased (Siddique *et al.* 2003c).

2.10.6. NaCl Tolerance

NaCl directly influence the population density of fluorescent *Pseudomonas*. The population was maximum at 0.25% NaCl. It was also found that the NaCl tolerant strains of the fluorescent pseudomonads have high survival and root colonizing activity in the rhizosphere, especially in the soils treated with high NaCl (Kalif *et al.* 2003). A concentration of 0.8 M NaCl was found optimum for *in vitro* survival and growth of

Pseudomonas aeruginosa while, nematocidal activity by *P. aeruginosa* was maximum when the bacterium was exposed to 0.4 M NaCl (Siddiqui *et al.* 2003c). The bacterium was highly sensitive to high (1.6 M) NaCl concentration. Culture filtrate of the bacterium resulting from the medium supplemented with 0.2 or 0.4 M NaCl showed the presence of secondary metabolite, hydrogen cyanide (HCN). Soil amendment with *P. aeruginosa* alone or in conjunction with up to 0.8 M NaCl enhanced bacterial efficacy against *M. javanica*.

2.11. COMPATIBILITY

2.11.1. Compatibility with agro chemicals

In the development of an integrated pest management (IPM) schedule in different crops, compatibility of biocontrol agents with chemical pesticides is an important prerequisite. Such compatibility is very much essential since farmers often prefer to use insecticides and fungicides together (Mathew 2003). It is also necessary that a biocontrol agent also compatible with pesticides to become an integral part of the IPM schedule (Thomason 1987).

P. fluorescens was found to be compatible with various pesticides like quinalphos (2000 ppm), triazophos (2000 ppm), imidacloprid (500 ppm), carbaryl 50 WP (4000 ppm), chlorpyrifos (2000 ppm), etofenprox (2000 ppm), mancozeb (2000 ppm), hexaconazol (1000 ppm), carbendazim (1000 ppm). The bacterium was highly compatible with mancozeb, carbendazim (fungicides), imidacloprid, etofenprox, chlorpyrifos and triazophos (insecticides) at the recommended dosages for field use (Senthilkumar & Ramakrishnan 2004). *In vitro* evaluation of selected pesticides revealed that formaldehyde, metham sodium [metam], carbendazim and copper oxychloride were highly inhibitory to *B. circulans* while carbofuran, phorate, mancozeb, ziram, zineb and 2-methoxyethylmercury chloride were not inhibitory (Sheela & Venkitesan 1997; Annapurna *et al.* 2000; Bano & Musarrat 2003c).

The compatibility of aromatic compounds like citral (3,7-dimethyl-2, 6-octadienal), furfural (2-furaldehyde) and benzaldehyde (benzoic aldehyde) which showed suppressive activity against *M. incognita*, with plant growth promoting rhizobacteria (PGPR) was studied by Bauske *et al.* (1997). Vapour of citral inhibited the growth of most bacterial strains *in vitro*, and vapour of furfural and benzaldehyde, killed almost all

bacteria tested, except *Bacillus* spp. And it can be combined with citral and benzaldehyde in the integrated management systems. *In vitro* investigations to explore the toxicity of fungicides for plant growth promoting rhizobacteria by Fathima *et al.* (2006) revealed the fungicides *viz.*, Alert Plus, Darosal and Mancozeb have inhibitory effect while Benlate have stimulatory effect on growth though the effect varies according to strain. No tested fungicides caused genotoxicity.

Parathion-methyl (methyl parathion) and permethrin were found to be highly compatible with *Bacillus thuringiensis* var. *kurstaki*. The higher concentrations of the chemical (1.88 and 3.75 ppm.) enhanced the germination and reproduction of the bacterium and concentrations of the compound of 1.44 ppm at pH 6.5 and 2.89 ppm at pH 8.6 stimulated bacterial germination (Habib & Garcia 1981). The compatibility of *B. thuringiensis* var. *kurstaki* was also studied with 27 chemical insecticides by Morris (1997). The most compatible insecticides were acephate (as Orthene), trichlorphon (as Dylox), methomyl (as Lannate), carbaryl (as Sevin), mexacarbate (as Zectran) and diflubenzuron (as Dimilin), and these can be recommendable for use in the integrated management schedules with *B. thuringiensis*.

Curley & Burton (1975) reported that *R. japonicum* is compatible with thiram at recommended rate and inoculation did not affect the survival rate, but carboxin and captan reduced survival by about 20% and PCNB killed 78% of the rhizobia after 1 hour and 83% in 4 hour.

In vitro evaluation of selected pesticides on the nematode pathogenic bacteria *B. circulans* by Sheela & Venkitesan (1997) revealed that formaldehyde, metham sodium [metam], carbendazim and copper oxychloride were highly inhibitory to *B. circulans* while carbofuran, phorate, mancozeb, ziram, zineb and 2-methoxyethylmercury chloride were not inhibitive. *Azospirillum* is compatible with fungicides carbendazim (0.1x) and Bordeaux mixture (1%), and insecticides carbofuran (0.75 kg a.i. ha⁻¹), phorate (1 kg a.i. ha⁻¹) and HCH (2 kg a.i. ha⁻¹) thiram caused growth reduction of the bacteria *in vitro* (Raji & Pillai 2000).

Akhtar *et al.* (1986) reported that *B. apiarius* and *Staphylococcus epidermidis* could tolerate DDT formulation up to 200 times of the normal field application rate (a

recommended dose of 1 kg ha⁻¹), while *B. megaterium*, *B. subtilis* and *B. circulans* showed minimal tolerance.

Das & Mukherjee (2000) studied the effect of soil application of HCH, phorate, carbofuran and fenvalerate at the recommended application rates (7.5, 1.5, 1.0 and 0.35 kg a.i. ha⁻¹, respectively) and found that all the insecticides in general, and HCH and phorate in particular, significantly increased the population of microorganisms in soil. The most predominant genera of microorganisms such as *Bacillus*, *Micrococcus* and *Aspergillus* were not affected by most of these insecticides. Incorporation of insecticides also significantly stimulated the mineralization and availability of organic C, N and P in soil.

Insecticides in general, and BHC and phorate in particular, stimulated the proliferation of aerobic non-symbiotic nitrogen fixing bacteria and phosphate solubilizing microorganisms and also their biochemical activities, such as non symbiotic nitrogen fixing and phosphate solubilizing capacities, which resulted in greater release of available N (NH₄⁺ and NO₃⁻) and P in soil (Das *et al.* 2000). All the insecticides were persistent in soil for a short period of time, and the rate of dissipation was highest for fenvalerate followed by phorate, carbofuran and BHC. Mahapatra *et al.* (2003) reported the management of root knot nematode in brinjal using carbofuran and compatible *P. fluorescens*. Although pesticides have negative effects on the growth of these fungi in laboratory tests, they would not necessarily be of significant importance under field conditions (Crump & Kerry 1986).

2.11.2. Compatibility with other microorganisms

Phenylacetic acid producing *Rhizoctonia solani* represses the biosynthesis of nematicidal compounds *in vitro* and influences biocontrol of *M. incognita* in tomato by *P. fluorescens* strain CHA0 and its GM derivatives (Siddiqui & Shaukat 2005). The inhibitory effect of PAA-producing *R. solani* on the biosynthesis of nematicidal agent(s) critical in biocontrol may reduce or even eliminate the effectiveness of fluorescent pseudomonads against root knot nematodes, both in nursery beds and in field conditions.

Differential impact of few *Aspergillus* species on *M. javanica* biocontrol by *P. fluorescens* strain CHA0 was studied by Siddiqui & Shaukat (2005). *Aspergillus niger* enhances the production of nematicidal and beta-galactosidase activities by *P. fluorescens*

in vitro and improves biocontrol potential of the bacterial inoculants in tomato while *A. quadrilineatus* reduces bacterial performance to suppress root knot nematodes.

Effects of *P. aeruginosa* on the diversity of culturable microfungi and nematodes associated with tomato and impact on root knot disease and plant growth was reported by Siddiqui & Shaukat (2003c).

Duponnois *et al.* (2002) reported that fluorescent *Pseudomonas* and a nematophagous fungus *Arthrobotrys* sp. T41 when added together to the soil, the multiplication of *R. reniformis* populations was inhibited. *T. harzianum* enhances the production of nematicidal compounds *in vitro* and improves biocontrol efficacy of *P. fluorescens* on *M. incognita* (Siddiqui & Shaukat 2004b). Considering the inconsistent performance of the biocontrol agents under field conditions, application of a mixture of compatible *T. harzianum* and *P. fluorescens* would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity with enhanced efficacy and reliability of control.

2.12. USE OF CONSORTIA

A greater thrust is given for the development of biological consortia with multiple modes of action to suppress both fungal and nematode pathogens since there is no spatial segregation of these pathogens in cropping systems, particularly in plantation crops (Sarma *et al.* 1994). Sarma & Anandaraj (1998) suggested the consortium approach for disease management in plantation and spice crops. The performance of biocontrol agents may be improved by applying mixtures of strains which are complementary in their capacity to suppress plant diseases (Maurhofer *et al.* 2004). One approach to overcome inconsistent biocontrol activity is the application of combinations of different biocontrol agents. Mutual compatibility of fungal and bacterial antagonists viz. *T. harzianum* and fluorescent pseudomonads was studied to establish an efficient consortium for the management of foot rot of black pepper caused by *Phytophthora capsici* and it was found that fungal and bacterial antagonists are compatible (Jisha *et al.* 2002a). Application of rhizobacteria and *T. harzianum* resulted in enhanced growth of black pepper. The combination of *Trichoderma* sp. (IISR-1369) and *P. fluorescens* (IISR-6) was found to decrease the root rot disease besides increasing the yield in black pepper. Sarma *et al.* (2000) has established the biocontrol consortium for black pepper, ginger and cardamom.

Mixtures of plant disease suppressive bacteria strains CHA0 (*P. fluorescens*), IE-6S+ (*P. aeruginosa*) and 569Smr (*Bradyrhizobium japonicum*) enhanced biological control of multiple pathogens on tomato. *Paecilomyces lilacinus*, *Bacillus subtilis* and green manuring with *Eichhornia crassipes* were used in combination for the successful management of *M. incognita* on tomato (Gautam *et al.* 1995; Siddiqui & Shaukat 2002c).

The effect of rhizobacteria like *P. fluorescens*, *Azotobacter chroococcum* and *Azospirillum brasilense*, alone and in combination with root symbionts, *Rhizobium* sp. and *Glomus mosseae*, on the growth of chickpea, and reproduction of *M. javanica* were studied by Siddiqui & Mahmood (2005). Siddiqui *et al.* (1998) used combination of *P. fluorescens*, *Glomus mosseae* and *Paecilomyces lilacinus* to control the wilt disease complex of pigeon pea caused by *H. cajani* and *F. udum* and reported increased plant growth, nodulation, phosphorus content and reduced nematode multiplication and wilting in infected plants.

P. aeruginosa and *P. lilacinus* when used alone or together significantly reduced infection of *M. javanica* (Perveen 1998). Combined action of *Bacillus subtilis* strain GB03, *B. amyloliquefaciens* strain IN937a and *B. subtilis* strain IN937b together with chitosan in suppressing nematodes was reported by Kloepper *et al.* (2004). Babu *et al.* (1998) reported the beneficial effects of *Azospirillum* and *Phosphobacterium* in combination with mycorrhizal fungus, *Glomus fasciculatum* in a field trial on maize, for the control of *Pratylenchus zeae* in rice field. *Fusarium solani* strains with increased nematicidal activity repress the biosynthesis of nematicidal compounds by *P. fluorescens* strains *in vitro* and greatly alter its biocontrol efficacy against root knot nematode under natural conditions (Siddiqui & Shaukat 2003b). Combined efficacy of *Pasteuria penetrans* and two soil fungi, *P. lilacinus* and *Talaromyces flavus* and a bacterium, *B. subtilis* for the control of *M. incognita* on okra was reported by Zaki & Maqbool (1991). *B. licheniformis* and *Pseudomonas mindocina* and two fungi namely, *Acrophialophora fusicarpa* and *A. flavus* (Siddiqui & Husain 1991), *Paecilomyces lilacinus* and *Bacillus subtilis* alone and in combination on chickpea (Siddiqui & Mahmood 1993), *Bradyrhizobium japonicum* with *Pachonia chlamydosporia*, *P. lilacinus* and *Talaromyces flavus* (Ehteshamul Haque *et al.* 1994) are excellent examples of consortia concept.

Some bacteria associated with *A. oligospora* were tested by Duponnois *et al.* (1998) for their potential effects on fungus development (saprophytic growth and trapping

activity), multiplication of *M. mayaguensis* on tomato plants and growth of the host plant. Three bacterial strains, called Nematophagous fungus Helper Bacteria (NHB), *in vitro* enhanced fungal activity against the nematode, which resulted in better control of the nematode and improved plant growth.

Plant health promoting rhizobacteria (PHPR) reduced the root knot nematode, *M. incognita*, and promoted root colonization of arbuscular mycorrhizal fungi on tomato under greenhouse conditions. Arbuscular mycorrhizal fungi and the nematode antagonistic rhizobacterium, *Rhizobium etli* G12, significantly increased mycorrhizal establishment and reduced root penetration of *M. incognita*. Rhizobacteria and symbiotic fungi in some combinations work synergistically in controlling root-knot nematodes. The potential of using such multitrophic treatment combinations in integrated management targeted first at reducing nematode early root penetration in young transplants and for sustainable biological control of root knot nematodes under field conditions were reported (Siddiqui & Shaukat 2003).

Siddiqui and Shaukat (2004b) reported that *T. harzianum* enhanced the efficacy of the antagonistic rhizobacterium *P. fluorescens* in suppressing the root knot nematodes both *in vitro* and under glasshouse conditions through increased production of nematicidal compounds by the bacterium.

Microbial consortia concept need to be coupled with an ideal and quality product development which the farming community can handle with ease and thereby realize the benefits of the PGPR technology for sustainability in the perennial cropping systems (Mahmood 1993). The potential for growth promotion and disease suppression in all the plantation crops through PGPRs is high and need to be exploited for increased productivity and sustainability.

2.13. INDUCED RESISTANCE

Induced disease resistance is the phenomenon by which a plant exhibits an increased level of resistance to infection by a pathogen after appropriate stimulation. One of the first published reports of systemic resistance in plants was by Chester (1933) who used the term “acquired physiological immunity”. Later Ross (1961a; 1961b), reported that tobacco plants exhibited “systemic acquired resistance” systemically throughout the plant and is effective against a broad spectrum of viral, bacterial, and fungal pathogens.

Other terms like “translocated resistance” (Hubert & Helton 1967), “induced systemic resistance” (Hammerschmidt *et al.* 1982), “plant immunization” (Ku 1987) etc. were also used to describe this phenomenon.

Effective utilization of induced resistance in crop protection will require a functional understanding of the physiological consequences of the “induced” state of the plant, coupled with the knowledge of the specificity and compatibility of the signaling systems leading to this state. This information may guide related strategies to improve crop performance in suboptimal environments, and define the limits of induced resistance in certain agricultural context (Yao *et al.* 1997; 2000; Bakker *et al.* 2003; Faina Kamilova *et al.* 2005).

Resistance against potato cyst nematode *G. pallida* systemically induced by the rhizobacteria *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43) was studied using split-root technique by Hasky-Guenther, *et al.* (1998). These studies showed that *B. sphaericus* strain B43 and *A. radiobacter* strain G12, selected from the potato rhizosphere, significantly reduced *G. pallida* juvenile penetration into potato roots. Sturtz *et al.* (2000), Hallmann (2001), Ramamoorthy *et al.* (2001) and Bashan & Bashan (2002; 2004) are of the opinion that the close association formed between endophytic bacteria and the plant through bacteria mediated induced resistance might result in a stronger and more persistent plant protection.

Non pathogenic root colonizing rhizobacteria also trigger disease resistance in the host plant and is denoted as induced systemic resistance (ISR). The ISR is found to be through a plant mediated mechanism that extends to the above ground plant parts and several chemicals involved in the resistance have been identified. Salicylic acid produced by turnip crinkle virus induced SAR against *Pseudomonas syringae* in *Arabidopsis* (Uknes *et al.* 1993). Accumulation of the salicylic acid is associated with the coordinate induction of a set of atleast nine SAR gene families, which include the genes encoding the PR proteins (Van Loon & Van Kammen 1970) and phytoalexins (Ramamoorthy *et al.* 2001; Ran *et al.* 2005). SAR is always accompanied by an increase in the concentration of salicylic acid and induction of pathogen related (PR) proteins. Exogenously applied salicylic acid and other chemicals such as salicylic analogues, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazoles (Gorlach *et al.* 1996) and various pesticides (Kessman *et al.* 1994) can also induce systemic acquired resistance. SAR is induced by necrotizing

plant pathogens (Kloepper *et al.* 1992c; Pieterse *et al.* 1998; Bano & Musarrat 2003; Durrant & Dong 2004) and is distinguished from ISR, induced by non pathogenic microorganisms (Ryals *et al.* 1996; Meena *et al.* 2000; Vidyasekharan *et al.* 2001). Rhizobacteria-induced systemic resistance triggers a signal transduction pathway different from common pathogen or chemical induced pathways. Pathogen and chemically induced systemic resistance in plants is characterized by enhanced accumulation of pathogenesis related (PR) proteins, increased peroxidase activity and cell wall modifications. In contrast ISR, induced by nonpathogenic microorganisms does not involve the salicylic pathway or induction of PR proteins (Viswanathan & Samiyappan 1999; Hoffland *et al.* 1995; Radiacommare *et al.* 2005). Jasmonic acid and ethylene activate the ISR signaling pathway. There are different signaling molecules that trigger resistance pathways in the plant. Induction of systemic resistance in potato by rhizobacterium *Rhizobium etli* strain G12 is not associated with accumulation of pathogenesis related proteins and enhanced lignin biosynthesis (Reitz *et al.* 2001). The bacterial determinants of ISR may be the lipopolysaccharides, O-antigen lipopeptide etc. (Yao & Tian 2005; Touere *et al.* 2004; Piater *et al.* 2004; Zehnder *et al.* 2000; Reitz *et al.* 2000; Leeman *et al.* 1995a, 1995b). Different bacterial determinants appear to be involved in triggering ISR, like lipopolysaccharides, siderophores like pyoverdine and pyochelin by *P. fluorescens* and flagella (Maurhofer *et al.* 1994). *Bacillus subtilis* strain GB03, *B. amyloliquefaciens* strain IN937a and *B. subtilis* strain IN937b together with chitosan produced antibiotics, while IN937a and IN937b elicit induced systemic resistance against nematode (Kloepper *et al.* 2004). Klement *et al.* (2003) have identified local early induced resistance of plants as the first line of defense against bacteria. Different signaling pathways of induced resistance in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae* were detected by Ryu *et al.* (2003c).

Barker *et al.* (2003) reported that various strains of *Pseudomonas* spp. induce broad spectrum disease resistance in plants in a bacterial strain/plant species specific manner. In *Arabidopsis thaliana* specific strains of nonpathogenic fluorescent *Pseudomonas* bacteria develop ISR, unlike pathogen induced systemic acquired SAR, independent of salicylic acid (SA), but requires sensitivity to jasmonic acid (JA) and ethylene (ET). Combining ISR and SAR increased resistance against pathogens that resisted both JA/ET and SA dependent defenses, but not against pathogens that are affected exclusively by either ISR or SAR. Samiyappan (2004) working on the genes

behind the resistance reported that ISR operates in the *P. fluorescens* (Pf-1) treated plants by activating the genes encoding PR proteins and genes involved in the Phenyl propanoid metabolic pathway. Major induced PR proteins include chitinases namely, chi3 and chi9, β -1, 3-glucanases gluac and glubas, thaumatin like proteins (TLP), PR1 and peroxidases (Zehnder *et al.* 2000). The phenyl propanoid pathway products viz., phenolics and lignin, which were induced in Pf-1 treated plants showed enhanced resistance to several pests and pathogens. Major enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) were also induced (Cal *et al.* 2000; Ongena *et al.* 2004; Ryu *et al.* 2005). Pf-1 strain treatment appears to induce systemic resistance in both monocot and dicot plants which is polygenic in nature, suitable for sustainable management of several insect pests and plant pathogens (Hoon *et al.* 2001). *Rhizobium etli* induced systemic resistance against potato cyst nematode through a signal transduction pathway different from common pathogens or chemical induced pathways (Murphy *et al.* 2000; Reitz *et al.* 2001). Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants were reported by Hamid *et al.* (2005 a, 2005 b). Macromolecules released by a plant growth promoting rhizobacterium as elicitors of systemic resistance in tomato to pathogens are reported by Romeiro *et al.* (2005). Systemic resistance in tomato induced by biocontrol bacteria against the root knot nematode, *M. javanica* is independent of salicylic acid production (Siddiqui & Shaukat 2004a). Hauschild *et al.* (2004) tested combinations of different bacteria strains to induce systemic resistance in plants. Induced changes in plant metabolism were assessed for rhizobacteria alone or in combination with *F. oxysporum*. Activities of enzymes related to defense against pathogens as catalase, beta -1-3-Glucanase or peroxidases were determined under different conditions. Phenol patterns, chlorophyll contents and cell wall structure were analysed. Transient alterations were found for peroxidases. Changes were also observed for phenols and chlorophyll. Plant responses differ between various rhizobacteria strains, even when resistance is induced against the same pathogen. These alterations are probably not alone responsible for reduced wilt symptoms or reduced nematode penetration. Plant growth promoting rhizobacteria systemically protect *Arabidopsis thaliana* against cucumber mosaic virus by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway (Ryu *et al.* 2004). Macromolecules synthesized by the PGPR and released into the environment act as elicitors of systemic resistance (Romeiro *et al.* 2005).

Benhamou *et al.* (1998) reported that chitosan, deacetylated derivative of chitin, induces systemic resistance in tomato plant against *Fusarium oxysporium* f.sp. *radicis-lycopersic*. This suggests that plant defense mechanism might contribute to the overall nematode suppression. Stimulation of the lipoxygenase pathway is associated with systemic resistance induced in bean by a nonpathogenic *Bacillus* strain (Ongena *et al.* 2004).

Cytological observations of cucumber plants during induced resistance elicited by rhizobacteria by Jeun *et al.* (2004) revealed that inoculation with the PGPR strains caused no visible toxicity, necrosis, or other morphological changes. Cytological studies using fluorescent microscopy revealed a higher frequency of autofluorescent epidermal cells, which are related to accumulation of phenolic compounds at the sites of fungal penetration in plants induced with PGPR and challenged by the pathogen.

Cluzet *et al.* (2004) worked on the stability of N gene that confers resistance to southern root knot nematode, *M. incognita* in bell pepper and reported that nematode resistant cultivar may be useful component of cropping system.

Split-root experiments, that guarantee a spatial separation of inducing agent and a challenging pathogen, showed that soil treatment of one half of the root system with cell suspension of *P. fluorescens* resulted in a significant systemic induced resistance leading to reduction of *M. javanica* infection of tomato roots in the non-bacterized nematode treated half. The results clearly suggest that the antibiotic 2,4-DAPG from *P. fluorescens* CHA0 act as the inducing agents of systemic resistance in tomato roots (Siddiqui & Shaukat 2003d).

While studying the similarities and differences in the mode of action of *Bacillus* sphaericus strain B43 and *Agrobacterium radiobacter*, antagonistic to *Globodera pallida* on potato, Hallmann *et al.* (1998a) reported that induced resistance was triggered by vital and heat-killed bacterial cells of both strains and in the case of *Bacillus* also by bacterial culture filtrates. Fluorescent pseudomonads induce systemic resistance against root knot nematode via a signal transduction pathway, which is independent of SA accumulation in roots (Siddiqui & Shaukat 2004).

Press *et al.* (2001) evaluated the role of iron in rhizobacteria mediated induced systemic resistance in cucumber by rhizosphere bacterium, *Serratia marcescens* strain 90-

166, and suggest that catechol siderophore biosynthesis genes in bacterium is associated with ISR but that this role may be indirect via a reduction in internal root populations. Induction of systemic resistance against root knot nematode by *P. fluorescens* in tomato was reported (Jyothi & Sivakumar 2003). *P. fluorescens* strains protect plants from various pathogens by activating the defense genes encoding chitinase, B1-3 glucanase, peroxidase, phenyl alanine ammonia lyase and other enzymes involved in the synthesis of phytoalexins (Van peer *et al.* 1991; Chen *et al.* 2000a; Zehnder *et al.* 2000). Induced systemic resistance once expressed, activates multiple potential defense mechanisms that induce increased activity of chitinase and peroxidases (Ramamoorthy *et al.* 2001; Hussain *et al.* 2003).

2.14. MASS MULTIPLICATION AND FORMULATION

Formulation of biocontrol agents involves the incorporation of the viable propagules with carrier substances like inert matrix, food substances or their combinations to facilitate various application and delivery requirements of the BCAs. The commercial use of biocontrol agent requires inoculum that retains high cell viability and can easily be transported and applied (Kloepper & Schroth 1981). In addition formulation may facilitate shipping and storage of the biological control agents. Economic viability demands a higher shelf life (Balaraman & Hoti 1984; Saravana kumar *et al.* 2003; Pramod & Sivaprasad 2003). Practicalities of developing and registering microbial biological control agents are put forward by Kiewnick (2007). Prospects and problems for commercialization of fungal and bacterial antagonistic organisms for Indian farmers are reviewed by Ghosh & Jayanth (2006). The nature of BCA, site of action against the target pathogen and delivery requirements are the major deciding factors in determining the type of formulation of a BCA (Lewis 1991; Chaluvvaraju *et al.* 2003). Hence, sustaining the viability of BCA is the major concern during various stages of formulation processes *i.e.* fermentation (mass production of biomass), inoculum production, formulation and storage (Baker & Dunn 1990; Lumsden *et al.* 1995; Warrior *et al.* 2002). Many BCAs have been formulated into various forms of liquids, solids and wettable powders (Green *et al.* 1998; Lewis 1991; Lumsden *et al.* 1995; Radjacommar *et al.* 2002). However, dry formulations *i.e.* solid or powder formulation, are generally preferred over liquid formulations because they contain dry propagules of BCAs and, thereby, tend to withstand environmental extremes, thus providing extended shelf life and ease of transportation and storage (Lewis 1991). Many solid or powder formulations can

also be made in to liquid or water based suspensions to fulfill the delivery requirements of drench, spray or dip treatments (Lumsden *et al.* 1995).

Most commercially available BCAs and plant growth promoting rhizobacteria have been formulated in granular or powder forms (Lumsden *et al.* 1995). Wttable powder of *Streptomyces griseoviridis*-Mycostop (Tavonen & Avikainen 1987); *Bacillus subtilis*, Quantum 4000 (Connick *et al.* 1990) *P. fluorescens*, Dagger-G (Currier *et al.* 1988) and *P. cepacia* - Blue Circle (Looughlin *et al.* 1992) were commercially made available. Powder formulation was successfully prepared using pyrophyllite clay (Pyrex) as carrier substance (Papavizas *et al.* 1984; Papavizas & Lewis 1989).

One of the most studied formulations of BCAs is the granular or solid formulation which involves the immobilization of wet or dry biomass of BCA with an inert organic polymers such alginate, polyacrylamide or carrageen with appropriate bulking agents (Connick *et al.* 1990; Lewis 1991). Plant growth promoting rhizobacteria have been formulated in to alginate beads for delivery along with seeds (Bashan 1986). Embedding propagules of BCAs in a matrix of sodium alginate and bulking agents such as clay, lignite or bran was found to be an effective way of delivering most bicontrol agents (Fravel *et al.* 1998; Papavizas *et al.* 1987; Lewis 1999). Depending on the nature of the BCA, these formulations may contain adjuvants to assist in the establishment of BCA at the site of action (Foy, 1991; Lumsden *et al.* 1995). Talc powder is the most commonly used carrier for microbial inoculant formulations and can be safely used as carrier without adding any amendments (Santhi *et al.* 1999; Pramod & Sivaprasad 2003; Mathivanan *et al.* 2005). *P. aeruginosa* survived for 244 days in the powdered formulation, and when applied to soil it suppressed root rot and root knot diseases and improved the growth of tomato plants in both greenhouse and field. *P. aeruginosa* multiplied on talc showed better rhizosphere colonization than bare root dip or soil drench of the bacterium (Siddique *et al.* 2001).

Formulations of bacteria with inert carrier materials like talc powder, lignite and wheat bran and two adhesives, carboxymethyl cellulose and gum arabic recorded sustained viable counts up to 30 days and thereafter, a decline in viable counts was recorded (Chaluvaraju *et al.* 2003; Vaithiyanathan *et al.* 2007). Use of wheat bran, an agricultural waste is a low cost technology for the production BCA formulations (Kanavade & Kapadnis 2003). Addition of food based adjuvants to granular formulations

can enhance the persistence of BCAs and facilitate controlled release of BCAs at the site of action (Foy 1989; Lumsden *et al.* 1995).

The adhesive carboxymethyl cellulose was found to be better in retaining the shelf life of the formulations (Pramod & Sivaprasad 2003). The feasibility of incorporating a biocontrol organism into various pre-gelatinized carrier substances such as starch, to form granular and paste like formulations are reported by Lewis & Papavizas (1987) and Lewis *et al.* (1995). Granular formulations of the BCAs, *T. harzianum* and *G. virens* have been made with pre gelatinized corn starch flour mixture and used in controlling damping off diseases (Lewis *et al.* 1995). *Azospirillum brasilense* (ATTC29710) and *Pseudomonas* sp. (strain 84313) were entrapped in sodium alginate skim milk based beads for slow release and effective colonization of these bacteria on plant roots (Bashan 1986).

Kloepper & Schroth (1981) reported that gum guar, gum locust bean, gum arabic, gum karaya, gum tragacanth and xantham gum can be used for effective formulation of bacteria. Saleh *et al.* (2001) reported the use of local gamma irradiated vermiculite as a substitute for the peat based inoculant of *Azorhizobium*. Survival of *Azorhizobium* in peat was the best compared to vermiculite and different combinations of peat and vermiculite. The results indicated that vermiculite and its combinations with peat could be used as carriers for *Azorhizobium* inoculants. The shelf life of peat inoculants extended to 40 weeks, while vermiculite inoculant recorded shelf life of 28 weeks. Jeyarajan *et al.* (2000) reported that the substrates for seed treatment such as gypsum, industrial wastes, diatomaceous earth granules, wheat bran: sawdust formulation, wheat bran: peat, vermiculite-wheat bran, alginate pellets, farmyard manure, gobar gas slurry, press mud, paddy chaff, rice bran and groundnut etc, can effectively be used.

Management of root knot nematode in brinjal using formulations of *P. fluorescens* was achieved by Mahapatra *et al.* (2003). Bio-efficacy of the formulation of fluorescent pseudomonads isolates against reniform nematode, *Rotylenchulus reniformis* infecting cotton was demonstrated by Jayakumar *et al.* (2003). Six formulations of plant growth promoting rhizobacteria were effectively used against *M. incognita* by Kokalis *et al.* (2003). Biological suppression of reniform nematode, *Rotylenchulus reniformis* infesting finger millet by formulations of bacteria was reported by Rajendran & Cannayane (2000). Warrior *et al.* (2002), Siddiqui *et al.* (2001) and Li *et al.* (2002) used various formulations of bacteria against nematodes.

Kanavade & Kapadnis (2003) pointed out the clogging of the pump nozzle when wheat bran, rice bran, corn starch etc. are used for the formulation of bacteria and suggested fairly good filtration of the bran must be accomplished before pumping the bacterial solution and felt that a water soluble substrate has to be developed. The survival of *P. aeruginosa* strain 78 was tested by Ali *et al.* (2001) on mung bean seeds coated with a variety of substrates/carriers and was found that talc amended with carboxymethylcellulose or on gum arabic was the best. *Albizia saman* (*Samanea saman*) and *Cordia myxa* gum gave poor survival. On all substrates, the antagonist populations declined dramatically at 120 days after coating.

Mathew (2003) reported that coconut water alone or supplemented with 0.5% peptone is an excellent medium for mass multiplication of *P. fluorescens*. The bacterial counts in these two media were on par with that in the Standard King's B medium. Mathew also reported the suitability of KBM, PBM, coconut water, coconut water supplemented with 0.5% peptone and coconut water amended with 1% sugar for the mass multiplication of *Pseudomonas*. All the five media produced yellowish green diffusible pigment after 48 hours of inoculation and the bacterial counts were above 20 at 10⁻⁷ dilution. Renymol (2000) reported that coconut water was an efficient medium for mass multiplication of *Trichoderma*.

Lokesh & Ramana (2003) reported that plant extracts of *Chromolaena odorata* and *Semecarpus kakhalekanensis* can be used for the mass production of *Pseudomonas fluorescens*. Ethanol extract of *S. kakhalekanensis* had maximum stimulatory effect followed by distilled water extract of *C. odorata*. Methanol and petroleum ether extracts of *C. odorata* and petroleum ether and distilled water extracts of *S. kakhalekanensis* showed moderate stimulatory effect on the growth of PGPR, where as butanol extract of *C. odorata* and chloroform extract of *S. kakhalekanensis* were ineffective in growth stimulation of PGPR. The problems and prospects of mass production of bio-agents and their use in integrated pest management were discussed in detail by Rabindra *et al.* (2002).

MATERIALS AND METHODS

B. Beena “Studies on bacterial antagonists of plant parasitic nematodes of black pepper (*Piper nigrum* L.) ” Thesis. Indian Institute of Spices Research Calicut , University of Calicut, 2008

Chapter 3

MATERIALS AND METHODS

3.1. COLLECTION AND PRELIMINARY EVALUATION OF BACTERIAL ISOLATES

3.1.1. Survey and sampling

Random surveys were undertaken in black pepper growing areas of different districts of Kerala for isolation of bacterial and fungal antagonists. Soil and root samples were collected from the rhizosphere of healthy and nematode infected black pepper vines. Samples of roots and rhizosphere soils were also collected from the nematode antagonistic plants like *Chromola odorata*, *Strychnos nux-vomica*, *Pimenta dioica* (allspice), *Piper colubrinum* and from highly nematode susceptible plants like coleus (*Coleus blumeri*) and tomato (*Lycopersicon esculentum*). The top 3-5 cm of soil and litter were removed from base of the plants and about 250 cc of soil and 10 g of feeder roots were collected up to a depth of 30 cm in polythene bags. These samples were used for the isolation of various antagonists (Table 3).

3.1.2. Isolation of bacteria from rhizosphere soil and roots

Soil sample (10 g) was suspended in 90 ml sterile water blanks and the resultant suspension was agitated at 150 rpm for 30 minutes in a shaker at room temperature. Isolation of bacteria was done by the dilution plate technique (Walksman & Fred 1922; Johnson & Curl 1972). Serial dilutions up to 10^9 were made by pipetting one ml into additional dilution blanks having 9 ml sterile water. One ml of these dilutions was poured into sterile petri plates. Approximately 15 ml of the respective media at 45⁰ C were added to these plates, shaken well and incubated at 28⁰ C. The emerging colonies were sub

cultured, purified and stored. In the case of root samples, small segments of feeder roots were separated from the soil, placed in sterile distilled water and shaken at 200 rpm for 30 minutes. The resulting water suspension was serially diluted and plated on appropriate media as described above. For the isolation of endophytic bacteria from the roots, the root segments (1 g) were washed in sterile water and surface sterilized by dipping in 0.1% mercuric chloride for one minute, then for two minutes in 70% ethanol, followed by three washes with sterile water. The samples were macerated in sterile mortar and pestle aseptically and the tissue was extracted in 10 mM MgSO_4 and the dilutions were plated on to King's B agar (King *et al.* 1954) amended with Nalidixic acid (40 µg/ml), Ampicillin (100 µg/ml) and Kanamycin (50 µg/ml) or on fluorescent pseudomonad selective media (Kluefel *et al.* 1993). The suspensions were heated up to 65°C for 30 minutes to kill non-spore forming mesophiles (Gordon *et al.* 1973) and plated on *Bacillus* specific medium (ATCC medium 552, Atlas 1995), after it reached ambient temperature for the specific isolation of *Bacillus* species.

3.1.3. Isolation of bacteria from nematodes

Bacteria were isolated from egg masses and eggs of *Meloidogyne* spp. Roots of black pepper, infected with root knot nematodes were collected and washed thoroughly. The galls in the roots were teased carefully with the aid of a stereomicroscope and female nematodes and egg masses were extracted. The eggs and female nematodes were surface sterilized by treating with 0.1% Mercuric chloride, followed by 0.1% Streptomycin sulphate and rinsed thoroughly with sterile water. These surface sterilized eggs and females were placed separately on sterile cover slips (24 mm square) placed over Nutrient agar (NA) or Muller Hinton agar (MHA) petri plates and the resulting suspension was diluted and plated on Nutrient agar or Muller Hinton agar and incubated at 28°C for two days. Bacteria growing out were purified by sub culturing and were stored (Crump 1987).

Nematode infected root samples were collected from healthy and diseased pepper plants. Different stages of nematodes (larvae and adult females) were picked out and examined for the symptoms of bacterial infection. The diseased specimens were then washed and aseptically transferred to petri plates having sterile Muller Hinton agar medium. The growth of bacteria was recorded after 48 hours and then were purified by sub-culturing.

Nutrient agar and Muller Hinton agar were used as general media for isolation. *Bacillus* spp. and *P. fluorescens* were isolated using specific, selective or semi-selective media. Kings B medium (King *et al.* 1954) and *Pseudomonas* isolation agar were used for isolating *P. fluorescens* and *Bacillus* specific medium (Atlas 1995) was used for isolating *Bacillus*. Corn Meal Agar (CMA) was used wherever sporulation was required. In order to avoid fungal contamination and to minimize yeast growth, suitable antibiotics were added to the media.

After incubation, randomly selected bacterial colonies were streaked again for purification and stored in yeast extract glucose calcium carbonate agar slants. For long-term storage, the bacterial isolates were kept at -80° C in 20% glycerol (Feltham *et al.* 1978). These bacteria were streaked on nutrient agar amended with TTC plates and the morphological features of the colony were recorded and grouped according to the gram staining reaction.

3.1.4. Isolation of bacteria by baiting with sodium alginate prills.

Sodium alginate solution (3% w/v) was prepared by blending medium density sodium alginate with warm (60° C) demineralised water in a blender for 10-15 seconds. The solution was heated to approximately to 80° C in a microwave oven and left at room temperature for 3-4 hours until a clear solution, free of air bubbles, was obtained. This solution was mixed with an aqueous suspension of *M. incognita* eggs (2:1 v/v) to obtain a final alginate concentration of 25 eggs per ml. A cleaned screen was then immersed in the alginate egg suspension, lifted with forceps and the excess liquid was allowed to run off. The screen was then pulled slowly through glass rods aseptically to produce a film of uniform thickness (Plate. 4.C). This film was dipped in 0.25 M CaCl₂ for 3-5 seconds to form a solid alginate gel and rinsed thrice in demineralised water contained in beakers. The concentration of the eggs in the aqueous suspension was adjusted to 3-5 eggs per grid area. A metal spatula was inserted in to the soil and pushed slightly to one side and the alginate screen pieces were buried in the black pepper rhizosphere at a depth of 2-3 cm. The screen was placed into the soil which was pressed lightly against the film to seal the hole. After four days, the films were removed from the soil and washed with sterile water. The soil particles adhering to the screen surface were removed by brushing gently with a soft brush. The films were washed again in demineralised sterile water and stored

aseptically. The screens were then plated on to the surface of Muller Hinton agar for the isolation of bacteria.

3.1.5. Nematodes, culturing and maintenance, extraction from roots

The root knot nematode *M. incognita*, originally collected from black pepper was routinely maintained on tomato (*Lycopersicon esculentum* cv. Local) in green house conditions (Plate 3.A). The burrowing nematode *R. similis* was also extracted from black pepper roots cultured on carrot discs placed on 2% water agar (O'Bannon & Taylor 1968) and were maintained at $28 \pm 1^{\circ}\text{C}$ in a BOD incubator (Plate 3.B). Eggs of *Meloidogyne* was extracted by modification of Hussey's NaClO method (Hussey & Barker 1973 modified by Kabana *et al.* 1981). Galled roots were washed and cut into 2-3 cm pieces. Approximately 20g of roots were homogenized with 100 mL of water for 30 seconds in a warring blender. The resulting suspension was transferred to 1L bottle. Water was added to 400 mL and 200 mL of 1.8 % NaOCl solution was added. The bottle was stoppered and shaken gently for 3 minutes and its contents were poured to a 4L beaker with 2L of water. The suspension was then passed successively through 250, 75 and 20U m mesh stainless steel sieves. The contents of all but the 20 um sieve were discarded and the eggs retained there were rinsed with water. Roots were washed with tap water and the egg masses were collected after staining with phloxin B (Holbrook *et al.* 1983) rinsed in tap water, followed by sterile distilled water. Suspension of nematode eggs was prepared by dispersing the egg masses of root knot nematodes in SDW (Hussey & Barker 1973). The collected nematode eggs and juveniles were surface sterilized in 0.01 per cent mercuric chloride, washed in sterile water and subsequently treated with 0.01 percent streptomycin sulphate and washed again with sterile water.

3.1.6. Laboratory screening

The efficiency of the bacterial strains in suppressing nematodes was evaluated by conducting a number of in vitro experiments. Suppressive action of the bacterial isolates on *M. incognita* and *R. similis* was assessed in these experiments

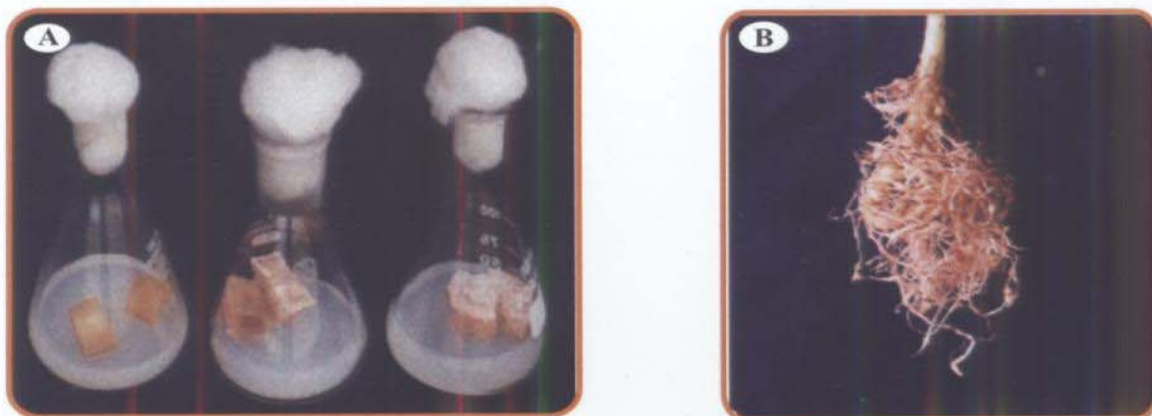


Plate 3. Culturing and maintenance of nematodes

- A. *Radopholus similis* cultured on carrot callus
 B. Culturing and maintenance of *Meloidogyne incognita* on tomato

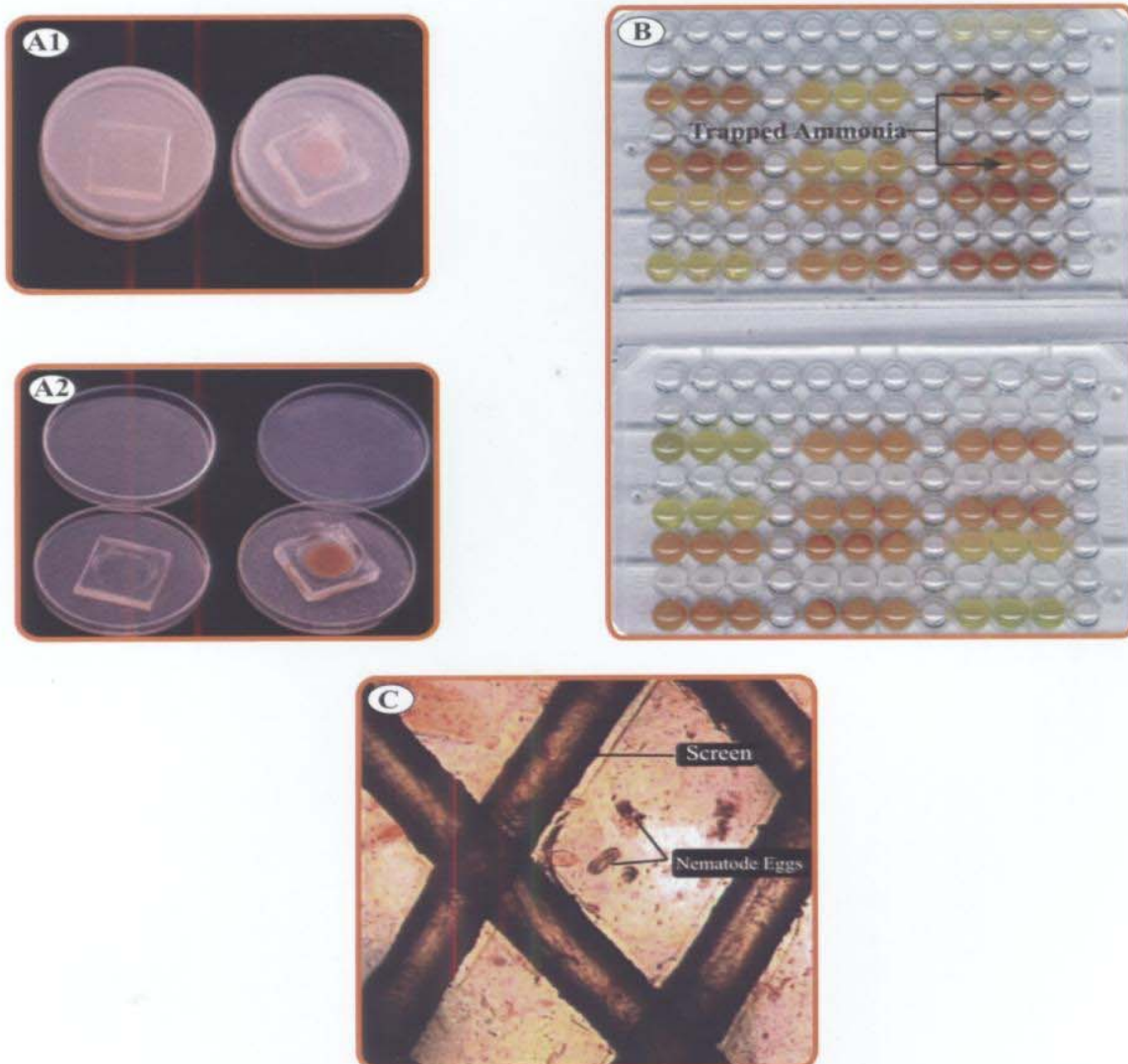


Plate 4. *In vitro* evaluation of bacterial isolates against nematodes

- A. Method of testing metabolites- i. Assembly of plates ii. Plates open
 B. Microtitre plates for testing culture filtrates
 C. Alginate prills used for isolation of bacteria

Screening against *M. incognita*

Bioefficacy of 108 bacterial isolates for suppressing *M. incognita* was evaluated *in vitro* by the following methods.

3.1.6.1. Buffer method

Total of 108 bacterial isolates were evaluated in this method (Cronin *et al.* 1997). Potassium phosphate buffer (pH 6.8) was taken in test tubes (5 ml each) and sterilized. In each of the tubes, a loop full of bacterial cells of the test isolate was added individually with a calibrated loop and maintained at 4° C for 10 days. Each of the buffers (100µl/well) was transferred to sterile 96 well microtitre plates. An aqueous suspension (100 µl) containing surface sterilized second stage juvenile of *M. incognita* (about 30-35 nematodes /100 µl) was added to each well. An uninoculated control was also maintained. Three replications were kept for each treatment. The microtitre plates were incubated at 28° C. After 48 hours, mortality of the juveniles was recorded with the aid of a stereomicroscope.

3.1.6.2. Assay of bacterial culture filtrates

Bacterial test stains were inoculated with a calibrated loop into 5 ml sterile peptone water aseptically, incubated at 28° C for 48 hours and then centrifuged at 13,600 g for 5 minutes. The supernatant was aseptically removed and placed in to 96 well microtitre plates (100 µl/well). Each of these wells was surrounded by wells containing 0.05 M sulfuric acid to trap toxic ammonia released by the culture filtrate (Plate 4.B). Controls consisted of 100 µl each of sterile peptone water and sterile distilled water. A suspension (100 µl) of surface sterilized second stage juveniles of *M. incognita* was added to each well. After 48 hours, mortality of the juveniles was recorded with the aid of a stereomicroscope. The trapped ammonia was detected with Nessler's reagent (Neipp & Becker 1999). Culture filtrates of 107 bacterial isolates were tested.

3.1.6.3. Direct action of bacterial cells (Nematicidal properties of bacterial suspension)

Direct action of the bacterial cells was assessed by adopting the procedure of Tian & Riggs (2000). Peptone water was prepared in 5 ml aliquots in test tubes, sterilized and

inoculated with 100 μ l of 24 hours old bacterial suspension and incubated at 28⁰ C for 24 hours. Bacterial suspensions (1 ml) were transferred to sterile cavity blocks and surface sterilized second stage juveniles of *M. incognita* were added to these suspensions. They were covered with sterile lids, sealed with aqueous solution of borax powder to eliminate contamination and were maintained at 28⁰ C. Mortality of juveniles was recorded with the aid of a stereomicroscope after 48 hours. Simultaneously uninoculated controls were also maintained. Total of 108 bacterial isolates were evaluated in this method.

3.1.6.4. Effect of volatile and non-volatile metabolites

Nematode toxic volatile and non volatile metabolites released by 71 bacterial isolates were evaluated by modifying the method of Tarabily *et al.* (2000). Nutrient agar media (50 ml each) was prepared in conical flasks and sterilized. To these 100 μ l of bacterial suspensions were added at 45⁰ C, mixed well and poured into sterile petri plates. After solidification, 50 μ l of juvenile suspension of *M. incognita* having 20-25 surface sterilized J2 were added to the bacteria seeded agar plates and also to nutrient agar plates without any bacteria. Two ml 0.05M H₂SO₄ was added to the sterile cavity blocks kept inside the seeded agar plates to trap the toxic ammonia liberated by the bacterial isolates (Plate 4.A). The lids of the plates were removed aseptically and each of the seeded agar plate was covered by the peritheca of the nutrient agar plates and were sealed with parafilm, and were incubated at 28⁰ C. Suitable control plate assemblies having no bacteria were also maintained. After 24 hours, 100 μ l of Nessler's Reagent was added to the cavity blocks having 0.05M H₂SO₄ and noted the intensity of the colour developed. The mortality rate of the juveniles both in the seeded agar plates was recorded with the aid of a stereomicroscope. Live juveniles were distinguished from dead ones by the addition of 2-3 drops of 1N NaOH.

Screening against *R. similis*

As the buffer method was found to be the most effective for the *in vitro* evaluation of bacteria against nematodes, this method was selected to evaluate some effective bacterial strains (46 isolates) against *R. similis*. The methodology was the same as described in 3.3.1.1, except that the nematode used were *R. similis* instead of *M. incognita*.

3.1.7. Greenhouse evaluation

Potential biocontrol agents of soil borne plant pathogens can be screened *in vitro* or in soil. *In vitro* screening usually selects organisms that produce toxic metabolites or parasitize the target pathogen. Furthermore, organisms that have good antagonistic activity *in vitro* often have no biocontrol activity in soil (Racke & Sikora 1992). Therefore, in this study the greenhouse screening was conducted in the heat treated soil in greenhouse because this would allow for selection of multiple mechanisms of antagonism including antibiosis, competition and parasitism.

The preliminary screening of the bacterial isolates was done by a series of greenhouse experiments using tomato as the test plant. Heat treated soil was used to reduce the risk of plant diseases from pathogens in the soil and also for eliminating the adverse effect of the other organisms on the introduced bacteria.

Purified bacterial strains were grown in 100 ml nutrient broth overnight (200 rpm at 28°C). Bacteria were pelleted (7,000 rpm, 20 minutes), re-suspended in 60 ml distilled water. The optical density was adjusted to 0.8 with sterile distilled water (SDW). Tomato seedlings (10 days old, local variety) raised in vermiculate were carefully uprooted, washed in sterile water and the roots were dipped in the respective bacterial suspension for 15-20 minutes. After inoculation, the seedlings were planted individually in plastic pots containing sterilized potting mixture. Additional bacterial suspension (10 ml) was pipetted into the soil surrounding each seedling. Control plants received only sterile water. There were three replications for each treatment and the pots were arranged in randomized design in the greenhouse. The seedlings were watered daily with boiled and cooled water. Nematode suspension (5 ml) containing 100 J2 of *M. incognita* was inoculated to each plant, 20 days after bacterial inoculation. The plants were uprooted carefully 30 days after inoculation of nematode. Growth parameters like height, shoot weight, root weight etc. were recorded. The root system was washed with water and stained with phloxin B (Fenner 1962; Holbrook *et al.* 1983). The number of galls and egg masses were recorded. Gall index was recorded according to the 0-5 galling index scale (Barker 1985). Nematode population in roots was estimated after staining the root system with lactophenol-acid fuchsin (Mc Beth *et al.* 1941; Franklin & Goodey 1959). The data were analyzed using ANOVA and means were separated by least significant difference. The nematode counts were square root transformed prior to statistical analysis.



Plate 5. Method of evaluating bacterial isolates for the suppression of nematodes in black pepper under greenhouse condition



Plate 6. Method of evaluating bacterial isolates for inducing resistance to nematodes under greenhouse condition

A. Split root Experiment
C. Control

B. Experiment of nodal runners overview
D. Treated with Ap4

Altogether 216 bacterial isolates were evaluated in eleven separate greenhouse experiments.

3.2. CHARACTERIZATION OF SHORT LISTED BACTERIAL ISOLATES AND THEIR MODE OF ACTION ON NEMATODES

3.2.1. Identification of bacteria

The isolated colonies were identified by studying colony characters, Gram staining, morphology and biochemical reactions. The bacterial isolate were grown on nutrient agar amended with 2,3,5-triphenyl tetrazolium chloride (TTC). Attempts were made only to identify *Pseudomonas* and *Bacillus* species as they were selectively isolated. Guide lines in the Bergey's Manual of Systematic Bacteriology were followed.

3.2.2. Molecular characterization

3.2.2.1. Isolation of bacterial DNA

1 ml of 24 hours old bacterial culture was taken and pelleted at 10,000 rpm for 10 minutes at 4°C. The Pellet was washed in sterile water two times by centrifugation. 675µl of DNA extraction buffer was added and incubated 37°C for 30 minutes. Then 20% SDS was added and incubated at 65°C for 2 hrs with constant agitation in water shaker, centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant was collected without disturbing the pellet. Equal volume chloroform: isoamyl alcohol (24:1) was added and recovered aqueous phase without disturbing the interphase between organic and aqueous phase. To this 0.6 volume of isopropanol was added and incubated for 1 hour or over night at 28°C. This was centrifuged at 10,000 rpm for 10 minutes with a temperature of 28°C, discarded the supernatant retaining the pellet. To this pellet added 70% ethanol, centrifuged at 10,000 rpm for 10 minutes in room temperature, discarded supernatant dried in vacuum for 30-60 min. The pellet was dissolved in 50µl of sterile distilled water kept at 37°C for 30 min and the DNA was stored at 4°C.

3.2.2.2. Quantification of DNA

The DNA pellet was dissolved in 50µl sterile distilled water and was further diluted to 1:10 using sterile distilled water. The concentration of DNA determined using

spectrophotometer. The quantified DNA was reconstituted to get 50ng/μl. The purity of DNA extracted was visualized on 0.7 % agarose gel.

3.2.2.3. Agarose gel electrophoresis

Agarose (0.7%) was prepared in 1x TAE with ethidium bromide (final concentration 0.5 μl/ml) and cast in Hoefer gel caster with well placed comb. 5μl of DNA sample was mixed with 2μl gel loading dye (6x) and loaded in each well. 2μl λDNA was loaded in the first well. The gel was electrophoresed at 60 volts for 1.50 hours in Hoefer HE33 mini horizontal submarine unit. The DNA band was visualized under UV transilluminator in alpha imager gel documentation system.

3.2.2.4. Polymerase chain Reaction

A PCR was performed to obtain DNA amplification of product by using 16s primer. 23μl mastermix and 2 μl DNA 50ng/μl was mixed in a PCR tube. (Ramesh *et al.* 2002)

Table 1. Primer sequences for amplifying r DNA gene

Primer	Sequence (5'-3')
16SF	AGAGTTTGATCCTGGCTCAG
16SR	AAGGAGGTGATCCAGCCGCA

These primers are expected to produce 1500 bp amplicon.

3.2.2.5. Thermo cycling conditions

PCR was done in an Eppendorf master cycler gradient under the following conditions:

STEP	TEMPERATURE	DURATION
1. Initial denaturation	92 °C	2 min, 10 sec
2. Denaturation	94 °C	1 min, 10 sec
3. Annealing	49°C	30 sec
4. Extension	72°C	2 min
Repeated for 34 cycles		
Final Extension	72°C	10 min
Holding	4°C	

3.2.2.6. Restriction *analysis*

The final PCR products were resolved in 0.7% agarose. PCR products were restricted with Taq 1, Sau3A1 and Alu1 adopting the manufactures instructions (GENEI). Master mix was prepared by mixing double distilled water (7.3µl), Restriction enzyme buffer - 2µl, BSA-0.2µl, Restriction Enzyme (4 unit) -0.5µl.

10µl of restriction mix and 10µl of 16S rDNA was taken in a 0.2ml microtube. It was restricted in a water bath shaker (Taq1 - 65°C for 3 hour, Sau3A1 -37°C for 4 hour and Alu 1 37°C for 16 hour incubation). The restricted product resolved in 3 % agarose gel. The gel was photographed on a UV transilluminator and the results were documented in alpha imager Analysis System for analysis

Comparison of banding pattern generated from each strain. Restriction profile was scored manually as 1 for the presence of band and 0 for the absence of band, assuming that the bands with molecular size in different individual were homologous. The final binary data was analyzed using NTSYS software and similarly coefficient was calculated and the tree plot was constructed by Unweighed Pair Group Arithmetic Average (UPGMA).

3.2.3. Hatching suppression

Root knot nematode egg masses were collected as described earlier and nematode egg suspension was prepared by treating the egg masses with sodium hypochlorite (Hussey & Barker 1973 modified by Kabana *et al.* 1994). Peptone was prepared in 5 ml

aliquots in test tubes, sterilized and inoculated with 100 µl of 24 hours old bacterial suspension and incubated at 28⁰ C for 24 hours. This bacterieial suspension (1 ml) was dispensed to cavity blocks containing root knot nematode eggs (100 numbers) to check the effect of hatching. Eggs treated with sterile water served as control. Short listed 25 bacterial isolates were screened to evaluate their effect on hatching.

After 14 days, the number of eggs hatched was counted at x 400 magnifications and per cent hatch was calculated using the following formula

$$\text{Hatch \%} = \frac{J_{14} - J_0}{\text{Eggs } 0} \times 100$$

Eggs 0

Where J 14 = the number of juveniles observed at day 14.

J 0 = the number juveniles observed at day zero.

Eggs 0 = the number intact eggs counted at day zero.

3.2.4. Phosphate solubilization

Pikovskaya's solid medium (Pikovskaya 1948) was prepared, sterilized and poured into sterile plates. One loop full of the respective bacterial culture was spot inoculated on the plates at three different spots on the plates. Calibrated loop was used for the inoculation to ensure uniformity of the cultures. Twenty five short listed bacterial isolates were tested for phosphate solubilization. The plates were incubated at 28⁰ C for 5-6 days. The plates were then observed for clearing zones around the colony and the diameter of the clearing zone was measured.

3.2.5. Effect of bacteria on the mycelial growth of *Phytophthora*

The effect of 25 selected nematode antagonistic strains on the suppression of *Phytophthora capsici*, the fungal pathogen causing foot rot disease in black pepper was evaluated by the dual culturing method (Dennis & Webster 1971; Utkhede & Rahe 1983). The virulent *P. capsici* isolate 99-101 was obtained from the National Repository of *Phytophthora* (NaRPh) maintained at the Indian Institute of Spices Research (IISR), Calicut. Plates containing 15 ml of Potato Dextrose agar medium (PDA) was centrally inoculated with 5 mm discs of 72 hour old culture of *P. capsici* growing on carrot agar

(Ribeiro1978). Bacterial culture was streaked on either side of the fungal disc at a distance of 2 cm and incubated in the dark for 96 hours at $25\pm 2^{\circ}\text{C}$. Plates without bacterial isolate served as check and the experiment was replicated five times. The inhibition zone between bacteria and the pathogen was also recorded (Jin & Hee 1989). The per cent inhibition of mycelial growth was calculated using the formula

$$I = \frac{100 (C-T)}{C}$$

Where I = inhibition of mycelial growth, C = growth (cm) of pathogen in the control plate and T = growth (cm) of pathogen in dual cultures.

3.2.6. Production of HCN

Ninety four bacterial strains were characterized for the production of HCN. Production of HCN was determined using a modified procedure of Wei *et al.* (1991). Bacteria were grown on King's B medium amended with of glycine in a petri plate, then filter paper strips soaked in picric acid solution was placed in the lid of each petriplate. The petriplates were sealed with parafilm and incubated for 2-4 days. HCN production was indicated by the change in colour of the filter paper strips from yellow to brown to red. Reaction was scored as low, medium and high, depending on the intensity of the colour.

3.2.7. Production of H₂S

Ninety four bacterial strains were also characterized for the production of H₂S . Sterile SIM agar slants were prepared and stab inoculated with the bacterial isolates to be tested. These inoculated SIM agar slants were incubated at 37°C for 24-48 hours. The slants were observed for the change in the colour of the media, if any. Positive reaction is indicated by the blackening of the media and the negative reaction by no change in the colour.

3.2.8. Production of ammonia

Toxic ammonia produced by the bacterial isolates were trapped by 0.05 M H₂SO₄ and were detected by Nessler's reagent as in 3.1.6.2

3.2.9. Detection of siderophores by UV- Fluorescence method

Twelve selected isolates were tested for the production of siderophores. Log phase culture (10 μ l) of *Pseudomonas* strains were streaked on to Pseudomonas agar for fluoresin (PAF) and Pseudomonas agar for pyocyanin (PAP) and incubated at 28⁰ C for 48 hours. The plates were observed on a UV trans-illuminator to record the fluorescence (Kloepper *et al.* 1980 c).

3.2.10. Detection of protease enzyme activity

Twenty five short listed bacterial isolates were screened for the production of protease enzymes. To test for proteolytic activity, colonies were streaked on peptone broth supplemented with skim milk powder (10%) and solidified with 1.5% of agar on plates. The plates were incubated at 28⁰ C for 96 hours until the zone of clearing could be seen around the colonies.

3.2.11. Detection of gelatin liquefying capacity of bacterial isolates

Gelatin media was prepared, dispensed in 20 aliquots in culture tubes and autoclaved. The tubes were inoculated with 10 μ l log phase cultures of the bacterial isolates to be tested and incubated at 28⁰ C for 48-72 hours. After incubation, these tubes were kept at 4⁰ C in a refrigerator for 4 hours. The tubes were observed for the liquefaction of gelatin. Uninoculated media kept in the same conditions served as the control. Twenty five short listed bacteria isolates were screened for detection of gelatin liquefying capacity.

3.2.12. Antibiotic sensitivity

Antibiotic resistant screening was performed for the identification of resistant markers in the short listed 25 bacterial isolates. Fourteen antibiotics (Table 2.) were dissolved in distilled water or in ethyl alcohol and filter sterilized by using membrane filtration (0.22 μ m) in a Millipore system. Nutrient agar was taken in 100ml conical flask and autoclaved at 121⁰C for 20 min at 15 lbs, required quantity of antibiotics were added to the medium to get the following final concentrations: Antibiotic amended medium was poured in Petri plates and inoculated with 2 μ l of 24 h old bacterial cultures in the marked columns. The plates were incubated for 24 – 48 h at 28⁰C and observations were taken. Colonies developed on plates which were comparable to non-amended plates were scored as resistant types.

Table 2. List of antibiotics tested

Sl No.	Antibiotic	Concentration ($\mu\text{g/ml}$)
1	Nalidixic acid	40
2	Ampicillin	100
3	Tetracycline	15
4	Kanamycin	50
5	Gentamycin	10
6	Chloramphenicol	30
7	Streptomycin	100
8	Cycloheximide	100
9	Rifampicin	100
10	Penicillin	100
11	Polymyxin	100
12	Spectinomycine	25
13	Bacitracin	100
14	Trichloro Tipheryl Tetrazolium chloride (TTC)	10

3.2.13. Utilization of carbon sources

In order to study the utilization of different carbon sources by the bacterial strains, the basal medium for oxidation/fermentation (O/F) was used (Block & Sweetmore 1994). The ingredients of the medium are given in annexure -1. Medium (100 ml) was prepared with 1% concentration of the different carbon sources *viz.* glucose, lactose, maltose, galactose, mannose, sorbitol, dulcitol and sucrose. Sterile carbon source amended medium (700 μl) was taken in sterile microfuge tube and stab inoculation was performed with a log phase culture of the bacteria to be tested and incubated at 27⁰ C for two weeks. The change in colour of the medium from olivacious green to blue/yellow indicated the capacity of the bacterium to utilize that particular carbon.

3.2.14. Suppression of nematodes infecting black pepper

Twenty five bacterial isolates, short listed based on their efficiency in both *in vitro* and *in vivo*, were further evaluated against *M. incognita* and *R. similis* under green house conditions.

The selected bacterial isolates were multiplied in peptone broth. Fresh stem cuttings of black pepper variety, Panniyur 1 were dipped in the bacterial suspension ($\times 10^{11}$ c.f.u. /ml) for 10 minutes and planted in sterilized potting mixture. Further, 10 ml of the respective bacterial suspension was poured on to the root zone. Two weeks after the bacterial inoculation, each plant was inoculated separately with *R. similis* and *M. incognita* @ 50 nematodes per plant. An absolute control and a nematode treated control were also maintained. There were three replications for each treatment and the plants under each experiment one for *M. incognita* and the other for *R. similis* were arranged separately in completely randomized design in a greenhouse (Plate 5). After seven months, the plants were uprooted and observations on plant biomass, root weight and height were recorded for each plant. The nematode population in roots was estimated using standard procedures. The data were analyzed using ANOVA and means were separated by least significant difference. The nematode counts were log transformed prior to statistical analysis.

3.2.15. Induced resistance

3.2.15.1. Split root experiments

Split root bioassays were performed for better understanding the mechanism of nematode suppression by the bacteria.

Tomato (*Lycopersicon esculentum* cv. Local) seedlings were raised in sterile vermiculite. One month old seedlings were uprooted carefully without damaging the root system. The root system was split into two equal halves and one half of the roots was dipped in the bacterial suspension ($\times 10^{11}$ c.f.u./ml, multiplied in peptone water) for 10 minutes. The treated seedlings were planted in such a way that the each half of the root system is placed in a separate poly bag filled with sterilized potting mixture. Further, 10 ml of the respective bacterial suspension was poured on to the root zone after planting in the polybag. Care was taken to avoid contact between these separated halves of the root system. The seedlings were properly supported with the help of a wooden pieces attached

to the cup. Two weeks after the bacterial inoculation, the other half of the seedling was inoculated with *M. incognita* @ 50 nematodes per plant. An absolute control (no bacteria and no nematode) and a nematode treated control were also maintained. There were three replications. The treatments were arranged in completely randomized design (CRD) in a green house and maintained for three months. The plants were watered with boiled and cooled water carefully to avoid cross contamination. After three months, the plants were uprooted and observations on plant height, root weight and plant biomass were recorded for each plant. The nematode population in two halves of the root system was estimated using standard procedures. The data were analyzed using ANOVA and means were separated by least significant difference. The nematode counts were log transformed prior to statistical analysis. Two separate bioassays were conducted in which a total of 25 bacterial isolates were tested.

The split root experiment was also conducted using black pepper seedlings following the same procedure as described above (Plate 6 A). Three month old black pepper seedlings raised in sterile vermiculite were used and the experiment was terminated after three months.

3.2.15.2. Nodal roots of runner shoots

As black pepper is a climber, it produces roots at every node. The induced resistance experiment was attempted in another way. The roots from each node were planted in separate polybags and bacterial suspensions were added to the first poly bag and *M. incognita* @ 50 nematodes per plant was added to the second poly bag. An absolute control (no bacteria and no nematode) and a nematode treated control were also maintained. There were three replications. The treatments were arranged in completely randomized design in a green house and maintained for three months (Plate 6. B). The plants were watered with boiled and cooled water carefully to avoid cross contamination. After three months, the plants were uprooted the nematode population in two halves of the root system was estimated using standard procedures. The data were analyzed using ANOVA and means were separated by least significant difference. The nematode counts were log transformed prior to statistical analysis.

3.3. STUDIES ON MOST PROMINENT ISOLATES

3.3.1. 16S rDNA sequencing for bacterial identification

16S rDNA of five short listed bacterial isolates were sequenced. The DNA of each isolate were isolated as in 3.2.2. The final PCR products of the five short listed bacterial isolates were resolved in 0.7% agarose in 1X TAE (Tris Acetate EDTA buffer) at room temperature for 2 hours. The gel was added with Ethidium Bromide (EtBr) and photographed on an UV transilluminator and the results were documented by Alpha imager analysis system for analysis. The 1500bp PCR product was excised from the gel and weighed. Gel elution was done using GENEI elution kit procedure. Lyophilized and send for sequencing in GENEI, Bangalore.

3.3.2. Effect of promising organisms on root knot nematodes infesting black pepper

The experiment was conducted in three different types of potting mixtures namely, normal potting mixture, sterilized potting mixture and potting mixture naturally contaminated with nematodes. Five selected isolates of bacteria were evaluated. Each isolate of bacteria was multiplied in peptone broth. Fresh cuttings of black pepper variety, Panniyur 1 were dipped in the bacterial suspension ($\times 10^{11}$ c.f.u. /ml) for 10 minutes and planted in the above mentioned three types of potting mixtures. Further 10 ml of the respective bacterial suspension was poured on to the root zone. Two weeks after the bacterial inoculation, each plant was inoculated with *M incognita* @ 50 nematodes per plant. An absolute control (no nematode, no bacteria) and a nematode treated control were also maintained. There were three replications. The treatments were arranged in completely randomized design in a greenhouse. After seven months, the plants were uprooted and observations on plant height, plant biomass and root weight were recorded for each plant. The nematode population in roots was estimated using standard procedures. The data were analyzed using ANOVA and means were separated by least significant difference. The nematode counts were log transformed prior to statistical analysis.

3.3.3. Effect on rhizobacteria in root initiation and proliferation

The selected five isolates were multiplied as described earlier to get suspensions of uniform turbidity. Three node cuttings of black pepper variety, Panniyur 1 were dipped in the bacterial suspension (25×10^9 cfu/ml 0.1M MgSO_4) for 10 minutes and planted in plastic pots filled with sterile potting mixture. There were 16 replications per each treatment. Black pepper cuttings dipped in sterile water served as the control. The pots were arranged in completely randomized design in a moisture chamber set up in a greenhouse. Plants from four replicates from each treatment were uprooted every month and observations were recorded. The experiment was terminated after four months. The root growth was graded visually and the endophytic nature of the bacteria was assessed at the end of the experiment.

3.3.4. Compatibility studies

3.3.4.1. Compatibility with other organisms

Compatibility among different bacterial isolates was analyzed according to Mariano *et al.* (2000) and modified agar disc method (Siddiqui 2000). The compatibility of bacteria with fungus was assessed by the dual culture plate method as described by Siddiqui (2001). The compatibility of the bacterial isolate with these fungal isolates was also tested in another way. These fungal isolates were grown on sterile cellophane sheet spread over potato dextrose agar (PDA) plates and carefully removed the fungal mycelium along with cellophane sheet. Disc (4 mm) were cut from the PDA plates and placed on the lawn plate culture of Ap4 and incubated for 4-5 days.

3.3.4.2. Compatibility with agrochemicals

The compatibility of the biocontrol agents with agrochemicals was studied by the poisoned food technique (Zentmeyer 1955) in nutrient agar medium. Nutrient agar medium was prepared in conical flasks (100 ml) and sterilized. The requisite quantity of the chemical to be tested was added separately to the medium so as to get the required final concentration of the agrochemical. A series of concentrations from 6 ppm to 48 ppm were prepared. The chemicals were thoroughly mixed by stirring. The media was cooled up to 45°C and 100 μl of 24 hour culture of the bacteria was added and mixed again and poured in to sterile petri plates aseptically. Suitable checks were also kept at the same condition on nutrient agar medium. The plates were incubated at the optimum

temperature. The colony forming units were counted after 48 hours and compared with those of the control.

3.3.5. Effect of pH

The ability of the test organisms to grow at various pH levels was studied using nutrient broth. Nutrient broth at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were used for the study. The pH of the medium was adjusted to the required level using NaOH or HCl. The nutrient broth having different pH was dispensed in 50 aliquots in 100 ml conical flasks and sterilized at 15 lbs for 15 minutes. The test organisms were inoculated from a 24 hour old culture and incubated at 30⁰ C for 24-48 hour in a rotary shaker at 150 rpm. The optical density of the cultures was measured at 6 hours intervals using a spectrophotometer. The population of the bacteria was calculated spectrophotometrically at 595 nm.

3.3.6. Combined inoculation of bacteria and antagonistic fungi

Pot culture experiments on the management of root knot nematodes through consortia of biocontrol agents were conducted under greenhouse conditions. The experiments were conducted in black pepper with field soil. Three fungal isolates exhibiting antagonistic property against nematodes viz. *Trichoderma harzianum*, *Paecilomyces lilacinus* and *Pochonia chlamydosporia* were obtained from the culture collection of Nematology Department, IISR, Calicut, and tested along with the potential bacterial isolates. The fungal and bacterial inocula were prepared separately as reported earlier. Fresh cuttings of black pepper variety, Panniyur 1 were dipped in the bacterial suspension (2.6×10^{11} c.f.u. /ml) for 10 minutes and planted in sterilized potting mixture. Further 10 ml of the respective bacterial suspension was added to the root zone. The fungal isolates, multiplied in rice grains (2.5×10^9 cfu/g of the carrier), were used as the fungal inoculum. After inoculation the seedlings were planted individually in plastic pots containing potting mixture. The plants were inoculated with J₂ of *M. incognita* @ 100 plant⁻¹ two weeks after the inoculation of the biocontrol agent as per the treatments given below.

Treatments

T1 - Control (No nematode; no antagonist)

- T2 - Nematode alone
- T3 - *Paecilomyces lilacinus* alone (P.l) Is.36
- T4 - *Trichoderma harzianum* alone (T.h) Is. 33
- T5 - *Pochonia chamydosporia* alone (P.c) Is.32
- T6 - *Pseudomonas aeruginosa* alone (P.a) Ap4
- T7 - P.l + P.a
- T8 - T.h + P.a
- T9 - P.c + P.a

Four replications were maintained for each treatment and the pots were arranged in a completely randomized design in green house. The experiment was terminated three months after nematode inoculation. Observations on nematode population, plant growth characters and microbial colonization were recorded at the time of termination of the experiment. For assessing the microbial colonization on the roots, the roots were washed in sterile water and plated on to PDA and nutrient agars amended with chloramphenicol and were observed for the growth of the respective organism after an incubating for 2-5 days depending upon the organism to be observed.

3.3.7. Rejuvenation of nematode infected black pepper

Five efficient strains of rhizobacteria, short listed in earlier experiments were tested for their efficacy in rejuvenating the black pepper seedlings infected with root knot nematodes. Black pepper seedlings having foliar yellowing with a yellowing index of 5 and roots showing symptoms of nematode infection like root galls were collected from the nurseries. Nematode populations of randomly selected seedlings were estimated after staining the root system of with lactophenol acid fuchsin (Franklin & Mc Beth 1941). The root systems of these selected seedlings were washed in sterile water. The bacterial strains were grown in 300 ml nutrient broth overnight (200 rpm at 28⁰ C). Bacteria were pelleted (7,000 rpm 20 minutes) and re-suspended in 180 ml of sterile distilled water. The optical density was adjusted to 0.8 with sterile distilled water. The roots of the seedling were dipped in the respective bacterial suspension for 10 minutes and were planted individually

in plastic bags containing potting mixture. Additional bacterial suspension of 10 ml each as per the treatments was pipetted into the soil surrounding each seedling. Control plants received only sterile water. There were ten replications for each treatment and the bags were arranged in a randomized design in the green house. The seedlings were watered daily with boiled and cooled water. Yellowing index of all the seedlings was recoded every month using the foliar index on 0-5 scale (No yellowing-0; 0-20 % yellowing-1; 20-40 % yellowing-2; 40-60% yellowing-3; 60-80% yellowing-4 and 80-100% yellowing-5).

The experiment was terminated after four months. At the time concluding the experiment, nematode populations in the root system were estimated (Franklin & Mc Beth *et al.* 1941).

3.3.8. Evaluation of biocontrol formulations

3.3.8.1. Sodium alginate beads

Sodium alginate solution was prepared by dissolving 20 g of dry sodium alginate in a minimum quantity (10 ml) of sterile distilled water. This mixture was poured in 1 litre of swirling, warm distilled water and allowed to mix on the stirrer for 30 minutes until a homogeneous suspension was obtained. The alginate solution was sterilized for 15 minutes at 121⁰ C. Ten ml of *P. aeruginosa* (Ap 4) suspension (3.1×10^9 cfu/ml) was added to the mixture and was allowed to swirl in the stirrer until ready for adding into the droplet device. A droplet forming device was constructed by attaching a 1 litre reagent bottle with a spout at the bottom to a T-valve outlet system. The entire device was sterilized for 15 minutes at 121⁰ C. The sodium alginate solution amended with the bacterial suspension and stirred continuously while the suspension was allowed to drip through an Eppendorff tip, attached to the T valve into a sterile solution of 0.1M CaCl₂. The resulting beads were then allowed to stand in fresh 0.1M CaCl₂ solution for 30 minutes, filtered through a sterile cheese cloth and washed three times with sterile distilled water. The resulting beads were stored as such and also in dried condition at 40⁰ C and also at room temperature.

Various additives were added to the sodium alginate solution to study their effect on the shelf life of these formulations. The additives tried were rice bran, ginger leaf

powder, tea waste, tapioca powder and coir compost. The resulting beads were also processed in the same way as described above and stored.

For determining the bacterial load in the sodium alginate beads, three randomly selected beads were homogenized separately in 10 ml sterile water with a homogenizer under aseptic conditions. One ml of the resulting homogenate was plated as pour plate method using King's B medium and incubated for 48 hours and the colony forming units were counted at 15 days intervals. Similarly the bacterial load in dried beads was also estimated.

3.3.8.2. Granular formulations

The granular formulations were prepared by mixing 200 g clay and 50 g gum arabic with 800 g of rice bran, processed in the mixer until fine powder was obtained and the carrier was sterilized at 70⁰ C for three days. The sterilized carrier was mixed aseptically with 300 ml of *P. aeruginosa* (Ap 4) suspension (3.1×10^9 cfu/ml) in a blender. The granules were aseptically dried overnight and passed through a 2 mm sieve.

Similarly, granular formulations with ginger leaf powder, tapioca powder and coir compost were prepared as described above instead of rice bran. These powder formulations were transferred into polypropylene bags, (100 g each) and stored at 4⁰ C and at room temperature. The shelf life of these granular formulations was compared by estimating the bacterial load in each formulation at different intervals. One gram of the formulation was withdrawn at 15 days interval for a period of 90 days, subjected to serial dilution plating and assessed for viable population (cfu/g) of bacteria.

3.3.8.3. Talc-based formulations

A loopful of *P. aeruginosa* (Ap 4) was inoculated into the Kings B broth and incubated in a rotary shaker at 150 rpm for 48 hours at room temperature (28±2°C). After 48 hours of incubation, the bacterial broth containing 3.1×10^9 cfu/ml was used for the preparation of talc-based formulation. To 400 ml of bacterial suspension, 1 kg of the commercially available talc, (sterilized at 105⁰ C for 12 hours), and calcium carbonate 15 g (to adjust the pH to neutral) were mixed under sterile conditions, following the method described by Vidhyasekaran & Muthamilan (1995). After shade drying for over night under sterile conditions, it was packed (100 g each) in polypropylene bags and sealed.

To evaluate the effect of adhesives on the shelf life of the bacterial strains gum arabic 10 g/kg and carboxymethyl cellulose (10g/kg) were used separately. These bags were stored at 4⁰ C and also at room temperature.

RESULTS

B. Beena “Studies on bacterial antagonists of plant parasitic nematodes of black pepper (*Piper nigrum* L.) ” Thesis. Indian Institute of Spices Research Calicut , University of Calicut, 2008

Chapter 4

RESULTS

4.1. COLLECTION AND PRELIMINARY EVALUATION OF BACTERIAL ISOLATES

4.1.1. Collection and maintenance of test organisms

Altogether 190 soil and root samples of black pepper and nematode antagonistic plants were collected and processed using standard techniques. Samples were drawn from all the pepper growing districts of Kerala. Maximum samples (65) were collected from black pepper. Besides black pepper, samples were also drawn from *P. colubrinum*, *Chromolena odorata*, *Strychnos nuxvomica*, Allspice and *Coleus* plants. In total 93 samples were collected from the above four antagonistic plants. Thirty two samples were taken from *Coleus* plants, which is highly susceptible to root knot nematodes. The soil and root samples collected from different plants yielded a large number of bacteria and those with distinct colony morphological features were selected and stored. In total 319 bacteria were isolated using different methods, purified and maintained for further screening. Seventy bacterial isolates were directly isolated from soil and isolation through the baiting of alginate prills yielded a total of 16 bacteria. Fifty two bacteria were isolated directly from different nematode stages. Roots of black pepper, coleus and nematode antagonistic plants yielded a total of 181 bacteria (Table 4, Plate 7).

Table 3. Soil and root samples collected from different districts of Kerala for isolating nematode antagonistic bacteria

District	Crop					Coleus	Total
	<i>Piper nigrum</i>	<i>Piper colubrinum</i>	<i>Chromolena odorata</i>	<i>Strychnos nuxvomica</i>	Allspice		
Ernakulam	6	-	5	7	5	5	28
Idukki	10	-	5	6	6	5	32
Kannur	5	-	5	-	-	5	15
Kasaragod	10	-	-	-	-	-	10
Kozhikode	12	10	5	5	5	10	47
Malappuram	7	-	6	6	5	7	31
Trichur	5	-	-	-	2	-	7
Wynad	10	-	5	5	-	-	20
Total	65	10	31	29	23	32	190

Table 4. List of bacteria isolated from samples collected from various parts of Kerala

	<i>Pseudomonas</i>	<i>Bacillus</i>	Unidentified	Total
<i>Isolation from soil</i>				
Direct isolation	25	7	38	70
Alginate prills	6	2	8	16
Total	31	9	46	86
<i>Isolation from nematodes</i>				
Egg masses	4	2	16	22
Juveniles	2	5	10	17
Females	3	4	6	13
Total	9	11	32	52
<i>Isolation from plant roots</i>				
<i>P. nigrum</i>	9	12	45	66
<i>P. colubrinum</i>	5	5	8	18
<i>C. odorata</i>	4	4	6	14
<i>S. nuxvomica</i>	5	4	11	20
Allspice	4	5	4	13
Coleus	10	5	35	50
Total	37	35	109	181
Grand total	77	55	187	319

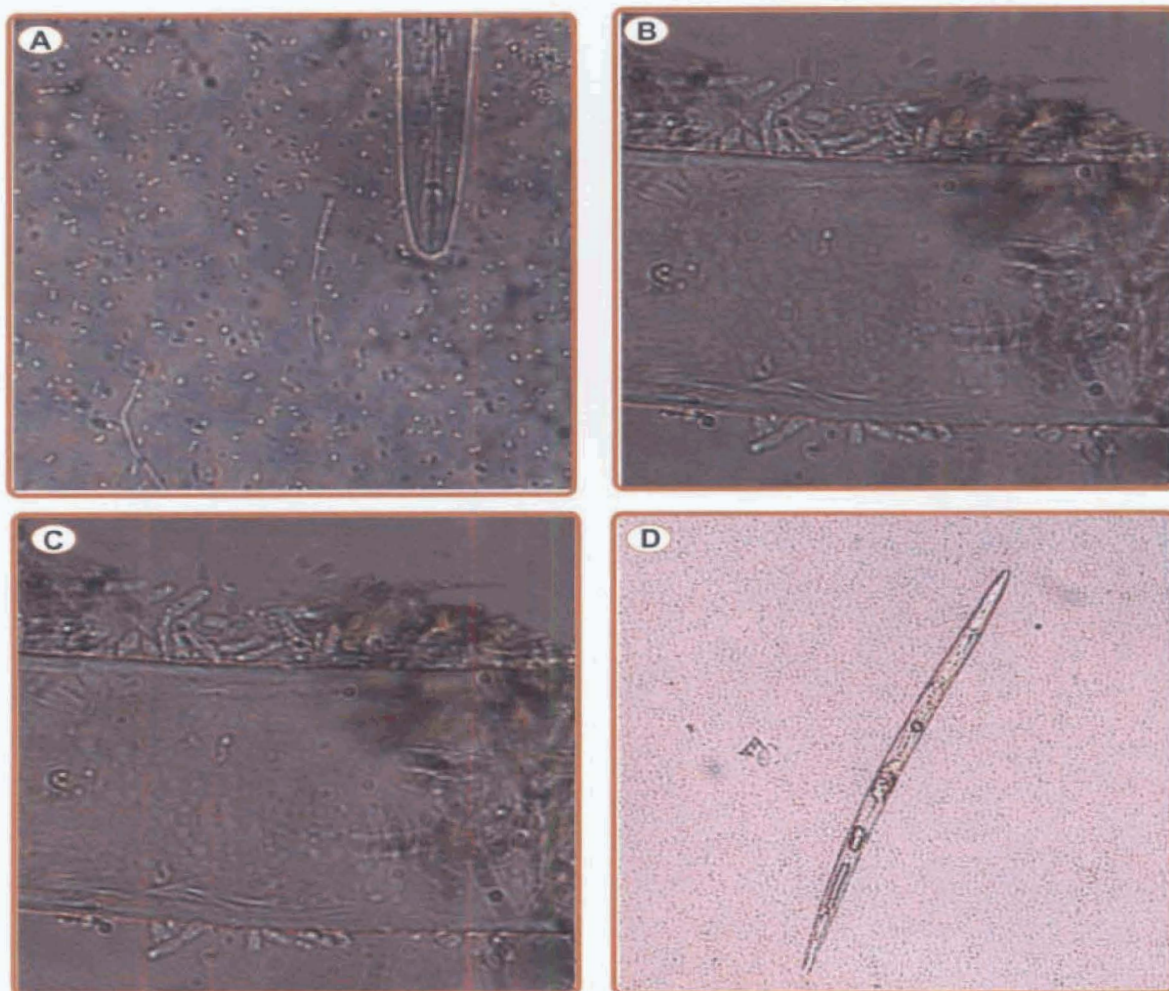


Plate 8. Suppression of *M. incognita* juveniles with bacterial isolates

A. Treated with Is. 136

B. Treated with Is. 127

C. Treated with B15

D. Treated with Is. 132

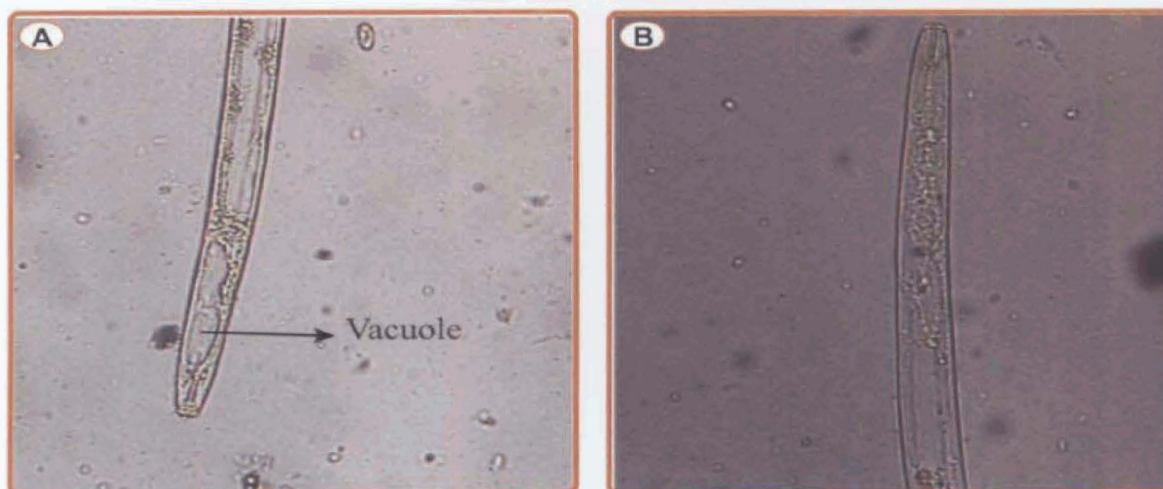


Plate 9. Suppression *R. similis* with bacterial isolates

A. Treated with Ap4

B. Treated with B2

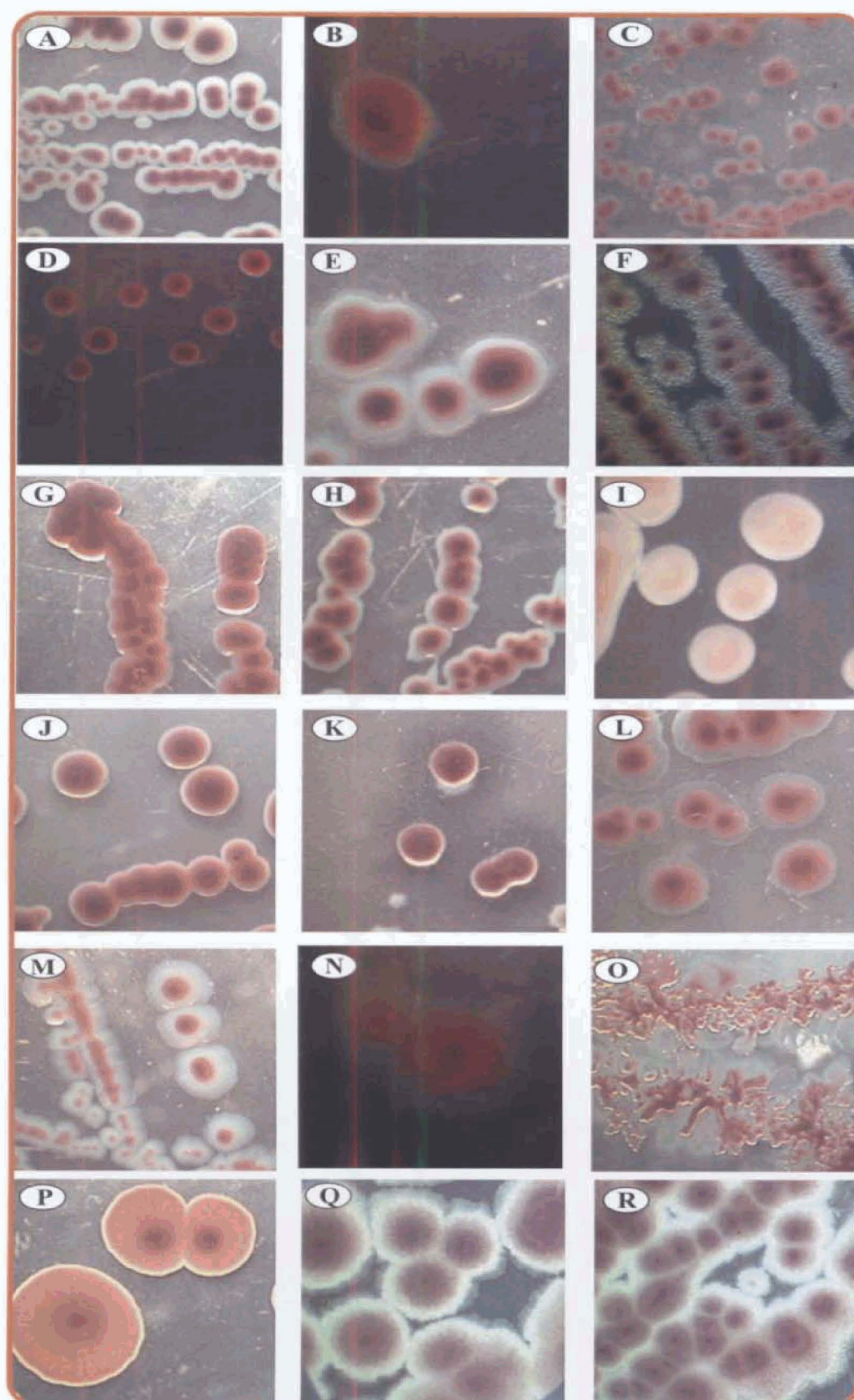


Plate 7. Colony morphology of some bacterial isolates in Nutrient agar media amended with TTC

A. Is. No.13 B. Ap4 C. B15 D. Ap8 E. 18 F. 24 G. B14 H 101 I. 117
 J. Is No.123 K. Is No. 127 L. Ap 5 M. Is No. 19 N. Ap.8 O. Is No. 136
 P. Is No. 113 Q. Is No. 118 R. Is No.119

4.1.2. Laboratory screening

4.1.2.1. Screening against *M. incognita*

A total of 108 isolates were screened against *Meloidogyne* in various *in vitro* experiments. Culture suspensions of 108 bacterial isolates were tested for their efficacy to control nematodes. In the buffer method, the metabolites released into the buffer suspension of the test bacteria were evaluated in 96 well microtitre plates (100 µl/well). Mortality of the juveniles of *M. incognita* was studied by adding *M. incognita* juveniles to this suspension of bacteria after 48 hours of incubation. The direct action of bacterial cells was also evaluated by adding nematodes directly to the bacterial cell suspension in peptone water. Similarly, cell free culture filtrates of 107 bacterial isolates were also evaluated by assaying the mortality of root-knot nematode juveniles. The toxic ammonia released from the culture filtrates was trapped by 0.05 M sulfuric acid so as to detect the effect of culture filtrates per se. Effect of bacterial metabolites (both total and volatile) of 71 isolates on *Meloidogyne* juveniles was also studied in bacteria seeded agar plates.

Mortality of nematodes was comparatively high in peptone water than in buffer solution. More than 90 per cent of bacterial isolates caused very high mortality (>90 %) to nematodes in peptone water (Table 5, Plate 8). The mortality ranged from 32.05% by Is. No. 114 to 98.03 % by Is. No. 109. However in buffer solution only three isolates (Is. 105, Is.110, and Is. 113) caused more than 80 per cent mortality to nematodes. Mortality was very high in culture filtrates too. Culture filtrates and total metabolites of majority of isolates were highly toxic to nematodes. Volatile metabolites of 27 isolates caused more than 90 per cent mortality to nematodes. The highest mortality was noticed in Is. 125 (99.19 %) followed by Is.113 (99.10%) and Is.110 (98.92 %). The results of various *in vitro* tests reiterated the superior biocontrol potential of the isolates tested.

4.1.2.2. Screening against *R. similis*

Altogether 46 isolates of bacteria were evaluated against *R. similis* by the buffer method. All the bacterial strains tested suppressed *R. similis* to varying extents (Table 6, Plate 9). The percentage of suppression ranged from 15.03% by the isolate B 12 to 80.43% by isolate No. Ap 4. More than 70% suppression was achieved by seven isolates (Is. 68, Is. 77, Is. 124, Is. 136, Ap 4, Ap10, Ap 16 and B2). It is highly significant that those strains effective against *Meloidogyne* were also effective against *R. similis* too.

Table 5. Mortality of root knot nematodes by different isolates of bacteria in different *in vitro* methods (Mean of three replications)

Is. No.	Nematode mortality (%)				
	In peptone broth	In phosphate buffer	In culture filtrate	Metabolites	
				Total	Volatile
2	81.77	23.65	62.11	NT ^b	NT
7	89.46	26.12	8.13	NT	NT
9	91.35	24.25	65.42	NT	NT
11	92.01	16.93	43.83	NT	NT
12	94.33	26.30	54.77	NT	NT
13	92.50	37.28	50.42	NT	NT
17	93.28	13.06	71.59	NT	NT
18	93.99	23.52	71.60	NT	NT
19	94.68	16.50	63.07	NT	NT
21	93.11	24.86	54.46	NT	NT
22	94.29	23.87	40.40	NT	NT
23	94.92	29.04	73.24	NT	NT
24	94.59	9.67	80.14	NT	NT
25	93.25	13.42	42.83	NT	NT
62	97.70	32.54	93.02	94.26	8.66
101	94.49	27.14	NT	NT	NT
105	89.06	80.42	59.60	28.43	2.04
106	89.10	26.50	92.78	97.03	98.91
107	91.21	79.78	85.53	98.75	69.22
108	87.58	51.87	92.72	58.99	9.17
109	98.03	28.54	91.80	98.46	4.48
110	87.16	87.83	92.15	98.23	98.92
111	89.27	48.14	92.21	96.18	96.00
112	87.66	46.81	91.98	87.83	97.29
113	87.10	84.63	82.64	84.88	87.08
114	32.05	19.26	90.90	52.42	40.19
115	88.42	61.23	91.25	95.10	97.71
116	94.76	18.52	58.05	96.12	39.34
117	95.83	25.66	89.19	95.61	47.48
118	96.43	27.31	79.10	95.82	97.01
119	90.32	66.38	94.44	53.62	95.45
120	90.22	55.32	92.92	95.10	97.71
121	93.60	46.28	93.49	41.47	51.83
122	93.95	32.84	93.43	97.83	68.18
123	88.60	28.20	85.91	98.16	89.12
124	90.33	63.18	87.49	98.09	96.33
125	95.35	21.65	94.88	98.09	99.19
126	55.34	34.25	92.72	96.08	95.18
127	94.69	24.12	88.64	NT	NT
128	94.68	11.74	95.93	NT	NT
129	94.94	17.94	93.89	95.10	97.71
130	93.42	13.73	95.25	NT	NT
131	94.51	33.17	93.90	97.64	35.38
132	92.67	25.46	90.81	NT	NT
133	93.50	18.47	92.15	98.26	99.10
134	93.42	23.13	90.50	NT	NT
135	93.50	16.76	91.55	NT	NT
136	95.40	36.32	90.67	98.87	97.64
Ap1	93.89	26.67	31.92	NT	NT
Ap2	94.52	27.39	49.70	NT	NT
Ap3	94.94	26.01	36.56	NT	NT
Ap4	93.60	34.10	95.15	NT	NT
Ap5	93.9	33.09	73.41	NT	NT

Table 5. Continued

Ap6	94.69	23.55	59.29	NT	NT
Ap7	94.67	26.59	83.20	NT	NT
Ap8	94.93	27.52	87.88	NT	NT
Ap9	92.29	26.08	84.67	NT	NT
Ap10	93.68	47.73	90.01	NT	NT
Ap11	92.67	12.05	77.56	NT	NT
Ap12	93.50	26.77	62.06	NT	NT
Ap13	92.39	22.04	18.04	NT	NT
Ap14	93.08	18.12	73.31	NT	NT
Ap15	95.40	58.79	85.62	NT	NT
Ap16	91.63	34.54	90.82	NT	NT
B1	94.77	12.97	93.60	52.42	40.19
B2	95.09	14.26	94.29	79.51	17.85
B3	94.07	57.36	91.60	98.26	99.10
B4	93.43	12.68	9.25	32.90	5.90
B5	94.26	23.71	93.25	78.16	64.58
B6	94.91	10.68	93.92	98.61	59.93
B7	92.78	8.01	93.51	97.48	98.93
B8	91.04	16.09	48.32	81.32	51.28
B9	69.91	20.48	94.01	21.11	16.88
B10	93.04	19.52	94.48	95.13	32.56
B11	89.17	13.12	74.02	92.98	31.38
B12	93.01	16.90	94.17	71.25	57.34
B13	92.45	33.66	94.31	66.56	12.77
B14	93.77	20.58	91.47	92.81	18.89
B15	92.98	13.96	88.02	98.70	2.63
B16	92.34	50.47	93.53	92.16	27.49
C1	96.01	18.88	30.28	89.88	86.29
C2	97.18	38.11	77.99	94.65	86.11
C3	95.09	29.24	40.68	97.61	94.64
C4	96.17	23.66	33.62	95.54	95.61
C5	96.36	12.74	94.95	96.68	95.91
C6	95.29	27.21	82.50	97.01	92.79
C7	95.09	19.96	88.34	96.60	95.34
C8	96.16	7.32	68.65	96.30	95.59
C9	95.39	36.41	68.59	98.26	93.14
C10	96.36	29.25	40.28	96.04	95.38
C11	95.99	26.02	94.28	97.05	10.67
C12	94.75	44.61	92.62	98.87	15.30
C13	94.92	24.76	92.89	99.09	95.46
C14	92.86	46.92	92.12	96.63	93.02
C15	95.70	31.48	92.37	98.57	81.74
C16	94.49	43.39	42.90	96.93	75.01
C17	93.97	63.78	98.19	97.38	97.83
C18	93.97	41.81	99.05	97.31	70.38
C19	97.98	9.43	98.87	98.25	7.55
C20	94.73	19.82	36.67	56.26	44.92
C21	93.56	25.56	98.00	98.09	78.38
C22	95.08	22.93	98.46	98.51	78.38
C23	95.00	20.74	94.77	98.78	88.94
C24	96.56	26.12	93.29	98.92	7.17
C25	97.98	37.48	83.20	33.97	23.47
C26	93.97	27.94	87.89	50.01	74.36
C28	95.70	22.11	84.68	98.60	6.39
C29	92.86	37.48	92.01	98.16	26.04
Control ^a	10.42	4.50	10.45	8.88	4.58

ANOVA was not performed because the bacteria were evaluated in different batches.

^a pooled mean of different set of experiments

^b Not Tested

Table 6. Mortality of *Radopholus similis* treated with different isolates of bacteria
(Mean of three replications)

Sl. No.	Is. No.	Nematode mortality (%)	Sl. No	Is. No.	Nematode mortality (%)
1	13	44.46ab	24	136	79.62
2	18	41.54abc	25	Ap 1	64.14ab
3	19	55.29ab	26	Ap 4	80.43a
4	24	41.33abc	27	Ap 5	60.48ab
5	25	54.24 ab	28	Ap 8	63.88ab
6	68	71.73a	29	Ap 10	76.96a
7	76	46.37ab	30	Ap 11	64.81ab
8	77	74.08a	31	Ap 16	73.68a
9	81	25.23abc	32	B1	44.32ab
10	83	38.53abc	33	B2	78.31a
11	85	53.83ab	34	B3	37.11abc
12	86	42.06abc	35	B4	53.83ab
13	89	43.90ab	36	B5	41.62abc
14	101	55.67 ab	37	B6	25.23abc
15	113	61.97 ab	38	B7	39.19abc
16	115	33.15abc	39	B8	48.22ab
17	116	42.51abc	40	B9	29.18abc
18	117	68.31ab	41	B10	24.47abc
19	118	29.33abc	42	B11	24.87abc
20	119	42.60abc	43	B12	15.03abc
21	123	53.08ab	44	B13	41.12abc
22	124	74.08a	45	B14	58.19ab
23	132	61.08ab	46	B15	38.20abc
Control		4.85c			

Data are arsine transformed prior to analysis. Means followed by the same alphabet in a column do not differ significantly according to Duncan's multiple range test.

Each of the bacterial isolate differed in their effect on suppressing nematodes in different methods. Though, *in vitro* evaluation of bacteria for nematode suppression is having different advantages over greenhouse methods, the tested isolates exhibited different level of nematode antagonism in different methods and thus it is found to be difficult to shortlist effective strains based on *in vitro* analysis.

4.1.3. Greenhouse evaluation

The efficiency of the bacterial isolates in controlling *M. incognita* was evaluated in a series of greenhouse experiments using tomato as the test plant. Bacterized tomato plants were challenge inoculated with *M. incognita* juveniles after 20 days of treating with bacteria. On assessing the growth parameters after 30 days of nematode inoculation, significant increase in plant growth was observed in most of the bacteria treated tomato plants as compared to the *M. incognita* inoculated and untreated control. The control plants showed the lowest growth in all the eleven set of experiments. Significant increase in height and biomass was also observed in most bacteria treated plants with shoot weight being more (Table 7).

Compared to the nematode alone treated control, most of the bacteria tested restricted the root gall development and reproduction of *M. incognita* (Table 7, Plate10a & 10b). However their ability varied considerably. The experimental results are highly significant. The results of the present study clearly indicated that the antagonists tested can be short-listed depending upon their bio efficacy in the greenhouse experiments.

In experiment No. 1, all the twelve bacterial isolates used, significantly increased the growth of tomato both in terms of total biomass and height of the plants (Table 7a).

All the treatments differed significantly from each other in increasing the growth of the tomato plants. Isolate No. 13 recorded maximum height of 36.50 cm (138.04 % increase over control) followed by isolate No. 11 (35.40 cm *i.e.* 130.87 % increase over control). Maximum biomass was achieved by isolate No. 11 (5.77 g *i.e.* 223% increase over control) followed by 220 % increase by isolate No. 3 (5.72 g). Maximum root weight was recorded with bacterial isolate No. 2 (1.27 g). Total nematode suppression was achieved with the bacterial isolate No. 13 which is very effective both in promoting the growth of the tomato plant and in suppressing nematodes.

In experiment No. 2, out of the 18 isolates used, 12 isolates resulted in significant increase in the height of the plants (Table 7b). Maximum growth promotion with respect to height was observed with Is. No. 86 (34.97 cm *i.e.* 79.83 % increase over control) followed by isolate No. 81 (32.27 cm. *i.e.* 66.91 % increase over control) and in biomass with Is. No. 84 (4.79 g *i.e.* 112.43 % increase over control) followed by Is. No. 74 (3.92 g *i.e.* 73.96 % increase over control). All the isolates caused significant nematode suppression but none of them gave complete control of nematodes. Maximum nematode suppression was achieved with isolate No. 78.

In experiment No. 3, none of the nine isolates used resulted in significant increase in the growth of tomato and some of them even decreased the height of the plants with respect to the untreated control plants (Table 7c). Isolate No. 93, 94 and 98 resulted in growth retardation of the plant with regard to height and isolate No. 95 reduced total biomass. Maximum growth of the tomato plant was observed in the treatment with bacterial isolate No. 92, which recorded a height of 26.33 cm and a shoot weight of 5.02 g and root weight of 3.28 g. But all the isolates used resulted in significant nematode suppression even though none of them could give 100% nematode suppression. Maximum nematode suppression was achieved by isolate No. 102 with respect to both number of egg masses and galls.

In experiment No. 4, where 21 isolates were tested, most of them increased the growth of the plant (Table 7d). Maximum height was reported with two isolates, Is. No. 121 and Is. No. 123 where a height of 31.50 cm was recorded (65.79 % increase over control) followed by Isolate No. 108 with 30.83 cm (an increase of 62.28 % increase over control). Maximum biomass was recorded for the isolate No.119 (3.61 g) followed by Is. No. 108. In this experiment, the increase in root weight was less compared to the other set of experiments and some isolates even retarded the root weight. Nematodes were significantly suppressed by all the isolates and 100% nematode suppression was achieved by seven isolates.

In experiment No. 5, all the 23 isolates used significantly increased the growth of tomato both in terms of height and total biomass of the plants. Majority of the bacterial isolates used in this study were *Pseudomonas* spp. (Table 7e). Maximum growth promotion was achieved by isolate P9 (34.74 cm) followed by P 12 (32.66 cm). Highest shoot weight was recorded for the isolate P 7 (4.78 g) followed by P 1 (3.95 g).

Maximum root weight was achieved in the treatment of bacterial isolate No. P1. Out of the 23 isolates, 18 suppressed nematodes significantly where as the other five isolates enhanced the nematode attack. Three isolates (P13, P16 and P17) resulted in 100% suppression of nematodes.

In experiment No. 6 and 7, where 16 and 15 isolates were used respectively, all of them significantly increased the growth of tomato plants and suppressed nematodes significantly (Table 7f and 7g). In experiment No. 6 the isolate No. 68 recorded the maximum growth of tomato (35.83 cm height) followed by isolate No. 28 (35.00 cm). Maximum shoot weight of 3.82 g was reported for the isolate No. 64 followed by isolate 30 with a shoot weight of 3.81 g. Maximum root weight of tomato was obtained when treated with bacterial isolate No. 19. Seven isolates resulted in 100 % nematode suppression. In experiment No. 7, the bacterial isolates used were originally isolated from the black pepper rhizosphere in Silent Valley biosphere. Maximum biomass and height of tomato was observed in the treatment of bacterial isolate No. B5 (35.66 cm *i.e.* 272.10 % increase over control) followed by B3 (34.56 cm) and B 14 (34.00 cm) while maximum shoot weight was obtained when treated with B5 (5.02 g) followed by B 14 (4.93 g). Maximum root weight is obtained with B 15 (2.99 g) followed by B 11 (2.95 g). Three isolates, B5, B14 and B 15, resulted in 100% nematode suppression too.

In experiment No. 8, all the 28 isolates used were effective in both increasing the growth of tomato plants along with nematode suppression (Table 7h). Maximum height of tomato plants was obtained with the use of bacterial isolate No. 53 which resulted in 35.33 cm height followed by isolate No. 39 and 55 recording 35.00 cm height each. Maximum increase over control in the total biomass of the plants was achieved by the use of bacterial isolate No. 51 (8.80 g) followed by Is. No. 46 (8.87 g). Most of the bacterial isolates resulted in uniform height of the tomato while the fresh biomass varied with bacterial strains. All the isolates were effective in controlling nematodes too. Ten isolates resulted in 100 % nematode suppression.

In experiment No. 9, out of the 28 isolates were used, majority of them belonged to *Pseudomonas* spp, and all the isolates improved the growth of the plants both in terms of height and total biomass and suppressed the nematodes (Table 7i). Maximum height of the tomato plants was obtained when Is.No.101 was used (37.61 cm) followed by the Is. No. 71 (35.00 cm). Maximum shoot weight was achieved by the Is. No 71(5.07 g)

followed by the Is. No. 132 (4.75 cm). Maximum root weight of 1.65 g was achieved by the Is. No. 101 and 1.56 g by Is. No. 71. All the isolates except two have suppressed nematodes significantly. Total nematode suppression was recorded with six isolates. They are Is. No. 101, 71, 59, 132, 133 and 136.

In experiment No. 10, 16 bacterial strains isolated from nematode antagonistic plants isolates were used and maximum height of the tomato plants was obtained by Isolate No. Ap 4 (38.31 cm) followed by Ap 16 (Table 7j). Maximum shoot weight was recorded by the Is. No. Ap. 4 (5.04 g) then by Ap. 16 (4.85 g). Maximum root weight was obtained with Is. No. Ap. 4 (2.92g) and Ap. 16 (2.82 g). Seven isolates resulted in 100% nematodes suppression.

In Experiment No. 11, where 30 strains isolated from Coleus, a highly nematode susceptible plant were used, maximum height of the plants was observed in plants inoculated with bacterial Is. No. C22, (45.01 cm) followed by C11 (47.17 cm) (Table 7k). Is. No. C 20 recorded a shoot weight 7.40 g. Three isolates (C9, C18 and C19) gave 100% nematodes suppression.

It was observed that the bacterial treatment in some plants resulted in minute uniformly round galls resembling *M. hapla* infection with profuse secondary and tertiary roots. In contrast, the control plants had larger galls typical of *M. incognita* infection. It was shown that some of the developing root galls had no nematodes associated with them indicating that the developing juveniles might have disintegrated. The number of eggs per eggmass was significantly low Based on the results in the preliminary greenhouse experiments, 25 bacterial isolates were short-listed. The details of these isolates are given in Table 8.

Among the 25 short listed bacterial isolates, 18 isolates were isolated from black pepper roots or rhizospheric soil or from the nematodes of black pepper. Seven isolates were from nematode antagonistic plants.

Table 7. a. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications) : Experiment 1

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	15.33a	1.35a	0.43a	1.99a	0.00(0.71)a	0.00(0.71)a
RKN	18.00ab	2.09ab	1.03bcd	3.12ab	7.33(2.79)f	23.00(4.83)d
1	26.80bcd	3.77cd	1.06bcd	4.83bcd	5.00(2.32)ef	22.67(4.65)cd
2	28.67cd	3.56bcd	1.27d	4.84bcd	3.00(1.86)cde	11.00(3.31)bc
3	31.73cd	4.64d	1.08bcd	5.72d	2.33(1.68)cde	16.33(4.10)bcd
7	32.00cd	4.49cd	0.89abcd	5.38cd	1.67(1.39)bc	12.67(3.44)bc
8	29.63cd	4.22bc	1.07bcd	5.29cd	3.00(1.86)cde	9.67(3.19)b
9	23.73abc	2.92bc	0.67abc	3.59abc	1.33(1.34)abc	9.00(3.08)b
11	35.40d	4.57d	1.20cd	5.77d	2.67(1.76)cde	9.00(3.07)b
12	29.57cd	3.87bc	0.86abcd	4.73bcd	3.67(1.97)cde	11.00(3.31)bc
13	36.50d	3.80cd	0.46a	4.26bcd	0.00(0.71)a	0.00(0.71)a
14	30.40cd	3.38bcd	0.40a	3.78bcd	2.00(1.56)cd	11.53(3.36)bc
15	28.00bcd	3.57bcd	0.56ab	4.13bcd	4.33(2.15)def	9.67(3.18)b
16	27.83bcd	3.44bcd	0.71abc	4.15bcd	4.33(2.20)def	12.33(3.56)bcd

Means followed by the same alphabets in a column are not significantly different.

Table 7. b. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications): Experiment 2

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	19.00 a	2.25ab	0.35a	2.60a	0.00(0.71)a	0.00 (0.71)a
RKN	25.50bcde	3.80cd	2.54cdef	5.35def	14.33 (3.85)i	22.67 (4.80)h
74	23.17 abc	3.92cd	1.78ef	5.70ef	3.67 (2.04)cde	14.00 (3.80)fg
75	25.67bcde	2.96abc	1.82f	4.78def	6.33 (2.56)efg	13.67 (3.75)efg
76	25.20 bcde	3.27abc	1.71def	4.98def	4.00 (2.10)cde	11.33 (3.42)cdefg
77	29.53 defg	3.58bcd	1.34bcdef	4.92def	1.67(1.39)bc	7.67 (2.80)c
78	21.32ab	2.03a	0.88ab	2.90ab	1.00 (1.17)ab	4.33 (2.16)b
79	28.00cdef	3.75cd	1.28bcdef	4.83def	11.33 (3.42)hi	15.33 (3.96)g
80	31.73 fg	3.08 abc	1.18bcde	4.51cde	4.33 (2.16)def	10.00 (3.24) cdef
81	32.27fg	3.35abc	1.54cdef	4.89def	4.00 (2.06)cde	10.00 (3.23)cde
82	30.00efg	2.63abcd	1.29bcdef	3.93abcd	3.00 (1.86)bcde	8.67 (2.99)cd
83	26.33bcdcf	2.62abc	1.21bcdef	3.83abcd	3.33 (1.95)cde	9.00 (3.08)cde
84	29.67defg	4.79ad	1.36bcdef	6.15f	11.00 (3.39)hi	12.00 (3.53)defg
85	27.33cdef	3.14abc	1.10bcd	4.26bcde	6.33 (2.59)efg	9.67 (3.17)cdef
86	34.97g	3.63cd	1.12bcd	4.75def	7.67 (2.86)fgh	12.67 (3.62)defg
87	31.00 efg	3.42bc	1.04bc	4.45bcde	4.33 (2.18)def	7.33 (2.80)c
88	24.00abcd	2.72abc	1.00ab	4.10bcde	6.33 (2.56)efg	9.33 (3.13)cdef
89	28.00cdef	3.15abc	1.42bcdef	4.25bcdef	2.33 (1.68)bcd	9.00(3.07)cde
90	26.33bcdcf	3.38bc	1.09bcd	4.51cde	8.00 (2.91)gh	11.00(3.39)cdefg
91	24.64abcde	2.96abc	1.15abc	2.96abc	2.67 (1.77)bcd	10.67 (3.34)cdefg

Means followed by the same alphabets in a column are not significantly different.

Table 7. c. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications): Experiment 3

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	18.00ab	3.12abc	0.74a	3.87a	0.00(0.71)a	0.00(0.71)a
RKN	16.33a	2.72abc	2.18bc	4.9abc	16.33 (5.75)f	45.00 (5.74)g
92	26.33b	5.02c	3.28c	8.3c	11.33 (3.35)de	14.67(3.81)ef
93	16.67a	2.20ab	1.97bc	4.18ab	6.67 (2.66)bcd	9.67 (3.14)bcd
94	16.67a	1.92a	2.22bc	4.14ab	3.67 (1.94)bc	5.00 (2.32)bcd
95	21.00ab	2.24ab	1.33ab	3.57a	13.33 (3.72)e	11.33 (3.40)def
97	24.33ab	4.93c	2.79c	7.72bc	5.33 (2.39)bcd	14.67 (3.87)ef
98	16.67a	2.80abc	2.62bc	5.42abc	12.33 (3.58)e	15.33 (3.97)f
99	22.00ab	3.83abc	3.28c	7.12abc	2.33 (1.54)abc	5.00 (2.04)bc
100	24.33ab	4.68bc	2.99c	7.67bc	7.00 (2.63)cde	6.67 (2.65) cde
102	21.33ab	3.84abc	2.59bc	6.43abc	1.33 (1.34)ab	1.33 (1.34)ab

Means followed by the same alphabets in a column are not significantly different

Table 7. d. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications) : Experiment 4

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	19.00ab	2.78abc	0.50ab	2.23ab	0.00 (0.71) a	0.00 (0.71)a
RKN	23.33abcde	3.09abc	1.37d	4.47cdefg	47.00 (6.67) d	35.00 (5.87)c
105	26.17abcde	3.55c	1.35cd	4.90fg	2.00 (1.56)ab	3.00 (1.82)ab
106	23.17abcde	2.77abc	0.80abcd	3.57abcdefg	1.67 (1.44)ab	1.33 (1.27)a
107	24.17abcde	3.26abc	1.37d	4.63defg	1.67 (1.39)a	2.33 (1.68)ab
108	30.83de	3.54c	1.33cd	4.87fg	0.67 (1.05)ab	1.00 (1.17)a
109	22.93abcde	2.62abc	0.87abcd	4.99bcdefg	1.67 (1.39)ab	2.67 (1.64)ab
110	28.43bcde	3.40bc	0.98bcd	4.39cdefg	0.33 (0.88)a	1.33 (1.29)a
111	26.00abcde	2.98abc	1.06bcd	4.04bcdefg	2.00 (1.58)e	2.67 (1.72)ab
112	23.77abcde	2.38abc	0.50ab	2.88abcdef	10.00 (3.22)c	8.67 (2.96)d
113	20.83abc	2.59abc	1.37d	3.96bcdefg	0.00 (0.71)a	0.00 (0.71)a
114	18.33a	2.21abc	0.72abcd	2.93abcdefg	1.33 (1.29)a	2.00 (1.48)ab
115	21.83bcde	2.60abc	0.70abc	3.30abcdefg	25.00 (4.15)b	26.67 (4.65)c
116	19.83ab	1.99abc	0.81abcd	2.80abcde	0.00 (0.71)a	1.00 (1.10)a
117	24.00abcde	2.08abc	0.46ab	2.54abcd	0.00 (0.71)a	0.00 (0.71)a
118	24.00abcde	2.00abc	0.45ab	2.46abc	0.00 (0.71)d	0.00 (0.71)a
119	29.50cde	3.61c	1.37d	4.98g	0.00 (0.71)a	0.00 (0.71)a
120	24.67abcde	2.54abc	0.54ab	3.08abcdefg	0.33 (0.88)a	0.33 (0.88)a
121	31.50e	3.61abc	0.97bcd	4.88abcdefg	1.33 (1.27)a	1.00 (1.10)a
122	24.67abcde	2.25bc	0.50ab	2.65abcde	0.33 (0.88)a	0.67 (1.00) a
123	31.50e	3.37bc	0.50ab	3.87abcdefg	0.00 (0.71)a	0.00 (0.71) a
124	29.77cde	2.64abc	0.52ab	3.15abcd	0.00 (0.71)a	0.67 (1.05)a
125	23.50abcde	1.74ab	0.52ab	2.26ab	6.33 (2.21)ab	5.00 (2.04)ab

Means followed by the same alphabets in a column are not significantly different

Table 7. e. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications: Experiment 5)

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	19.66a	2.46a	1.99cd	3.43ab	0.00(0.71) a	0.00(0.71)a
RKN	25.5bed	2.61ab	2.10d	4.72abcd	11.67(3.43)d	25.33(5.01)d
127	32.00efg	3.58abc	1.71abcd	5.29bcd	0.00(0.71)a	0.00(0.71)a
128	24.33abc	2.50a	1.13ab	3.63a	0.00(0.71)a	0.00(0.71)a
129	30.00cdefg	3.75bc	1.41abcd	4.83abcd	0.00(0.71)a	0.00(0.71)a
130	33.73efg	3.07abc	1.17abc	4.65abc	0.00(0.71)a	0.00(0.71)a
P1	23.83ab	3.95cd	2.31d	6.08cd	6.67(2.37)ab	10.00(2.83)c
P2	27.13bcdef	3.21abc	1.67abcd	4.89abcd	4.67(2.22)acc	48.00(6.91)d
P3	28.66bcdef	3.27abc	1.86bcd	5.13bcd	58.00(7.34)g	98.33(9.54)d
P4	32.26efg	3.59abc	1.68abcd	5.28bcd	0.67(1.00)ab	14.00(2.64)ab
P5	30.00cdefg	2.77ab	1.29abcd	4.06ab	1.67(1.39)ab	1.67(1.39)ab
P6	26.33bcde	2.62ab	1.20abc	3.83ab	15.00(3.84)def	16.67(3.99)b
P7	29.66bcdefg	4.78d	1.36abcd	6.15d	4.00(1.91)abc	16.00(3.72)b
P8	27.33bcdef	3.13abc	1.12ab	4.26ab	5.67(2.41)bcd	11.67(3.34)ab
P9	34.76g	3.63abc	1.12ab	4.75abcd	6.00b(2.55)cde	125.00(11.20)d
P10	31.31defg	3.41abc	1.03ab	4.45ab	15.00(3.94)ef	12.00(3.54)ab
P11	24.00 ab	2.71ab	1.00a	4.10ab	9.00(3.08)cedf	11.00(3.38)ab
P12	32.66 g	3.15abc	1.42abcd	4.24ab	2.33(1.74)ab	4.67(1.74)ab
P13	30.33defg	3.38abc	1.09ab	4.51ab	0.00(0.71)a	0.00(0.71)a
P14	26.66bcde	2.96abc	1.13ab	4.31ab	2.00(1.47)ab	3.67(1.86)ab
P15	27.70bcdef	3.55abc	1.35abcd	4.90abcd	13.67(3.76)def	14.67(3.89)b
P16	32.00defg	2.77ab	1.16abc	3.93ab	0.00(0.71)a	0.00(0.71)a
P17	24.33abc	3.25abc	1.37abcd	4.63ab	0.00(0.71)a	0.00(0.71)a
P18	30.83defg	3.54abc	1.48abcd	5.02abcd	0.67(1.00)ab	2.67(1.44)ab
P19	32.00defg	2.77ab	1.16abc	3.93ab	19.67(4.44)f	14.67(3.86)b

Means followed by the same alphabets in a column are not significantly different

Table 7. f. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications): Experiment 6

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	15.50a	1.35a	0.64a	1.99a	0.00 (0.71)a	0.00(0.71) a
RKN	18.00ab	2.08ab	1.03abc	3.11ab	37.67 (6.12)c	151.00(12.16) d
17	30.00de	3.61cd	1.15abcd	4.76cd	27.00 (4.72)c	114.33(9.23) d
18	33.16ef	3.92d	1.78ef	5.70d	0.00 (0.71)a	0.00 (1.70)a
19	25.66cd	2.96bcd	1.82f	4.78cd	0.00 (0.71)a	0.00 (0.71)a
20	31.33def	3.25cd	1.67def	4.94cd	0.00 (0.71)a	0.00 (0.71)a
21	31.60def	3.05bcd	1.44cde	5.02cd	1.67(1.39)ab	15.00(3.43)ab
23	21.33abc	2.02ab	0.87ab	2.90ab	7.00(2.02)ab	29.33(3.94)ab
24	33.00ef	3.99d	1.28bcde	5.21cd	0.00 (0.71)a	0.00 (0.71)a
25	33.73ef	3.39cd	1.32bcdef	4.94cd	0.00 (0.71)a	0.00 (0.71)a
26	32.60ef	3.35cd	1.43cdef	4.70cd	6.00(2.55)b	100.00(10.02)d
27	30.00de	2.63bc	1.29bcdef	3.96bc	2.67(1.64)ab	16.00(3.49)ab
28	35.00ef	3.67cd	1.37bcdef	5.04cd	0.00 (0.71)a	0.00(0.71)a
29	30.33de	3.73d	1.17bcd	4.90cd	4.00(2.12)ab	3.00(1.87)ab
30	37.33ef	3.81d	1.29bcdef	5.10cd	0.00(0.71)a	13.33 (2.59)ab
64	33.99ef	3.82d	1.17bcd	4.98cd	2.67(1.74)ab	27.67(4.88)ab
65	31.00def	3.41cd	1.03abc	4.45cd	9.00(3.08)b	30.00(5.52)bc
68	35.83ef	3.75d	1.27bcde	5.03cd	0.00(0.71)a	0.00(0.71)a

Means followed by the same alphabets in a column are not significantly different

Table 7. g. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications) : Experiment 7

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	17.00a	1.68a	1.31a	2.99a	0.00 (0.71)a	0.00 (0.71)a
RKN	19.33a	2.08a	2.03b	4.11ab	15.33(3.95)c	20.33(4.50)f
B1	30.00b	3.77ab	1.05ab	4.82abc	14.00 (3.78)c	7.00 (5.14)abcd
B2	32.33bcd	3.56ab	1.27ab	4.83abc	4.00(1.84)ab	5.33(2.12)cd
B3	34.56cd	4.64b	1.08ab	5.72abcd	4.33(1.94)ab	6.00(2.41)ab
B4	33.66bcd	4.04ab	0.88ab	5.38abcd	0.33(0.88)ab	0.33(0.88)ab
B5	35.66d	5.02b	3.28d	8.30d	0.00(0.71)a	0.00(0.71)a
B6	30.06b	2.92ab	0.67a	3.59ab	3.00(1.86)ab	2.67(1.74)abc
B7	31.56bc	4.57b	1.20ab	5.77abcd	2.33(1.54)ab	2.00(1.43)abc
B8	31.33bc	3.87ab	0.86a	4.73abc	1.67(1.44)ab	4.00(1.96)abc
B9	30.33bc	3.80ab	0.46a	4.26ab	3.67(2.00)c	10.67(3.34)bc
B10	32.33bcd	3.84ab	2.03b	6.43bcd	1.00(1.17)ab	1.33(1.29)bc
B11	33.33bcd	3.50ab	2.95cd	6.45bcd	1.67(1.39)ab	4.67(2.21)bcd
B12	32.83bcd	3.44ab	0.71a	4.15ab	10.67(3.33)c	14.33(3.76)e
B13	33.00bcd	2.79ab	2.59cd	5.41abcd	3.00(1.87)b	2.67(1.77)abc
B14	34.00bcd	4.93b	2.07bcd	7.72cd	0.00(0.71)a	0.00(0.71)a
B15	33.00bcd	4.68b	2.99cd	7.64cd	0.00(0.71)a	0.00(0.71)a

Means followed by the same alphabets in a column are not significantly different

Table 7. h. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications): Experiment 8

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	18.00a	2.33ab	1.24a	3.55a	0.00(0.71)a	0.00(0.71)a
RKN	21.66a	2.26ab	1.31ab	3.57a	45.67(6.78)g	66.00(8.15)i
31	33.66b	5.02bc	3.28cd	8.30abc	2.00(1.56)cde	4.67(2.16)defgh
32	34.00b	2.20ab	1.97abc	4.17ab	6.00(2.54)efg	6.33(2.60)efghi
33	33.66b	1.92a	2.22abc	4.14ab	0.00(0.71)a	0.00(0.71)a
34	33.66b	2.24ab	2.10abc	4.34ab	3.67(2.00)ef	2.00(1.56)bcde
35	33.66b	4.93abc	2.78abc	7.72abc	0.33(0.88)ab	0.33(0.88)a
36	34.33b	3.24abc	2.62abc	5.86abc	1.00(1.17)abcd	1.33(1.27)abcd
37	34.33b	3.83abc	3.28cd	7.11abc	0.00(0.71)a	0.00(0.71)a
38	34.33b	4.68abc	2.99bcd	7.67abc	0.00(0.71)a	0.00(0.71)a
39	35.00b	3.83abc	2.59abc	4.43abc	0.00(0.71)a	0.00(0.71)a
40	32.66b	4.93abc	2.78abc	7.72abc	0.00(0.71)a	0.00(0.71)a
41	34.00b	3.62abc	2.62abc	4.24abc	6.67(2.66)efg	13.33(3.67)hi
42	31.66b	3.83abc	3.28cd	7.11abc	6.00(2.50)efg	8.76(3.01)ghi
43	33.66b	3.83abc	3.28cd	7.11abc	1.67(1.46)bcde	2.00(1.56)bcde
44	34.00b	4.68abc	2.99bcd	7.67abc	0.67(1.00)abc	1.00(1.10)abc
45	33.00b	3.84abc	3.56abc	6.43abc	0.00(0.71)a	0.00(0.71)a
46	30.00b	5.07bc	2.71abc	7.79abc	0.67(1.00)abc	1.67(1.39)abcde
47	33.66b	4.72abc	2.68abc	7.40abc	0.00(0.71)a	0.00(0.71)a
48	30.00b	2.79abc	2.62abc	5.41abc	2.33(1.54)cde	4.00(1.94)defgh
49	33.66b	3.81abc	3.28cd	7.11abc	0.00(0.71)a	0.00(0.71)a
50	32.66b	3.95abc	2.99bcd	6.94abc	3.67(2.00)ef	6.00(2.50)defghi
51	32.33b	5.51c	3.28cd	8.80c	5.33(2.35)efg	7.00(2.67)fghi
52	30.00b	4.68abc	2.99bcd	7.68abc	0.00(0.71)a	0.00(0.71)a
53	35.33b	4.95bc	3.83d	8.79c	2.33(1.68)de	2.67(1.77)cdef
54	34.33b	3.38abc	2.44abc	5.82abc	2.00(1.56)cde	3.00(1.87)cdefg
55	35.00b	4.40abc	3.90d	8.30bc	2.00(1.56)cd	1.67(1.46)abcd
56	32.66b	2.30abc	2.44abc	5.44abc	2.00(1.58)cde	2.33(1.68)bcde
57	34.00b	3.95abc	3.27cd	7.22abc	0.33(0.88)ab	0.33(0.88)ab
58	31.66b	4.47abc	2.44abc	7.24abc	0.00(0.71)a	0.00(0.71)a

Means followed by the same alphabets in a column are not significantly different.

Table 7. i Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications) : Experiment 9

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Con	19.00a	1.53a	0.87abcd	2.40a	0.00(0.71)a	0.00(0.71)a.
trol						
RK	23.33ab	2.16ab	1.37abcdef	3.54b	46.67(6.84)h	57.67(7.61)g
N						
59	31.00cdef	3.71cdefg	1.35abcdef	5.06cdefg	0.00(0.71)a	0.00(0.71)a
60	31.33cdefg	3.33cdef	1.08abcdef	4.46bcdef	2.00(1.56)abcd	4.61(2.23)abc
61	28.33bcde	3.25cdefgh	1.37bcdef	4.63bcdefg	4.67(2.26)abcdef	8.33(2.94)bcde
62	26.66bcde	3.54cdefgh	1.16abcdef	4.70bcdefg	14.00(3.80)g	20.00(4.53)d
63	29.00bcdef	3.60cdefgh	1.44def	5.05cdefg	1.00(1.17)abc	2.00(1.43)ab
67	33.33efg	3.40cdefgh	1.38cdef	4.78bcdefg	5.33(2.27)bcdef	8.00(2.88)bcde
69	34.00fg	3.78cdefgh3	1.38cdef	5.13defg	2.00(1.56)abcd	4.67(2.23)abcd
70	33.33efg	4.13fghi	1.22abcdef	5.36efgh	3.67(2.00)abcde	6.33(2.56)bcd
71	35.00fg	5.07j	1.56ef	6.63j	0.00(0.71)a	0.00(0.71)a
72	32.66bcdefg	4.02fgh	1.44def	5.11efg	9.00(2.73)defg	13.00(3.22)cde
101	37.67fg	4.35ghij	1.65f	6.01ij	0.00(0.71)a	0.00(0.71)a
102	28.67bcdefg	2.88bcde	1.31abcdef	4.19bcde	0.67(1.00)ab	2.00(1.32)ab
103	26.33bcd	2.63bcd	0.97abcde	3.60d	3.00(1.86)abcde	2.67(1.77)abc
104	29.33bcdef	2.58bc	1.21abcdef	3.80bc	1.33(1.27)abcd	3.33(1.73)abc
132	34.33fg	4.75ij	0.99abcde	4.74fg	0.00(0.71)a	0.00(0.71)a
133	34.66fg	4.42hij	1.37cdef	5.94g	0.00(0.71)a	0.00(0.71)a
134	32.00cdefg	3.29cdef	1.09abcdef	4.41bcde	1.00(1.17)abc	3.00(1.71)abc
135	27.00bcde	3.23cdef	0.86a	3.98bcd	4.67(2.18)abcde	8.33(2.94)bcde
136	34.64fg	4.28ghij	1.23abcdef	5.50efgh	0.00(0.71)a	0.00(0.71)a
P29	33.00defg	3.97efgh	1.33abcde	5.11efgh	5.33(2.29)abcdef	12.00(3.24)cde
P30	33.00defg	3.53cdef	1.09abcdef	4.74bcdefg	30.00(5.51)h	36.33(6.06)f
P31	33.33efg	3.68dfgh	1.11abcdef	4.79bcdefg	33.00(5.72)h	31.00(5.90)ef
P32	29.50bcdef	3.50cdef	1.18abcdef	4.68bcdefg	9.00(2.62)cdefg	12.67(3.28)cde
P33	31.33cdefg	3.60cdef	0.98abcdef	4.61bcdefg	10.00(3.19)defg	12.67(3.56)cde
P34	26.00ab	3.02bcdef	0.87abc	3.80bc	4.67(1.94)abcd	5.33(2.17)abcd
P35	34.00fg	3.09bcdef	1.35abcdef	4.43bcd	13.00(3.67)fg	13.00(3.66)cde
P37	28.78bcdef	3.22cdef	1.00ab	3.98bcd	6.33(2.60)cdef	11.00(3.34)cde
P38	31.33cdefg	3.64cdef	1.34abcdef	4.97cdefg	2.67(1.44)abcd	4.00(1.65)abc

Means followed by the same alphabets in a column are not significantly different.

Table 7. j. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications): Experiment 10

Is. No.	Plant growth character				Nematode development	
	Height (cm)	Shoot wt. (g)	Root wt. (g)	Total wt. (g)	No. of egg masses	No. of galls
Control	17.67a	2.63a	0.62a	3.24a	0.0 (0.71)a	0.0(0.71)a
RKN	19.23a	3.24bcd	1.21bc	4.46bc	21.00(4.62)f	22.33(4.77)d
AP1	34.98ghi	4.62ghi	2.20gh	6.82hi	0.0(0.71)a	0.00 (0.71)a
AP2	32.4efg	3.44cde	1.91defg	5.34ef	5.33(2.34)bcd	5.33(2.39)abc
AP3	28.83cde	3.05abcd	2.18fgh	5.24def	3.33(1.95)bc	4.33(2.16)bc
AP4	38.33i	5.04i	2.92j	7.96k	0.0(0.71)a	0.0(0.71) a
AP5	34.77gh	4.3fgh	2.47hi	6.78hi	0.0(0.71)a	0.0(0.71)a
AP6	25.9c	2.85abc	1.6cd	4.45bc	9.33(3.13)de	9.33(3.09)c
AP7	22.13b	2.74ab	1.18b	3.91a	12.67(3.58)e	16.33(4.08)d
AP8	34.17fg	4.15fg	2.03efg	6.18gh	0.0(0.71)a	0.0(0.71)a
AP9	30.5de	3.58de	1.76de	5.34ef	2.00(1.56)ab	4.67(2.24)bc
AP10	34.7gh	4.76hi	2.45hi	7.22ij	0.0(0.71)a	0.0(0.71)a
AP11	30.2de	3.29bcd	1.35bc	4.64cd	0.00(0.71)a	0.0(0.71)a
AP12	30.5de	3.4cde	1.29bc	4.69cde	2.33(1.49)ab	5.33(2.15)bc
AP13	27cd	2.88abc	1.22bc	4.09bc	7.33(2.78)d	8.33(2.94)c
AP14	26.5c7	3.12abcd	1.37bc	4.49bc	6.66(2.67)cd	7.33(2.78)c
AP15	30.87ef	3.92ef	1.8def	5.72fg	2.33(1.54)ab	3.00(1.71)ab
AP16	37.97hi	4.85hi	2.82ij	7.68jk	0.00(0.71)a	0. 0(0.71)a

Means followed by the same alphabets in a column are not significantly different.

Table 7. k. Effect of different isolates of bacteria on root-knot nematode suppression and growth of tomato plants (Mean of three replications): Experiment 11

Is. No.	Plant growth character				Nematode development	
	Height(cm)	Shoot wt.(g)	Root wt.(g)	Total wt.(g)	No. of egg masses	No. of galls
Control	18.00a	2.27abcd	1.36abc	3.63ab	0.0(0.71)a	0.00(0.71)a
RKN	17.66a	2.33abcd.	1.26ab	3.63ab	19.67(4.4)e	41.00(6.43)f
C1	31.67cdefgh	2.33abcd	1.79 abcd	4.12ab	0.67(0.99)ab	1.33(1.27)ab
C2	38.33fghi	4.69efghi	3.27abcdef	7.96bcde	0.33(0.88)ab	0.67(0.99)ab
C3	33.33dfgh	6.73hi	4.27g	11.00e	3.67(1.83)bcde	4.67(2.18)bcde
C4	23.5abcde	2.40 abc	1.47 abcd	3.87ab	4.67(2.16)cde	6.33(2.55)cde
C5	29.17abcdefg	2.40abcde	1.87abcd	4.27abc	2.33(1.54)abcd	2.67(1.61)abcde
C6	21.67abcd	1.35abc	0.87a	2.21a	1.67(1.35)abcde	2.67(1.61)abcde
C7	17.50a	2.11a	1.07abc	3.17ab	0.33(0.88)ab	0.67(0.99)ab
C8	21.17abc	3.43abcde	1.67abcd	5.09ab	1.67(1.46)abcde	2.00(1.56)abcde
C9	21.17abc	2.83abcd	0.87abc	3.69ab	0.0(0.71)a	0.0(0.71)a
C10	20.50abc	2.76abcd	0.93abc	3.69e	0.67(0.99)ab	2.00(1.56)abcde
C11	41.17hi	6.09hi	5.40cdefg	11.49cde	1.67(1.25)abcd	2.67(1.61)abcde
C12	29.00abcde	5.73fghi	3.67defg	9.40abc	1.0(1.17)abc	1.67(1.39)abc
C13	27.00abcdef	3.35abcde	3.20abcdef	2.55cde	2.00(1.54)abcde	3.00(1.71)abcde
C14	33.83efgh	6.40efg	3.92cdefghi	10.32cde	0.67(0.99)ab	0.67(0.99)ab
C15	30.00bcdefgh	5.28fghi	5.13bcdefg	10.41ab	1.33(1.18)abc	1.33(1.17)ab
C16	27.75abcdef	1.43abc	2.33abcd	3.76ab	1.00(1.22)abc	1.33(1.34)ab
C17	27.17abcdef	3.09abcdef	2.80abcd	5.89abcd	1.00(1.17)abc	1.00(1.17)ab
C18	38.33fghi	6.44hi	5.40cdefg	11.84e	0.00(0.71)a	0.67(0.99)ab
C19	39.00ghi	5.81ghi	4.87bcdefg	10.68de	0.00(0.71)a	0.33(0.88)a
C20	30.00bcdefgh	7.40j	4.65efghi	12.05e	3.33(1.90)bcde	4.67(2.18)bcde
C21	29.00abcdefg	3.79bcdefgh	1.60ab	5.39abc	5.00(1.27) e	6.67(2.67)e
C22	45.00i	6.53fg	5.24fghi	11.77e	1.33(1.27)abcd	2.33(1.57)abcde
C23	22.50abcde	3.00abcd	1.63abc	4.63ab	1.00(1.17)ab	2.00(1.47)abcd
C24	23.50abcde	2.40abcd	2..08abcd	4.48ab	1.00(1.17)ab	1.67(1.39)abc
C25	21.67abcd	1.88abcd	2.07abc	3.95ab	1.00(1.22)ab	1.67(1.46)abcd
C26	33.33defgh	2.03abcd	1.33abcd	4.35ab	0.33(0.88)ab	0.33(0.88)a
C27	26.67abcdef	2.60abc	1.87abcd	5.47abc	1.00(1.10)ab	1.00(1.09)ab
C28	19.17ab	2.03abcd	1.07abcde	5.09ab	0.33(0.88)ab	0.33(0.88)a
C29	30.50bcdefgh	3.31abcdefg	2.53abcd	5.84abcd	4.67(2.25)de	6.33(2.61)de
C30	23.75abcde	2.55abcde	2.13abc	4.68ab	1.67(1.39)abcde	4.00(1.91)abcde

Means followed by the same alphabets in a column are not significantly different.

Table 8. Details of the bacterial isolates short-listed as promising isolates for nematode control based on preliminary screening.

Is. No.	Date of collection	Location	Crop	Sample	Taxonomic identity
13	21.09.2000	Pulpally	Black pepper	Root	<i>Pseudomonas</i>
18	21.09.2000	Idukki	Black pepper	Soil	<i>Pseudomonas</i>
19	12.09.2000	Idukki	Black pepper	Soil	<i>Pseudomonas</i>
24	28.08.2000	Moolamattam	Black pepper	Root	<i>Pseudomonas</i>
25	28.08.2000	Thodupuzha	Black pepper	Soil	<i>Pseudomonas</i>
101	15.11.2000	Piravom	Black pepper	Soil	<i>Bacillus</i>
113	15.11.2000	Wayanad	Black pepper	Root	<i>B. megaterium</i>
117	16.11.2000	Wayanad	Black pepper	Root	<i>Bacillus</i>
118	16.11.2000	Mulanthuruthy	Black pepper	Root	<i>Bacillus</i>
119	16.11.2000	Aluva	Black pepper	Root	<i>Comamonas testosterone</i>
123	29.11.2000	Wayanad	Black pepper	Root	<i>Serratia</i>
124	29.11.2000	Wayanad	Black pepper	Nematode	Unidentified
127	19.02.2001	Pulpally	Black pepper	Nematode	<i>Pseudomonas</i>
132	01.03.2002	Pulpally	Black pepper	<i>M.i</i> juveniles	<i>Bacillus</i>
133	07.03.2002	Chelavoor	Black pepper	<i>M.i</i> juveniles	<i>Bacillus</i>
136	01.05.2002	Cheruthony	Black pepper	Female <i>Meloidogyne</i>	<i>Alcaligenes</i>
Ap1	01.11.2001	Calicut	<i>P. colubrinum</i>	Root	<i>Pseudomonas</i>
Ap4	01.11.2001	Calicut	<i>Chromolena odorata</i>	Root	<i>Pseudomonas aeruginosa</i>
Ap5	01.11.2001	Pariyaram	<i>Chromolena odorata</i>	Root	<i>Pseudomonas</i>
Ap8	01.11.2001	Malappuram	Allspice	Root	<i>Pseudomonas</i>
Ap10	01.11.2001	Kottakkal	Nuxvomica	Soil	<i>Pseudomonas</i>
Ap11	07.11.2001	Calicut	Nuxvomica	Soil	<i>Pseudomonas</i>
Ap16	21.11.2001	Calicut	Nuxvomica	Root	<i>Pseudomonas</i>
B14	05.01.2001	Silent valley	Black pepper	Soil	<i>Pseudomonas</i>
B15	05.01.2001	Silent valley	Black pepper	Soil	<i>Bacillus</i>

M.i- Meloidogyne incognita

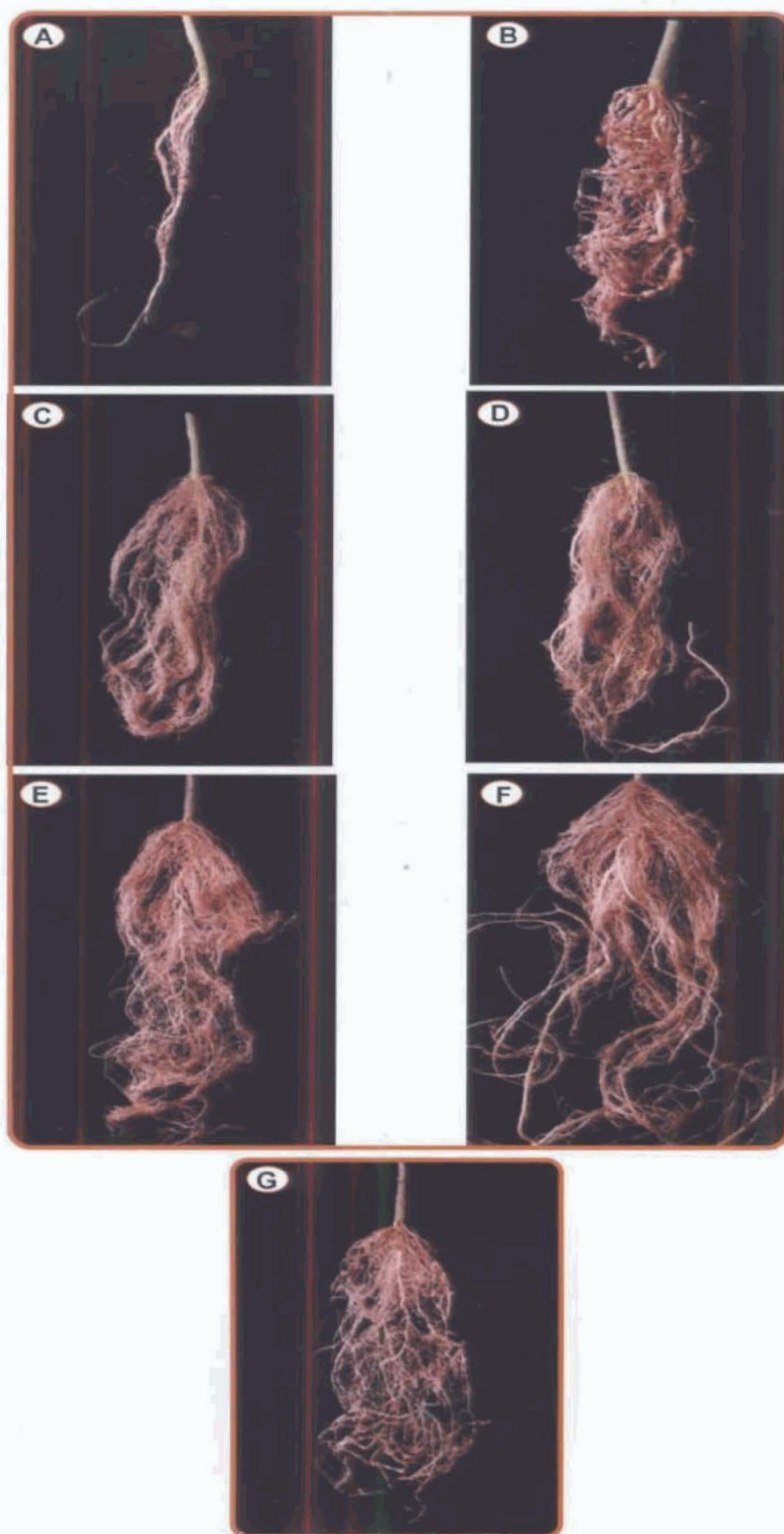


Plate 10a. Evaluation of bacterial isolates for the suppression of *Meloidogyne* under greenhouse conditions using tomato as the test plant

A. Control B. *Meloidogyne* C. Treated with Ap.16 D. Treated with Ap.4.
E. Treated with Is.101 F. Treated with Is.136 G. Treated with Is.13

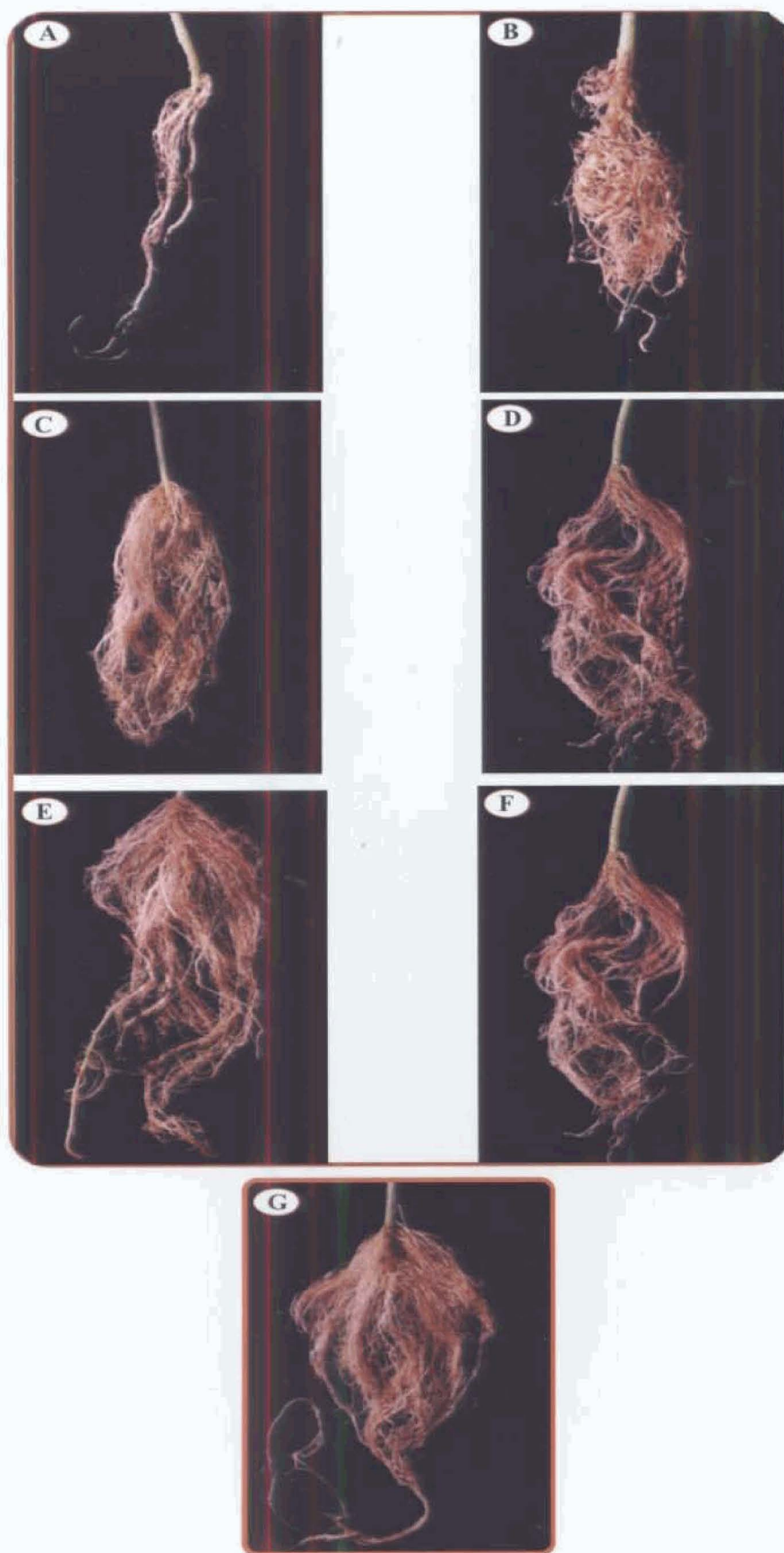


Plate 10b. Evaluation selected bacterial isolates for the suppression of *Meloidogyne* under green house conditions using tomato as the test plant.

A. Control **B.** *M. incognita* **C.** Treated with Ap.10 **D.** Treated with Ap.8

E. Treated with B14 **F.** Treated with B15 **G.** Treated with Is.113

4.2. CHARACTERIZATION OF SHORT LISTED BACTERIAL ISOLATES AND THEIR MODE OF ACTION ON NEMATODES

4.2.1. Identification of bacteria

An attempt was made mainly for the identification of *Pseudomonas* and *Bacillus* strains based on the morphological and biochemical reactions. Out of the 319 bacteria isolated, 77 were tentatively identified as *Pseudomonas* spp. 55 as *Bacillus* spp. and 187 bacterial isolates could not be identified. Out of the 70 bacterial isolates directly isolated from soil, 25 belonged to *Pseudomonas* spp. and seven were *Bacillus* spp. Sixteen bacteria isolated through the baiting of alginate prills yielded a total of 16 bacteria, with 6 and 2 *Pseudomonas* spp. and *Bacillus* spp., respectively. Out of 181 bacteria from roots of black pepper, coleus and nematode antagonistic plants, 37 were *Pseudomonas* spp. and 35 were *Bacillus* spp. (Plate 7).

4.2.2. Molecular characterization

Out of the 25 short listed bacterial isolates, molecular characterization of the short listed 19 bacterial strains was attempted through Unweighed Pair Group Arithmetic Average (UPGMA). DNA was isolated from the bacterial isolates using CTAB + SDS method and amplification was done using 16-S primer. PCR was done and the final PCR products were resolved in 0.7% agarose and were restricted with Taq 1(Plate11), Sau3A1 and Alu1 adopting the manufactures instruction (GENEI). 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. The tree plot (Fig. 1) reveals that the test strains are different from each other.

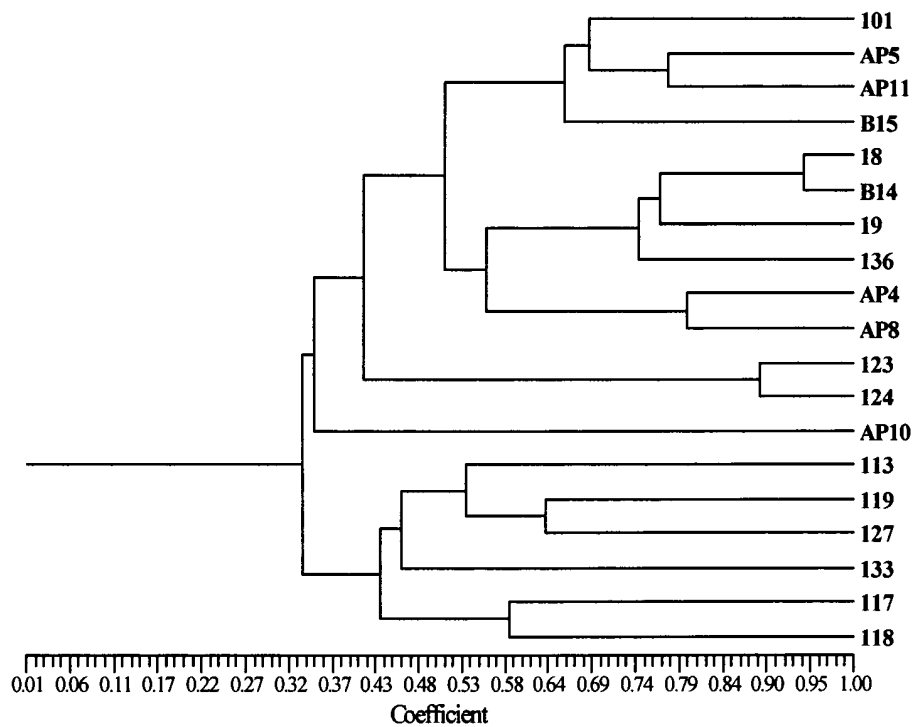


Fig. 1. Dendrogram showing the grouping of short listed bacterial isolates based on their molecular characterization



NB-5509

583-25 BCT/13

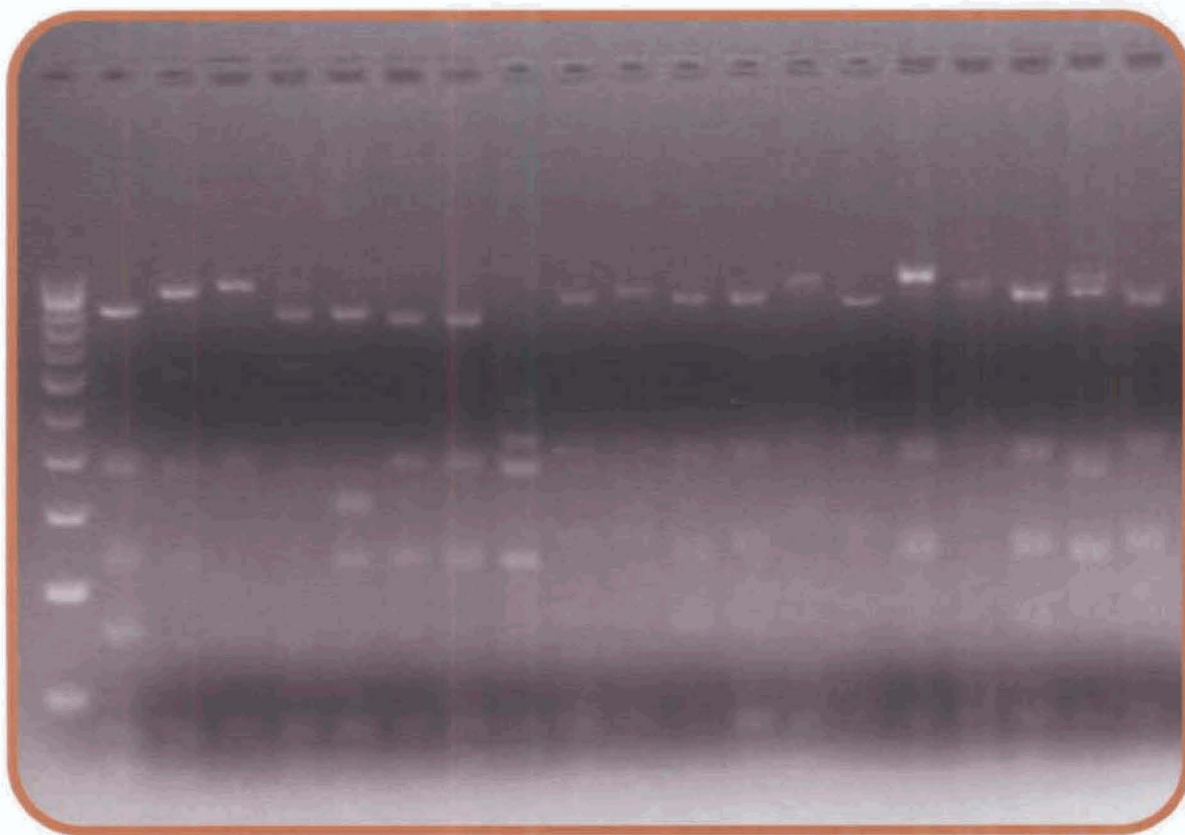


Plate 11. Restriction analysis of rDNA of selected isolates of bacteria with TaqI

Line 1. 100bp marker line 2. Is.101, Line 3.Is113, Line 4. Is. 117, Line 5 Is. 118
Line 6. Is.119, Line 7. Is. 123. Line 8. Is. 124, Line 9. IS.127 Line10. Is. 18,
Line 11. Is.19 Line12. Is. 136, Line 13 B.14, Line 14 B15
Line 15 Ap4,,Line 16.Ap5, Line 17 Ap10,
Line 18. Ap16. Line 19Is.133,Line 20 Ap8

4.2.3. Hatching suppression

Among the 25 short listed bacterial isolates evaluated for the nematode suppression, all the isolates significantly suppressed the hatching of the nematode eggs (Table 9, Plate 12). None of them caused 100% reduction in hatching of nematode eggs. The percentage of nematode suppression ranged from 33.96 % by Ap11 to 91.66% by B15. Isolate B15 caused the maximum nematode hatching suppression (91.66%) followed by B14 (87.89%). Six isolates caused more than 80% suppression in hatching. Twenty one isolates caused more than 50% hatching suppression. Nine isolates caused 60-80 % hatching suppression. High percentages of the remaining eggs were spherical in shape rather than ovoid. The internal integrity of most of the eggs exposed to this concentration of bacteria was completely lost by the end of two week incubation. None of the isolates showed any stimulatory effect on the hatching of the nematodes. There was no sign of egg parasitism by any of the bacterial strains. Agglutination of the bacterial cells was also observed in the presence of nematode eggs (Plate 12 G). Dead juveniles and abnormal eggs were seen in plenty. The abnormal eggs were observed to be bursting spontaneously (Plate 12 D). Vigorous movements of bacteria were seen inside the eggs. In comparison to this, all except 3-4 eggs hatched in the control. Advanced stages of egg development were observed along with actively moving J1 inside the eggs in the control.

4.2.4. Phosphate solubilization

The short listed 25 bacterial isolates were cultured on Pikovaskaya's solid medium to test their phosphate solubilization ability. The bacterial isolates produced clearing zones of various dimensions indicating their ability to solubilize phosphates. The maximum phosphate solubilization was reported by the Is. 13 (2.5 cm) (Plate13b. A) followed by Is.. B15 (2.4 cm) (Fig 2). Four isolates were unable to solubilize phosphates in the study conducted.

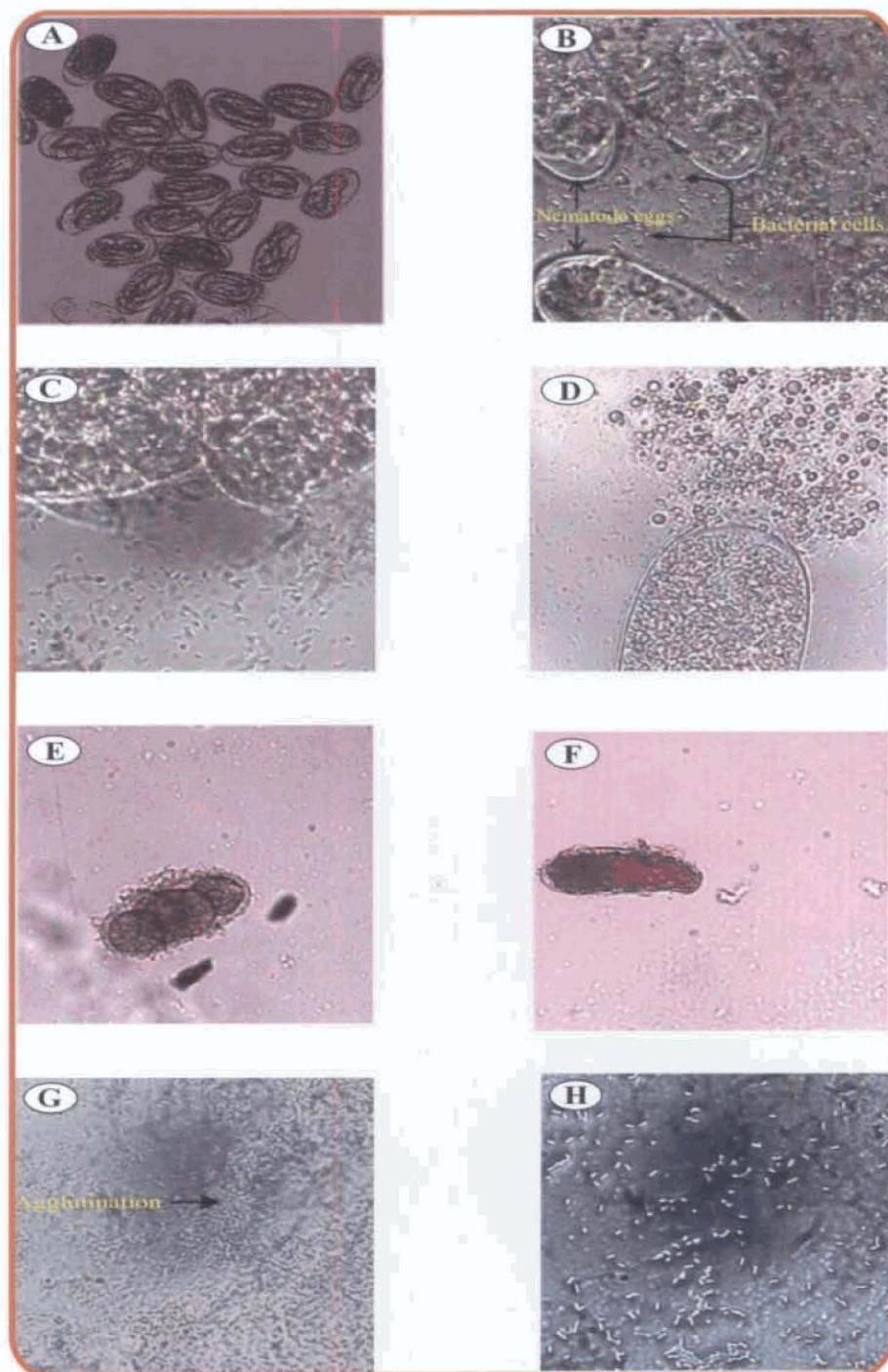
4.2.5. Effect on mycelial growth of *Phytophthora*

The effect of the bacterial isolates on the mycelial growth of *Phytophthora* was evaluated in dual culture method. The bacterial isolates varied in their inhibition to *P. capsici*, the inhibition varying from 0 to 73.33 % (Fig 3, Plate.13b. D). Out of the 25 isolates tested, 17 were able to inhibit the pathogen to different extends. The maximum inhibition was achieved with the Is. 127 (73.3 %) followed by Is.123 (60.4%). There was no inhibition of *Phytophthora* with eight bacterial isolates.

Table 9. Suppression of root-knot nematode egg hatching after 48 hours of exposure to 25 selected bacterial isolates (Mean of three replications)

Sl. No.	Isolate No.	Total no. of eggs	No. hatched	Hatching suppression (%)
1	13	106.67	25.33	75.53ghij
2	18	108.33	48.33	50.06bcdefg
3	19	97.00	15.67	84.05ij
4	24	108.33	26.33	75.60ghij
5	25	110.33	66.00	39.27bcde
6	101	88.66	31.33	64.47fgh
7	113	97.00	35.67	62.85fgh
8	117	98.67	43.00	55.62cdefg
9	118	109.33	51.67	52.47bcdefg
10	119	100.33	46.00	52.00bcdef
11	123	92.67	49.66	46.49bcdef
12	124	88.33	26.66	69.76fghi
13	127	110.00	19.33	82.52j
14	132	96.00	36.67	61.80efgh
15	133	98.66	41.33	58.85defgh
16	136	103.33	47.33	55.07bcdefg
17	Ap 1	91.67	56.33	38.12bcd
18	Ap 4	110.00	19.33	82.38ij
19	Ap 5	100.00	31.00	68.97fghi
20	Ap 8	112.33	45.67	61.33efgh
21	Ap 10	111.67	44.33	61.27efgh
22	Ap 11	97.00	63.33	33.96bc
23	Ap 16	96.00	19.00	80.22hij
24	B 14	100.67	12.33	87.89j
25	B 15	96.00	08.00	91.66j
26	Control	91.67	85.00	7.32a
	Mean	100.79	38.26	61.52efgh

Means followed by the same alphabet in a column do not differ significantly.



¹²
Plate 8. Effect of bacterial isolates on the hatching suppression of *Meloidogyne incognita* eggs

- A. Normal eggs, B. Treated with Is.136, C. Treated with Is.132,
- D. Bursting of eggs treated with Is.135
- E. Agglutination of bacterial cells on nematode eggs
- F. Bacteria infected nematode egg with distorted morphology
- G. Agglutination of bacterial cells in presence of nematode egg
- H. No agglutination in the absence of nematode eggs

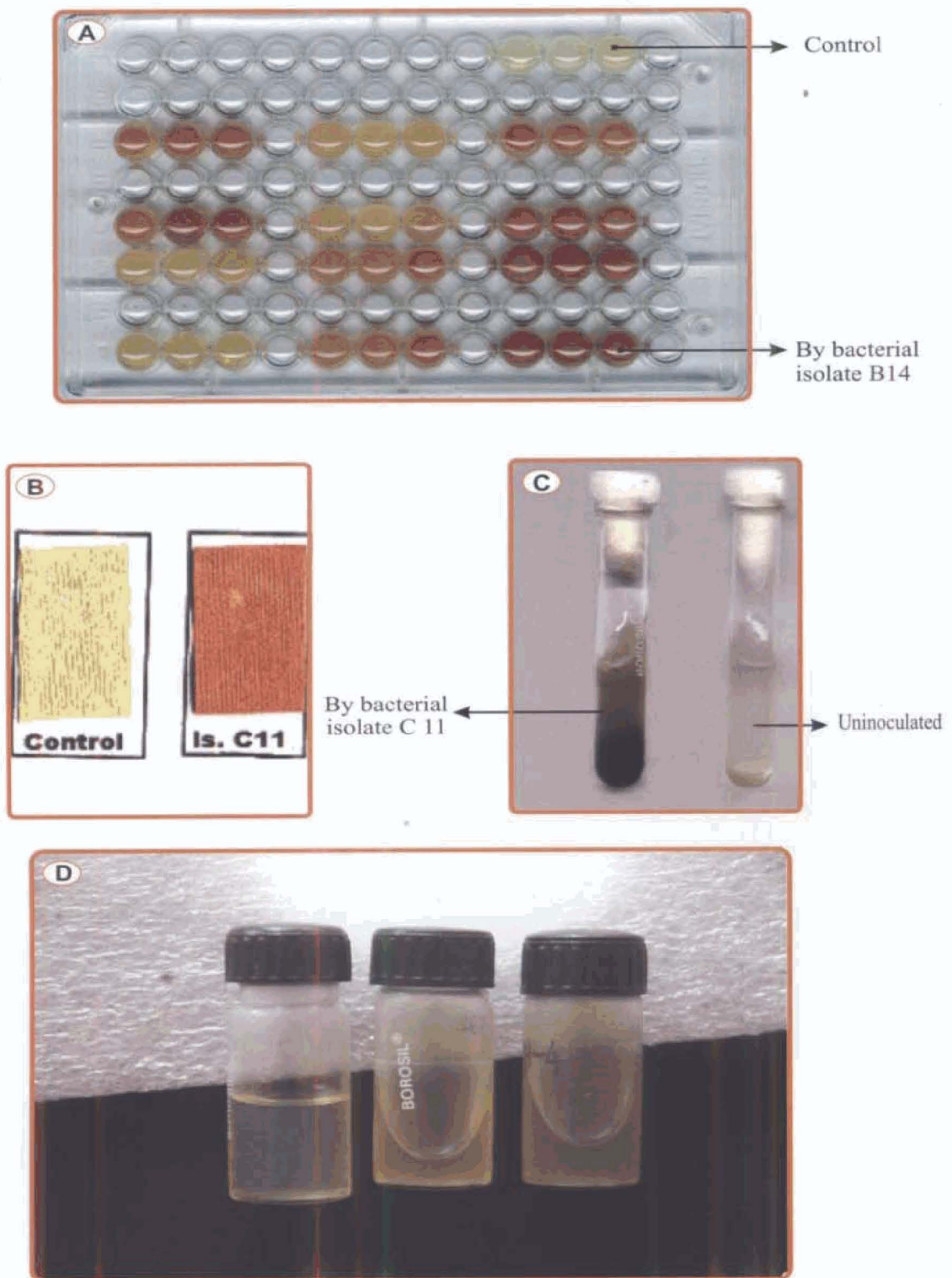


Plate13a. Mode of action of bacterial isolates in suppressing nematodes

- A. Microtitre plate showing ammonia produced by bacteria
- B. HCN production by bacteria as evidenced by the color change in filter paper soaked in picric acid solution
- C. H_2S production by bacteria in SIM Agar slants
- D. Gelatin liquefying capacity of Ap4 and Is.123

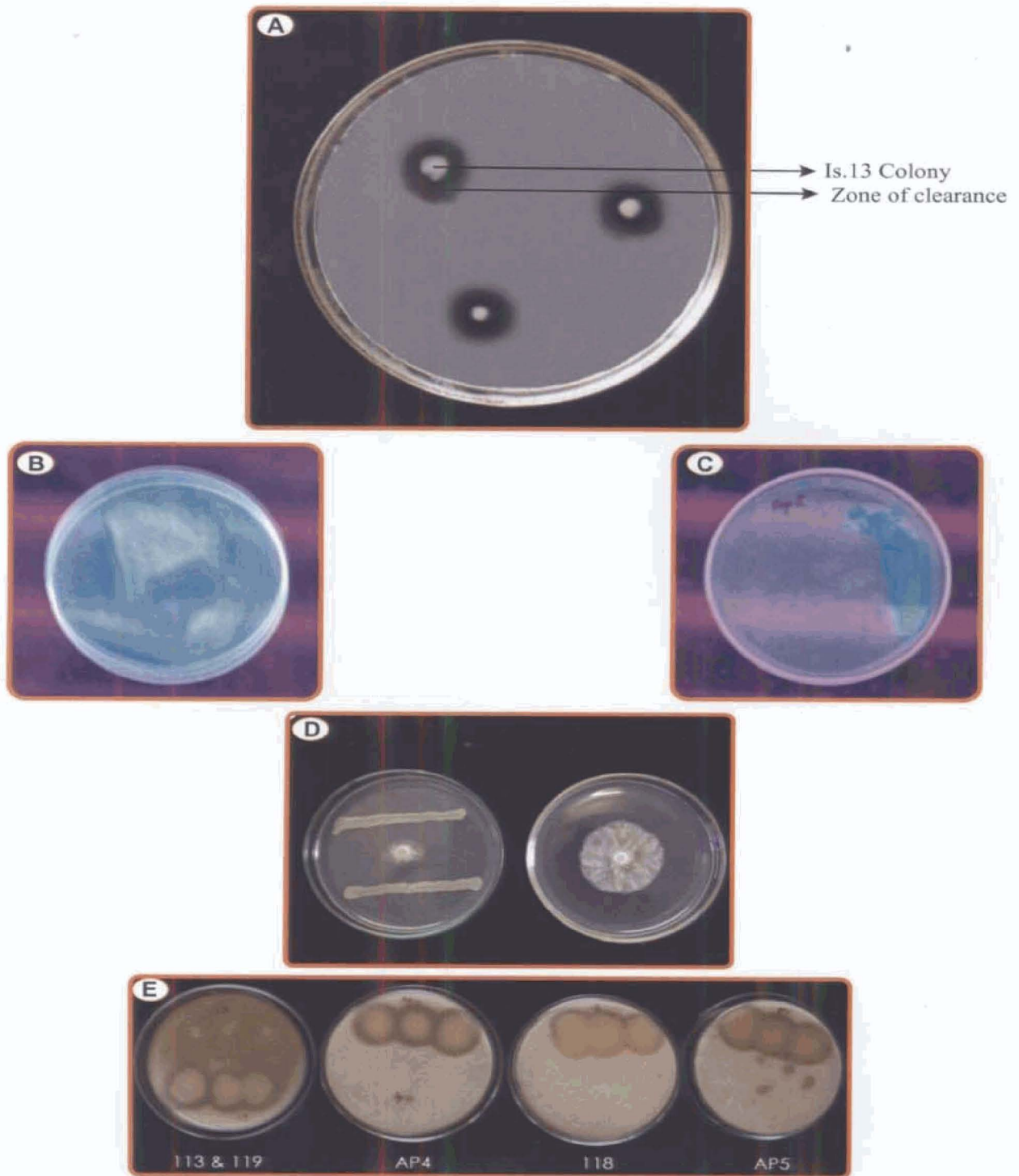


Plate 13b Mode of action of bacterial isolates in suppressing nematodes

- A. Phosphate solubilization in Pikovaskaya's media
- B. Siderophore production by Ap 4
- C. Siderophore production by Ap 8
- D. Inhibition towards *Phytophthora* by *Pseudomonas* Ap 4
- E. Production of Protease enzyme by bacterial isolates in Skim milk agar as evidenced by zone of clearance around the bacterial colonies

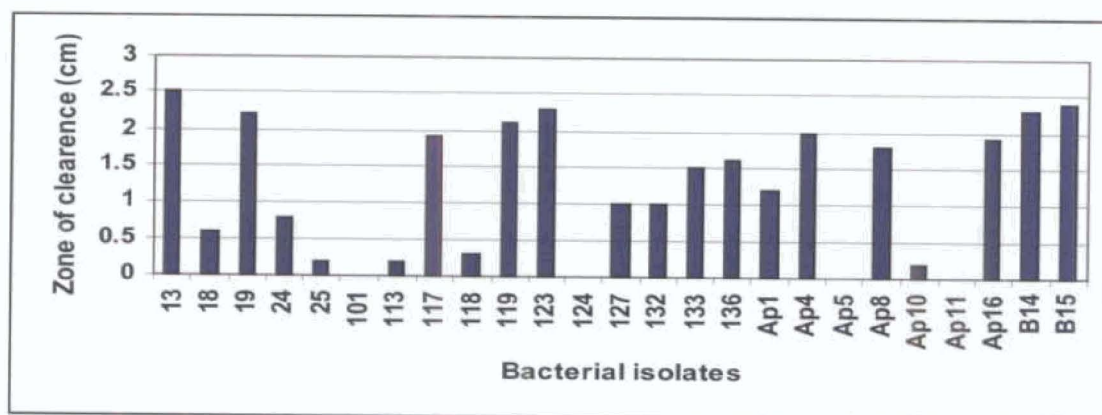


Fig. 2. Ability of short-listed bacterial isolates to solubilize phosphate in Pikoyaksya's Agar Medium

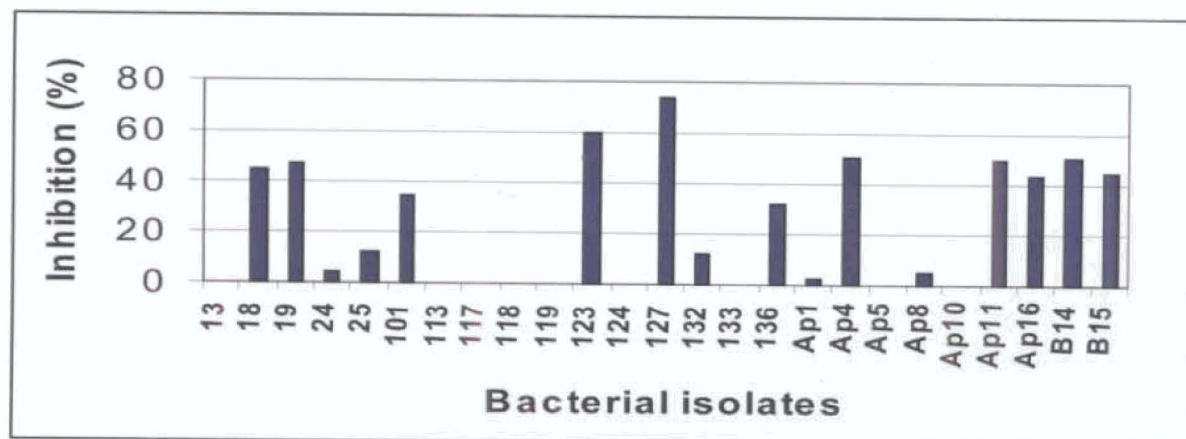


Fig3. Inhibition of mycelial growth *Phytophthora capsici* by short-listed bacterial isolates

4.2.6. Production of HCN

Production of HCN by different strains of bacteria by assessing the colour change in filter paper strips soaked in picric acid solution. Out of 94 isolates screened including the short listed 25, only eight isolates produced HCN, which is good nematocide. Out of these, five were found to be strong HCN producers; two were moderate HCN producers while one isolate was a weak producer. The rest of isolates did not produce any HCN (Table 10; Plate 13 a. B).

4.2.7. Production of H₂S

Similarly the production of hydrogen sulphide by 94 bacterial isolates was studied in another *in vitro* experiment. The results showed that 21 isolates were able to produce H₂S (Table 10; Plate 13a. C).

4.2.8. Production of ammonia

The production of ammonia by 94 bacterial isolates was studied. All of the 94 bacterial isolates produced varying levels of ammonia. Among these, 14 isolates were very strong producers of ammonia, and 36 were strong producers (Table 10, Plate 13a. A.).

4.2.9. Detection of siderophores by UV- fluorescence method

Siderophores are very crucial in the availability of iron in the rhizosphere which ultimately influences the survival of pathogens. In the present study an attempt was made to detect the production of siderophores in 12 bacterial isolates out of the 25 short listed, using UV- fluorescence. Among the twelve isolates studied Ap 4 and Ap 8 fluoresced under UV light indicating their production (Plate.13b B&C). Only one isolate (Ap 4) was positive for pyocyanin production.

4.2.10. Detection of protease enzyme activity

As proteins are integral parts of nematode body, strains of bacteria having proteolytic activity can be better nematode antagonists. On screening the short listed 25 bacterial isolates for production of protease enzyme, 13 were found to produce the above enzyme as evidenced by the zone of clearance around the colonies (Plate13b.E) in skim milk agar medium.

4.2.11. Detection of gelatin liquefying capacity of the bacterial isolates

Several nematodes like root knot and cyst nematodes lay their eggs in a gelatinous matrix. Degradation of this matrix can make them vulnerable to attack by other microbial agents. Therefore, the gelatin liquefying capacity of 25 bacterial isolates were tested. The study has revealed the ability of two isolates, Ap 4 and Is.123 to liquefy gelatin (Plate13a.D).

4.2.12. Antibiotic screening

The short listed 25 isolates were subjected to screening for antibiotic resistance to 14 antibiotics. Several strains were found to have intrinsic antibiotic resistance to different antibiotics used (Table 11). Among the antibiotics tested, the isolates were resistant to majority of the antibiotics. Is.13, Is. 127, Ap5, Ap8, Ap16, B14 and B15 were resistant to all of the antibiotics tested where as Is. 18 and Is.124, were susceptible to nalidixic acid only. Is. No. 19, Is.113, Is.119, Is.123, AP1 and Ap10 were susceptible to polymyxin only. Ap11 was susceptible only to a single antibiotic, cycloheximide while Is. 24, Is.101, Ap4 were susceptible to nalidixic acid and polymixin. Is.No. 25 was susceptible to kanamycin and polymixin. Is.136 was susceptible to cycloheximide and polymyxin. Is. No 118 is susceptible to four antibiotics tested while Is. No. 132 was susceptible to five antibiotics tested.

4.2.13. Utilization of carbon sources

The ability of 25 short listed bacterial isolates to utilize various carbon sources was tested in the basal medium for oxidation/fermentation (O/F).

Table 10. Production of hydrogen cyanide, hydrogen sulphide and ammonia by different isolates of bacteria under *in vitro* conditions

Is. No.	HCN	H ₂ S	Ammonia	Is. No.	HCN	H ₂ S	Ammonia	Is. No.	HCN	H ₂ S	Ammonia
62	-	-	++	135	+++	-	+++	B14	-	-	++++
101	-	-	++	136	-	+	+	B15	-	+	++
105	-	-	++	Ap1	-	-	+++	B16	-	+	++
106	+++	-	+++	Ap2	-	-	++	C1	-	-	+
107	-	-	++	Ap3	-	-	++	C2	-	-	++++
108	-	-	+++	Ap4	-	-	+++	C3	-	-	+++
109	-	-	++++	Ap5	-	-	++++	C4	-	-	++
110	+++	+	++	Ap6	-	-	++	C5	-	-	++++
111	+	+	++++	Ap7	-	-	+	C6	-	-	++++
112	-	-	+	Ap8	-	-	+++	C7	-	-	+++
113	-	+	+++	Ap9	-	-	+++	C8	-	-	++
114	-	-	+	Ap10	-	-	+++	C9	-	-	+++
115	-	-	++	Ap11	-	-	+++	C10	-	-	+++
116	-	-	++	Ap12	-	-	+++	C11	++	+++	++
117	-	+	++++	Ap13	-	-	+++	C12	-	-	+++
118	-	+	++	Ap14	-	-	++	C13	-	-	+++
119	-	+	+++	Ap15	-	-	+	C14	-	-	++
120	-	-	+++	Ap16	-	-	+++	C15	-	-	++
121	-	+	+++	B1	-	-	++	C16	-	-	+++
122	-	-	+++	B2	-	-	+++	C17	-	-	++
123	-	+	+	B3	-	-	++	C18	-	-	++
124	-	-	+++	B4	-	+	+	C19	-	-	+++
125	-	-	+++	B5	-	+	++	C20	-	-	++++
126	+++	-	++	B6	-	+	+++	C21	-	-	++
127	-	+	++	B7	-	+	++	C22	-	-	+
128	-	-	++	B8	-	+	++	C23	-	-	+++
129	-	-	+	B9	-	+	++	C24	-	-	+++
130	-	-	++	B10	-	-	++	C25	-	-	+++
131	-	-	++	B11	-	-	+	C26	-	-	+++
132	-	-	++++	B12	-	-	++	C28	-	-	+++
133	++	-	+++	B13	-	-	++	C29	-	-	+++
134	-	-	+++								

++++ very strong, +++ strong, ++ moderate, + weak, - negative

Table 11 . Growth of 25 short- listed bacterial strains in media nutrient agar amended with 13 antibiotics

Culture No.	Strep (100µg/ml)	Amp (100µg/ml)	Rif (50µg/ml)	Kan (50µg/ml)	cyclo (30)	Nal (40µg/ml)	Gen (10µg/ml)	TTC (10µg/ml)	Pen (100µg/ml)	Poly (100µg/ml)	Bac (100µg/ml)	Spec (25µg/ml)	Tetra (15µg/ml)	Control ^a
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	-	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	-	+	+	+	+
24	+	+	+	+	+	-	+	+	+	-	+	+	+	+
25	+	+	+	-	+	+	+	+	+	-	+	+	+	+
101	+	+	+	+	+	-	+	+	+	-	+	+	+	+
113	+	+	+	+	+	+	+	+	+	-	+	+	+	+
117	+	+	+	+	+	-	+	+	+	+	+	+	+	+
118	+	+	-	-	-	+	+	+	+	+	+	+	-	+
119	+	+	+	+	+	+	+	+	+	-	+	+	+	+
123	+	+	+	+	+	+	+	+	+	-	+	+	+	+
124	+	+	+	+	+	-	+	+	+	+	+	+	+	+
127	+	+	+	+	+	+	+	+	+	+	+	+	+	+
132	+	-	-	+	-	-	+	+	+	+	+	+	-	+
133	+	+	+	+	+	+	+	+	+	+	+	+	+	+
136	+	+	+	+	-	+	+	+	+	-	+	+	+	+
Ap1	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Ap4	+	+	+	+	+	-	+	+	+	-	+	+	+	+
Ap5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ap8	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ap10	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Ap11	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Ap16	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B14	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B15	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ represents bacterial growth, - represents inhibition of bacterial growth

^a no antibiotics added

Nine isolates were able to metabolize all the carbon sources tested. All the strains of bacteria tested metabolized glucose and mannitol. Four isolates were not able to metabolize lactose, while three isolates were not able to metabolize galactose, 10 isolates not able to metabolize maltose, 11 isolates not able to metabolize sorbitole and ten isolates not able to metabolize dulcitol. Bacterial isolates Ap1 Ap4 and Ap11 could not utilized four carbon sources out of seven evaluated. Bacterial isolates retrieved from antagonistic plants had poor ability to utilize carbon sources other than glucose and mannitol. In general, the tested bacterial strains were found to have multiple nutritional spectrums utilizing most of the substrates tested (Table 12).

4.2.14. Suppression of nematodes infecting black pepper

The short listed 25 bacterial isolates were further evaluated for their effect on root knot and burrowing nematodes using black pepper (Panniyur 1) as the test plant. The results showed that all the tested bacteria suppressed *R. similis* population and the range of suppression varied from 87.93 to 99.80 % (Table 13, Plate 16). Highly significant nematode suppression was noticed with isolates Ap 4, Ap16, Is. 119 and Is.136. Out of 25 bacterial isolates evaluated, 11 isolates enhanced the growth of black pepper rooted cuttings. Among them Ap4, 101, 133, 136 and Ap16 were superior in increasing the height of the plants. Almost all isolates increased the root biomass, which is one of the mechanism responsible for combating the nematode damage. Very profuse root proliferation was observed in plants treated with Is.136, Ap4 and Ap16. However, comparatively less number of isolates increased the shoot biomass. Notable among these are Is.13, Is.19 and Is.101 and Is. Ap16. Highest total biomass was observed in plates inoculated with Is.13, 19, 136 and Ap16. On the basis of the above green house study isolates 101, 133, 136 Ap4 and Ap16 can be considered as excellent growth promoters.

All the short-listed 25 bacterial isolates significantly suppressed (above 90%) root knot nematodes too (Table 14, Plate 17). The range of suppression was from 91.26 to 100 %. 100 % nematode suppression was achieved with isolates Ap4 and Is.136. The growth of the black peppers cuttings were also increased significantly. Maximum height of black pepper was achieved by Is.136 followed by Ap4. Significantly increased biomass of black pepper was achieved with the application of several bacterial isolates. Notable among them are Is. 13, 101, 136, Ap4, Ap11, Ap16 and B14. Maximum root biomass was achieved by the application of bacterial isolate Ap16 followed by Is.136.

Table 12. Characterization of bacterial isolates based on utilization of carbon

Culture No.	Glucose	Lactose	Maltose	Galactose	Mannitol	Sorbitole	Dulcitol
13	+	+	+	+	+	+	+
18	+	+	+	+	+	-	+
19	+	+	+	-	+	-	+
24	+	+	-	+	+	-	+
25	+	+	+	+	+	+	-
101	+	+	+	+	+	+	+
113	+	+	+	+	+	+	+
117	+	+	+	+	+	+	+
118	+	+	+	+	+	+	+
119	+	+	+	+	+	+	+
123	+	+	+	+	+	+	-
124	+	+	-	+	+	-	-
127	+	-	-	+	+	+	-
132	+	+	-	+	+	+	-
133	+	+	+	+	+	+	+
136	+	+	-	+	+	-	-
Ap1	+	-	-	+	+	-	-
Ap4	+	-	-	+	+	-	-
Ap5	+	+	+	+	+	-	+
Ap8	+	+	-	-	+	-	+
Ap10	+	+	-	-	+	-	+
Ap11	+	-	-	+	+	-	-
Ap16	+	+	+	+	+	+	-
B14	+	+	+	+	+	+	+
B15	+	+	+	+	+	+	+

(+ represents growth, - represents no growth)

Table 13. Effect of short listed bacterial isolates on growth of black pepper rooted cuttings and suppression of *Radopholus similis* (Mean of three replications)

Treatment	Height (cm)	Fresh Biomass (g)			Nematodes/ g pepper root
		Root	Shoot	Total	
Control	54.00ab	4.84abc	24.28abcd	29.12abc	0.00a
<i>R. similis</i>	67.00abc	3.87a	16.25a	20.11a	948.25d
13	59.33abc	4.67abc	54.09hi	58.75efg	65.84ab
18	43.67a	4.44ad	19.04ab	23.48ab	45.43ab
19	76.33abcd	4.45abc	56.81i	61.36fg	83.06ab
24	46.67ab	6.20abcd	27.16abcdef	33.36abcd	44.30ab
25	48.33ab	4.83abc	44.03bcdefghi	48.87cdef	44.36ab
101	102.00bcd	5.84abcd	52.06ghi	57.90efg	63.92ab
113	87.67abcd	5.66abcd	23.81abcd	29.51abc	18.30ab
117	83.33abcd	5.62abcd	26.24abcdef	31.86abcd	106.67ab
118	61.66abc	6.45abcd	22.72abc	29.18abc	24.49ab
119	85.66abcd	9.27def	31.46abcdefg	40.73abcdef	7.00a
123	65.67abc	7.34abcde	30.46abcdef	37.80abcde	24.60ab
124	62.00abc	6.66abcd	25.19abcde	31.85abcd	114.47c
127	52.67abc	4.78abc	19.81ab	24.60ab	89.35ab
132	67.00abcd	5.66abcd	23.85abcd	29.51abc	33.58ab
133	100.66bcd	8.07cde	44.63defghi	53.33defg	53.67ab
136	122.00d	12.87fg	46.97fghi	59.75efg	4.89a
AP 1	84.33abcd	5.45abcd	33.53abcdefgh	38.98abcdef	51.82ab
AP4	94.33abcd	11.00ef	40.01bcdefghi	51.02cdefg	1.92a
AP5	65.00abc	8.36cde	29.15abcdef	37.51abcde	38.73ab
AP8	84.33abcd	6.57abcd	33.66abcdefgh	40.24abcdef	87.57ab
AP10	82.66abcd	5.93abcd	33.27abcdefgh	39.21abcdef	52.90ab
Ap11	75.66abcd	5.52abcd	26.75abcdef	32.27abcd	30.82ab
Ap16	109.00cd	14.00g	53.91hi	67.92g	3.07 a
B14	70.00abcd	5.34abcd	32.67abcdefg	38.02abcde	37.99ab
B15	67.33abcd	6.01abcd	46.00efghi	52.01cdefg	64.56ab
Mean	74.75	6.66	33.99	40.67	79.32

Means followed by the same alphabet in a column are not significantly different.

Table 14. Effect of short listed bacterial isolates on growth of black pepper rooted cuttings and suppression of *Meloidogyne incognita* (Mean of three replications)

Treatment	Height (cm)	Biomass (g) fresh			Nematodes/g root
		Root	Shoot	Total	
Control	58.66 ab	4.38 a	21.32 ab	25.07a	0.0a
RKN	57.66 ab	7.39 abc	18.11 a	25.50a	3841..0c
13	59.33 abc	5.35 abc	45.83 de	51.18cde	56.27a
18	65.00abcde	4.56 a	39.0 cde	43.57bcd	105.15 a
19	76.33 bcdefg	4.55 a	44.41de	48.96bcd	106.84 a
24	62.66 abcd	6.45 abc	26.90abc	33.36ab	40.00a
25	67.00abcdef	4.83ab	44.03de	48.87bcd	56.72a
101	102.00defgh	6.07abc	51.40e	57.67de	73..33a
113	89.33 bcdefgh	7.85abcd	38.50cde	46.36bcd	53..33a
117	83.33abcdefgh	5.63abc	26.24abc	31.86ab	118.89a
118	61.66 abcd	6.85abcd	25.75abc	32.61ab	26.66a
119	104.00 efgh	10.09de	30.64abcd	40.73abcd	13.33a
123	79.00 abcdefg	8.16bcde	35.21bcd	43.37bcd	200.73ab
124	82.66abcdefgh	6.88abcd	31.53abcd	38.41abc	153.3ab
127	62.66 abcd	4.78ab	27.03abc	31.82ab	335.83ab
132	67.00 abcdef	5.66abc	31.58abcd	37.24abc	105.14a
133	100.66 defgh	8.60cde	42.00de	50.60cde	100.00a
136	122.00h	13.23fg	51.49e	64.72e	0.0a
AP 1	81.00 abcdefg	5.42abc	33.55abcd	38.98abc	53.44a
AP4	111.00gh	11.23ef	45.15de	56.38de	0.0a
AP5	65.00abcde	8.36cde	29.15abc	37.51abc	73.30a
AP8	84.33abcdefgh	6.57abc	40.62cde	47.19bcd	160.84ab
AP10	107.00fgh	8.52cde	30.68abcd	39.21abc	20.00a
Ap11	75.67abcdefg	5.22abc	26.75abc	32.27ab	73.30a
Ap16	100.00cdefgh	14.54g	39.62cde	54.17bcd	6.67a
B14	67.33abcdef	5.89abc	45.69de	51.58cde	60.00a
B15	70.00abcdef	5.79abc	35.51bcde	41.31abcd	40.00a
mean	80.08	7.14	35.47	42.61	217.55

Means followed by the same alphabet in a column are not significantly different

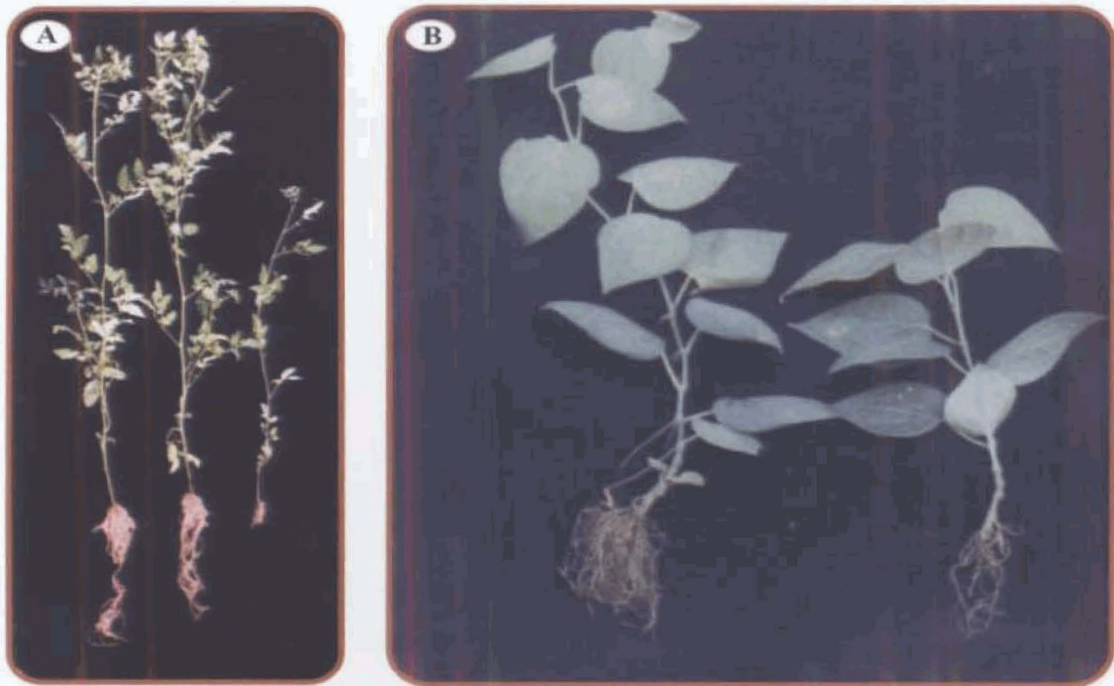


Plate 14. Effect of bacterial isolates on growth promotion
A. In tomato B. In black pepper



Plate 15 . Effect of bacterial isolates in root proliferation of black pepper

A. Treated with Ap4. B. Unoculated control

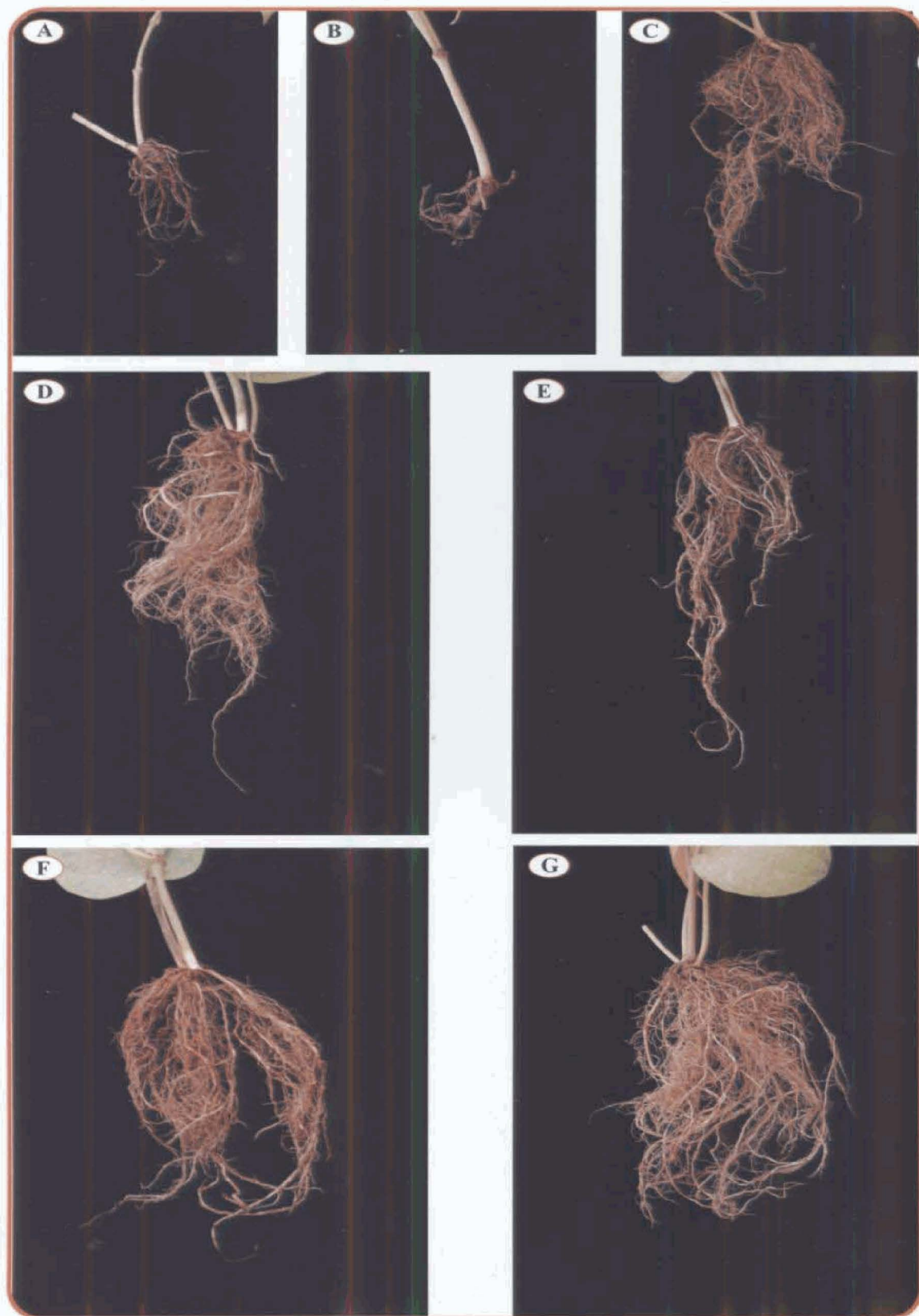


Plate 16. Evaluation of selected bacterial isolates for the suppression of *Radopholus similis* infecting black pepper

A. Control B. *R. similis* alone C. Treated with Ap4 D. Treated with Ap16
E. Treated with Is.113 F. Treated with Is.119 G. Treated with Is.136



Plate 17. Evaluation of selected 25 bacterial isolates for the suppression of *Meloidogyne incognita* infecting black pepper

A. Control B. *Meloidogyne* C. Treated with Ap4 D. Treated with Ap16

4.2.15. Induced resistance

The ability of bacterial isolates to induce resistance inoculated plants was detected in this experiment.

25 bacterial isolates were evaluated for their effect to induce resistance against root knot nematode in tomato in two different set of experiment. In the first experiment, out of the seven bacteria tested, five isolates were able to reduce the nematode population significantly in the nematode inoculated root half. Total nematode suppression was obtained with isolate No. 25. Isolate No.25 also achieved maximum height and biomass of the plant (Table15)

In the second experiment out of the 18 bacteria used, 11 were able to reduce the nematode population in the nematode inoculated root half of the plant and 100% nematode suppression was achieved with five isolates. They are AP4, AP 10, AP16, 136 and 118. Majority of the isolates resulted in growth promotion of black pepper. Maximum height of tomato was recorded with Is.136 (51.94g) and maximum biomass was recorded with the bacterial isolate Ap4.

Resistance to root knot nematodes were evaluated in black pepper too. Out of the 20 bacterial isolates tested, four resulted in 100% nematode suppression (Table 16, Plate 18).

The evaluation of bacterial isolates in black pepper were also carried out in an experiment of nodal runners too. Out of the twenty isolates used, 17 isolates reduced the nematode population significantly and 100% nematode suppression was achieved with seven isolates (Table 17, Plate 19). Though the nematode and biocontrol agnets were spatially separated from each other, these bacterial isolates induced resistance in black pepper against the attack of nematodes.

Table 15. Evaluation of bacterial isolates to induce resistance against *M. incognita* in tomato plants in split root experiment.

Treat ment	Plant growth character			Root knot nematode development				
	Height (cm)	Biomass (g)	EM n	EM b	Galls n	Galls b	Index n	Index b
<i>Experiment 1</i>								
13	51.33b	18.81ab	2.67bc	0.0b	1.33b	0.0b	0.67cd	0.00b
18	66.33a	18.44ab	7.0ab	0.0b	8.00a	0.0b	2.00ab	0.00b
19	51.33b	16.01bc	1.67c	0.0b	1.00b	0.0b	0.67cd	0.00b
24	70.00a	19.26ab	1.00c	0.0b	1.00b	0.0b	0.00d	0.00b
25	77.00a	22.28a	0.0c	0.0b	0.00b	0.0b	0.00d	0.00b
101	44.00bc	19.54ab	1.00c	0.0b	1.00b	0.0b	0.00d	0.00b
113	37.30bcd	15.72bc	11.00a	3.0 b	5.33a	0.33b	1.33bc	1.00a
RKN	30.33cd	12.56cd	12.33a	2.0a	10.33a	1.67a	2.67a	1.00a
control	25.00d	10.39d	0.0c	0.0b	0.00b	0.0b	0.00d	0.00b
<i>Experiment 2</i>								
Control	30.60a	12.2ab	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
RKN ^a	28.57a	12.0a	14..3d	1.3bc	19.7d	1.67ab	3.6e	1.0b
B14	45.82defg	20.8cde	3.33 bc	0.0a	1.67a	0.0a	1.0bc	0.0a
B15	44.92cdefg	19.9cde	1.33 ab	0.0a	1.0a	0.0a	0.67a	0.0a
Ap10	50.37ef	25.4ef	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
Ap1	45.67defg	20.6cde	2.33 bc	0.0a	1.0a	0.0a	0.67a	0.0a
124	40.71bcd	15.7abc	11.0d	0.67ab	5.0b	0.33a	1.33bcd	0.67b
Ap8	43.92bcdef	18.9bcde	14.0d	1.33 bc	15.0bc	1.67ab	2.67e	1.0b
Ap4	50.37efg	25.4ef	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
Ap11	43.31bcde	18.3abcd	4.0 c	1.33bc	5.0b	1.67ab	1.33bcd	1.0b
Ap16	50.98efg	26.0f	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
136	51.94g	24.1def	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
117	37.56b	12.6ab	12.33d	1.0bc	17.7d	1.33ab	3.0e	1.0b
118	45.54cdefg	20.5cde	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
119	44.85defg	19.9cde	3.0 bc	0.0a	5.67b	0.0a	1.33bcd	0.0a
123	45.56cdefg	20.6cde	2.33 bc	0.0a	1.0a	0.0a	0.67a	0.0a
Ap5	40.72bcd	15.7abc	11.00d	0.67ab	12.3c	0.33a	1.67cd	0.67b
127	39.78bcd	14.8abc	14.67d	1.33bc	17.7d	1.67ab	2.67e	1.0b
132	39.28bcd	15.7abc	12.33d	0.67ab	14.0bc	0.33a	2.0de	0.67b
133	38.00bc	14.0abc	14.00d	2.0c	16.3bc	1.67ab	2.67e	1.0b

n and b denotes the results of nematode inoculated and bacteria inoculated halves of the roots respectively. The values on nematode population are square root transformed before statistical analysis.

ANOVA was carried out experiment wise

^a Root knot nematode

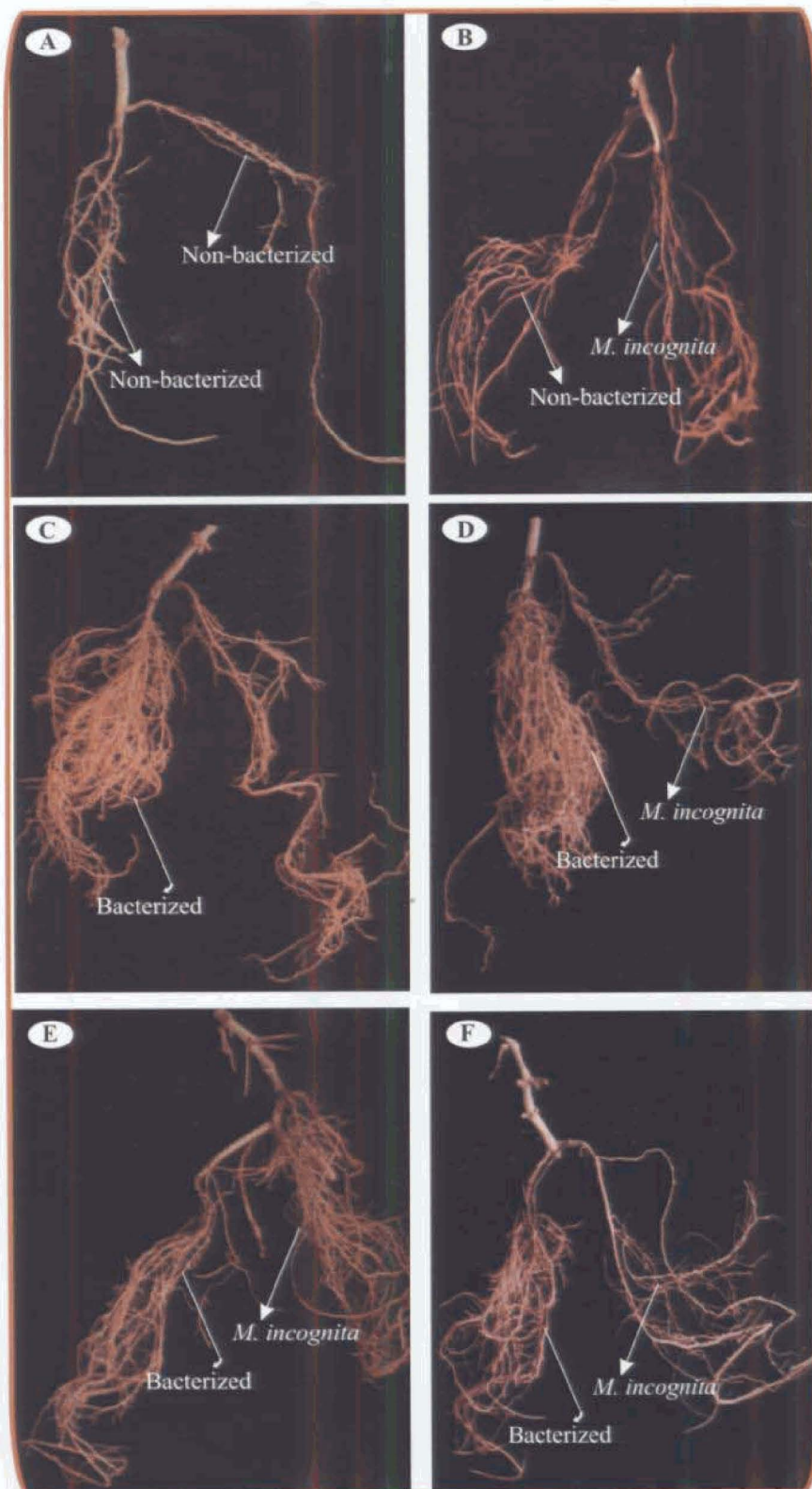


Plate 18. Induction of systemic resistance against root knot nematodes by bacterial isolates in Black pepper in split root experiment

A. Control, B. Treated with *Meloidogyne* alone, C. Treated with Ap16
D. Treated with Ap11, E. Treated with Ap4, F. Treated with Is. 25

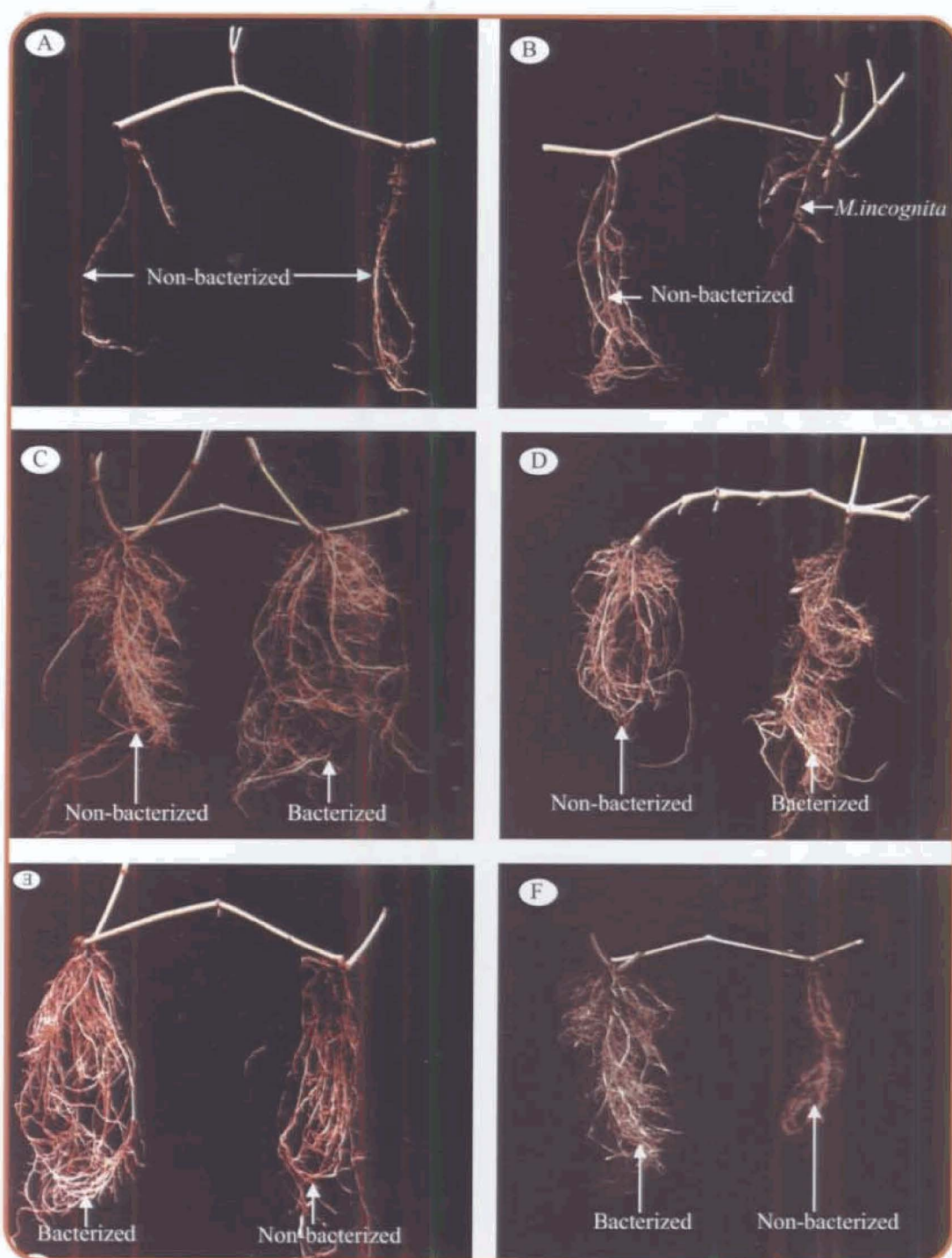


Plate 19. Induction of systemic resistance against root knot nematodes by bacterial isolates in Black pepper in an experiment of nodal roots.

A. Control B. Nematode alone C. Treated with Ap4 D. Treated with Ap16
E. Treated with Is113 F. Treated with Ap11

Table 16. Evaluation of bacterial isolates to induce resistance against *M. incognita* in black pepper in split root experiment

Treatment	Root Knot Nematode population					
	Eggs n	Eggs b	Juveniles n	Juveniles b	Females n	Female b
13	50.0cde	16.67ab	0.0a	0.00	0.0a	0.0a
25	0.00a	0.0a	16.67a	0.00a	0.0a	0.0a
113	566.67e	83.33bc	480.0cd	0.00	33.33a	0.0a
117	116.67abc	66.67abc	33.33abc	0.00a	0.0a	0.0a
118	16.67ab	0.0a	33.33abc	0.00a	0.0a	0.0a
119	150.00abc	33.33abc	66.67bcd	0.00a	0.0a	0.0a
123	50.00cde	0.0a	0.0a	0.00a	0.0a	0.0a
124	116.67cde	0.0a	33.33abc	0.00a	17.0a	0.0a
127	150.00cde	33.33abc	0.0a	0.57b	0.0a	0.0a
133	0.00a	0.0a	0.0a	0.00a	0.0a	0.0a
136	50.00cde	16.67ab	0.0a	0.00a	0.0a	0.0a
Ap1	16.67ab	0.0a	16.67a	0.00a	0.0a	0.0a
Ap4	16.67ab	0.0a	16.67	0.00a	0.0a	0.0a
Ap5	0.00a	0.0a	0.0a	0.00a	0.0a	0.0a
Ap8	33.33bcd	16.67ab	33.0ab	0.00a	0.0a	0.0a
Ap10	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
Ap11	16.67ab	16.67ab	0.0a	0.00a	0.0a	0.0a
Ap16	0.00a	0.0a	0.0a	0.00a	0.0a	0.0a
B14	266.67cde	16.67ab	83.33bcd	0.00a	0.0a	0.0a
B15	416.67de	16.67ab	50.0bcd	0.00a	17.0a	0.0a
Control	0.00 a	0.00a	0.00a	0.00a	0.0a	0.0a
RKN ^a	1116.7 e	150.00c	633.33d	33.30c	100.0a	0.0a

The figures are log transformed values.

^a Root Knot Nematode

n and b denotes the results of nematode inoculated and bacteria inoculated halves of the roots respectively

Table 17. Evaluation of bacterial isolates to induce resistance against *M. incognita* in black pepper in an experiment of nodal runners

Treatment	Root knot Nematode population					
	Eggs n	Eggs b	Juveniles n	Juveniles b	Female n	Female b
Control	0.00 a	0.00a	0.00a	0.00a	0.00 a	0.00a
RKN ^a	2733.3 g	533.3c	816.67 a	33.3 _b	100 c	0.00 a
25	0.00 a	0.00a	0.00 a	0.00a	0.00 a	0.00a
101	216.67 def	66.67 b	50.00 ab	16.67 a	16.67 bc	0.00a
113	6.67 bcde	33.33 ab	0.00 a	0.00a	0.00 a	0.00a
117	216.67 def	1.30 ab	33.33 ab	0.00a	0.00 a	0.00a
118	16.67 ab	0.00a	33.33 ab	0.00a	0.00 a	0.00a
119	233.33 defg	1.14 ab	83.33 bc	0.00a	0.00 a	0.00a
123	0.00a	0.00a	0.00 a	0.00a	0.00 a	0.00a
124	50.00 bed	0.00a	16.67 a	0.00a	0.00 a	0.00a
127	150.00 cdef	1.14 ab	0.00 a	0.00a	0.00 a	0.00a
133	33.33 ab	0.57 ab	16.67 a	0.00a	0.00 a	0.00a
136	0.00 a	0.00a	16.67 a	0.00a	0.00 a	0.00a
Ap1	33.33 ab	0.00a	16.67 a	0.00a	0.00 a	0.00a
Ap4	0.00 a	0.00a	0.00 a	0.00a	0.00 a	0.00a
Ap5	0.00 a	0.00a	0.00 a	0.00a	0.00 a	0.00a
Ap8	50.00 bcd	0.57 ab	33.33 ab	0.00a	0.00 a	0.00a
Ap10	16.67ab	0.57ab	0.00 a	0.00a	0.00 a	0.00a
Ap11	0.00a	0.00a	0.00 a	0.00a	0.00 a	0.00a
Ap16	0.00a	0.00a	0.00 a	0.00a	0.00 a	0.00a
B14	283.33 efg	0.57ab	83.33 bc	0.00a	0.00 a	0.00a
B15	300.00 fg	0.57ab	33.33 ab	0.00a	0.00 a	0.00a

The figures are log transformed values.

^a Root knot nematode

n and b denotes the results of nematode inoculated and bacteria inoculated halves of the roots respectively

4.3. STUDIES ON MOST PROMINENT ISOLATES

Based on the previous studies, five bacterial isolates viz. Is. 113, Is. 119, Is.136, Ap4 and Ap16 were identified as the most promising isolates for suppressing nematodes of black pepper. Further studies were carried out to characterize and evaluate their biocontrol efficacy. The results are given below.

4.3.1. 16S rDNA sequencing

16S rDNA of the selected bacterial isolates was amplified and sequenced. The sequences on comparing with NCBI database have clearly revealed the species identity of the chosen microorganisms Is. 113 was identified as *Bacillus megaterium*, Is. 119 was identified as *Comamonas testosteroni*, Is. 136 as *Alcaligenes faecalis* Ap4, as *Pseudomonas aeruginosa* and Ap16 as *Pseudomonas* Sp.

4.3.2. Effect of promising organisms on root knot nematodes infesting black pepper

Five promising bacterial isolates were further tested for suppressing root knot nematodes infesting black pepper rooted cuttings planted in three different potting mixtures viz. potting mixture, sterile potting mixture and soil contaminated with root knot nematode. The results are given in Table 18.

Table 18. Effect of three types of potting mixture on the efficiency of a few bacterial biocontrol agents (Mean of three replications)

Treatment	Growth		Nematode development ^c			
	Height (cm)	Rot wt. (g)	Shoot wt. (g)	Juveniles	Eggs	Female
Potting mixture						
Abs control ^a	152.67 a	8.61a	29.79 a	0.0 a	0.0 a	0.0 a
RKN ^d	142.33 a	17.49 bc	46.99 b	563.05 b	700.82 b	32.61 b
113	225.00 b	16.59 b	82.36 c	5.99 a	8.04 a	0.0 a
119	210.33 b	15.78 b	81.08 c	6.55 a	6.6 a	0.0 a
136	289.33 c	19.03 c	85.36 c	0.0 a	0.0 a	0.0 a
Ap4	292.33 c	19.55 c	89.19 b	0.0 a	0.0 a	0.0 a
Ap16	285.00 c	17.71 bc	89.18 c	1.94 a	1.94 a	0.0 a
Nematode infected soil						
Abs control ^b	94.33 a	3.75 a	22.54 a	549.53 c	698.81 c	56.06 b
RKN ^d	80.33 a	5.63 bc	28.92 a	1195.6 c	1404.99 c	130.54 c
113	134.00 c	5.01 b	51.96 b	81.86 b	57.22 b	0.0 a
119	126.67 b	6.35 cde	54.34 b	52.34 b	47.08 b	0.0 a
136	160.67 c	6.85 de	53.61b	9.54 a	4.72 a	0.0 a
Ap4	165.23 c	7.24 c	60.20 b	9.39 a	4.06 a	0.0 a
Ap16	154.64 bc	5.18b c	58.60 b	51.79 b	45.20 b	0.0 a
Sterile potting mixture						
Abs control ^a	105.00 a	4.29 a	33.57a	0.0 a	0.0 a	0.0 a
RKN ^d	108.33 a	8.33 b	39.94 a	1069.9 c	1007.74 d	68.08 ab
113	164.00 b	8.97 b	62.98 b	7.63 bc	11.19ab	0.0 a
119	161.67 b	9.32 b	65.36 bc	14.84 c	18.42 c	0.0 a
136	179.67 cd	10.53 cd	68.98 bc	0.0 a	0.0 a	0.0 a
Ap4	189.33 d	11.13 d	70.97 c	0.0 a	0.0 a	0.0 a
Ap16	169.33 bc	10.20 cd	69.64 bc	3.26 ab	3.26 a	0.0 a
Table of means						
Abs control	117.33	5.55	26.17	183.18	232.94	18.69
RKN ^c	110.33	10.48	38.62	879.33	1037.9	77.08
113	174.33	10.19	65.77	31.83	25.48	0.0
119	166.22	10.48	66.93	24.58	24.03	0.0
136	209.89	12.14	69.32	3.18	1.57	0.0
Ap4	215.63	12.64	80.08	3.13	1.35	0.0
Ap16	202.99	11.03	72.47	19.00	16.8	0.0

Means followed by the same letter in column are not statistically different.

^a Absolute control in which no nematode or bacterial treatment. The rooted cuttings were dipped only in sterile water

^b Absolute control in which no nematode or bacterial treatment. The rooted cuttings were dipped only in sterile water but planted in nematode infected soil

^c figures on nematode population were log transformed before statistical analysis and converted to original mean

^d Root Knot Nematode

In normal potting mixture, the growth of black pepper plants had increased and there was significant suppression of nematodes with all the bacterial isolates tested. Maximum height (292.33 cm) of the pepper was recorded with isolate No. Ap4 (i.e. 91.48 % of increase over control) followed by Is. No. 136 (289.33 cm i.e. 89.52% increase over control). The biomass of the plants both in terms of root weight and shoot weight had also increased. Maximum root weight (19.55g) and shoot weight (89.19 g) were reported with Ap4. All the treatments resulted in efficient suppression of nematodes (98.93-100%). Two isolates, AP 4 and 136 resulted in 100 % nematode suppression.

In soil contaminated with nematodes, the experimental results clearly showed that application of these bacterial biocontrol agents are efficient in suppressing the nematode infection. The bacterial treatments resulted in growth promotion and nematode suppression though the growth of the pepper cuttings was comparatively less when compared to the normal potting mixture. Though 100% nematode suppression could not be achieved with any of the isolates, all the isolates significantly suppressed the nematode population. The nematode suppression ranged from 93.15-99.71%. The maximum height (165.23 cm i.e. 75.27 % increase over control) was reported by isolate Ap 4, followed by 160.67 cm (i.e. 70.32 % increase over control) reported with isolate No. 136. Maximum root weight (7.24g) and biomass (60.20 g) was also reported by the application of Ap 4.

In sterile potting mixture the increase in growth of the plants was higher than that in contaminated soil and lesser than that in normal potting mixture. Here also the maximum height of the pepper seedlings was reported with isolate No. Ap. 4 (189.33 cm i.e. 80.32 % increase over control) followed by isolate No. 136 (179.67 i.e. 71.11 % increase over control). All the treatments resulted in significant suppression of root knot nematodes and 100 % suppression could be achieved with two isolates (Ap 4 and 136).

4.3.3. Effect of bacteria in root initiation and proliferation of black pepper

The five bacterial isolates when tested for their efficiency for rooting of black pepper, all the isolates significantly induced the proliferation of roots (Plate 15.) The bacterial strains significantly increased the root biomass of the plants, the highest increase among the bacterial strain was noticed with Is. No. Ap 4 (19.7 g, i.e. 155.20% The effect of selected isolates of bacteria on rooting of black pepper increase over control) followed by an increase of 144.61 % over control by the isolate 136. All the strains increased the root length in the treated plants, highest being Is. 136 (74.83 % increase over control)

which was on par with Ap4 (72.39% increase over control). It was noticed that bacteria treated plants had higher number of feeder roots as evident from the increase in number of root tips in the treated plants (Fig 4).

At the end of the experiment, all the bacterial strains were reisolated from the roots, indicating the colonization potential of these bacterial strains.

4.3.4. Compatibility studies

4.3.4.1. Compatibility with other organisms

Compatibility among bacterial isolates

Most promising five bacterial isolates Is. 113, Is. 119, Is. 136, Ap4 and Ap16 were tested for their mutual compatibility. Among the five isolates of bacteria tested, it is found that these bacteria are compatible with each other. There was no sign of inhibition among the bacterial isolates.

Compatibility with fungal isolates

Compatibility of the most promising bacterial isolate Ap4 was evaluated with three efficient fungal biocontrol agents of nematodes viz. *Trichoderma harzianum*, *Paecilomyces lilacinus*, *Pachonia chlamydosporia*. The combined inoculation of *T. harzianum* and Ap4 in potato dextrose broth resulted in 24×10^6 cfu/ml of Ap4 and 6×10^5 cfu/ml of *T. harzianum*. The combined inoculation of *T. harzianum* and Ap4 in nutrient broth resulted in 3×10^8 cfu/ml of Ap4 and 4×10^8 cfu/ml of *Trichoderma*. When bacterial isolate Ap4 was co inoculated with *P. lilacinus* in potato dextrose broth, the cfu/ml of Ap4 was 29×10^6 and 4×10^8 cfu/ml of *Trichoderma* (Plate 21)

The compatibility of the bacterial isolate with these fungal isolates was also tested in another way. These fungal isolates were grown on sterile cellophane sheet spread over potato dextrose agar (PDA) plates and carefully removed the fungal mycelium along with cellophane sheet. Disc (4 mm) were cut from the PDA plates and placed on the lawn plate culture of Ap4 and incubated for 4-5 days. There was no sign of inhibition among the bacterial and fungal biocontrol agents (Plate 20)

4.3.4.2. Compatibility with agro chemicals

The Compatibility of three agrochemicals with *Pseudomonas aeruginosa* (Ap4), one of the most promising isolates in this study was tested. The result clearly indicated that the bacterial isolate (Ap4) tested was highly compatible with the three pesticides tested after 48 hours of incubation (Fig. 6).

4.3.5. Effect of pH

The result indicates that the bacterial isolate (Is No. Ap 4) is adapted to grow at the different pH levels tested (Fig. 5)

4.3.6. Combined inoculation of bacteria and antagonistic fungi

Five selected bacterial isolates viz. Ap 4, Ap 16, Is. 113, Is. 119, and Is. 136 were tested for their efficacy of controlling nematodes when combined inoculated with nematode antagonistic bacteria. All the treatments increased the growth of the plants along with nematode suppression (Table 19, Plate 22). The height and biomass increased significantly by both the fungi and bacteria when treated alone and in combination. The increase in growth of plants was significantly higher when *P. aeruginosa* was applied along with the fungi. Combined application of *P. aeruginosa* (AP4) with *T. harzianum* (IISR 1369.) recorded maximum plant height of 111.33 cm (67.01% increase over control) followed by Ap 4 + *P. lilacinus* (108.33 cm i.e. 62.57% increase over control). All the three fungi and the bacteria significantly reduced the nematode population level in roots, the combined application being superior to the treatments where the fungi and bacteria were used independently.

Total nematode suppression was achieved by the combination of *P. lilacinus* and Ap 4 followed by the combined application of *P. chlamydosporia* and the bacteria (99.70%). The nematode suppression ranged from 95.31% to 100%. All the bacteria and fungi applied to the tomato plant were re isolated from the roots after termination of the experiment.

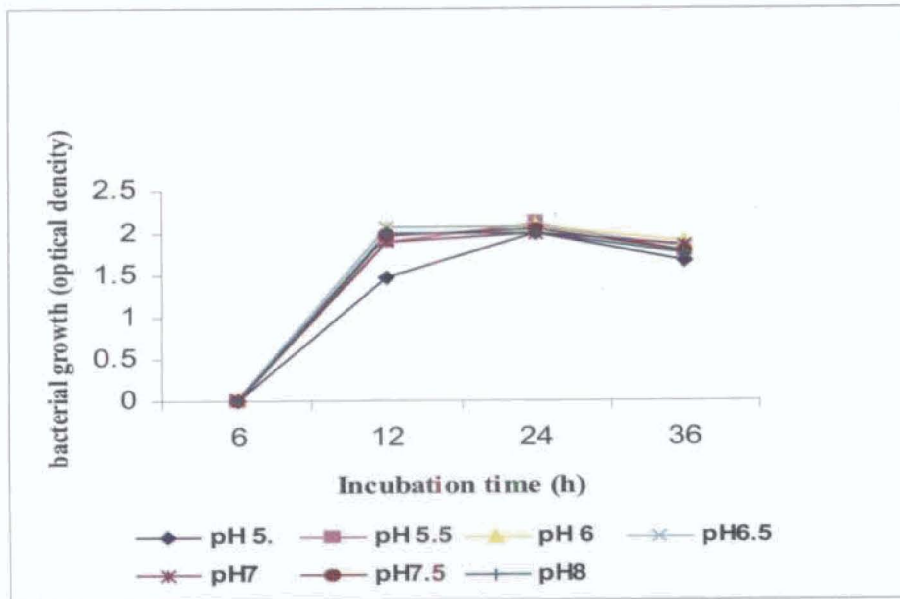


Fig 5. Effect of pH on the growth of bacterial isolate Ap4 (Means of three replication)

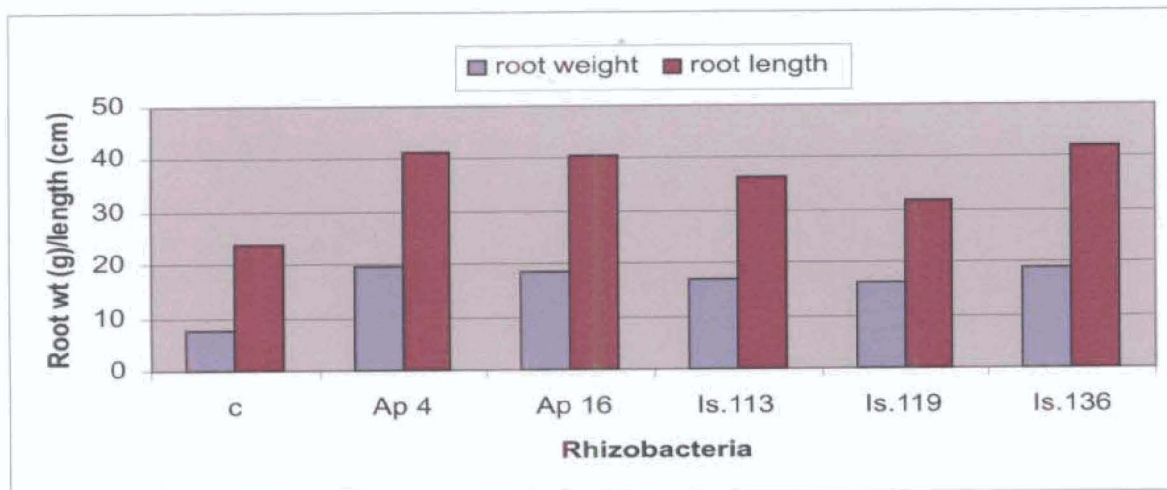


Figure 4. The effect of selected isolates of bacteria on rooting of black pepper

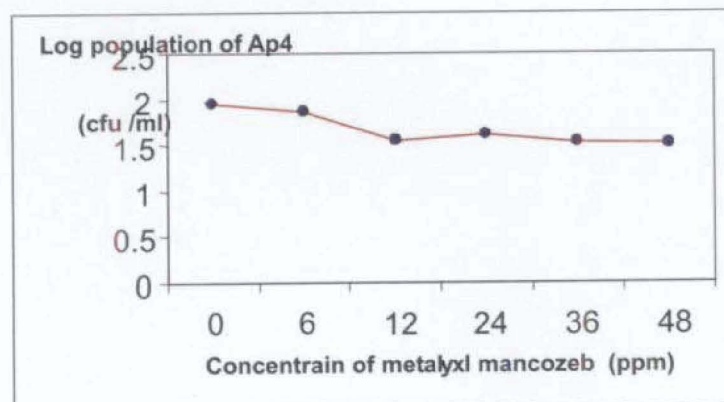
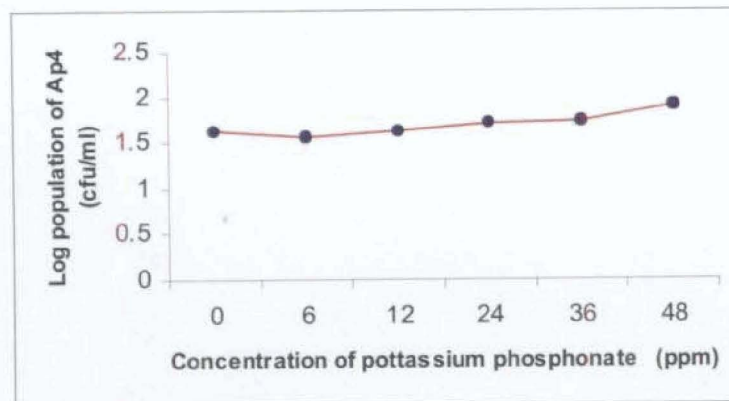
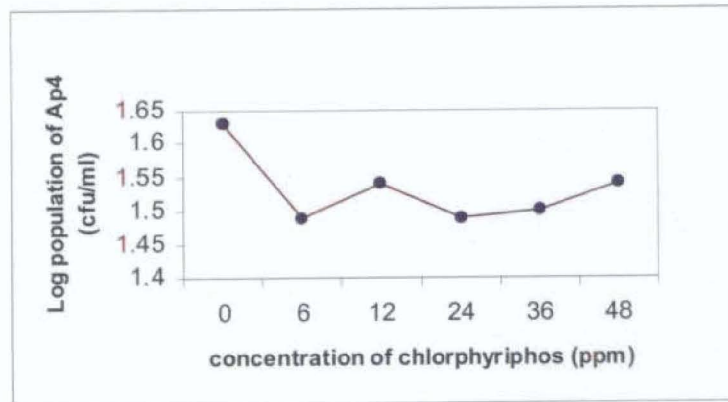


Fig. 6. Effect of various concentrations of agrochemicals on the growth of Ap 4

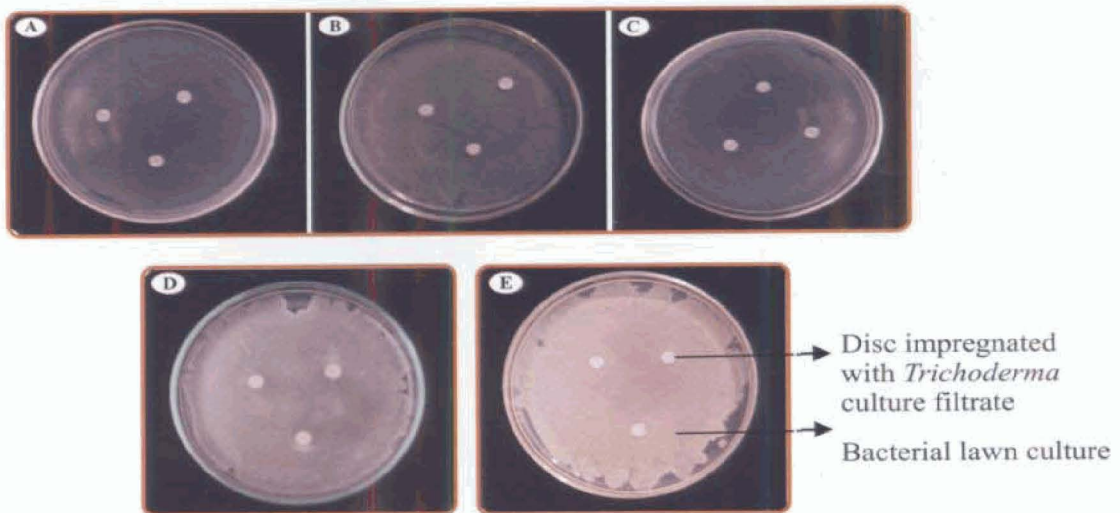


Plate 20. Compatibility of bacterial isolates with *Trichoderma harzianum* in paper disc method

A. Is.136 B. Ap 4 C. Ap 16. D. Is. 119 E. Is. 113.

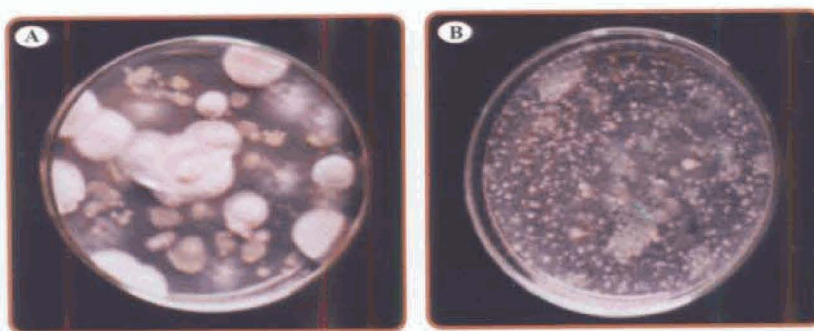


Plate 21. Compatibility of *Pseudomonas aeruginosa* (Ap4) with antagonistic fungi in dual culture method

A. *Paecilomyces lilacinus*

B. *Trichoderma harzianum*



Plate 22. Evaluation of combined application of bacteria and fungi on black pepper

- A. Control, B. Treated with *Meloidogyne* alone, C. Treated with *P. Lilancinus* alone
 D. Treated with *T. harzianum* alone, E. Treated with *P.chlamydosporia* alone
 F. Treated with Ap4 alone, G. *PI*+ Ap4, H. *Th*+Ap4, I. *Pc*+Ap4

4.3.7. REJUVENATION OF ROOT KNOT NEMATODE INFECTED BLACK PEPPER

Severely nematode infected black pepper rooted cuttings (Panniyur 1) were treated with five short listed bacterial isolates in order to test their rejuvenative capacity on the nematode infected black pepper. Severely nematode infected black pepper rooted cutting were uprooted and washed carefully and, dipped in the respective bacterial suspensions and replanted. The experiment was terminated after four months. All the five isolates of bacteria tested, rejuvenated the nematode infected black pepper cuttings to varying levels (Table 20, Plate23). After 4 months, the plants treated with Ap 4 resulted 100% survival of plants. Fifty percent survival of the plants was achieved with the treatment of bacteria Ap 16. Though, 60% survival was recorded with Is.119, the yellowing index was high in the plants survived. None of the plants in the control have survived at the end. However 100% nematode suppression was not achieved with any of the bacteria. Maximum suppression of nematodes was observed with the treatment with Ap4. Though, the survival rate in the treatments with Is. 113 and Is. 119 was high, still a high population of nematodes was detected in the roots of the survived black pepper plants.

4.3.8. Formulations

4.3.8.1. Sodium alginate beaded formulations

Ap4, one of the most efficient bacterial isolates in the present study was formulated. Different types of formulations like sodium alginate beads, granular and talc based formulations were prepared and the shelf life of the bacterial isolate in these formulations were assessed.

Initially the population increased in all the formulations. On the fifteenth day, a general increase in the population was observed. In the beads in which sodium alginate alone is used, mean cfu/gram of the beads increased from 62×10^9 at the initial day to 87.67×10^{11} at the 15th day. One month after packing, the population trend was almost the same as that recorded on the fifteenth day. By the second month onwards, there was a steady decline in the population. On the 90th day of storage only 48×10^5 bacterial population survived in the alginate beads (Table21, Plate 24.).



**Plate23. Rejuvenation of nematode infected black pepper
by bacterial isolates**

A. Control B. Treated with Ap4 C. Treated with Ap16 D. Treated with Is.136
E. Treated with Is. 119 F. Treated with Is.113

Table 19. Effect of combined inoculation of bacteria and fungus on nematode suppression and growth promotion of black pepper (Mean of three replications)

Treatment	Plant Growth Character		Nematode /g root ^a		
	Height (cm)	Biomass (g)	Eggs	Juv	Total
Control	66.67 ab	25.31 a	0.0 a	0.0 a	0.0 a
RKN alone	58.00 a	20.78 a	170.70 b	170.73 b	341.42 b
<i>P.l</i> alone	76.67 bc	44.32 b	6.81a	7.53a	14.33a
<i>T.h.</i> alone	87.00 cd	47.54 b	9.01a	6.99a	16.01a
<i>P.c</i> alone	79.33 c	50.04 bc	6.00a	6.69a	12.70a
<i>P.a</i> alone	84.67 c	51.09 bc	3.76a	2.89a	6.64a
<i>P.l</i> + Ap4	108.33 e	65.68 d	0.0 a	0.0 a	0.0 a
<i>T.h</i> + Ap4	95.67 d	60.30 cd	1.13a	1.69a	2.83a
<i>P.c</i> + Ap4	111.33 e	65.01d	0.52a	0.52a	1.03a
Mean	85.30	47.79	21.99	21.89	43.88

P.l= *Paecilomyces lilacinus*, *T.h*= *Trichoderma harzianum*, *P.c*= *Pochonia chlamydosporia*, Means followed by the same alphabet in a column are not significantly different

^a figures on nematode population were log transformed before statistical analysis and converted to original values

Table 20. Effect of promising bacterial isolates on rejuvenation of nematode infected black pepper

Treatment	Survival (%)					Yellowing index					Nematode population ^b (per g root)			
	1 month	2 month	3 month	4 month	Mean	1 month	2 month	3 month	4 month	Mean	Eggs	juv	Females	Total
Control ^a	30	20	10	0	15	5	5	5	*	5	*	*	*	*
113	30	30	10	10	20	5	3.67	1	1	2.42	176.99d	132.74d	0.0a	309.74d
119	90	80	70	60	75	4.11	3.25	1.7	0	2.52	95.12c	62.58c	0.0a	158.33c
136	90	70	50	40	62.5	4	3.14	1.4	0	2.32	22.01a	0.00a	0.0a	22.01a
Ap4	100	100	100	100	100	4	3.1	0.8	0	1.98	23.98a	0.0a	0.0a	23.98a
Ap16	80	70	60	50	65	4.5	3.43	1.8	0	2.43	46.37b	23.18b	0.0a	69.55b
Mean	70	61.67	50	43.33	56.25	4.35	3.60	1.95	0.2	2.78	72.89	43.7	0	116.72

* None of the plants survived at the end. ^a Plants dipped in sterile water only.

^b Figures on nematode population were log transformed before statistical analysis and converted to original mean. Means followed by the same letter in column are not statistically different.

In the sodium alginate beads amended with various substrates, there was significant increase in cfu/gram and better survival of the bacteria up to the 90th day of storage. The survival of the bacteria varied depending upon the substrate used. Significantly high percentage of the bacterial population survived in formulations amended with rice bran and ginger leaf powder.

When amended with rice bran, in sodium alginate beads, the bacterial population increased from 59.63×10^9 cfu to 88×10^{11} and 88.33×10^{13} cfu /g at 15th and 30th days, respectively. But on the 45th day the population decreased to 84×10^{12} , and thereafter a general decline in the population was observed, finally yielding, 53×10^7 cfu on the 90th day of storage.

When amended with ginger leaf powder, the bacterial population increased from 60.67×10^9 cfu to 70.33×10^{11} and 65.33×10^{13} cfu /g at 15th and 30th days, respectively. The decline of bacterial population was 82×10^{12} on 45th day. On the 90th day of storage, only 33×10^7 cfu/g was observed in this formulation.

The same trend is observed with tapioca powder amendments also. In the formulation with tapioca powder, the bacterial population was increased from 58.67×10^9 cfu to 54.00×10^{11} and 58.33×10^{13} cfu /g at 15th and 30th days respectively. On the 45th day the population decreased to 56.6×10^{11} and on 90th only 48.67×10^6 cfu/g was observed.

In the formulations with coir compost the cfu/g was 55.67×10^9 on the initial day which increased to 72.33×10^{10} on the 15 day. On the 30th day also the cfu was found to be increasing (59.67×10^{12}). The cfu/g on the 45th day was 40.33×10^{10} and on the 90th day it was 44.67×10^6

When the sodium alginate beads are dried and stored both at room temperature and at 4^o C, the same pattern was observed. The dried beads which were kept refrigerated, the cfu/g was found to be increasing from the 15th day to 30th day was declining afterwards. When sodium alginate alone was used, the initial cfu/g was 63.33×10^9 and increased up to 81.33×10^{10} on 15th day and 55.67×10^{11} on the 30th day. Then it gradually decreased and only 43.33×10^5 cfu/g were found on the 90th day (Table 21).

Those beads which were amended with various substrates, the bacteria was better adapted to survive. When rice bran was used, the initial cfu/g of 62.00×10^9 increased to 83.00×10^{11} on the 15th day and up to 81.67×10^{13} on the 30th day. The decline in the cfu was less when compared to the unamended sodium alginate beads and 37.00×10^7 cfu were able to survive at the end of third month.

Same pattern was observed when other substrates were used, when ginger leaf powder was used, the cfu increased to 63.00×10^{11} on the 15th day and 64.67×10^{13} on the 30th day. At the end of the third month the bacterial load was 27.33×10^7 cfu.

Lesser survival rate compared to rice bran and ginger amended formulations were observed in formulations with tapioca powder and coir compost where, 38.67×10^6 and 36.33×10^6 respectively. Storage of dried alginate beads at room temperature decreased the survival rate compared to refrigerated conditions

The dried beads when stored at room temperature the survival rates were lesser to that were kept refrigerated. When sodium alginate alone is used, the initial population of 58.33×10^9 gradually increased to 46.67×10^{10} at the end of the first month. At the end of the third month, only 44.33×10^4 were able to survive.

Among various amendments rice bran and ginger leaf powder recorded the maximum growth and survival of the bacteria.

4.3.8.2. Granular formulations

In the case of granular formulations, the formulations stored under refrigerated condition recorded more cfu/g when compared to that stored at room temperature. Here also the bacterial population increased gradually in the beginning and then started to decline. Starting with a cfu of 65.33×10^9 , the tapioca clay mixture retained cfu of 50.33×10^5 at the end of third month. In the case of clay ginger leaf powder mixture, the initial population of 68.00×10^9 increased in the beginning to 83.67×10^{13} at the end of one month and retained 43.00×10^7 at the end of third month. In the case of granular formulation with rice bran, the initial cfu of 65×10^9 increased to 70.66×10^{13} after one month. 33.33×10^7 cfu was recorded at the end of third month. When coir compost was used, the initial cfu was 64.33×10^9 and the final cfu at the end of third month was 42.33×10^6 (Table 22, Plate26)

The granular formulations when stored at room temperature, the initial cfu of the formulation of tapioca clay mixture was 66.33×10^6 and it was declined to 41.33×10^4 to after storage of three months. When ginger leaf powder was used instead of tapioca powder, the initial cfu was 65.67×10^9 retaining 34×10^6 at the end. When rice bran used, the. In the case of coir compost initial and final cfu was 68.66×10^9 and 33.33×10^4 respectively (Table 22).

4.3.8.3. Talc based formulations

Formulations of Ap 4 was also prepared with talc alone and in combination with two adhesives viz. carboxy methly cellulose and gum arabic. The formulations prepared using talc alone recorded lower viable counts compared to the formulations with addition of adhesives carboxy methyl cellulose and gum arabic. When talc was used without any additional amendments the population ranged from 84.67×10^{10} cfu per gram on the first day of observation to 43.66×10^5 after 90 days of storage. The cfu increased to 83×10^{11} fifteenth day and one month after packing, the population trend was almost the same as that recorded on the fifteenth day (81.66×10^{11}). Then the population started declining by the end of second month (67.66×10^{11}). (Table 23, Plate 27)

When talc was mixed with the adhesives like carboxy methyl cellulose and gum arabic there was an increase in shelf life of the bacterial populations. The population increased initially as in the case of talc alone, and there was only slight decrease in the cfu per gram carrier afterwards. When carboxyl methyl cellulose was used as adhesive the initial population of 91.33×10^{10} decreased to 37.00×10^6 cfu on 90th day. When gum arabic was used instead of carboxymetyk cellulose, the initail population of 87.67×10^{10} decreased to of 42.33×10^6 cfu on 90th day of storage.(Table 23.....Plate27)

Table 21. The survival of the bacterial isolate Ap4 in various formulations of sodium alginate beads (wet and dry) stored at room temperature and at 4°C up to 90days (Mean of three replications)

Treatment		Bacterial population (log cfu /g)				
		Sodium alginate alone	sodium alginate + rice bran	sodium alginate + Ginger leaf powder	sodium alginate + Tapioca powder	sodium alginate+ Coir compost
Wet formulation at 4°C						
	0day	10.79	10.77	10.78	10.77	10.75
	15 day	12.94	12.94	12.85	12.73	11.86
	30 day	12.78	14.95	14.81	14.77	13.77
	45 day	11.69	13.92	13.91	12.75	11.61
	60 day	8.88	11.80	11.82	11.74	9.76
	75 day	7.72	9.67	8.74	8.65	8.68
	90 day	6.68	8.72	8.52	7.69	7.65
Mean		10.21	11.83	11.63	11.30	10.58
Dry formulation at 4°C						
	0day	10.80	10.79	10.77	10.76	10.76
	15 day	11.91	12.62	12.80	12.61	11.86
	30 day	12.74	14.92	14.81	14.70	13.75
	45 day	11.56	13.90	13.89	12.72	11.57
	60 day	8.83	11.77	11.74	11.68	9.71
	75 day	7.63	9.55	8.62	8.53	9.39
	90 day	6.64	8.55	8.44	7.59	7.55
Mean		10.02	11.77	11.58	11.23	10.66
Dry formulation at room temp.						
	0day	10.77	10.78	10.78	10.80	10.74
	15 day	10.91	11.91	11.79	11.68	10.88
	30 day	11.99	13.86	13.82	13.73	12.78
	45 day	11.55	12.89	12.88	11.75	10.60
	60 day	7.87	10.74	10.76	10.68	8.74
	75 day	6.54	8.58	7.99	7.55	7.69
	90 day	5.64	7.55	7.10	6.62	6.55
Mean		9.33	10.90	10.3	10.40	9.71
Table of means						
	0day	10.79	10.78	10.78	10.78	10.75
	15 day	11.92	12.49	12.48	12.34	11.53
	30 day	11.99	14.58	14.48	14.4	13.43
	45 day	10.66	13.57	13.56	12.41	11.26
	60 day	8.53	11.10	11.44	11.37	9.40
	75 day	7.30	9.27	8.38	8.24	8.59
	90 day	6.32	8.26	8.02	7.3	7.25

LSD carrier 0.05663, LSD wet dry conditions=0.03548, LSD Carrier x condition 0.09388, LDS Days =0.054
 Figures are log transformed

Table 22. The survival of the bacterial isolate Ap4 in various granular formulations stored at room temperature and at 4°C up to 90days(Mean of three replications).

Carrier ^a	Temperature of storage	Bacterial population (log cfu /g)							Mean
		0 day	15 day	30 day	45 day	60 day	75 day	90 day	
Tapioca powder	4 ⁰ C	10.81	12.27	12.48	11.62	8.53	7.35	6.70	9.97
	Room temperature	10.82	11.89	12.00	10.85	7.74	6.60	5.61	
	mean	10.82	12.08	12.24	11.24	8.14	6.98	6.16	
Ginger leaf powder	4 ⁰ C	10.83	12.95	14.92	13.93	11.81	9.62	8.62	11.8
	Room temperature	10.82	12.90	13.87	11.88	10.74	8.51	7.52	
	mean	10.83	12.93	14.40	12.91	11.28	9.07	8.07	
Rice bran	4 ⁰ C	10.81	12.84	14.85	13.92	11.78	9.35	8.52	11.7
	Room temperature	10.82	12.78	13.79	12.87	10.71	8.59	7.39	
	mean	10.82	12.81	14.32	13.40	11.26	8.97	7.96	
Coir compost	4 ⁰ C	10.80	11.89	13.79	11.63	9.76	8.76	7.62	10.6
	Room temperature	10.84	11.84	12.72	10.53	9.02	7.70	5.51	
	mean	10.82	11.87	11.26	11.08	9.39	8.23	6.57	

LSD carrier = 0.1413, LSD days = 0.1267, LSD Carrier x Days = 0.1278

^a The granular formulations were prepared by mixing 200 g clay and 50 g gum arabic with 800 g of rice bran or ginger leaf powder or tapioca powder or coir compost with bacterial suspension

Table 23. Survival of bacterial isolate Ap 4 in different formulations of talc stored up to 90 days (Mean of three replications)

Carrier	Bacterial population (log cfu /g)							
	Days of storage							
	0 day	15 day	30 day	45 day	60 day	75 day	90 day	Mean
Talc alone	11.93	12.92	12.24	9.90	8.83	7.68	6.60	10.01
Talc with CMC ^a	11.96	13.98	13.27	11.92	10.88	8.67	7.55	11.18
Talc with GA ^b	11.94	13.96	13.30	11.93	10.85	8.73	7.62	11.19
Mean	11.94	13.62	12.94	11.25	10.18	8.36	7.26	

LSD Carrier = 0.1014, LSD days = 0.2262, LSD Carrier x Days = 0.3919

Figures are log transformed. ^a carboxy methy cellulose, ^b Gum Arabica .

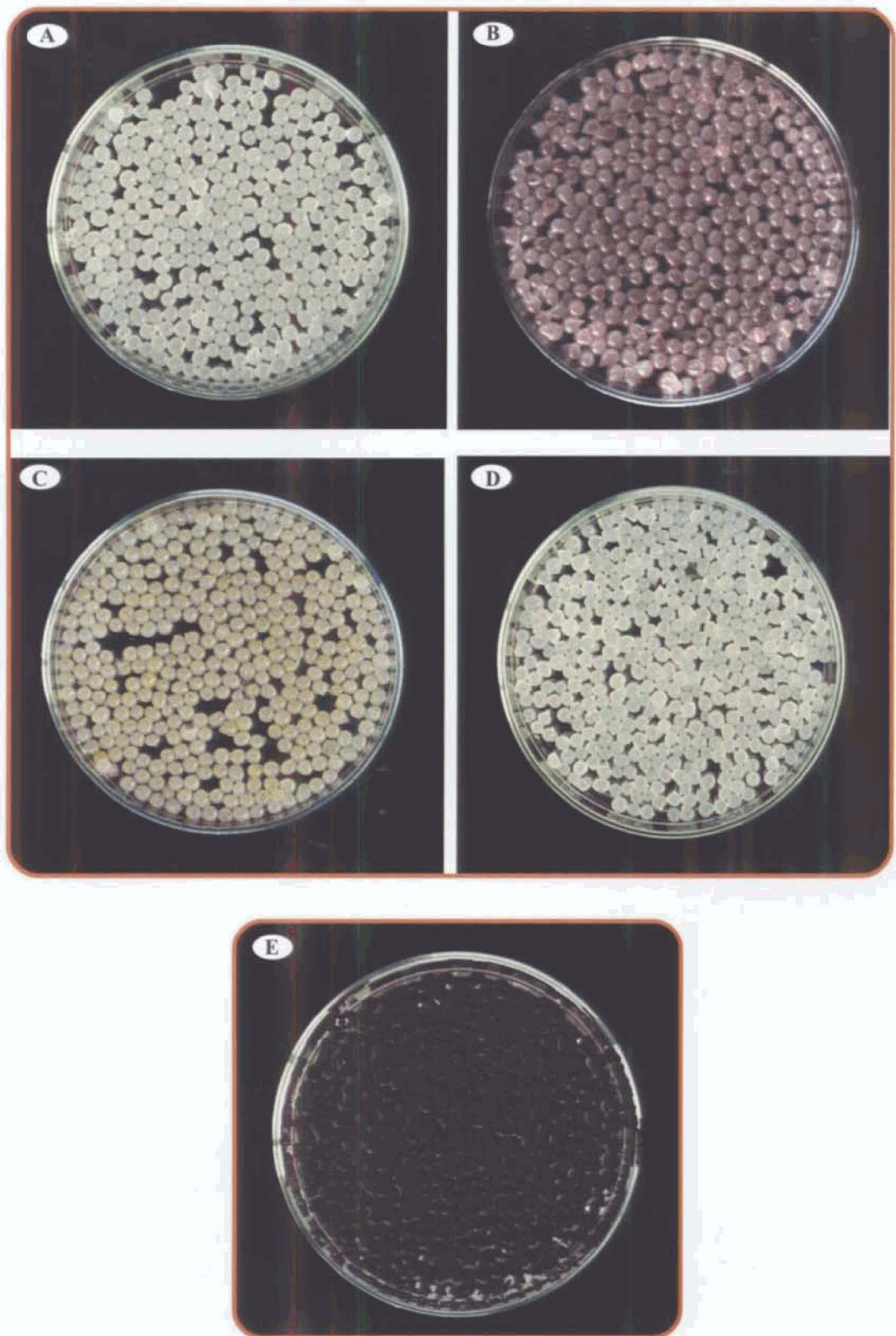


Plate 24. The survival of the bacterial isolate Ap4 in various formulations of sodium alginate beads in wet condition stored at 4°C

A. SA alone, B. SA with rice bran, C. SA with ginger leaf powder
D. SA with tapioca, E. SA with coir compost



Plate 25. The survival of the bacterial isolate Ap4 in various dry formulations of sodium alginate beads stored at room temperature

- | | |
|------------------------------------|--------------------------------|
| A. Sodium alginate alone | B. Amended with rice bran |
| C. Amended with ginger leaf powder | D. Amended with tapioca powder |
| E. Amended with coir compost | |

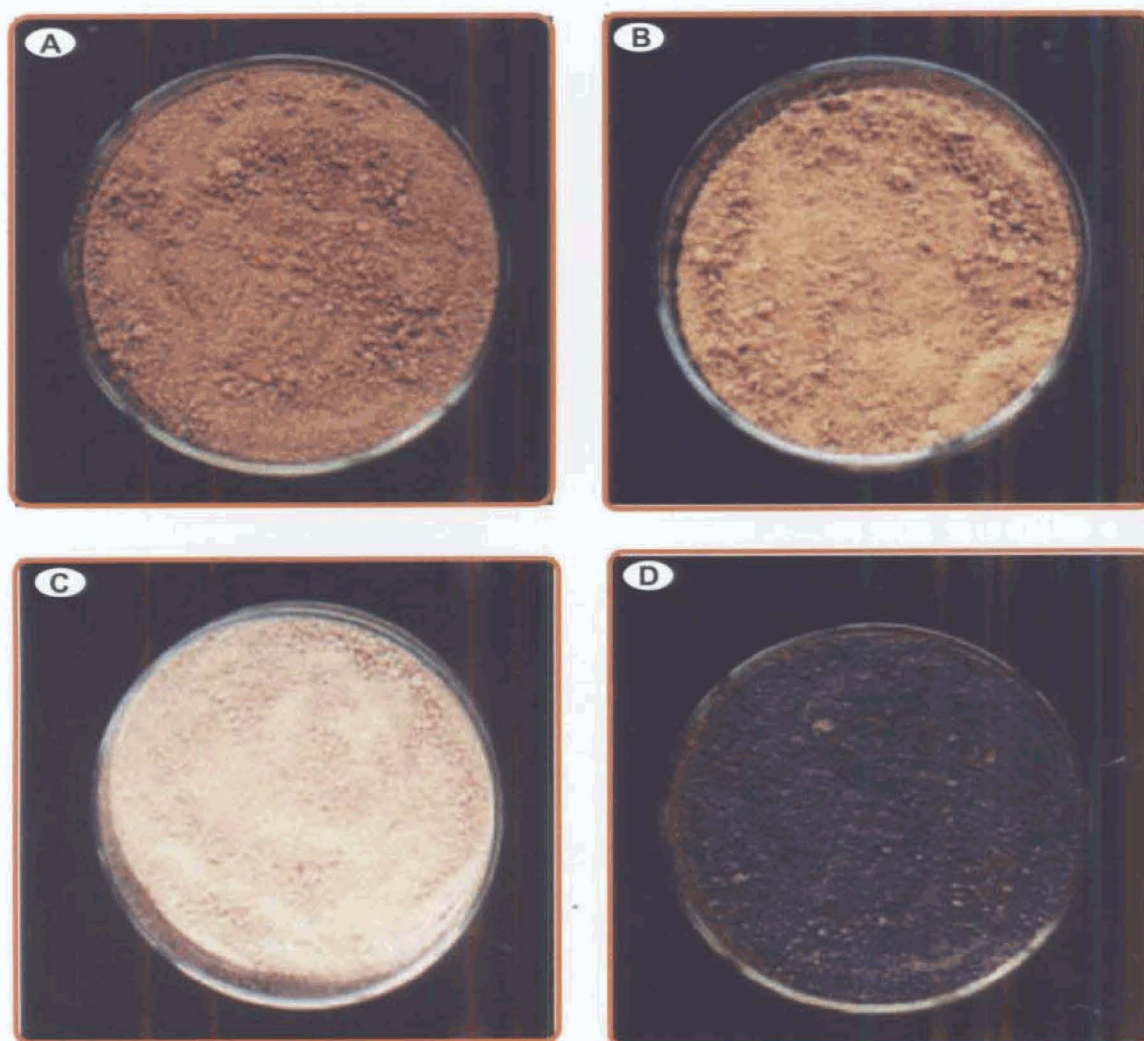


Plate 26 . The survival of *Pseudomonas aeruginosa* (Ap4) in various granular formulations of clay stored at room temperature

A. With rice bran

B. With Ginger leaf powder

C. With tapioca powder

D. With Coir Compost

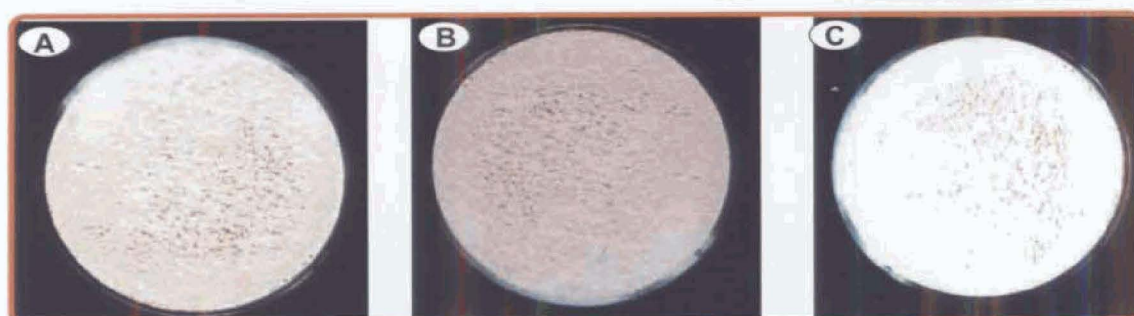


Plate 27. The survival of the bacterial isolate Ap4 in various formulations of talc stored at room temperature

A. With Carboxy Methyl cellulose

B. With Gum Arabic

C. Talc alone

DISCUSSION

B. Beena “Studies on bacterial antagonists of plant parasitic nematodes of black pepper (*Piper nigrum* L.) ” Thesis. Indian Institute of Spices Research Calicut , University of Calicut, 2008

Chapter 5

DISCUSSION

Black pepper is the most important as well as most widely used spice in the world and is one of the major export earning crops of India (Ravindran 2000). The production of black pepper is limited by heavy crop losses caused by insect pests, pathogens and nematodes (Sarma *et al.* 1994). Among the various nematode pests that affect this crop, burrowing nematode (*R. similis*) and root knot nematode (*Meloidogyne* spp.) are more destructive (Ramana & Eapen 2000). As chemical control of these nematodes may lead to various health problems biological control a proven and effective measure against major pathogens and nematodes was attempted.

Rhizospheric bacteria collected from different locations were extensively studied for their efficiency to suppress major nematodes of black pepper. In the present study several bacterial strains were isolated from black pepper as well as nematode antagonistic plants and also from the body of nematodes and their multimode of antagonism against plant parasitic nematodes of black pepper was established through various experiments. The ability of these strains to induce systemic resistance to black pepper against nematodes is also proven in this study. Developing formulations of one of the most promising bacterial isolate was also attempted.

5.1. COLLECTION AND PRELIMINARY EVALUATION OF BACTERIAL ISOLATES

5.1.1. Isolation

Rhizosphere samples collected from black pepper and nematode antagonistic plants yielded a total of 319 bacterial isolates. Seventy isolates of bacteria were obtained from the direct isolation from soil samples. Sodium alginate baiting yielded 16 bacterial isolates. Fifty two bacteria were isolated from various life stages of nematodes. The isolation from roots of black pepper and nematode antagonistic and susceptible plants yielded a total of 181 bacterial isolates. In the present study, bacteria were isolated from the rhizosphere of healthy black pepper plants from diseased gardens too keeping in view that the suppressiveness of the soil is brought about by the beneficial organisms. Collection from various agro-climatic regions resulted in strains capable of thriving in diverse environmental conditions. Collection from Silent Valley biosphere reserve was also included. The isolates from Silent Valley biosphere were proven to be very effective against *Phytophthora* (Lisha *et al.* 2002; Diby Paul 2004). Isolation made from varied crops is expected to work on a broad spectrum of hosts, which would ultimately lead to a product that is suitable for all the crops existing in a garden with multicropping system (Jisha *et al.* 2002a).

In the present study, bacteria were isolated mainly from the rhizosphere of black pepper so that there was more chance for these strains to be effective in colonizing the roots of black pepper when used as biocontrol agents. Apart from the rhizosphere of black pepper, the isolation of biocontrol agents was also done from the rhizosphere of nematode antagonistic plants too. The percentage of nematode suppressive bacteria is comparatively high in the nematode antagonistic plants. The nematode antagonistic plants supported microflora distinct from that of black pepper in physiological and biochemical reactions. The isolation from nematode antagonistic plants enhanced the chance of getting efficient strains (Kloepper *et al.* 1991). In comparison, Gram-negative isolates occurred at higher frequencies from antagonistic plants (Kloepper *et al.* 1992b). They also reported that the closely related species like *P. cepacia* and *P. gladioli* represented the dominant group of isolates from mature plants of all antagonistic plants. The biological activity of bacterial isolates towards nematodes was affected by the source plants. Supporting this, the most promising strains in this study like Ap4, Ap8, Ap10, Ap16 etc were isolated from the antagonistic plants.

Alahm *et al.* (1990) reported the importance of the host plant on the population structure of microbial communities in the rhizosphere. Rhizobacterial isolates reacted differently to different plant species and to different genotypes within species (Aström 1991, Aström & Gerhardson 1988). The frequency with which bacterial isolates exhibited biocontrol activity was affected by the plant from which the bacteria is originally isolated. The plant genome determined the root exudates and enriches for specific microbial colonists of root (Randall *et al.* 2001) and plant species as well as different cultivars within plant species directly affected the overall ability of naturally occurring or introduced PGPR in the rhizosphere (Lugtenberg *et al.* 1990).

Isolation of bacterial strains from the body of nematodes also increased the chance to get effective candidate strains of nematode suppressive bacteria. Supporting this, five bacterial isolates out of the short listed 25 were originally isolated from various developmental stages of nematodes. Bacterial isolate No. 136, later identified as *Alcaligenes faecalis* was isolated from the female *Meloidogyne*, is one of the five prominent isolates of this study. A total of 52 bacterial isolates were directly isolated from the body of nematodes out of which 22 were from the egg masses. Egg masses of root knot nematodes were reported as more densely populated microbial niche than the rhizosphere (Popert & Kok 2000; Kok *et al.* 2001a, 2001b). Percentage occurrence of bacteria was found very high in females and egg masses when compared to larvae in all the locations studied (Sheela & Venkitesan 1992; Sheela *et al.* 1992). The DNA profile of the egg mass bacterial community was different from that of the rhizosphere community. The egg mass has a distinct microbial community that may have a function in protecting the eggs from antagonists. *Meloidogyne* egg masses is congenial niche for microbe and microflora and would be an important factor in determining the success of nematode antagonists (Kok *et al.* 2001). But it was difficult to make a meaningful comparison of population density between the bacterial communities of the rhizosphere and the egg masses, because the gelatinous matrix is a three dimensional porous structure whereas the root surface resembles more a two dimensional habitat. Different workers like Kok *et al.* (2001) Ansari *et al.* (2002) and Papert and Kok 2000 reported the size and community metabolic profile of the bacterial population of *Meloidogyne* egg masses. Moreover dilution plating detects only culturable microorganisms. The chemical composition of the gelatinous matrix would cause the metabolic profile of bacterial egg mass population to differ from the metabolic profile of the bacterial population of the rhizosphere.

Different methods of isolation *viz.* dilution plating methods for root and soil samples, direct plating methods for the isolation from infected nematodes and sodium alginate gel impregnated with nematode eggs were used in this study. The techniques used here allowed the isolation of endoparasitic species more efficiently. It permitted isolation of a range of different species as well as a number of strains of single species in pure culture with comparative ease. Alginate films supported on a screen were easy to prepare and provide many advantages for delivery of inoculum and for studying the dynamics of soil organism over other methods and helpful to isolate microorganisms affecting nematodes. Microscopic observations were made directly on the films. Quantitative information on egg development can be obtained with relative ease (Rodríguez-Kabana *et al.* 1994).

Dilution plating detected only culturable microorganisms and direct examination of soil by microscopy usually revealed populations at least 100 times larger than this. There are many faults and problems with methods like dilution plates and most probable number techniques. These methods usually detected only 1-10% of the organisms known to be present, as the media used were inevitably selective to some extent and unicellular organisms which spread rapidly on the surface of the culture medium and organisms that produced many propagules were favored. Therefore direct isolation from nematode stages and soil baiting were also attempted in this study. In the present study appropriate culture media amended with suitable antibiotics were used to eliminate the over growth of fungi and yeast. Apart from nutrient agar, the general purpose media, isolation of bacteria was carried out by utilizing various specific or selective media for the selective isolation of bacteria of interest. Soil bacteria belonging to different phylogenetic groups were poorly studied in the past because their representatives were rarely isolated in routine cultivation studies. Part of the reason for the failure to cultivate these bacteria was the low frequency with which bacterial cells in soil form visible colonies when inoculated on to standard microbiological media. Inoculum size, growth medium, incubation time etc. were reported to be having effects on viable counts, assessed as numbers of cfu on solid media, and on the phylogenetic groups to which the isolated colony-forming bacteria belong. Decreasing the inoculum size resulted in significant increases in the viable count but did not appear to affect colony formation by members of rarely isolated groups. Some media that are traditionally used for soil microbiological studies returned very low viable counts and did not result in the isolation of members of rarely isolated groups.

Bacteria were the most abundant microorganisms in the rhizosphere, with roots of most plants favoring Gram-negative, nonsporing, and rod shaped genera. Gram positive nonsporing rods and cocci, pleomorphic rods and aerobic spore forming bacteria were relatively less abundant adjacent to roots. Different approaches like ACC deaminase activity were also being adopted (Pal *et al.* 2002). The microbial compartment of the soil community can be characterized through assessing total microbial biomass (using fumigation-extraction), density of bacteria (using colony forming units counts), microbial activity (using alkaline phosphatase) and genetic structure of soil microbial community (using denaturing gradient gel electrophoresis) phospholipid fatty acids (PL-FA) in the determination of rhizosphere specific microbial communities -RSMC (Arab *et al.* 2001).

Microorganisms growing in the rhizosphere were reported as ideal for use as biocontrol agents, since the rhizosphere provided the frontline defense for roots against attack by pathogens (Weller 1988). In disease suppressive soils microbial antagonism of the pathogen was especially great and chances of finding effective strains were more (Schneider *et al.* 2003). The chance of selecting effective strains could be improved initially by first isolating the bacteria from the same environment in which they will be used (Cook & Baker 1983).

The entire Kerala stretch is identified among the top eight bio- diversity hot spots in the world and is has perhaps the healthiest soil in the country blessed with sufficient amount of organic matter and microflora. Studies showed that, on an average one acre of land in Kerala contains 30-35 tons of bacteria, 15 tons of actinomycetes and 10 tones of fungi (Rajesh 2003; Sainnamol Kurian 2003).

Known nematode antagonistic bacteria like *Pseudomonas* and *Bacillus* spp. was isolated in abundance in the present study too. But most of the bacteria isolated were saprophytes which may be due to the fact that the isolation was done mainly from egg masses and rhizospheric soil. Specific isolation of *Pseudomonas* and *Bacillus* were conducted keeping in view that most efficient nematode biocontrol agents belong to these two genera. The most efficient isolates, viz Ap4, Ap8, Ap10 Ap16 etc. in this study were *Pseudomonas* and Is. 113, one among the five short listed isolate was *Bacillus megaterium* supports this view. Samiyappan (2003) had reported the prevalence of fluorescent pseudomonads in several parts of India and the predominance of *P. fluorescens* (>90 %) followed by *P. putida* and *P. aeruginosa*. In this study also

Pseudomonas spp., particularly fluorescent pseudomonads, were the most abundant bacteria in the rhizosphere. The bacteria needed lesser generation time in the rhizosphere than that in the nearby soil as was evident from the study of Bowen (1980). *Pseudomonas* sp. and *Bacillus* sp. had generation times of 5.2 and 39 hours respectively in the rhizosphere compared with 77 hours and greater than 100 hours respectively in the nearby soil. The rapid growth of *Pseudomonas* on the root surface might explain why these bacteria are one of the most important groups of organisms in the rhizosphere. The persistence of *Pseudomonas* was only for short periods in natural soil (Wessendorf & Linger 1989). The decline in population of pseudomonads was higher in soils without plant as they are typically rhizosphere colonizers and the decline is slower in presence of plant roots.

There existed a correlation between the population of nematodes and bacteria. Flaherty *et al.* (2003) had proved that nematodes had a greater impact on the rhizosphere population of a susceptible host than a poor one and the nematode infested roots were colonized by a different subpopulation of soil microbes compared to that on plants where infection was controlled.

5.1.2. Laboratory screening

Number of laboratory bioassays were conducted to evaluate the effect of the bacterial isolates on root knot and burrowing nematodes. One hundred and eight bacterial isolates out of 216 which were screened in the greenhouse bioassays were subjected to *in vitro* bioassays against root knot nematode. The selection of the bacterial isolates for *in vitro* experiments was random. In order to find out the mode of action of these bacterial isolates, the same isolates were subjected to various bioassays and the results indicated that these isolates differed in their action against root knot nematodes. Direct action of the bacterial cell suspensions was determined in the first set of bioassays in peptone water. The percentage mortality of nematodes was high in this set of experiment. More than 90 % of the bacterial isolates caused nematode mortality above 90%. The bacterial isolate No. 109 caused maximum mortality of nematode (98.03 %). The high percentage of nematode mortality may be due to the fact that ammonia which is a common nematicide may be released by the bacterial isolates in this nutritionally rich media (Table 5).

When the nematicidal effect of these isolates was tested in phosphate buffer, the mortality of nematodes was lesser than that in peptone water. Majority of the bacterial

isolates caused in mortality of nematodes below 80 %. Maximum mortality of nematode in this method was achieved by the bacterial isolate No. 110 (87.83 %) followed by Is. No.113 (84.63%). One more isolate having high nematode mortality is Is.105 (80.42%) (Table 5). Use of phosphate buffer offered better evaluation of the test strains of bacteria as use of rich broth cultures to detect activity was rejected by many workers because some bacteria did not produce antibiotics in broth and the richness might inhibit production of the desired compound (Meyer *et al.* 2000; Siddiqui *et al.* 2003). Use of solid or liquid media also influenced the nematicidal property of test strains of bacteria. Becker *et al.* (1988) reported the production of detectable amounts of antibiotics in agar media but not in broth cultures with the same nutrient composition.

The culture filtrates of these bacterial isolates were also subjected to evaluation against root knot nematodes and as in the case of bacterial cell suspension, the mortality of nematodes was high (Table 5). Many workers pointed out that the short-term effects of cell-free bacterial filtrates (CFCF), namely toxicity and repellency, were almost entirely due to ammonia (Rodriguez- Kabana 1986; Spiegel *et al.* 1991; Grewal *et al.* 1999; Velusamy 2003). In order to eliminate the toxic effect of ammonia, the released ammonia was trapped by sulphuric acid. Even after the toxic ammonia was trapped, the high percentage nematode mortality indicated the production of metabolites other than ammonia that were toxic to nematodes. Various nematicidal compounds might be present in the bacterial culture filtrate such 2, 4-diacetylphloroglucinol (2, 4-DAPG) and pyoluteorin (Siddiqui & Shaukat 2003d). The culture filtrate concentration and exposure period (Jayakumar *et al.* 2002b), temperature (Walker *et al.* 1966) etc. might also influence the nematicidal effect. Fresh culture suspension or old culture filtrate are found to be superior to old culture suspension or fresh culture filtrate. In the first case the surface colonization of the bacteria encountered the pathogen attack whereas in the second case, the inhibitory principle was the antibiotic produced by the bacteria, which acted as a curative agent (Walker *et al.* 1966). Nematicidal activity of culture fluids from different isolates varied with temperature and length of incubation of the cultures emphasizing the importance of the environmental conditions and the age of culture in the production of metabolites affecting nematodes. The production of such metabolites appeared to be independent of total growth, as greatest nematicidal activity did not always occur in cultures, which produced the maximum growth. The absence of ideal conditions for the growth and production of nematicidal compounds had resulted in lower production

of active substance (s), instability of the substance(s), and (or) their rapid utilization after synthesis at higher temperatures (Walker *et al.* 1966). Suitable controls were used in all the *in vitro* experiments to detect whether the components of media used had any adverse effect or not on the nematodes as Meyer *et al.* (2000) reported. But in the present study, in the control, there were no significant nematode suppression in all the *in vitro* methods (Table 5).

When 71 randomly selected bacterial isolates were evaluated for their production of nematicidal metabolites including volatile metabolites, 52 bacterial isolates caused more than 90 % mortality of nematodes (Table 5). Naturally occurring volatile compounds produced by bacterial isolates with nematicidal activities are of significant economical importance in agriculture. Volatile and non volatile organic compounds (VOCs) produced by the bacterial isolates caused suppression of nematodes. Antibiosis with volatile substances would diffuse through air filled pores in soil and thus actual physical contact between the antagonist and the pathogen might not be necessary for inhibition of the pathogen as reported by Padmodaya & Reddy (1996). In the present study 27 bacterial isolates produced volatile metabolites causing more than 90 per cent mortality to nematodes. The volatile metabolites produced by Is. 125 caused nematode mortality as high as 99.19 % followed by Is.113 (99.10%) and Is.110 (98.92 %). But the use of micro detection plates as designed by Walker *et al.* (1996) which allows a two way diffusion of antimicrobial substances applied would have increased the sensitivity of detection rather than with the use of conventional petri dishes which allows a three way diffusion.

To find a successful biocontrol agent, many species and strains of biocontrol agents needs to be screened. This challenge had resulted in the development of *in vitro* bioassays for antagonism to nematodes which are rapid, space efficient and repeatable. The *in vitro* method enabled numerous bacterial strains to be processed with minimum requirements of equipment and space (Becker *et al.* 1998). *In vitro* pathogenicity does not ensure that a bacterium will be an effective biocontrol agent, but screening in the greenhouse and field consumes much time and space. As a consequence prior selection of bacteria based on laboratory tests is very critical. Furthermore, laboratory studies must be conducted even on known pathogens to determine mechanism of pathogenicity. *In vitro* bioassays Random screening of microorganisms for biocontrol of plant parasitic

nematodes without regard to characteristics related to nematode antagonism has a low probability of success because of the large number of organisms that must be tested (Schisler *et al.* 2004). Antibiosis is only one of the many characteristics that may be important to a strain enabling it to provide biological control (Ali *et al.* 2002). Virtually all well described rhizobacteria with excellent biocontrol activity had shown *in vitro* production of antimicrobial metabolites, which is of major importance in the biocontrol of pathogens of major importance in the biocontrol of pathogens. In this present study efforts were made to study the mode of action of the bacterial isolates on nematodes through various laboratory screening methods. Each isolate differed in its nematode suppressive action in each method. So short listing of effective isolates was done only after subjecting these isolates to greenhouse evaluation also.

To understand clearly the mechanisms involved in the suppression of plant parasitic nematodes by biocontrol organisms, knowledge of the ultra structure of the nematodes is a prerequisite. Plant parasitic nematodes though aquatic in nature, had evolved protective structures and metabolic adaptations which allow them to survive and flourish in what is often a harsh and competitive soil environment. The nematode body is protected by a multilayered, proteinaceous cuticle which functions as a flexible skeleton and barrier to undesirable elements in the environment and is not easily destroyed by chemicals or biocontrol agents (Sterling 1991). Eggs of the nematodes are also well protected with shell consisting of chitin as its major component. Hence, the biocontrol agents face additional barrier of the cuticle of the unhatched juvenile, where the embryonic development is completed besides the eggshell. This probably explains why the eggs in the later stages of development are not readily parasitized by the biocontrol agents (Sterling & Mankau 1979; Irving & Kerry 1986). Some nematode species lay the eggs in gelatinous matrix or retained within the body of the female. The gelatinous matrix protects the eggs from desiccation and from soil microorganisms (Bird & Bird 1991). The compounds of the gelatinous matrix though affect a number of rhizobacteria serve as a nutrient source for several bacteria (Orion & Kirtzman 1991). Egg masses supported greater bacterial populations than that of rhizosphere soils. Hence, an understanding of the bacterial populations of the egg masses is important since the resident bacterial population could affect the eggs and/or egg parasitic microorganisms. High reproductive capacity, short life cycle with a number of developmental stages results in a varied and ever changing targets for biocontrol agents (Stirling *et al.* 1979).

The greatest problem in screening microorganisms for biocontrol of nematodes is not having the necessary information on phenotypic characters that most likely are associated with desired ecological and physiological behavior patterns. Thus most researchers generally base their initial isolation procedures on some characteristics like antibiotic or siderophore production. But our first approach to find bacteria for nematode control was to select rhizosphere bacteria at random, using no predetermined bias in the isolation procedure and screening them *in vivo*.

Culture media was reported to be influencing the percentage of eggs parasitized by biocontrol agents (Kim *et al.* 1998; Meyer *et al.* 2000). Tian and Riggs (2000) reported that the Tryptic Soy broth control immobilized J2 suggesting that some chemical property of the medium contributed to the immobilization of nematodes. The organisms therefore, found to be producing toxins needs to be cultured on root exudates of black pepper to test whether the toxin production is nutrient dependent or not. The production of metabolites is independent of total growth of bacteria (Fravel 1988). Bacterial traits essential for nematode antagonism might had been lost due to spontaneous mutations on artificial media (Duffy & Difago 2000). Variable results with biocontrol agents applied to soil had long been recognized but little research has been done to elucidate the contributing factors. The fact that the cell suspensions of most of the bacteria tested, regardless of whether they suppressed or enhanced nematode numbers in soil, immobilized J2 but did not reduce their viability or infectivity suggests a general phenomenon such as low oxygen levels. Therefore, immobilization of J2 by bacterial suspension might not be a reliable indicator of potential biocontrol agents (Tian & Riggs 2000). But in the present study the juveniles treated with the cell suspensions were weaker in infecting tomato plants on subsequent inoculation indicating the destructive action of the juveniles by the bacterial cell suspension. Beside from possessing high specific activity and target selectivity, biologically-active secondary metabolites are generally biodegradable (Oyekanmia *et al.* 2007). The nematocidal activity of the bacterial isolates differed with the strength of the media Liquid culture carbon, nitrogen and inorganic phosphate source regulated nematocidal activity by bacterial isolates *in vitro* condition (Siddiqui & Shaukat 2004a).

Distinguishing live and dead nematodes *in vitro* bioassays was quite difficult. Due to the effect of bacteria the juveniles showed convulsive movements and paralysis.

Nematodes which were both non-motile and in the typical death position (straight) were considered dead by Walker *et al.* (1966) and they claimed that these criteria were satisfactory for determining nematode mortality. According to Cayrol *et al.* (1989) the juveniles could be considered dead when they were not moved on probing with a fine needle. Staining with the vital stain bromothymol blue facilitated the distinguishing of dead nematodes from live ones i.e., those staining deep blue can be classified as dead (Samliet *et al.* 2000). In the present study the dead and live juveniles were distinguished based on their non motility and typical death position and when probed with a fine needle and confirmed by the addition of 2-3 drops of 1N NaOH.

For initial *in vitro* evaluation of bacterial antagonists, several methods are followed such as evaluation of bacterial suspension in peptone water, bacterial suspension in phosphate buffer, cell free culture filtrate, total and volatile metabolites of bacteria were followed. But every method has certain disadvantages. For instance in the bacterial suspension in peptone high percentage of nematode mortality was noticed where as if the liberated ammonia are rapped, the mortality is totally altered. The phosphate buffer method appears to be more reliable as the results of the *in vitro* selection are consistent with the results obtained from greenhouse evaluation. In the present investigation among the *in vitro* methods tried some of the isolates such as Is. 113, Is. 119 and Is. 136 showed consistent results with all the methods tried. It is suggested to have at least more than one *in vitro* tests to short list bacterial isolates Though, *in vitro* evaluation of bacteria for nematode suppression is having different advantages over greenhouse methods, the tested isolates exhibited different level of nematode antagonism in different methods and thus it is found to be difficult to shortlist effective strains based on *in vitro* analysis.

5.1.3. Greenhouse evaluation

A total of 216 bacterial isolates were evaluated in greenhouse including the 108 bacterial isolates tested *in vitro* in a series of 11 experiments using tomato as the test plant. The results clearly indicated the superior ability of most of the test strains both in nematode biocontrol and in improving the vigor and growth of tomato. Total nematode biocontrol was achieved by 40 isolates but four isolates viz Is. No. 93, 94 95and 98 had retarded the growth of tomato (Table7) may be due to the competition for nutrients. In this study the growth promotion was achieved by the bacterial isolates in spite of the

nematode infestation. No separate experiments were conducted to find out the action of bacterial isolates alone in promoting the growth of test plants.

Potential biocontrol agents of soil borne plant pathogens can be screened *in vitro* or in soil (Merriman & Russell 1990). Greenhouse screening though, consumes time and space, would allow understanding multiple mechanisms of antagonism such as antibiosis, competition and parasitism and to study the adaptability of potential biocontrol agents in soil. The greenhouse tests were also useful in detecting strains that had a negative effect on plant growth (Neipp & Becker 1999) and helped to study the adaptability of potential biocontrol agents in soil (Racke & Sikora 1992b). In the greenhouse studies, number of bacterial isolates were having the ability of promoting the growth of tomato (Table 7) and these strains would have missed if *in vitro* screening only was used a criteria for selection of effective strains. Furtherer, these strains effective in growth promotion of tomato was also found to be effective in growth promotion of black pepper in later studies (Table 13, 14 & 18).

The choice of tomato as the test plant was on the basis of its high germination rate (~98%), fast growth, convenient plant size, root growth pattern, growth rate and susceptibility to *M. incognita*. As suggested by Tyler in 1933, bioassays utilizing suitable host plants had much to offer in managing certain nematodes. Each combination of nematode and host is different, and plant species, host age and host genotype were found to influence the biocontrol of nematodes by bacteria (Siddiqui & Shaukat 2003).

Greenhouse trials were conducted in plastic pots with sterile soil with tomato plant. Sterile soil was used in the preliminary screening experiments to reduce the risk of other plant pathogenic organisms that may be present in the soil and also for avoiding the adverse effect of other organisms on the introduced bacteria. Though sterile soil did not mimic the natural environmental conditions with dynamic interactions among the micro and macro organisms, it has permitted the selection of potential biocontrol agents based on their efficacy. Greenhouse structure and surface water for irrigation could also serve as infection sources (Godoy *et al.* 1983a). Wilting or too frequent watering could also have interfered with nematode infection or the activity of bacteria. High moisture content in polybags might lead to cross contamination and decay of the roots. Care was taken to use only adequate amount of water and to prevent cross contamination. The water for irrigation was boiled and cooled before application. Appropriate level of cleanliness was

maintained in the greenhouse through out period of study. All the experiments were conducted in the same greenhouse during different periods maintaining constant temperature and moisture.

In the initial screening tests, the effects of bacteria on the nematode multiplication ranged from inhibition to stimulation. In one set of greenhouse experiment, out of the 23 isolates, 18 suppressed nematodes significantly where as the other five isolates enhanced the nematode attack (Table 7e). Similar phenomenon had been reported with other bacteria and other plant parasitic nematodes (Becker *et al.* 1988, Oostendorp & Sikora 1989; Racke & Sikora 1992; Tian & Riggs 2000; Mahdy 2002). Significant suppression in root galling was observed in most of the bacteria treated tomato plants. Bacterial treatment in some plants resulted in minute uniformly round galls resembling *M. hapla* infection with profuse secondary and tertiary roots. In contrast, the control plants had larger galls typical of *M. incognita* infection. This altered tomato root gall size and shape could be due to induced systemic resistance or multiple potential defense mechanisms consequent to the interaction between the host, the rhizobacteria and the nematode. Similar findings were reported by Wei *et al.* (1996) and Jonathan *et al.* (2000). Root knot nematode gall size could be a factor in determining the efficiency of biocontrol agents, because root knot nematode eggs embedded in plant tissue were generally protected from biocontrol agents (de Leij *et al.* 1992; de Leij & Kerry 1991; Meyer 1990). The small size of the galls observed at harvest on roots in some treatments might have enhanced the ability of the applied agents to act against the nematode. The number of eggs per egg mass was significantly suppressed by bacteria as Khan *et al.* (2002b) reported. It was shown that some of the developing root galls had no nematodes associated with them indicating that the developing juveniles might have disintegrated. Plant health promoting rhizobacteria adversely influenced the intimate relationship between the plant parasitic nematodes and its host by regulations of the nematode behavior during the early root penetration phase of nematode parasitism.

Significant growth promotion of the treated tomato plants was observed with many bacterial isolates. There are several reports on the favorable influence of PGPR on plant growth and plant biomass (Bloemberg & Lugtenberg 2001; Barka *et al.* 2002; Kloepper 2003). Plant growth promoting activities had since been reported in the strains of soil bacteria including *Azotobacter*, *Bacillus*, *Hydrogenophaga* and *Pseudomonas* (Kloepper *et al.* 1989, Murphy *et al.* 2000; Amruthesh *et al.* 2003). Plant growth

promotion by PGPR is a multi-task phenomenon. There are several determinants for mechanisms of growth promotion that include bacterial synthesis of the plant hormones viz. indole-3-acetic acid (IAA), cytokinins and gibberellins; breakdown of plant-produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and increased mineral and N availability in the soil (Kloepper *et al.* 1992; Glick 1995) or indirectly changing the microbial balance in the rhizosphere in favor of the beneficial microorganisms (Nowak 1998). In addition, the bacterization induced an enhancement of the fresh weight of shoot and roots as well as the number of nodes per shoot (Barka *et al.* 2002). The increased biomass in the PGPR treated plants might also be attributed to the production of antibiotics, lytic enzymes, HCN, volatile organic compounds (Ryu *et al.* 2003a) and siderophores produced by these strains thus inhibiting the growth of deleterious microorganisms in the rhizosphere or having high lignin content (Anderson & Guerra 1985; Luz 2001). Some of the bacterial isolates having very good growth promoting capacity are Is. No. 13, Is. No. 11, Is. 113, Is. 119, Is. 136, Ap4, Ap16 etc. It is an added advantage to utilize the multifarious capabilities of these biocontrol agents.

Different mechanisms had been postulated to explain how rhizobacteria suppressed nematode populations. Antagonism mediated by specific or non-specific metabolites of microbial origin, by lytic enzymes, volatile compounds or other substances (Fravel 1988; Ryu *et al.* 2003) might contribute to the nematode suppressive action. The interference with the host finding process of nematodes was by receptor blockage of the lectin carbohydrate recognition sites (Sikora & Hiffman-Hergarten 1989). Gram negative rhizobacteria have lectin binding structures in the lipopolysaccharide layer of the cell wall membrane. The ability of plant health promoting rhizobacteria to envelop or bind to root-surface lectins interfered with nematode host recognition and therefore root penetration (Oostendorp & Sikora 1989). Some bacteria altered the root exudates that influenced nematode hatch, attachment and penetration into the host tissues (Oostendorp & Sikora 1989; van Loon *et al.* 1998). Competition for nutrients supplied by roots and seed exudates probably occurs in most interactions between bacteria and pathogens on the roots and is responsible for at least some small degree for the observed biocontrol by the introduced bacteria (Siddiqui & Shaukat 2004). Large populations of bacteria established on planting material and roots becomes a partial sink for nutrients in the rhizosphere, thus reducing the amount of carbon and nitrogen available to stimulate the growth of

pathogens (Khan *et al.* 2002). Duffy *et al.* (2003) suggested that niche exclusion as a potentially an important mechanism of antagonism.

Considerable variability was found among the isolates both in growth promotion and nematode suppression when further screened against nematodes using black pepper. For example Is. No 18 was very effective in enhancing both height and of fresh biomass of tomato (Table. 7f) but not so much effective in promoting the growth of black pepper (Table 13). This variability could be due to selective adaptation to various edaphic factors such as soil type or ambient temperatures. Besides a suitable rhizosphere environment, successful long term root colonization requires that introduced bacteria possessed rhizosphere competence traits involved in attachment, distribution, growth and survival. Bacterial traits that contribute to rhizosphere competence are reported be surface polysaccharides, fimbriae, flagella, chemotaxis, osmotolerance, and ability to utilize complex carbohydrates (Glick 1995).

While comparing the nematode suppressive effects of different bacterial isolates under *in vivo* and *in vitro* and considerable variation was observed. Besides environmental conditions such as moisture, temperature, and plant nutritional factors may also contribute to this inconsistencies (Weller, 1988; Kloepper *et al.* 1989). It is well known that *in vitro* assays may be poor predictors of *in vivo* performance and, in particular, that antibiosis under *in vitro* conditions may not necessarily correlate with field level biocontrol (Fravel 1988). Since antibiotics production is greatly affected by the environmental factors, particularly nutrition, it is not surprising that the assay conditions affect results of *in vitro* studies. There are several reports of lack of correlation between *in vitro* antibiosis and biocontrol (Becker *et al.* 1988; Oosterdorp & Sikora 1989; Racke & Sikora 1992; Neipp & Becker 1999; Tian & Riggs 2000). One factor that contributes to the variable result is the degree of establishment of bacteria on roots (Becker *et al.* 1988). Through *in vitro* screening, organisms that produce toxic metabolites or those parasitize target organism are usually selected. Bacteria that suppress nematodes by modification of root exudates or by induced systemic resistance cannot be identified by this method. It is well accepted that biocontrol agents cannot be selected only on the basis of an agar plate bioassay.

5.1.4. Hatching suppression

Twenty five short listed bacterial isolates were evaluated for their effect on hatching suppression of root knot eggs (Table 9, Plate 12). Significant suppression in the hatching of nematode eggs was observed with all of the tested bacterial strains (33.96% Ap11 to 91.66% by B15). Majority of bacterial isolates caused significant hatching suppression (>50 %). Maximum hatching suppression was achieved with the treatment of bacterial isolate B 15. None of the tested isolates stimulated the hatching of eggs of root knot nematodes. The integrity of most of the bacterial affected eggs was completely lost as it was noticed that the abnormal eggs bursted spontaneously. The test strains of bacterial isolates might have produced lytic enzymes capable of degrading the cuticle of eggs. Bacterial isolates like Ap4, Is. 123 etc. were capable of liquefying gelatin (Plate 13 a D) which might have helped these bacterial isolates to dissolve the gelatinous matrix of egg masses and to suppress their hatch. It was proved in the *in vitro* analysis. Higher suppression percentage by Ap4 (82.38%) confirmed the role of gelatinase enzyme in hatching suppression. The isolate Ap4 is also having the ability to produce protease enzyme. It was proved in the *in vitro* analysis that Ap 4 is having high potential in suppressing root knot nematode egg hatch (Table 9). Hatching suppression by these bacterial isolates needs further investigations. Reports are plenty on the use of bacteria to suppress hatching of nematode eggs (Dunne *et al.* 1998a; Sharma 1998; Sharma & Gomes 2000; Niknam & Dhawan 2002a; Ansari *et al.* 2002). Different mechanisms were reported for the suppression of nematode egg hatch by bacteria. Cronin *et al.* (1997b) and Mercer *et al.* (1990; 1992) reported the role of chitinase enzyme and 2,4-diacetylphloroglucinol (DAPG) in hatching suppression of nematodes by bacteria. Exogenous chitinases from bacterial sources caused premature hatch of nematode eggs and could be used as an aid in the control of nematodes (Mercer *et al.* 1992). The interference of hatching signal from host plant by the bacterial isolates may also contribute to the suppression of hatching. Reports are available in literature on the hatching suppression of eggs of other nematodes like *Heterodera schachtii* (Neipp & Becker 1999), *H. glycines* (Sharma & Gomes 1996, 2000) *Rotylenchulus reniformis* (Niknam & Dhawan 2002a, 2002b) *Globodera rostochiensis* (Cronin *et al.* 1997a) by *Serratia marcescens* (Mercer *et al.* 1992), *P. fluorescens* (Dunne *et al.* 1998) and by *Bacillus*, *Pseudomonas*, *Variovorax* and *Arthrobacter* (Neipp & Becker 1999).

5.2. CHARACTERIZATION OF EFFECTIVE STRAINS

5.2.1. Identification of bacteria

The selected bacterial isolates were identified by microscopic and biochemical tests and also through molecular techniques. The occurrence of bacteria belonging to the genus *Pseudomonas* and *Bacillus* having high potential in nematode suppression and growth promotion in the rhizosphere of both black pepper and nematode antagonistic plants was well established through this investigation. Such isolates were also present in the body of various life stages of nematodes. Molecular characterization of the short listed 19 bacterial strains was attempted through Unweighed Pair Group Arithmetic Average (UPGMA). The banding pattern in the restriction profile was subjected to NTSYS analysis. The tree plot (Fig1) reveals that the test strains are different from each other.

5.2.2. Phosphate solubilization

The ability of the selected 25 bacterial isolates to solubilize phosphate is evaluated on Pikovaskaya's agar media and it is found that the selected isolates solubilized phosphorus to various extents (Plate 13b. A). The potential of these strains to release soluble inorganic phosphates from fixed phosphate sources for plant uptake is of greater importance when the bacterial isolates are used as both growth promoters and biocontrol agents. Phosphorus, one of the key nutrients, stimulates growth and development of roots making plants more resistant to drought and facilitates more nutrient absorption. Microorganisms are critical for the transfer of P from poorly available forms and are important for maintaining P in readily available pools. The present study proved the bacteria mediated P solubilization and there by enhanced uptake by plants, which resulted in increased plant biomass. The isolates capable of solubilizing phosphate viz. Is.13, 19 123, Ap 4, Ap16, B14, B15 etc. were very effective in increasing the biomass of both tomato (Table 7) and black pepper (Table 13&14). Phosphate solubilizing bacteria had been isolated from roots of black pepper by different workers (Diby Paul *et al.* 2005; Ramachandran *et al.* 2007). The microbe mediated P mobilization in plants could be through i) an increase in the surface area of the roots, ii) by displacement of sorption equilibria that results in increased net transfer of phosphate ion in soil solution or an increase in soil mobility of organic forms of P and iii) through stimulation of metabolic process that are effective in directly solubilizing and mineralizing P from poorly available

forms of organic P (Jones 1998; Kundu & Gaur 1980; Şahin *et al.* 2004). Solubilization of tri-calcium phosphate by inoculation of plant growth promoting rhizobacteria was reported by Pal *et al.* (2004). The phosphate solubilization capacity of bacteria was attributed to the production of gluconic acid by direct oxidation of the glucose via membrane bound quinoprotein glucose dehydrogenase (Gcd) enzyme (Divya 2003) or by 2-ketogluconic acid or 2,5- diketogluconic acid (Goldstein 1995).

Reports are available on the solubilization of other forms of water insoluble forms of phosphate *viz.* aluminium phosphate (AlPO_4), ferric phosphate (FePO_4) and fertilizer forms of phosphate *i.e.*, Mussoorie rock phosphate and Rajphos by *P. fluorescens* (Randal *et al.* 2001). The phosphate solubilisation by bacterial isolates was mainly due to various organic acids and phosphatase enzyme produced by the bacterial isolates. Kpombrekou and Tabatabai (1994) had shown that the phosphate solubilizing ability was related to the nature of the organic acid and the kind of phosphate ore also influenced the type and amount of organic acid requirement. P release depended on the strength of the organic acid in the order of tricarboxylic acid > dicarboxylic acid > monocarboxylic acid (Bolan 1994).

The strains of *Pseudomonas* spp (Ap4 and Ap8). used in this study were found to be producing siderophores which might in turn released P from complex forms of P through ligand exchange reactions by chelating metal ions associated with the bound P. Processes such as rhizosphere acidification, exudation of organic acids and secretion of phosphorous from plant roots were reported to occur in response to P deficiency and are established mechanisms by which plants acquire P (Randal *et al.* 2001). A strong relationship was reported to exist between the metabolic activity of the rhizobacteria and its P solubilizing capacity which would determine the efficacy of the rhizobacteria in response to carbon substrates released by root exudates (Siddiqui & Shaukat 2003).

5.2.3. Effect of bacterial isolates on mycelial growth of *Phytophthora*

Strains of 25 selected rhizobacteria were screened against *P. capsici* *in vitro* by the dual culture technique. Among the 25 strains, 16 were able to inhibit the pathogen, the inhibition varying from 0 to 73.33 % (Figure 3 Plate 13b. D). The most efficient bacterial isolates in suppressing *Phytophthora* are Is. 127 (73.33%) Is.123 (60.4%), B14 (50.6 %) and Ap4(50.39). This study clearly indicated that these organisms could effectively be used against *Phytophthora* too. Inhibition of *Phytophthora* by rhizobacteria was reported

in black pepper by various workers like Lisha (2002); Anith *et al.* (2003); Susheela Bhai and Sarma (2003); Saju (2004), Dinu *et al.* (2007) and Diby Paul & Sarma (2005). There are different mechanisms by which the bacterial isolates inhibited *Phytophthora*. The antifungal metabolites involved are 2,4, diacetyld phloroglucinol (Keel *et al.* 1992), Oomycin-A, Phenazine (Thomashow & Weller 1995), Phenazine-1-carboxylic acid (PCA), Pyoluteorin and Pyrrolnitrin (Laville *et al.* 1992). Bacterial or actinomycete antagonists may produce antibiotics, other toxic metabolites, or enzymes (Lee *et al.* 2003; Kim 2006) that also may inhibit growth or sporulation and /or lyse hyphae or spores of the pathogen. These antagonists are thought to be attracted to and nourished by substances exuded by the pathogen hyphae or spores. Once present, these same microbes can produce lytic enzymes that destroy the pathogen. Some microbes can produce metabolites that enter plant tissues and induce defense reactions by the host plant without direct contact with the pathogen. Malajczuk (1983) reported antibiotic zwittermicin A, produced by the biocontrol agent *Bacillus cereus* UW85, which was important in biocontrol of *Phytophthora* root rot. Apart from *Phytophthora*, Kumar *et al.* (2004) reports the effective utilization of these strains of bacteria for the control of *Fusarium*, *Pythium* and *Ralstonia*, the major constraints of black pepper. The most effective strains in this investigation are thus having the potential of suppressing multiple pathogens of black pepper.

5.2.4. Production of HCN

The production of HCN was demonstrated by bacterial isolates like Is. 106, 110, 126, 135, and C11 etc. which were proven to be efficient in the biocontrol of nematodes (Table 10). In the present investigation the varying amounts of HCN production were not quantified, instead, they were grouped into strong, moderate and weak producers. With those strains incapable of production of HCN, no colour change was detected. HCN production has been postulated to play an important role in biocontrol of pathogens (Defago 1990). HCN production by antagonistic bacteria has been reported by other workers as well (Saxena *et al.* 1996; Jousset *et al.* 2006; Ahmad *et al.* 2006; Afsharmanesh *et al.* 2005; Fisher *et al.* 2006; Zlosnik *et al.* 2006). Threshold ion-pair production spectroscopy of HCN is reported by Hu *et al.* (2006). HCN was reported to inhibit the electron transport disrupting the energy supply to the cells ultimately leading to the death of the pathogen (Knowels 1976). Many researchers suggested that cyanogenic antagonists could be potential biocontrol agents (Laville *et al.* 1998;

Gallagher & Manoil 2001). Hydrogen cyanide is a typical pseudomonad secondary metabolite, a compound which is not required for growth, energy storage, or primary metabolism but which may provide some ecological advantage to the organism (Vining 1990). In *Pseudomonas* species, HCN was released by the decarboxylation of glycine (Wissing 1974). *Pseudomonas* strains produced cyanide by oxidative decarboxylation of glycine by the three-subunit membrane-bound flavoenzyme encoded by *hcnABC* (Blumer *et al.* 2000). *P. aeruginosa* produces HCN maximally in the late exponential and early stationary phases under microaerophilic conditions (Blumer *et al.* 2000), and transcription of the *hcn* genes appears to depend directly on the quorum sensor regulators *LasR* and *RhlR*, as well as the anaerobic regulator *Anr* (Brint & Ohman 1995; Pessi & Haas 2000; Whiteley *et al.* 1999). Additional components of the complex regulatory circuitry controlling the production of cyanide and other secondary metabolites had been identified (Albus *et al.* 1997; Chugani *et al.* 2001; Voisard *et al.* 1989). Way in 1984 explained the cyanide intoxication and its mechanism of antagonism. Thus the bacterial isolates like Is. 106, Is.110, Is.111, Is.126, Is.135 and C11 also possess HCN production ability (Table 10) which makes it potential biocontrol agents of nematodes of black pepper. However they are not among the efficient isolates when other parameters were also considered.

5.2.5. Production of H₂S

Laboratory trials had demonstrated the production of H₂S by a number of bacterial isolates (Table 10). These bacterial isolates had the ability to decompose sulphur containing amino acids producing H₂S among the products. When culture media containing ferric ammonium citrate or ferrous acetate was used they turned black or brown indicating the production of H₂S. Several workers reported that H₂S was lethal to nematodes (Rodriquez-Kabana 1965; Lopper *et al.* 1991). Strikingly three bacterial isolates viz. Is. 113, 119 and 136, capable of producing H₂S were found to be the most efficient strains in this investigation.

5.2.6. Production of siderophore

The selected bacterial isolates were evaluated for the production of siderophores. and out of which two bacterial isolates, Ap4 and Ap8 when grown in pseudomonas agar for fluorescein and viewed under UV irradiation showed yellow green fluorescing pigments and were found to be siderophores positive. Ap 4 when grown in pseudomonas agar for pyocyanin produced bluish green phenazine pigment (Plate 13b. B & C). The

fluorescing pigments that functions as siderophores are pyoverdins or pseudobactins (Mayor & Abdallah 1978). Pyocyanins were produced by *P. aeruginosa* only where as fluorescein may be produced by many other species also. Siderophores and antibiotic production by fluorescent pseudomonads were often proposed as the mechanism of action in reduction of soil borne plant pathogen or in plant growth stimulation (Kloepper & Schroth 1981). Nematode activity was greatly influenced by production of antibiotics than siderophores production (Eapen *et al.* 1997). But, when these strains were applied to the rhizosphere, siderophores were produced under conditions far different than those *in vitro*, and for some strains these conditions might not be suitable. Thus, short listing of biocontrol agents based on a particular mode of action might not be ideal. In the present investigation, *in vivo* results were confirmed the results obtained *in vitro* studies. The most effective strain in the present investigation Ap 4 and Ap 8 were found to be producing siderophores were better proven to be efficient nematode antagonists. Evidence for the production of pyocyanine by nonproliferating suspensions of *Pseudomonas* spp was put forward by Halpern *et al.* (1962). Reports are plenty in literature regarding the production of siderophores by bacterial isolates (Paton 1959; Kloepper *et al.* 1980; Ahl *et al.* 1986; Duffy & Defago 1999; Rubio *et al.* 2000; Manwar *et al.* 2001).

Formation of pigments by *Pseudomonas* bacteria in relation to the ratio of medium components was demonstrated by Kolesnikova *et al.* (1971). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria was reported by Kloepper *et al.* (1980).

5.2.7. Detection of protease

Out of 25 strains of bacteria were screened for the production of protease enzymes and 13 out of them were shown to be producers of protease enzyme. Bacterial isolates having proteolytic activity had a great deal to do in the nematode biocontrol as the proteins are integral part of the nematodes. Those strains with proteolytic activities were better adapted to suppress the nematodes.

The condition and abundance of protein component in the eggshell would determine its degradability by protease. In nematode eggshells the protein content is reported to be variable, and it was 59% in *G. rostochiensis* (Clarke *et al.* 1967), while it was 40% in *M. incognita* (Bird & McClure 1976). When protein content in the eggshell

was higher than chitin, proteases may be more important than chitinases. Proteases play a critical role in many physiological and pathophysiological processes. Since proteases are enzymes of metabolic as well as commercial importance, there is a vast literature on production of protease by bacteria (Dancer & Mandelstam 1975; Boguslawski *et al.* 1983; Braunagel & Benedik 1990; Barrett 1994; Bhosale *et al.* 1995; Binnie *et al.* 1996). Different workers had characterized bacterial proteases and demonstrated their importance in biocontrol of nematodes (Boguslawski 1983; Purva *et al.* 1998; Bhosale *et al.* 1995; Singh *et al.* 1999; Kumara *et al.* 1999; Kawai *et al.* 1999; Siddiqui *et al.* 2005). Protease genes from bacteria, fungi, and viruses had been cloned and sequenced (Safarik & Safarikova 1994; Rao 1998; Raaijmakers *et al.* 2002; Haas & Keel 2003; Haas & Defago 2005). Protease *aprA* from *P. fluorescens* CHA0 against *M. incognita* (Siddiqui *et al.* (2005z); neutral protease from *Bacillus nematocida* (*Bacillus* B16) against *Panagrellus redivivus* and *Bursaphelenchus xylophilus* (Niu *et al.* 2006); alkaline serine protease by *B. nematocida* (*Bacillus* B16) against *P. redivivus* (Niu *et al.* (2005); Neutral protease by *Brevibacillus laterosporus* against *P. redivivus* (Tian *et al.* (2007) etc. were reported as bacterial extra cellular proteases acting against nematodes. Thus the short listed isolates viz. Is.113, Is, 119, Is.118 Ap4 Ap5 etc also possess protease enzyme production ability (Plate 13b.E) which makes them potential biocontrol agents of nematodes of black pepper

5.2.8. Detection of gelatin liquefying capacity

The ability of 25 short listed bacterial isolates to liquefy gelatin was also investigated. It was found that two most efficient strains in this investigation, Ap 4 and Is. 123 were capable of liquefying gelatin. This test indicates whether a strain of bacteria could produce hydrolytic enzymes called gelatinases to digest and liquefy gelatin. Gelatinase allowed the organisms to break down gelatin into smaller polypeptides, peptides, and amino acids that could cross the cell membrane and be utilized by the organism. Gelatin is a potential energy source which is too large for most of the bacterial cells to allow into their semi permeable membranes. As a result these bacteria produced gelatinases to break down the gelatin and use it for energy. These strains may have the capacity to invade the gelatinous matrix of the root knot nematode egg masses. When gelatin was broken down, it could no longer solidify. If an organism can break down gelatin, the areas where the organism has grown will remain liquid even if the gelatin was refrigerated. Ap 4 the most important isolate in this study capable of producing gelatinase

enzyme was also effective in hatching suppression of nematode eggs (Table 9). The gelatinase produced by Ap4 helped in dissolving the gelatinous eggmasses of root knot nematodes.

There were various reports on the liquefaction of gelatin by bacterial isolates like *Klebsiella* (Lautrop 1956a; Edmondson *et al.* 1980), *Salmonella* (Lautrop 1956b) *Pseudomonas* (Kiprianova *et al.* 1972), *Corynebacteria* (Cook & Jebb 1952) etc.

The results of various *in vitro* studies indicated substantial production of H₂S, HCN as well as inorganic P solubilization by the tested strains. The data clearly suggest the wide spectrum of biological activities performed by these strains under *in vitro* conditions. The strains with multifarious activities could be perceived as super-bioinoculants. It is therefore considered essential to assess the performance of these strains under natural environment condition (Bano & Musarrat 2003). The inhibitory volatile and nonvolatile metabolites produced by these bacterial strains are capable of inhibiting the nematode population. Among the five short listed isolates viz. Is. 113, Is. 119, Is.136, Ap4 and Ap16, all the strains significantly suppressed the hatching of root knot nematode eggs; Is. 119, Is.136 Ap4 and Ap16 solubilized phosphate; Is.136 Ap4 and Ap16 suppressed *Phytophthora* ; none of them produced HCN; Is. 113, 119 and 136, were capable of producing H₂S; Ap 4 produced siderophores and gelatinase ; short listed isolates viz. Is.113, Is, 119, Is.118 Ap4, Ap5 etc. produced protease enzyme.

5.2.9. Antibiotic sensitivity

Screening for antibiotic resistance was performed for the identification of resistant markers in the short listed 25 bacterial isolates. Intrinsic antibiotic resistance assay of bacterial isolates revealed that many strains were resistant to many of the antibiotics tested in this study. In general all the strains were found to grow in the presence of most of the antibiotics tested except nalidixic acid (Table 11). This multiple antibiotic resistance can be exploited as markers in the selective isolation of the strains from the environment where they are released for disease control or growth promotion. Monitoring of introduced bacteria in the natural environment requires rapid differentiation of the introduced strain from a background of genetically similar and dissimilar indigenous bacteria (Kloepper & Beauchamp 1992; Kluepfel 1993). In addition, investigations into the population dynamics of introduced bacteria add the extra burden of accurate quantification. Antibiotic-marked strains frequently had pleiotropic

phenotypes (Kluepfel 1993) and might not express the resistance phenotype upon growth on selective media (Mossel & Netten 1984). Rifampicin resistance can be used as a reliable marker for ecological studies on rhizosphere pseudomonads (Glandor *et al.* 1992). Reports are plenty in literature on the antibiotic sensitivity of various bacteria (Bucknell *et al.* 1997; Parveen *et al.* 1997; Ash *et al.* 2002; Costanzo *et al.* 2005; Watkinson *et al.* 2007; Ashish *et al.* 2007). Ap4 were susceptible to nalidixic acid and polymyxin and Is.136 was susceptible to cycloheximide and polymyxin. 113 and Is.119 were susceptible to polymyxin only. Ap16 was resistant to all the 14 antibiotics tested (Table 11). The antibiotic resistance patterns of the short listed isolates were helpful in the ecological studies and also for re-isolating them from the inoculated rhizosphere.

A greater understanding of the ecology and modes of action of nematode antagonistic rhizobacteria might lead to a more consistent biological control. Since the infective juveniles are susceptible to a number of abiotic factors including temperature extremes, rapid desiccation, and ultraviolet light and biotic factors such as antagonists and competitors, an understanding on the biology and ecology of these nematodes is the first step to an effective pest control programme (Kaya *et al.* 1999). It is reported that the biosynthesis of antagonistic factors in biocontrol agents is influenced by environmental factors including biotic and abiotic factors (Duffy & Deffago 1999; Pierson *et al.* 1998; Teplitski *et al.* 2000; Someya *et al.* 2003). The sensitivity of pseudomonads to soil environmental factors was shown by Callghan (2003). Establishment of *P. fluorescens* was significantly affected by both soil moisture content and temperature and the decline was higher at higher temperature. Reduced reproducibility of results with some isolates might be due to the loss of survival dependent characteristics. For example, bacterial isolates which give 100 per cent nematode suppression in tomato like Is. 124 and Is. 127 were not so efficient in the experiments with black pepper. The loss could be caused by the influence of out side factors and through nonreversible spontaneous loss (Stainer *et al.* 1970). Factors such as low colonization (Weller 1988) and environmental factors like soil type, plant nutritional status, moisture and temperature might also cause inconsistent results (Kloepper *et al.* 1989).

5.2.10. Utilization of carbon sources

Among the short listed 25 bacterial isolates it is found that nine isolates out of 25 were able to metabolize all the carbon sources tested. The ability of bacteria to utilize

various carbon sources makes it an adaptable candidate for utilization of diverse and even complex nutritional requirements in the nature. The complex C utilization pattern revealed the diversity among the bacterial strains. The source from which the bacteria were isolated might have influenced their ability to utilize carbon sources as the bacterial isolates from antagonistic plants mainly utilized glucose and mannitol. The carbon utilization patterns of the bacterial isolates had direct influence on their other metabolic and biocontrol activities. Recent reports are available in plenty on the differential utilization of carbon sources by bacterial isolates (Suzuki *et al.* 2007; Park *et al.* 2007; Schwab *et al.* 2007 and Das & Mukherjee 2007).

Ji & Wilson (2002) reported correlation between reduction in disease severity and similarity in carbon source utilization, suggesting that similarity in carbon source use was significant in determining ability to suppress any disease. Similarity in carbon source utilization was a more important component of biological control ability for the catabolic mutants than for the nonpathogenic bacteria. Response of *P. aeruginosa* to carbon-starvation on general cross protection and production of some nematocidal compounds *in vitro* for the biocontrol of *M. javanica* in tomato was reported by Siddiqui & Shaukat (2003). Source of carbon, nitrogen and inorganic phosphate in liquid culture can regulate nematocidal activity by fluorescent pseudomonads *in vitro* (Siddiqui & Shaukat 2004). Developing bacterial strains more responsive to certain environmental signals could be exploited for increased secondary metabolite production in pharmaceutical fermentations and offers new avenues to improve biocontrol.

The carbon utilization patterns of the bacteria could be used for the identification of bacteria (Konopka *et al.* 1998; Garland 1996; Gamo & Shoji 1999). Miller & Rhoden (1991) reported the use of 'Biolog' identification system, a versatile and easy to use system of identification based on the C utilization pattern. The nature of C source also influenced the P solubilizing ability of the organism (Tsay & To 1987; Cerezine *et al.* 1988 ; Siddiqui & Shaukat (2003) as the nature of organic acid produced under various C source conditions varied.

5.2.11. Suppression of nematodes infecting black pepper

The effect of 25 short listed bacterial isolates to suppress pathogenic nematodes black pepper was evaluated. In the first set of experiments where, the inhibitory action of the bacterial isolates on burrowing nematodes was evaluated, the bacterial isolates were

proven to be efficient in both suppressing nematode and promoting the growth of black pepper (Table 13). Bacterial isolates viz. Ap 4, Ap16, Is. 119 and Is.136 were having high potential of suppressing *R. similis* and bacterial isolates like Is. 101, 133, 136 Ap4 and Ap16 are excellent growth promoters. In the second set of experiment in which the bacterial isolates were evaluated for their suppression of root knot nematode, the bacterial isolates were proven to be efficient in both suppressing nematode and promoting the growth of black pepper (Table14). Total nematode suppression was achieved by Ap4 and Is. 136. Here also the isolates were effective in growth promotion of black pepper.

Recently, interactions among the microorganisms, nematode target, plant and environment had been well reviewed and emphasized (Kerry 2000; Barker 2003; Davies 2005; Dong & Zhang 2006). The damage due to nematode attack was reported to be severe as the plant-parasitic nematodes and pathogenic organisms form complex association within the host plant. In black pepper also slow decline is the result of feeder root damage by *P.capsici*, *R.similis* and *M. incognita* either or in various combinations. (Anandaraj *et al.* 2000). Nematodes could 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 resulted in an environment beneficial for pathogenic organisms (Sitaramaiah & Pathak 1993). Ferraz *et al.* (1989) found that chlorophyll content in the leaves of the nematode infested black pepper vines was significantly low affecting the growth. In cases where the bacteria were able to suppress the nematode to a lesser extent (Table 13 & 14), the reason might be poor establishment of the bacteria or the juveniles might have escaped infection because of their high density or the mobility of the nematode J2 may be greater than the growth rate of the bacteria in the soil. The nature of the host plant (black pepper), also might have contributed to this. Juvenile penetration into roots of black pepper was reduced only at the beginning of experiment. The galls due to root knot nematode infection in black pepper are big in size and therefore the egg masses embedded in the gall tissues are not accessible to the bacteria (Eapen 2003). The other determinants for growth promotion in black pepper were enhanced production of feeder roots in the plant and also the increased absorptive surface area of the roots (Sarma *et al.* 2003). Production of nematode antagonistic compounds *in vitro* does not necessarily mean that such compounds will be produced in the soil. But in the present investigation the effective bacterial isolates in *in vitro* experiments were also efficient in soil. The percentage of female *Meloidogyne* developing

was very low indicating that the bacterium might have delayed the development of nematodes. The bacteria used induced physiological modifications of the plant roots that interacted negatively with the development of root-knot nematodes. A similar report was by Saliev *et al.* (2000), the motile cells of *P. oryzihabitans* attached themselves to the roots of the host plant which they penetrated; releasing toxins that may act directly on developing juveniles in the galls. This might explain why many galls were without developing juveniles in this study too. Thus the organisms with multifarious mode of action is likely to be more efficient as various mechanisms are activated based on requirement and change in the microenvironment of roots.

5.2.12. Induced resistance

Besides direct antagonism, activity by way of producing various bacterial metabolites, induction of systemic resistance etc. had been reported as new mechanisms by which the plants defend themselves from the pathogen attack. To understand the mechanism involved in rhizobacteria-mediated suppression of root-knot nematode in tomato, bacterial performance was assessed in a split root trial in which one-half of the root system was treated with bacterium, while the other was inoculated with the nematode. The split root technique to demonstrate ISR developed by Kloepper *et al* (1991) had been tested in the present study. Split-root experiments that guarantee a spatial separation of inducing agent and a challenging pathogen, clearly showed that bacterial treatment in one half of the root system with a bacterial cell suspension resulted in a significant induced systemic resistance leading to reduction of *M. incognita* infection in the non-bacterized nematode treated roots (Table. 15 & 16). The spatial separation of the pathogen and antagonists could be achieved in several ways while designing the experiment to detect the induced resistance. The foliar spray of the antagonists like *Pseudomonas*, *Bacillus* and *Trichoderma* significantly triggered the defense enzymes in banana cultivars as reported by Kannan *et al.* (2003). In this study, the spatial separation was made by separating the roots in different polybags and introducing the test organism.

The ISR was characterized by the significant reduction in root galling and poor development of nematodes in comparison with non bacterized roots (Table 15, 16 Plate 18&19). This bio protection was directly linked to the root colonization by the introduced bacteria. Induction of systemic resistance by selected strains of PGPR against plant

disease and nematodes had been proved by spatially separating the pathogen and PGPR in the plants (Van Loon 1997).

The present investigation thus clearly showed the two fold benefits of using microorganisms for disease management in that they not only improved the plant growth but also induced better resistance to infection by soil borne plant pathogens. The total number of root-knot galls and egg masses were recorded. Treatment with most of bacteria significantly reduced the number of root-knot galls when compared with the non-treated control. Induced systemic resistance is considered a possible control mechanism of endophytic bacteria against root-knot nematodes.

SAR is expressed to a maximum level when the inducing organism causes necrosis (Cameron 1994) where as ISR by PGPR did not cause any necrotic symptoms on the host plants (Van loon *et al.* 1998). Both SAR and ISR are the activation of latent resistant mechanisms that are expressed upon subsequent or challenge inoculation with a pathogen (Van Loon 1997). Induced resistance is a state of enhanced defensive capacity developed by a plant when stimulated (Kuc 2001). Endophytic bacteria are known to control plant pathogens by induction of systemic resistance and other defense response including the production of phytoalexins, accumulation of pathogenesis related proteins, deposition of structural barriers in the cell wall of the host plant, and by producing antimicrobial compounds and siderophore (Manjula 2002). Bacterial treatment in combination with chitosan at the appropriate concentration induced enhanced physiology and biochemical changes at the site of the attempted pathogen penetration. Some of the bacteria produced a wide range of secondary metabolites, such as siderophores, HCN and antibiotics, which are known to induce pathogen growth inhibition (Dowling & O’Gara 1994). Further the interaction of endophytic bacteria on the seed surface with seeds or sprouting seedlings lead to a certain threshold level necessary to initiate the defense mechanism (Hallmann 2001).

For mechanisms of ISR, previous workers demonstrated that several bacterial determinants such as siderophores, SA, and lipopolysaccharides (LPS) contributed to ISR (van Loon *et al.* 1998). Because siderophores sequester the limited supply of iron (III) in the rhizosphere, they limited its availability to pathogens and ultimately suppressed their growth (Manjula 2002). The availability of iron (III) in the soil declined logarithmically with increasing soil pH. Thus siderophore mediated suppression should be grater in

neutral and alkaline soils (Cameron 1994) than in acid soils (Dowling & O’Gara 1994). Pathogen related enzyme peroxidase was found to be responsible for the lignification of the plant cell walls (He *et al.* 2002).

In a study by Siddiqui & Shaukat (2004) clearly suggested that the antibiotic 2,4-DAPG from *P. fluorescens* CHA0 act as the inducing agents of systemic resistance in tomato roots..

Presence of phenolic compounds in plants or their synthesis in response to pathogen infection had been associated with resistance (Ingham 1972). Kavino *et al.* (2003) showed the increase in the phenolic content in fluorescent *Pseudomonas* treated plants. Accumulation of phenolic compounds had been reported by other workers like Chen *et al.* (2000), Kandan *et al.* (2002); Kannan *et al.* (2003) and Radhaeyalakshmi *et al.* (2003). Plants had defense genes that need appropriate stimuli or signals to activate them. Inducing the plants own defense mechanisms by prior application of a biological inducer is a novel technique for plant protection. Jyothi and Sivakumar (2003) reported the efficiency of salicylic acid producing fluorescent *Pseudomonas* in inducing systemic resistance against root knot nematodes in tomato. In *in vitro* studies the water soluble fraction (WSF) of the bacteria increased the mortality of root knot nematodes as the time increased. Induced systemic resistance once expressed, activated multiple potential defense mechanisms that induce increased activity of chitinases and peroxides which showed resistances to various plant pathogens Xue *et al.* 1998). The mechanism by which these bacterial treatments induced resistance against the nematodes was not determined in the present study. However, ISR induction have been proved by the split root experiment and among the isolates tested short listed isolates viz. Is.. 25, Is.118, Is. 136, AP4, AP 10, AP16 etc. proved to be the best (Table 15, 16 and 17).

5.3. STUDIES ON MOST PROMINENT BACTERIAL ISOLATES

5.3.1. 16S rDNA sequencing

16S rDNA sequence has long been used as a taxonomic "gold standard" in determining the phylogenies of bacterial species. 16S rDNA-based PCR assays provide rapid, simple, and reliable identification. Commercial test systems and other phenotype-based identification methods may misidentify the organisms (Shelly *et al.* 2000; Valinsky *et al.* 2002). To facilitate species identification of the efficient bacterial isolates, 16S

ribosomal DNA (rDNA) sequence data to design PCR assays intended to provide genus- or species-level identification. Amplified 16S rDNA was purified from reaction mixture by agarose gel electrophoresis. The 16S rDNA nucleotide sequence obtained were aligned with the most similar sequences. The sequences on comparing with NCBI database had clearly revealed the species identity of the chosen microorganisms. The DNA sequences that encode for RNAs had been extensively used to study the taxonomic relationships and genetic variations in microorganisms (Lane 1991, Hibbett 1992; Stackebrandt & Goebel 1994; Anzai *et al.* 2000). Effect of promising organisms on root knot nematodes infesting black pepper

5.3.2. Effect of promising organisms on root knot nematodes infesting black pepper

Five promising bacterial isolates when evaluated for suppressing root knot nematodes infesting black pepper rooted cuttings planted in three different potting mixtures *viz.* potting mixture, sterile potting mixture and soil contaminated with *Meloidogyne* and found to be efficient both growth promotion and nematode suppressions in the three types of potting mixtures (Table 18). Better results were observed in the normal potting mixture. The normal potting mixture, which mimics the real situation in the nature, allowed the interaction of all the rhizospheric organisms like plant growth promoting rhizobacteria and other beneficial fungi. When compared with the other two types of potting mixture *i.e.* the soil contaminated with nematodes and the sterile potting mixture, the applied bacteria were better adapted to the natural soil situations.

In the experiments with normal potting mixture the isolates were efficient in both growth promotion and nematode suppression. Maximum height of the pepper was obtained in the treatment with Ap4 when planted in normal potting mixture *i.e.* 292.33 cm height (91.48 % increase over control) followed by planting in sterile potting mixture (189.33 cm *i.e.* 80.32 % increase over control). The treatment of Ap4 in the soil previously contaminated with nematodes the increase in height over control was only 75.27 % (*i.e.* 165.23 cm) due to initial root damage. Same trend was noticed with all the five bacterial isolates. The treatment with Is. 136 resulted in 289.33 cm height of black pepper in normal potting mixture, 179.67 cm in sterile soil and only 160.67 cm in nematode infected soil. Maximum root weight of 19.55 g was recorded by Ap4 in the in normal soil and in sterile soil the root weight was 11.13 g followed by 7.4 g in nematode

infected soil. The growth promotive attributes of these bacterial isolates can be correlated with the nematode suppressive capacity of these bacterial isolates in these three types of soil. Maximum nematode suppressive action of these bacterial isolates were in normal potting mixture which in turn resulted in better growth of black pepper.

The sterilization of the soil prior to the treatments resulted in killing not only the pathogenic organisms, but also the beneficial organism. Though, the sterile system is useful for finding out the efficiency of a particular organism, it is always better to adapt to the normal potting mixture so that its efficiency can be evaluated in the actual environmental conditions. The soil system is very complex and complicated with its biotic and abiotic components and the balanced interactions will lead to the better efficiency of the applied organism. In the present study total nematode suppression obtained in the experiments with sterile potting mixture also, indicating the efficiency of the bacterial isolate like Ap4.

In the soil previously contaminated with nematodes, the nematodes were already present and before the bacterial isolates could colonize the roots and act against, the nematodes were able to infect the roots of the plants. It is already reported by different workers that it is very difficult to control the nematode once the infection started. (Ali and Kamal 1998; Bansal *et al* 1999; Chen *et al.* 1999; Cannayane *et al.* 2001; Hallmann *et al.* 2001 Ansari *et al.* 2002). But in the present experiments, though 100 % nematode suppression could not be achieved, all the five isolates were significantly efficient in suppressing the root knot nematodes. In our previous experiment severely nematode infected rooted cuttings of black pepper were rejuvenated with the same bacterial isolates (Table 18). This is due to the growth promoting activities of these isolates and offsetting the damage caused by nematodes before introduction of biocontrol agents.

5.3.3. Effect of bacterial strains on rooting of black pepper

Five bacterial strains efficient in nematode suppression strains were evaluated for their efficiency in root proliferation and elongation. The bacterial strains under the present investigation significantly increased the total root biomass of black pepper. The number of roots and the length of roots increased significantly over the uninoculated control plants (Figure 4). These strains were also found to be better colonizers of black pepper roots. The growth promotion of roots increases the root length, number of root

hairs, number of root branches, and root surface area. Highest increase in root biomass was achieved by the treatment of bacterial isolate Ap4 and the highest root length is obtained by the treatment of Is.136. Many investigators indicated that the plant growth substances produced by bacterial isolates induce root elongation and proliferation of lateral roots and root hairs concomitant with enhanced water and mineral uptake which in turn results in enhanced plant growth. For a PGPR strain to be effective biological control agents, they must colonize root. The introduced bacterium must colonize plant roots and it was important to demonstrate rhizosphere competence before its further utilization as biological control and plant growth promoting agents.

The introduced bacteria must establish and grow in the ecological habitat that includes indigenous microorganisms (Schroth & Becker 1990). Hence root colonization is a competitive process that is affected by both the PGPR and the host. Individual PGPR strains may be crop specific, cultivar specific, or nonspecific for root colonization (Chanway *et al.* 1991). An introduced organism having better multiplication and persistence is always known to yield the desired effect in the rhizosphere. The better multiplication and persistence of an organism depends upon the competitive ability and adaptability of the organism (Brooks *et al.* 1994). Most of the strains used in this investigation were actually isolated from the rhizosphere of black pepper enhancing the chance of better colonization potential in the black pepper. Those strains isolated from other nematode antagonistic plants too were found to be better colonizers of the black pepper roots.

The extent and thoroughness of colonization determines whether sufficient bacterial metabolite will be produced to inhibit, interrupt or stimulate certain processes in the nematode's life cycle or specific root exudates necessary to complete the nematode life cycle will be metabolized (Eapen *et al.* 1997). Any strain that has a negative effect on the nematodes is likely to thrive in the environment. Intergeneric and interspecific competition may influence the bacteria colonization on the root surface (Racke & Sikora 1992a).

One approach in selecting candidate bacteria for biocontrol was to screen strains for their ability to colonize roots irrespective of their biocontrol activity (Hinton & Bacon 1995). Variable root colonization by the introduced bacteria, including colonization from plant to plant, and root to root on a given plant, was probably the main reason for

inconsistent control by biocontrol agents (Schroth & Becker 1990). In the present investigation, only the short listed bacterial isolates were tested for their efficiency in root colonization. Strains effective in biocontrol of a root pathogen also must be able to colonize roots and avoid displacement by the indigenous microorganism. The methodology by which the colonization potential was evaluated was also critical. Spatial and temporal colonization patterns of introduced bacteria on individual roots offer the best analysis of a strain's colonization. Such patterns are rarely determined because the studies were extremely labor intensive (Brooks *et al.* 1994). Instead bacterial populations on the roots were usually determined from pooled samples of roots (Schroth & Becker 1990). All the five test strains used in this investigation were reisolated from the rhizosphere of black pepper.

The ecological fitness of these strains as demonstrated by their root colonizing capacity and coexistence with the soil communities further suggests their agronomic significance. The microbial complexity of soil, and the extensive interactions among organisms will also determine the root colonizing capacity as Knox *et al.* (2003) revealed that nematodes are important vectors for bacteria rhizosphere colonization in the absence of percolating water.

5.3.4. Compatibility studies

5.3.4.1. Compatibility with other organisms

Both antagonism (Varshney *et al.* 2000) and compatible interactions (Duffy *et al.* 1996) occur between biocontrol agents and the types of interaction vary with different agents. Hence proper evaluation of interaction is a pre-requisite before recommending a consortium of biocontrol agents. Rhizosphere harbors a variety of micro-organisms including bacteria, fungi and viruses. Recently, interactions among the microorganism, nematode target, plant and environment had been well reviewed and emphasized (Kerry 2000; Barker 2003; Davies 2005; Dong & Zhang 2006). While benefits may be considerable there are also potential drawbacks to application of a combination of organisms. Antagonism between biological control agents through competition and antibiosis may lead to decreased performance in the field (Paulitz 1990). The potential benefits and pitfalls must be examined so that efficacious combinations can be identified. The mutual compatibility among the bacterial isolates and the compatibility of the selected bacterial isolates with nematode antagonistic fungi were evaluated. It is found

that the test strains of bacteria and fungal isolates are compatible to each other (Plate 20,21).

5.3.4.2. Compatibility with agrochemicals

The compatibility studies with three agrochemicals revealed that Ap4 was highly compatible with all the chemicals tested. Fungicides like metalxyl mancozeb and potassium phosphonate and insecticide like chlorpyrifos were included in this study. Chlorpyrifos is a non-systemic organophosphorus insecticide (diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxy-phosphorane(C₉H₁₁Cl₃NO₃PS).

It is found that the *P. aeruginosa* isolate Ap4 is highly compatible with all the three chemicals tested at the recommended dose (Figure 6). Various pesticides were used to protect different crops from pest attack but very little is known about the damaging effects of pesticide application on non-target, beneficial soil microflora. The effect of agro chemicals on beneficial microorganisms had been investigated for a number of chemicals and biocontrol agents. This area is of such importance in biocontrol work that it was studied both by independent researches and by groups that organize tests and reports jointly on the results (Hassan & Oomen 1985). Studying the compatibility of the bacterial strains was important and strains of the nematode antagonistic microorganisms could be genetically manipulated to decrease the sensitivity of useful chemicals (Gaspard 1986). As reports say, tolerance to chemicals varies among the bacterial isolates. (Annapurna *et al.* 2000; Fathima *et al.* 2006). Difference in the tolerance levels occurred within one species, one strain could not tolerate the chemical whereas the other grew at high concentrations of the chemical. As the tolerance can vary greatly among different strains of a species each strain of interest should be tested individually. So one of the most important strains in this present investigation was selected for the compatibility studies.

However, the sensitivity or tolerance to a particular agrochemical never reflected its biocontrol activity. Meyer *et al.* (1991) reported that a strain of *V. chlamydosporium* that had no tolerance to benomyl reduced the viability of *H. glycines* eggs in laboratory study whereas the strain with high tolerance to benomyl did not affect the egg viability.

It is a general belief among farmers that fungi or bacteria used as biocontrol agents should not be mixed together with any chemical pesticides. Such a

recommendation will definitely limit the use of biocontrol agents like *Pseudomonas* which are recommended along with chemical pesticides as combined spray. Hence, reports on such compatibility is very much encouraging. The compatibility of this effective bacterial isolate with agrochemicals makes it very efficient in integrated pest management schedule (IPM).

Similar results are reported by Laha & Venkataraman (2001) Diby *et al.* (2003) and Joseph *et al.* (2003). Thus the effective strains could be integrated with other modes of controlling pathogens of black pepper.

5.3.5. Effect of pH

The ability of Ap 4, one of the most promising bacterial isolates in the present study to grow at different pH levels were evaluated. This isolate was better adapted to a wide range of pH (5-8). In this investigation the tolerance of the bacteria only to pH 5-8 was tested keeping in view studying its adaptability to the black pepper growing soil. The ability of the common bacterial biocontrol agents to grow in Kerala soil which is generally acidic in nature and this may be a limiting factor in their establishment in low pH soils. Liming prior to the application of biocontrol agents will help to eliminate this problem. However, if the introduced bacterial isolates are capable of tolerating different ranges of pH, it will be an added advantage of those strains. In general, the soils of Kerala where black pepper is grown are acidic, kaolintic and gravelly with low CEC, low water holding capacity and high phosphate fixing capacity. Alkaline soils are characterized by high pH values up to 11.5 (Sane 1987) and bacteria isolated from those alkaline soils were well adapted to grow in pH up to 12 (Kulkarni *et al.* 1990). Studies on the growth of the bacteria to grow in acidic and alkaline pH will reveal its adaptability to varying pH levels.

5.3.6. Combined inoculation of bacteria and fungi

Pot culture experiments on the management of root knot nematodes through the combined application of nematode antagonistic fungal agents and selected bacterial isolate were conducted under greenhouse conditions in black pepper with field soil. Three fungal isolates exhibiting antagonistic property against nematodes viz. *T. harzianum*, *P. lilacicus* and *P. chlamydosporia* and *P. aeruginosa* (Ap4) were used in this study.

The combined application of nematode antagonistic fungus and selected bacterial isolates resulted in better growth promotion and nematode suppression than the fungus or bacteria used alone. The synergetic action of the bacteria and fungi resulted in better protection of the plants. The nematode suppression ranged from 95.31 to 100%. The combined application of Ap4 with *P. lilacinus* resulted in cent per cent nematode suppression (Table 19) Combination of *P. chlamydosporia* and Ap4 give 99.70% nematode suppression which is on par with the combined application of *T. harzianum* and AP4 (99.17%). The result indicates that the combination of strains resulted in better nematode suppression than applied alone. The plant growth characters were also enhanced by the combination of strains than applied alone. The maximum height of black pepper was recorded by the combined application of Ap4 with *P. chlamydosporia* (111.33 cm) and the combination of *P. lilacinus* and Ap 4 resulted in a height of 108.33 cm. Fresh biomass of black pepper increased to its maximum by the combination of Ap4 and *P. lilacinus* (Table 19).

The isolation of the applied biocontrol agents from the rhizosphere of the plants after termination of the experiment suggested the compatibility of the organisms tested to various extends. Population of fungi like *Trichoderma*, *Paecilomyces*, *Pochonia* and others along with and *P. auriginosa* showed the co existence of these species in soil ecosystem and they were able to present in large numbers. Since nature harbors a mixture of these agents for natural biocontrol, combination or mixture of compatible organisms would be an added advantage over single species application. Field soil instead of sterile soil was used in this experiment so as to closely resemble the soil ecosystem and the effect of these organisms in the complex soil ecosystem could be reflected.

The combination of biocontrol agents had a greater potential for increased activity against one or more plant pathogens and provide for expression of traits under a wider range of environmental conditions. Additionally a combination of biocontrol agents may more effectively colonize the rhizosphere. Considering the inconsistent performance of the biocontrol agents under field conditions, the combined application of biocontrol agents are of great importance. Applying a combination of biocontrol microorganisms is one of the possible means of increasing consistency and efficacy of biocontrol agents. For this goal to be achieved, however, accurate knowledge is needed of the ecology, biology, and mechanisms of action of the populations of nematophagous bacteria.

In this experiment the fungal isolates were proven to be compatible with each other and also with the bacterial isolate and hence there was no sign of antagonism between the isolates. The beneficial interactions among the isolates may lead to increased suppressive action of the biocontrol agents against nematodes as Siddiqui & Shaukat (2004) reported *T. harzianum* enhances the production of nematocidal compounds *in vitro* and improves biocontrol of *M. javanica* by *P. fluorescens* in tomato. In the present study too, the combined use of the isolates was superior to their single application. The synergistic effect of *T. harzianum* on the production of nematocidal compound(s) is critical in biocontrol as it may improve the efficacy of biocontrol bacteria against plant-parasitic nematodes. Application of a mixture of compatible *T. harzianum* and *Pseudomonas* would closely mimic the natural situation and might broaden the spectrum of biocontrol activity with enhanced efficacy and reliability of control. There are several reports on the beneficial effect of combination of microorganism for biocontrol. Differential impact of some *Aspergillus* species on *M. javanica* biocontrol by *P. fluorescens* strain CHA0 was reported by Siddiqui *et al.* (2004). Two nonrelated pseudomonads stimulate each other in the expression of an antimicrobial compound important for biocontrol Maurhofer *et al.* (2004). This interpopulation communication occurs in the rhizosphere, i.e., at the site of pathogen inhibition, and is mediated by the antimicrobial compound itself acting as a signal exchanged between the two pseudomonads.

5.3.7. Efficiency of bacteria to rejuvenate nematode infected black pepper plants

The efficacy of the selected bacterial isolates to rejuvenate severely nematode affected black pepper cuttings was evaluated. The five biocontrol strains used were found to be capable of rejuvenating black pepper vines already infected with nematodes. Interestingly, out of the five isolates, one isolate Ap4, resulted in 100 % survival of the seedlings (Table 20). The survived seedlings were free from nematode infection and were healthy with green leaves and profuse root system. The rest of the isolates are also found to be capable of rejuvenating black pepper vines already infected severely with nematodes. The growth parameters of the rejuvenated plants also improved with the application of these bacteria in the present study. The competitive antagonistic ability of these bacteria on nematodes, in the present study, arrested the further growth and proliferation of nematodes already present in the infected area reducing its number to a

minimum threshold. These bacterial antagonists were proved to be effective in controlling nematodes in both *in vitro* and *in planta*. The different modes of antagonism of these strains might contribute to the reduction of pathogen. The plant health promoting attributes of these strains further boosted up the resistance mechanism within the plant to resist further attack by nematodes. The capacity of these strains to induce systemic resistance against nematodes might also contributed to the overall rejuvenation processes. The result of the present study clearly indicates that these strains can effectively be utilized to save or protect plants where nematode infection already started. Since black pepper is a perennial crop and initial damage to feeder roots often goes unnoticed (Anandaraj 2000) The rejuvenative property of efficient strains could be used in reviving the infected vines, Similar observations were found by Diby Paul, (2004) when he successfully utilized *Pseudomonas* strains to rejuvenate black pepper severely infected with *Phytophthora*. It is well known that it is difficult to control nematode population once the infection started and nematode biocontrol measures are mainly focused on the prevention of nematode infection. This finding requires special attention and further evaluation of the exact mechanisms involved.

5.3.8. Evaluation of biocontrol formulations

P. aeruginosa (Is. No. Ap 4), one of the most effective bacterial strains in this investigation was formulated using different carriers and its shelf life is evaluated for a period of 90 days. Different types of formulation like beads, granules and powder formulations using various amendments were tested in this study.

In all the formulations the cfu decreased as the storage time increased (Table 21,22 &23). Formulations of sodium alginate beads were prepared and stored both in wet and dry conditions. Various additives like rice bran, ginger leaf powder, tea waste, tapioca powder and coir compost were added to the sodium alginate solution to study their effect on the shelf life of these formulations. When different formulations of *Pseudomonas* in sodium alginate based beads were evaluated for the viability and shelf life of the bacterial isolate, the result was very good. The cfu of the bacterial isolate varied significantly between the different carriers. The sodium alginate without any amendments supported the lowest growth of bacteria. Among the various additives added to sodium alginate beads, rice bran resulted in maximum cfu and the beads amended with ginger leaf powder was on par with. The incorporation of biocontrol agents into alginate prills with or with

out an extra nutrient source has been studied (Ali *et al.* 2001). Further more encapsulation of BCAs in natural carrier substances such as flour starch or bran is environmentally friendly (Lewis *et al.* 1995; Lumsden *et al.* 1995) and can also serve as nutrient sources for BCAs and there by help in the establishment of BCA at the application site (Lewis *et al.* 1995). Thus the results are consistent with the previous reports that addition of food base enhances the biocontrol efficiency Lewis and Papavizas (1985) and Kundensen and Bin (1990).

On the other hand, several researchers had studied the influence of storage conditions on the viability in soil of antagonists formulated as alginate prills (Fravel *et al.* 1998; Lewis *et al.* 1995). It was early in the formulation of alginate gels with biocontrol fungi that certain genera of *Trichoderma* and *Gliocladium*, required a food base rather than inert clay as a carrier (Lewis & Papavizas 1987; Knudsen & Bin 1990).

Sodium alginate is an anionic polysaccharide extracted principally from giant kelp *Macrocystis pyrifera* as alginic acid and neutralized to sodium salt. It is soluble in hot or cold water and gives some what hazy solutions at neutral pH (Kaya & Nelson 1985). Coimmobilization of the freshwater microalga *Chlorella vulgaris* and the plant-growth-promoting bacterium *Azospirillum brasilense* in small alginate beads resulted in a significantly increased growth of the microalga (Luz *et al.* 2000). Alginate beads of various forms and shapes are convenient inoculant carriers for use in numerous industrial, environmental, and agricultural applications (Bashan 1986; 1998; Cassidy *et al.* 1996; Fages 1992; Leenen *et al.* 1996. Coimmobilization of mixed microorganism cultures in sodium alginate beads is reported by O'Reilly & Scott (1995).

In the case of granular formulations, the formulations stored under refrigerated condition recorded more cfu/g when compared to that stored at room temperature (Table 22). Preparation of talc powder and granular formulations involved simple technology and are time and cost effective compared to alginate beads formulation.

The present investigation was undertaken to study the multiplication and persistence of selected native *Pseudomonas* isolate in talc based formulations (Table 23). The talc based formulations resulted in enhanced shelf life. Initially the population increased and then started decreasing. The study indicates that the isolate Ap 4 has been surviving in talc based formulation without any additional amendments. But addition of

adhesives like carboxymethyl cellulose and gum arabic resulted in excellent growth and shelf life (Table 23).

Talc powder is the most commonly used carrier for microbial inoculant formulations. Talc is a natural mineral (hydrated magnesium silicate, $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$). Talc is an industrial raw material in powder form which is used as a commodity or specialty in a wide range of applications. The main characteristics of talc that make it a unique material for industrial and domestic applications are its lamellarity (made of platelets that easily slide on each other), softness (unctuous and not abrasive), chemical inertness, affinity for organic chemicals and whiteness. Talc's organophilic properties are used to be great benefit in number of applications. It is neither explosive nor flammable. The chemical inertness of talc is of obvious interest for carrier applications. Adding talc to the active ingredients results in a well managed release, allowing the practical and safe handling of very small and very large quantities of active substance. Hence the talc can be safely used as carrier for these isolates without adding any amendments. Pramod and Sivaprasad (2003) reported a population of 10^8 cfu of *Pseudomonas* isolates even after five months of storage in talc based formulation without any adhesives.

The formulations prepared using carrier materials in the absence of adhesives recorded lower viable counts compared to the formulations with addition of adhesives carboxymethylcellulose and gum arabic. The adhesives used were efficient in maintaining the shelf life of the organisms. Formulations with stickers (adhesives) are associated with better adhesion of organism to seeds. This may result in better efficacy of formulations when applied to plants. Stickers also protect the organism from desiccation and therefore contribute to the shelf life and efficacy of inoculants (Elegba & Rennie 1984). Gum arabic (Acacia) is an anionic polysaccharide collected as the dried exudates from acacia tree (*Acacia senegal*) it is low viscosity gum with possible concentrations of up to 50% in water.

Over the past twenty years a large number of studies had been undertaken to investigate the use of microorganisms as biocontrol agents against nematode pests. More and more bacteria have been identified as pathogens of plant-parasitic nematodes and had shown suppression effects on nematode pest populations. However, only a few commercial biocontrol products from the bacteria with nematicidal potentials had been developed and used in the agriculture system (Whipps & Davies 2000; Gardener 2004;

Schisler *et al.* 2004). The development of biocontrol agents is often unpredictable and too variable for large-scale implementation (Meyer 2003). No matter how well suited a commercial nematode antagonist is to a target host in a laboratory test, in order to realize ideal biocontrol effects in practice an intensive exploration of the mechanisms of the antagonist against nematode populations, and a thorough understanding of the interactions among biocontrol strains, nematode target, soil microbial community, plant and environment must be developed.

The present study has shown feasible and economical way to enhance the efficacy of the test strain. Numerous biotic and abiotic factors may affect the extent and patterns of the biocontrol agents when these formulated bacterial isolates are applied to soil. Identification and quantification of these variables may make biocontrol more predictable and effective. The logical next step will be to extend this study to the field level. Thus, it is possible that the interactions with other soil microbes will be taken in to consideration.

Gram negative bacteria such as *Pseudomonas* spp. are generally formulated as frozen cell pellets that must be kept at low temperature until application, which remain a major obstacle for their large scale use (Chelvaraju *et al.* 2003) However, despite considerable research efforts on the development of new biological control agents the number of such products on the market is still extremely low Kiewnick (2007).

Formulations of microorganisms help to overcome some of the adverse conditions encountered by microbial inocula entering into the soil. The formulations may include large amounts of carriers, selective food sources or buffers that can transiently alter the micro physiological environment of the soil to provide a temporary safe heaven (Paau 1998). Thus the formulation provides the inocula with time and space to reactivate, adjust physiologically to the new environment and propagate. The formulation can enhance the biocontrol agent in several ways. Formulation can overcome the environmental constraints such as dew requirement, that is the requirement by most of the biocontrol agents for free water or an extended dew period for optimal infection (Connick *et al.* 1990).

Biocontrol agents must compete with conventional chemical nematicides in terms of efficacy and persistence. Effective formulation will narrow the gap between chemical

pesticides and microbial pesticides in this regard as well as lengthen their shelf life and ease of use. It will offer the additional advantage of providing selective control of target pathogens without many of the environmental concerns one encounter with conventional pesticides. Development of improved methods to formulate and apply is a prerequisite to the economical use of the biocontrol agents. Regardless of the qualities of the biocontrol agents, the methods used to produce, formulate, and deliver these organisms may profoundly influence their efficacy (Chaluvaraju *et al.* 2003). The major consideration of for successful biocontrol is the application of the effective antagonists to the appropriate ecological niche at proper time Connick *et al.* 1990). Pure culture treatment to seeds is not a desirable practice since the organism cannot withstand the environmental factors when stored for further application. For practical utility and application of the organism at the farm level, formulated carriers like wheat bran, talc powder and lignite as well as adhesive materials are better (Chaluvaraju *et al.* 2003).

Aqueous solutions of sodium alginate and other univalent cation salts of alginic acids form a gel when in contact with calcium or other divalent cations. This property has been used to prepare beads containing microbial (Schuster & Sikora 1992) and nematode inocula (Kaya & Nelson 1985), enzymes and various substrates.

5.3.9. Identity of short listed isolates of bacteria

The most effective strains of bacteria in nematode suppression in this study are Is. 113 identified as *Bacillus megaterium*, Is. 119 as *Comamonas testosteroni*, Is. 136 as *Alcaligenes* Sp., Ap4, as *Pseudomonas aeruginosa* and Ap16 as *Pseudomonas* sp. *Bacilli* are considered to be good biocontrol agents for soil borne pests such as nematodes since, under unfavorable conditions, they formed long lived spores capable of germinating into active cells under more conducive conditions. The ability to survive as spores is also a desirable trait in view of commercial shelf life requirements for biocontrol products (Preasad & Thilak 1971). Various strains of *Bacillus megaterium* produce antibiotic compounds. Neipp & Becker (1999) demonstrated *B. megaterium* has a potential as a promising biocontrol candidate against nematodes. There are different reports on the effective utilization of bacterial isolates belonging to the genus *Pseudomonas*, against *Meloidogyne* and *Radopholus* like *P. fluorescens* and *P. putida* and this is the first report *Pseudomonas aeruginosa* for the suppression of *R. similis*. The *P. aeruginosa* isolate (Ap4) in the *in vitro* studies resulted in 93.60, 34.10 and 95.15 % mortality of

Meloidogyne in peptone broth, phosphate buffer and in culture filtrate respectively. This isolate resulted in the maximum mortality of *R. similis* (80.43 %) among the 46 isolates tested. In the greenhouse studies with tomato as the test plant this isolate was very effective both in nematode suppression and growth promotion. Maximum height and fresh biomass both in terms of root and shoot weight was obtained with the application of this isolate. It resulted in 82.38 % hatching suppression of root knot nematode eggs. It effectively solubilised phosphate and controlled *Phytophthora*. Though, ammonia was produced by this isolate Ap4 doesn't produced HCN or H₂S but produced fluoresin and pyocyanin pigments. It produced protease enzyme and liquefied gelatin. Having multiple antibiotic resistance and was found to be suppressive only to nalidixic acid and polymyxin among 14 antibiotics tested. It was also found effective against root knot and burrowing nematode infesting black pepper, effective in growth promotion in black pepper and was even capable of rejuvenating black pepper plants severely infected with root knot nematode. This isolate is equally effective inducing resistance to nematodes and it can easily be formulated. *Bacillus megaterium*, (Is 113) is another important isolate in the present investigation is also having multifarious effects on pathogens of black pepper. In the *in vitro* evaluation, the isolate 113 exhibited more than 80 % suppression of *Meloidogyne* in all the five methods studied. When screened against *R. similis*, it resulted in 61.67 % suppression and was found to be effective in suppressing the hatch of *Meloidogyne* eggs (62.85 %), produced H₂S and ammonia. The advantage of bacillus strains over gram negative bacteria are their ability to withstand adverse conditions and because of its formulation and long term survival characteristics, the effective utilization of the bacillus strain will also be equally important. The other strains like *Comamonas testosteroni* (Is. 119), *Alcaligenes* (Is.136) *Pseudomonas* (Ap16) can also be utilized not only for the suppression of phytonemtododes but also for other pathogens of black pepper.

Considering the multifarious beneficial traits of these isolates, to commercialize these strains a few more studies such as toxicological data and field data of formulation need to be generated.

SUMMARY

B. Beena “Studies on bacterial antagonists of plant parasitic nematodes of black pepper (*Piper nigrum* L.) ” Thesis. Indian Institute of Spices Research Calicut , University of Calicut, 2008

Chapter 6

SUMMARY

Plant-parasitic nematodes cause serious crop losses worldwide and are among the most important agricultural pests. Although chemical nematicides are effective, they are being withdrawn from the market in some developed countries owing to concerns about public health and environmental safety. The search for novel, environmentally friendly alternatives with which to manage plant-parasitic nematode populations has therefore become increasingly important. The use of suitable bacteria for suppression of plant parasitic nematodes of black pepper, provide an inexpensive, non-polluting strategy for plant protection.

COLLECTION AND PRELIMINARY EVALUATION OF BACTERIAL ISOLATES

Sample collection and isolation

Altogether 190 soil and root samples were collected from black pepper and nematode antagonistic plants like *Chromola odorata* (Eupatorium), *Strychnos nuxvomica* (nux-vomica), *Pimenta dioica* (allspice), *Piper colubrinum* (wild species) and from highly nematode susceptible plant *Coleus blumeri* (coleus) and processed using standard techniques. Isolation of bacteria was carried out from the soil and root samples and various life stages of root knot nematodes. Isolation was also carried out by baiting with sodium alginate prills. Bacterial isolates with distinct morphological features were

purified and maintained at -80°C for further screening. A total 319 bacteria were isolated using different methods.

Laboratory screening

The efficiency of the bacterial strains in suppressing nematodes was evaluated by conducting a number of *in vitro* experiments. Suppressive action of the bacterial isolates on *M. incognita* and *R. similis* was assessed in these experiments. Five different methods were used for assessing the nematode antagonistic potential of the bacterial strains. They are bacterial suspension in peptone water, bacterial suspension in phosphate buffer, cell free culture filtrate total and volatile metabolites.

Screening against *M. incognita*

Bioefficacy of 108 bacterial isolates for suppressing *M. incognita* was evaluated *in vitro* by different methods. In the buffer method, the mortality of nematodes was comparatively less. Out of 108 bacterial isolates tested, only 11 bacterial isolates caused nematode mortality more than fifty per cent, maximum being the treatment with bacterial isolate Is. No. 110 (87.83 %) followed by Is. No. 113 (84.63%) and Is.119 (66.38%). The direct action of bacterial cells was also evaluated by adding nematodes directly to the bacterial cell suspension in peptone water. More than 90 per cent of bacterial isolates caused very high mortality ($>90\%$) to nematodes in peptone water. The mortality ranged from 32.05% (Is. No. 114) to 98.03 % (Is. 109) and maximum nematode suppression was obtained by the treatment of bacterial isolate Is. No. 109 (98.03%) followed by C25 (97.98%). Cell free culture filtrates (CFCF) of 107 bacterial isolates were also evaluated by assaying the mortality of root-knot nematode juveniles and the mortality was very high in culture filtrates too. Maximum nematode suppression was achieved by the treatment of C18 (99.05%) followed by C19 (98.87). Toxic effect of bacterial metabolites of 71 isolates on *Meloidogyne* juveniles was also studied in bacteria seeded agar plates. Culture filtrates and total metabolites of majority of isolates were highly toxic to nematodes. Volatile metabolites of 27 isolates caused more than 90 per cent mortality to nematodes. The highest mortality was noticed in Is. 125 (99.19 %) followed by Is. 113 No. (99.10%) and Is. No. 10 (98.92 %).

Screening against *R. similis*

Forty six bacterial isolates were evaluated for their effect on *R. similis* by the buffer suspension method. The bacterial isolates were found to be inhibitory to *R. similis* to varying extents. The per cent of suppression ranged from 15.03% (B 12) to 80.43% (Ap4). Nematode suppression above 70 % was achieved by seven isolates. Those bacterial strains effective against *M. incognita* were also effective against *R. similis*.

Green house evaluation

A total of 216 bacterial isolates were evaluated in greenhouse including the 108 bacterial isolates tested *in vitro*, in a series of 11 experiments using tomato as the test plant in heat treated soil. Plant growth parameters and nematode population in the roots were assed using standard procedure on termination of the experiment after 30 days. Significant increase in height and biomass of tomato was observed in most bacteria treated plants with shoot weight being more compared to the *M. incognita* inoculated and untreated control. Significant nematode suppression was obtained with a number of bacterial isolates. Total nematode suppression was achieved with the application of 40 bacterial isolates. Out of the 216 bacterial isolates evaluated, only four resulted in growth retardation of tomato.

Based on the efficiency to suppress nematodes and to enhance growth of tomato, 25 bacterial isolates were short listed and further evaluated for their mode of action on nematodes.

CHARACTERIZATION OF SHORT LISTED BACTERIAL ISOLATES AND THEIR MODE OF ACTION ON NEMATODES

Bacterial identification

An attempt was made mainly for the identification of *Pseudomonas* and *Bacillus* strains based on the morphological and biochemical reactions. Out of the 319 bacteria isolated, 77 were tentatively identified as *Pseudomonas* spp. 55 as *Bacillus* spp. and 187 bacterial isolates could not be identified. Out of the 70 bacterial isolates directly isolated from soil, 25 belonged to *Pseudomonas* spp. and seven were *Bacillus* spp. Sixteen bacteria isolated through the baiting of alginate prills yielded a total of 16 bacteria, with 6

and 2 *Pseudomonas* spp. and *Bacillus* spp., respectively. Out of 181 bacteria from roots of black pepper, coleus and nematode antagonistic plants, 37 were *Pseudomonas* spp. and 35 were *Bacillus* spp.

Out of the 25 short listed bacterial isolates, molecular characterization of the short listed 19 bacterial strains was attempted through Unweighed Pair Group Arithmetic Average (UPGMA) and the results revealed the test strains of bacteria are different from each other.

The effective strains of bacteria were further characterized for the mode of action of nematodes and growth promotion. Nematode egg hatching suppression, phosphate solubilization, production of nematicidal metabolites like HCN, H₂S, ammonia and production of proteolytic enzyme, gelatin hydrolyzing capacity etc. of the test strains were evaluated.

Phosphate solubilization

The short listed bacterial isolates were evaluated for their efficiency to solubilize insoluble phosphate by spot inoculating Pikovskaya's solid medium. Twenty one out of 25 bacterial isolates solubilised phosphates to varying extents and the maximum solubilization was obtained by Is. 13 (2.5 cm). Bacteria mediated phosphate solubilization helps in enhanced uptake by plants and may result in increased plant biomass. The potential of these strains to release soluble phosphates for plant uptake is of greater importance when the bacterial isolates are used as both growth promoters and biocontrol agents.

Hatching suppression

Twenty five short listed bacterial isolates were evaluated for the root knot nematode egg hatching suppression and all the isolates significantly suppressed the hatching of *M. incognita* eggs though none of them caused 100 per cent hatching suppression. There was no sign of egg parasitism by any of the bacterial strains. B14 (87.89%) and B15 (91.66%) recorded maximum hatching suppression.

Characterization of bacterial isolates.

Out of 94 isolates screened only eight isolates produced HCN, five being strong HCN producers, two were moderate HCN producers, while one isolate was a weak producer of HCN. Ninety four bacterial strains were characterized for the production of H₂S and 21 isolates were found to be producing H₂S. Twelve bacterial isolates were evaluated for the production of siderophores and two isolates were found to be producing fluorescent pigments identical to siderophores. All of the 94 bacterial isolates produced varying levels of ammonia. Among these, 14 isolates were very strong producers of ammonia, and 36 were strong producers. The short listed 25 bacterial isolates were evaluated for their ability to produce protease enzyme. Bacterial strains capable of producing protease enzymes can be better nematode antagonists as proteins are integral parts of nematode body. Out 25 bacterial isolates screened for production of protease enzyme, 13 were found to produce protease enzyme as evidenced by the zone of clearance around the colonies. The short listed 25 bacterial isolates were screened for the production of gelatinase enzyme and two isolates, Ap 4 and Is. 123 were able to liquefy gelatin.

Antibiotic screening

The short listed 25 isolates were subjected to screening for antibiotic resistance to 14 antibiotics. Most of the strains were found to have intrinsic antibiotic resistance to different antibiotics tested.

Characterization of bacterial isolates based on utilization of carbon

The ability of the bacterial isolates to utilize eight carbon sources viz. glucose, lactose, maltose, galactose, mannose, sorbitol, dulcitol and sucrose was evaluated and bacterial strains were found to have multiple nutritional spectrums utilizing most of the substrates tested. Out of the 25 bacterial isolates evaluated nine isolates were able to metabolize all the carbon sources tested. Bacterial isolates retrieved from antagonistic plants had poor ability to utilize carbon sources other than glucose and mannitol.

Suppression of nematodes infecting black pepper

The short listed 25 bacterial isolates were further evaluated for their effect on *M. incognita* and *R. similis* using black pepper cv. Panniyur 1 as the test plant in two

separate experiments. In the first experiment, highly significant suppression of *R. similis* was obtained by all of the bacterial isolates used notable being Ap4, Ap16, Is.119 and Is.136. Isolates 101, 133, 136 Ap4 and Ap16 are excellent growth promoters of black pepper.

In the second experiment where, the suppressive action of bacterial isolates on *Meloidogyne* was evaluated, all the short-listed 25 bacterial isolates significantly suppressed (above 90%) root knot nematodes too. Total nematode suppression was achieved with isolates Ap4 and Is.136. The growth of the black peppers cuttings were also increased significantly by the application of bacterial isolates.

Induced resistance

The short listed bacterial isolates were evaluated for their ability to induce resistance against root knot nematode both in tomato and in black pepper. It is found that 16 bacterial isolates were able to induce resistance against nematode in tomato. They include Is. No. 25, AP4, AP 10, AP16, 136 and 118. The ability of the short listed isolates to induce resistance in black pepper was assessed in two different set of experiments; one split root experiment and an experiment of nodal runners. Out of the 20 bacterial isolate tested in black pepper, two resulted in 100 % nematode suppression by inducing resistance towards nematodes in split root experiment. In the experiment of nodal roots too 17 out of 20 were able to suppress nematode.

STUDIES ON MOST PROMINENT ISOLATES

16S rDNA sequencing

Five promising bacterial isolates were identified based on 16S rDNA sequencing. Is. 113 was identified as *Bacillus megaterium*, Is. 119 was identified as *Comamonas testosteroni*, Is. 136 as *Alcaligenes* Sp, Ap4 as *Pseudomonas aeruginosa* and Ap16 as *Pseudomonas* Sp.

Effect of bacteria in root initiation and proliferation

The five bacterial isolates, viz. Ap 4, Ap 16, Is. 113, Is. 119, and Is. 136 when tested for their efficiency for rooting of black pepper, all the isolates significantly induced the proliferation of roots. The bacterial strains significantly increased the root biomass of

black pepper. Maximum root biomass was recorded with Is. No. Ap 4 and maximum root length was noticed with Is 136.

Compatibility studies

Most promising five bacterial isolates Is. 113, Is. 119, Is. 136, Ap4 and Ap16 were tested for their mutual compatibility. Among the five isolates of bacteria tested, it is found that these bacteria are compatible with each other. One of most promising bacterial isolate Ap4 was found to be compatible with three fungal biocontrol agents of nematodes viz. *Trichoderma harzianum*, *Paecilomyces lilacinus*, and *Pachonia chlamydosporia*. *Pseudomonas aeruginosa* (Ap4) is found to be compatible with three agrochemicals viz. potassium phosphonate, chlorpyrifos and metalaxyl mancozeb after 48 hours of incubation

Combined inoculation of bacteria and nematodes antagonistic fungi

Three promising fungal isolates exhibiting antagonistic property against nematodes viz *Trichoderma harzianum*, *Paecilomyces lilacinus* and *Pachonia chlamydosporia* were tested along with the potential bacterial isolate Ap4. Black pepper, treated with both bacterial and fungal biocontrol agents were challenge inoculated with J2 of *M. incognita* @ 100 plant-1 two weeks after the inoculation of the biocontrol agent. The experiment was terminated after three months and plant growth parameters and nematode population in the roots were assed. The increase in growth of plants was significantly higher when bacterial isolate Ap4 was applied along with the fungi. All the three fungi and the bacteria significantly reduced the nematode population level in roots, the combined application being superior to the treatments where the fungi and bacteria were used independently. The inoculated bacterial and fungal biocontrol agents were reisolated from the roots after termination of the experiment.

Rejuvenation of nematode infected black pepper

Five efficient strains of bacteria, short listed in earlier experiments were tested for their efficacy in rejuvenating the black pepper seedlings infected with nematodes. Black pepper rooted cuttings severely infected with root knot nematodes were up rooted carefully, washed in running water and treated with five short listed bacterial isolates and replanted. The experiment was terminated after four months and nematode populations in

the root system were estimated. All the five isolates of bacteria tested, rejuvenated the nematode infected black pepper cuttings to varying levels. The plants treated with Ap4 resulted 100% survival of plants. Fifty per cent survival of the plants was achieved with the treatment of bacteria Ap16.

Evaluation of biocontrol formulations.

P. aeruginosa (Ap4), one of the most effective bacterial strains in this investigation is formulated using different carriers and its shelf life is evaluated for a period of 90 days. Different types of formulation like beads, granules and powder formulations using various amendments were tested in this study.

Sodium alginate beads were prepared incorporating the bacterial isolate Ap4. Various additives like rice bran, ginger leaf powder, tea waste, tapioca powder and coir compost were added to the sodium alginate solution and the resulting beads were stored at various conditions to study their effect on the shelf life of these formulations. It is found that the bacterial isolates able to survive in the sodium alginate beaded formulations with maximum survival obtained with the addition of rice bran and dry ginger leaf powder. Initially the population increased in all the formulations. When the sodium alginate beads are dried and stored both at room temperature and at 4⁰ C, the same pattern was observed. Storage of dried alginate beads at room temperature decreased the survival rate compared to refrigerated conditions. Granular formulations of bacterial isolate Ap4 was also prepared and were stored at 4⁰ C and also at room temperature in polypropylene bags (100 g each). The formulations stored under refrigerated condition recorded more cfu/g when compared to that stored at room temperature. In the granular formulations also, the survival of Ap4 was found to be more in the amendments with rice bran and ginger leaf powder. Formulations of the isolate Ap4 were also prepared with talc alone and in combination with two adhesives viz. carboxy methly cellulose and gum Arabic and the viability and shelf life of Ap4 was found to be better in formulations with adhesives

Identity of short listed bacterial isolates

The most effective strains of bacteria in nematode suppression in this study are Is. 113 (*Bacillus megaterium*), Is. 119 (*Comamonas testosterone*), Is. 136 (*Alcaligens*) Ap4, (*Pseudomonas aeruginosa*) and Ap16 (*Pseudomonas* sp). These isolates are proven to be effective in both in the greenhouse and laboratory studies and also posses multifarious

mode of action on other pathogens of black pepper. They differed in their mode of action towards nematodes. The *P. aeruginosa* isolate (Ap4) in the *in vitro* studies resulted in 93.60%, 34.10% and 95.15 % mortality of *Meloidogyne* in peptone broth, phosphate buffer and in culture filtrate respectively. This isolate resulted in the maximum mortality of *R. similis* (80.43 %) among the 46 isolates tested. In the greenhouse studies with tomato as the test plant this isolate was very effective both in nematode suppression and growth promotion. Ap4 suppressed hatching of nematode eggs (82.38 %). It effectively solubilized phosphate and controlled *Phytophthora*. Though, ammonia was produced, Ap4 doesn't produced HCN or H₂S. It was found to be producing fluoresin and pyocyanin pigments. It produced protease enzyme and liquefied gelatin. This isolate was having multiple antibiotic resistance and was found to be suppressive only to nalidixic acid and polymyxin among 14 antibiotics tested. It was also found effective against root knot and burrowing nematode infesting black pepper, effective in growth promotion of black pepper and was capable of even rejuvenating black pepper plants severely infected with root knot nematode. This isolate is equally effective inducing resistance to nematodes and it can easily be formulated. There are different reports on the effective utilization of bacterial isolates belonging to the genus *Pseudomonas*, against *Meloidogyne* and *Radopholus* like *P. fluorescens* and *P. putida* and this is the first report *Pseudomonas aeruginosa* for the suppression of *R. similis*. *Bacillus megaterium*, (Is 113), another important isolate in the present investigation is also having multifarious effects on pathogens of black pepper. In the *in vitro* evaluation, the isolate 113 exhibited more than 80 % suppression of *Meloidogyne* in all the five methods studied. When screened against *R. similis*, it resulted in 61.67 % suppression and was found to be effective in suppressing the hatch of *Meloidogyne* eggs (62.85 %), produced H₂S and ammonia. The advantage of bacillus strains over gram negative bacteria are their ability to withstand adverse conditions and because of its formulation and long term survival characteristics, the effective utilization of this bacillus strain will also be equally important. The other strains like *Comamonas testosteroni* (Is. 119), *Alcaligenes* (Is.136) *Pseudomonas* (Ap16) can also be utilized for the suppression of nematodes and other pathogens infesting black pepper.

Judicious development of these bacterial isolates will lead to effective control of major pathogens of black pepper.

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Chapter 7

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MEDIA COMPOSITION

(Ingredients per liter of media)

Bacillus specific medium (ATCC Medium 552)

Peptone	:	10.0 g
Lactose	:	5.0 g
NaCl	:	5.0 g
Beef extract	:	3.0 g
KH ₂ PO ₄	:	2.0 g
pH	:	7.2±0.2

Carbon minimal media

MgSO ₄ 7H ₂ O	:	1.0 g
K ₂ HPO ₄	:	0.9 g
KCl	:	0.2 g
NH ₄ NO ₃	:	1.0 g
Fe ²⁺	:	0.002 g
Mn ²⁺	:	0.002 g
Zn ²⁺	:	0.002 g

Corn Meal Agar (CMA)

Corn meal extract	:	2 g
Agar	:	15 g
Final pH (at 25 °C)	:	6±0.2

Carrot agar

Carrot	:	200.0 g
Agar	:	18.0 g

Carrot broth

Carrot	:	200.0 g
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Gelatin liquefying medium

Gelatin	:	40.0 g
Peptone	:	5.0 g
Beef extract	:	3.0 g

King's B medium

Protease peptone	:	20.0 g
K ₂ HPO ₄	:	2.5 g
Glycerol	:	15.0 ml
MgSO ₄ · 7H ₂ O	:	6.0 g
Agar	:	15.0 g
Ampicillin	:	40 µg
Chloramphenicol	:	13 µg
Cyclohexamide	:	100 µg
Final pH (at 25 °C)	:	7.2

Muller Hinton agar

Casein acid hydrolysate	:	17.5 g
Beef extract infusion	:	2.0 g
Starch soluble	:	1.5 g
Agar	:	17.5 g
Final pH (at 25 °C)	:	7.0±0.2

Media for the detection of HCN

a. King's B broth supplemented with 4.4g l⁻¹ of glycine

b. Picric acid solution for detection of HCN

picric acid	:	2.5 g
Na ₂ CO ₃	:	12.5 g

Nutrient agar

Peptone	:	5.0g
Beef extract	:	1.0g
Yeast extract	:	2.0g
Sodium chloride	:	5.0g
Agar	:	18.0g
Final pH (at 25 °C)	:	7.0±0.2

Oxidation / Fermentation Media

Peptone	:	1.0 g
Ammonium dihydrogen phosphate	:	1.0 g
Potassium chloride	:	0.2g
Magnesium sulphate	:	0.2g
Agar	:	3g
Bromothymol blue	:	0.08g
Final pH (at 25 °C)	:	7.00 ±0.2 with 40% NaOH

Peptone water

Peptone	:	10 g
Sodium chloride	:	5 g
Final pH (at 25 ⁰ C)	:	7.2±0.2

Pikovskaya's agar

Glucose	:	10.0g
Ca (PO ₄) ₂	:	5.0g
(NH ₄) ₂ SO ₄	:	0.5g
KCl	:	0.1g
MgSO ₄	:	0.1g
MnSO ₄	:	Trace
FeSO ₄	:	Trace
Yeast extract	:	0.5g
Agar	:	15.0g
Final pH (at 25 ⁰ C)	:	7.0±0.2

Potato dextrose agar

Potato	:	200.0g
Dextrose	:	20.0g
Agar	:	18.0g

Potassium phosphate buffer

- a. Solution A (0.2 M KOH solution)
KOH : 11.22 g
- b. Solution B (0.2 M KH₂ PO₄)
KH₂ PO₄ : 27.22 g
- c. Mix 24 ml Solution A and 50 ml Solution B
Final pH 6.8

Pseudomonas agar for fluorescin

Casein enzymatic hydrolysate	:	10.0g
Protease peptone	:	1.0 g
Dipotassium phosphate	:	1.5 g
Magnesium sulphate	:	1.5 g
Agar	:	15 g
Glycerol	:	10 ml
Final pH (at 25 ⁰ C)	:	7.0±0.2

Pseudomonas agar for pyocyanin

Peptic digest of animal tissues	:	20.00 g
Potassium sulphate	:	10.0 g
Magnesium chloride	:	1.4 g
Agar	:	15.0 g
Glycerol	:	10 ml
Final pH (at 25 ⁰ C)	:	7.0±0.2

Skim milk Agar

Skim milk powder	:	100.0 g
Peptone	:	5.0 g
Agar	:	15.0 g

SIM Agar

Ammonium ferric citrate,	:	0.2 g
Casein peptone	:	20.0
Meat peptone	:	6.6
Sodium thiosulfate	:	0.2
Agar	:	3.0
Final pH (at 25 ⁰ C)	:	7.3 ± 0.2

Yeast extract Glucose Calcium Carbonate A gar (YGCA)

Yeast extract	:	10.0 g
Ultra fine CaCO ₃	:	20.0 g
Agar	:	20.0 g
Distilled Water	:	800 ml
Autoclave and add 200 ml 10% sterilized Glucose		

Recipe for PCR Amplification of 16S rDNA

Components	Stock Concentration	Working concentration	Required Concentration	Required volume
Water	1X	1X	1X	15.63µl
Buffer	10X	10X	1X	2.5µl
MgCl ₂	25mM	25mM	3.0mM	3µl
dNTPs	25mM	2.5mM	400µM	0.5µl
BSA	50µg/µl	50µg/µl	2µg/reaction	0.2µl
F.primer	100pM	100pM	5pM	0.5µl
R.primer	100pM	100pM	5pM	0.5µl
Taq polymerase	3u/µl	3u/µl	0.51u	0.17µl
DNA		50ng/µl	100ng	2µl