# PLANT GROWTH-PROMOTION AND ROOT KNOT NEMATODE MANAGEMENT IN TOMATO BY *Piriformospora indica* AND RHIZOBACTERIA

*by*

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### THESIS

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# DECLARATION

I, hereby declare that this thesis entitled **"PLANT GROWTH-PROMOTION AND ROOT KNOT NEMATODE MANAGEMENT IN TOMATO BY** *Piriformospora indica* **AND RHIZOBACTERIA."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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# CERTIFICATE

Certified that this thesis entitled **"PLANT GROWTH-PROMOTION AND ROOT KNOT NEMATODE MANAGEMENT IN TOMATO BY** *Piriformospora indica* **AND RHIZOBACTERIA."** is a record of bonafide research work done independently by Ms. Shilpa Varkey (2014-11-185) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associate ship to her.

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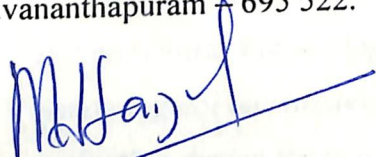
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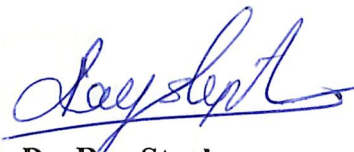
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| | |
|---|---|
| % | Per cent |
| @ | At the rate of |
| °C | Degree Celsius |
| CD | Critical difference |
| CFE | Cell free extract |
| CWA | Coconut water agar |
| cm | Centimeter |
| *et al.* | And other co workers |
| Fig. | Figure |
| g | Gram |
| Hrs | Hours |
| *i.e.* | that is |
| L | Litre |
| μm | Micro meter |
| ml | Milli litre |
| min | Minutes |
| mg | Milli gram |
| nm | Nanometer |
| NS | Non-Significant |
| sec | Seconds |
| sp or spp. | Species (Singular and plural) |
| *viz.* | Namely |
| pH | Negative logarithm of hydrogen ions |
| PDA | Potato dextrose agar |

| rpm | Revolution per minute |
|-----|----------------------|
| OD | Optical density |
| No. | Number |

# Introduction

# 1. INTRODUCTION

Tomato is a major vegetable crop that has achieved tremendous popularity over the last century. It is grown in practically every country of the world - in outdoor fields, greenhouses and net houses. Cultivation of tomato is limited by both biotic and abiotic stress factors. Root-knot nematode, *Meloidogyne incognita*, is one of the major pathogens of tomato throughout the world, affecting both the quantity and quality of marketable yields. It is an economically important polyphagous, highly adapted obligate plant parasite, distributed worldwide and parasitizes nearly every species of higher plant. Chemical nematicides that are applied in large quantities to tackle this pest not only very toxic to the mammals and beneficial soil micro fauna/flora, but also leaves toxic residue on farm produce. Therefore management of root knot nematode adapting alternative approaches including biological control is getting momentum.

Biological control of nematodes has long been considered as an alternative to managing nematodes with pesticides. Plant growth-promoting rhizobacteria (PGPR) like *Psuedomonas fluorescens, Bacillus subtilis, Azospirillum, Azotobactor* etc., are having protection potential in modern agricultural system. PGPR are capable of improving the plant growth in many plants and they also act as biological control agents against various soil borne plant pathogens including root infecting nematodes. Biological amendment with PGPR inoculants in transplanted vegetables has been reported by several workers (Gagne *et al.*, 1993; Nemec *et al.*, 1996., Kokalis-Burelle *et al.*, 2002; Russo, 2006; Russo and PerkinsVeazie, 2010).

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Biological agents used as consortium, or as mixture, is advantageous than when they are used separately (Schisler *et al.*, 1997; Slininger *et al.*, 2010). However, their *in vitro* and *in vivo* interactions are to be studied for efficient use. The fungal endophyte, *P. indica* has been applied together with other beneficial bacterial agents such as PGPR strains as mixed inoculum in mung bean and tomato and improved growth response and disease suppression ability of the consortium was reported (Sarma *et al.*, 2011; Kumar *et al.*, 2012). Improved plant growth promotion by combined inoculation of *P. indica* and phosphate solubilizing bacteria *Pseudomonas striata* in chick pea has been reported by Meena *et al.*, (2010). Anith *et al.* (2011) have reported dual inoculation of *Piriformosproa indica* and the mycoparasitic fungus *Trichoderma harzianum* and its effect on plant growth in tissue cultured black pepper. It is therefore hypothesized that utilizing the plant growth promotional and nematicidal effect if any, of *P.    indica* and PGPR will help enhance the growth and yield of tomato plants and suppression of root knot nematode. The current study was undertaken with the objective of assessing the potential of the root endophytic fungus *Piriformospora indica,* and plant growth-promoting rhizobacteria in improving plant growth and suppressing root knot nematode infection in tomato.

# Review of Literature

# 2. REVIWE OF LITERATURE

## 2.1 TOMATO

*Solanum lycopersicum* L., the commercially cultivated tomato belongs to the family of Solanaceae, which includes important crops such as eggplant, pepper, tobacco and potato. According to FAO (2010) about 125 million tonnes of fresh tomatoes were produced in the world in 2008. China is leading in production with 25 per cent of the global output followed by the United States. The crop is consumed and distributed in almost all the countries and considered as one of the most important horticultural crops worldwide. Tomato is the second most cultivated vegetable in the world, after potato. It is grown in practically every country of the world - in outdoor fields, greenhouses and net houses.

The fruit is consumed in diverse ways, as raw, as an ingredient in many dishes, sauces and in drinks. The beneficial health effects are attributed to lycopene present in the fruit. Lycopene is a very powerful antioxidant which can help prevent the development of many forms of cancer. The fruit is a good source of vitamins such as vitamins A, C, thiamine, riboflavin, niacin as well as minerals such as potassium and sodium (Smith, 2004). The antioxidant is known to prevent prostate cancer and improve the skin's ability to guard against harmful ultra-violet radiation (Rao and Rao, 2007).

## 2.2 ROOT KNOT NEMATODE (*Meloidogyne incognita*)

The rhizosphere is the imperative ecological zone of intense microbial activity where a vast array of organisms, including microbial phytopathogens and nematodes, live together. In the immediate root zone area nematodes are found to be significantly prominent than to root free soil (Bazin *et al.*, 1990). These include obligate parasites, sedentary endoparasites, ectoparasites that cause dramatic yield

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losses in many agricultural crops. Plant parasitic nematodes cause global losses to crop plants with an estimated loss of $ 125 billion per year in the tropics (Chitwood, 2003).

Root knot nematodes are sedentary obligate endoparasites that cause major economic damage to crops around the world (Williamson and Hussey, 1996). *Meloidogyne* spp. cause significant constraints in production of vegetables and other commercially cultivated crops. It has been considered the most damaging of ten important genera of plant parasitic nematodes (Sasser and Freckman, 1987) and cause 5 per cent of worldwide crop loss (Hussey *et al.*, 2002). The devastating and deleterious effect on economically important crop plants gives root knot nematodes the status of most serious pests group among the phytonematodes in India (Sharma and Pankaj, 2002).

*Meloidogyne incognita*, the most important root knot nematode species causes dramatic yield losses in tropical and subtropical agriculture attacking more than 2000 species of cultivated plants, particularly vegetables (Sikora and Fernandez, 2005). Second stage juveniles ($J_2$) penetrate the roots and migrate to the vascular cylinder, induce severe root galling and ravage the utilization efficiency of water and nutrients and greatly affect photosynthetic products (McClure, 1977). Consequently the nematode infection of plants leads to foliage symptoms including stunted growth, wilting, and poor fruit yield.

Root knot nematodes are one of the major parasites of tomatoes worldwide. In India the estimated damage to tomato production due to *Meloidogyne* spp. is around 40 to 46% (Bhatti and Jain, 1977; Reddy, 1985). *Meloidogyne incognita, Meloidogyne javanica, Meloidogyne hapla and Meloidogyne arenaria* are the four major species of root knot nematode infecting crops among which the one seriously damaging tomato plants is reported to be *M. incognita* and has been found to be an important limiting factor in the tomato production (Maqbool *et al.*, 1988). The

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destructive potential of *M. incognita* in tomato is very high indicating a yield loss of up to 7.2% in India (Jain *et al.*, 2007). Tomato plants infected with *Meloidogyne* shows nitrogen deficiency symptoms or chlorosis, stunted growth and profused root galling. This disease is a major constraint to the successful cultivation of tomato crop (Reddy, 1985).

## 2.3. MANAGEMENT OF ROOT KNOT NEMATODE

Several control strategies, such as host plant resistance, rotation with non-hosts, destruction of residual crop roots and use of nematicides have been reported to effectively control root-knot nematodes (Whitehead, 1998). An imperative nematode management strategy is the use of chemicals but with some serious constraints. Fumigation of the soil with approved chemical nematicides achieves the best control of root-knot nematodes. However, the residual effect and slow degradation rate of chemical nematicides may lead to environmental and human health concerns (Atkins *et al.*, 2003). The deleterious effects of chemical nematicides are unavoidable since they are highly toxic to the mammals and beneficial soil micro fauna/flora. Pollution of groundwater and residual effect on farm produce could not also be excluded. Integrated nematode management approaches by the use of plant extracts and antagonistic microorganisms are now getting fast momentum.

## 2.3 BIOLOGICAL MANAGEMAENT OF ROOT KNOT NEMATODE

Microbial pathogens, endophytes and antagonists are extremely important in the regulation of plant parasitic nematode populations, irrespective of agroecosystems. Biological control activity is omnipresent in soil and can range from negligible to complete nematode suppression, with the degree of biological control determined by the diversity and density of communities and / or individual antagonistic microorganisms present in a specific soil.

The potential of microbial pathogens, endophytes and antagonists for biological control of *Meloidogyne* spp. is great in a suppressive soil –a soil that totally suppresses nematode multiplication. Nematode suppressive soils have been detected, and their activity has been shown to be driven by a diverse spectrum of microbes: fungal pathogens of eggs, rhizobacteria, generalized fungal antagonists, mutualistic fungal endophytes and obligate nematode parasitic bacteria (Whipps and Davies, 2000). Methodologies have been developed to measure and monitor specific microbial agents comprising the antagonistic potential of a soil suppressive to root-knot nematodes (Hirsh *et al.*, 2001).

## 2.4. BIOLOGICAL CONTROL OF ROOT KNOT NEMATODES WITH BACTERIAL AGENTS.

Endoparasitic bacteria are known to affect nematodes, the most studied of which are from the genus *Wolbachia* (Taylor, 2003). The *Pasturia* group of bacteria is hyperparasites of plant parasitic nematodes and water fleas. All the economically important genera of plant parasitic nematodes are parasitized by *Pasturia* spp. Todate, five species of *Pasturia* that differ in their host ranges and pathogenicity have been described, among which *Pasturia penetrance* is parasitic on *Meloidogyne* spp. (Sayre and Starr, 1985). Parasitized females of root knot nematode produce only a few eggs (Davies *et al.*, 2008) and this bacterium has been associated with root knot nematode suppressive soil (Turdgill *et al.*, 2000).

Rhizosphere bacteria form a complex assemblage of species and among the dominant bacterial genera, *Bacillus* and *Pseudomonas* are able to antagonize plant parasitic nematodes (Sikora, 1992). Other rhizosphere bacteria expressing antagonistic potential against *Meloidogyne* include members of the genera *Agrobacterium, Alcaligens, Aureobacterium, Chryseobacterium, Corynebacterium,*

*Enterobacter, Klebsiella, Paenibacillus, Phyllobacterium, Rhizobium* and *Xanthomonas.* (Klopper *et al.*, 1992; Duponnois *et al.*, 1999; Krechel *et al.*, 2002; Oliveira *et al.*, 2007). Following intensive *in vitro* and *ad planta* screening procedures, several endophytic bacteria have been identified as antagonists of *Meloidogyne* spp.(Siddique and Mahmood, 1999).

## 2.5. PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

Plant growth-promoting rhizobacteria (PGPR), are rhizosphere bacteria that significantly facilitate plant health, by improved plant growth and crop yields under greenhouse and field conditions. Many of them also act as biocontrol agents against various plant diseases, (Kloepper *et al.*, 1980; Kloepper *et al.*, 2004; Hass and Defago, 2005; Lugtenberg and Kamilova, 2009; Kavino *et al.*, 2010; Lee *et al.*, 2014)). The amendment of soil with beneficial rhizobacterial strains may help promote plant growth in various ways, such as phosphate utilization (Rodriguez and Fraga, 1999), enhancing indole-3-acetic acid (IAA) production (Gupta *et al.*, 2000), and making atmospheric nitrogen available to the plants (Bashan and Holguin, 1997). Most of the PGPR strains are able to protect the plants through direct mechanisms by the production of antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes, bacterial allelochemicals, including iron-chelating siderophores (Raupach *et al.*, 1996).

Many marketable biofertilizers are mainly based on plant growth promoting rhizobacteria (PGPR) that exert beneficial effects on plant development often related to the increment of nutrient availability to host plant (Vessey, 2003). PGPR seem to promote plant growth through suppression of plant pathogens (Zehnder *et al.*, 2001; Ji *et al.*, 2006; Veerubommu and Kanoujia, 2011), production of antimicrobial substances, competition for space, nutrients and ecological niches, or through production of phytohormones and peptides acting as bio stimulants without negative

effects on the user, consumer or the environment (Glick *et al.*, 1998; Johnsson *et al.*, 1998; Jimenez-Delgadillo, 2004).

Alternatively PGPR act against phytopathogens through induced systemic resistance (ISR), production of antimicrobials and bacteriocins (Kloepper *et al.*, 2004). Research efforts all over the world have led to the successful development of biotic and abiotic agents that can induce systemic resistance in host plants against infectious agents and promote plant growth (Kloepper *et al.*, *2004*; Catinot *et al.*, *2008*).

PGPR can also protect plants against nematodes (Oliveira *et al.*, 2007; El-Hadad *et al.*, 2010). Approximately, 7–10% of all rhizobacteria display antagonistic potential against nematodes through antibiosis and induced resistance (Burkett-Cadena, 2008). Several *Bacillus* and *Pseudomonas* species are effective in managing Root-Knot Nematode diseases in pot experiment (Siddiqui and Akhtar, 2009; Singh and Siddiqui, 2010). Consortium of biocontrol agents with different plant colonization patterns and mode of action may be useful for suppression of different plant pathogens (Akhtar and Siddiqui, 2007). Moreover, mixtures of taxonomically different biocontrol agents that require different optimum temperature, pH, and moisture conditions may colonize roots more aggressively, improve plant growth and efficacy of disease suppression (Siddiqui, 2006).

Upregulated activity of defence related enzymes such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and total phenol content was observed in the consortium of *P. fluorescens* (pf 123) and *B. subtilis* (Bs 214) black pepper cv. Panniyur 1against root knot nematode, *Meloidogyne incognita* (Devapriyanga et al., 2011). Bioagents such as *Trichoderma harzianum* and *P. fluorescens* was found to have significant effect on control of *M. incognita-Rhizoctonia solani* complex on okra (Bhagwati *et al.*, 2009). Several rhizophere

bacteria, such as *Bacillus sphaericus* B43, *Rhizobium elti* G12 and *P. fluorescens* pfl, have been shown to induce systemic resistance towards several plant parasitic nematodes, including species of *Meloidogyne* (Siddiqui and Shaukat, 2004; Sikora and Fernandez, 2005).

### 2.5.1 *Bacillus* spp.

The genus *Bacillus* includes organisms which are often considered as microbial factories for the production of a wide variety of biologically active molecules against phytopathogens, and the beneficial interactions stimulates the host defense mechanisms in the colonized plants (Ongena and Jacques, 2007). The beneficial effect as a growth promoter by the genus is attributed to its good colonization ability and production of growth hormones (Weller, 1988). *Bacillus* species have been reported to possess growth promotional effect on a wide range of plants (De Freitas *et al.*, 1997; Kokalis-Burelle *et al.*, 2002) and are very effective in the biological control of many plant diseases. *Bacillus* spp. are helps the plant to evade diseases through pathogen inhibition by diffusible or volatile compounds, induction of resistance in plants and aggressive root colonization (Kloepper *et al.*, 1980; Weller, 1988; Siddiqui and Mahmood, 1999; Siddiqui, 2000). They produce a wide variety of antibiotics, growth promoting hormones and can solubilize phosphorous (Rodriguez and Fraga, 1999). *Bacillus* spp. is capable of producing endospores that allow them to survive in adverse environmental conditions for extended periods. Moreover, a few members are diazotrophs *B. subtilis* was isolated from the root zone of a wide range of plant species at concentrations as high as $10^7$ per gram of rhizosphere soil (Wipat and Harwood, 1999). *Bacillus megaterium* KL39, a biocontrol agent of red-pepper Phytophthora blight, produces an antifungal antibiotic active against a broad range of plant pathogenic fungi (Jung and Kim, 2003). The species such as *B. cereus*, *B. lentimorbus* and *B. licheniformis* were found to be the best isolates to inhibit *Fusarium roseum var. sambucinum* (Sadfi *et*

*al.*, 2001). The enhancement of plant growth and suppression of plant pathogenic organisms in the rhizosphere was reported to be contributed by a strain of *B. amyloliquefaciens*, FZB42 (Koumoutsi *et al.*, 2004).

The cell free extracts of *Pseudomonas* spp.RKP-33 and *Bacillus* spp. RKB-91 containing the toxic metabolites has significantly delayed egg hatching and nematode mobility to the extent of 64-77 % of the treated juveniles within 24 houres (Pankaj *et al.*, 2011). In the package of practice KAU, nursery treatment with *Bacillus macerans/Paecilomyces lilacinus* at the rate of 25 g/ m$^2$ and drenching the same at the rate of 3% solution seven days after sowing is recommended for managing root knot nematode. BioNem-WP and BioSafe are two biological nematicides based on lyophilized *Bacillus firmus* supplemented with non-toxic additives intended mainly for controlling *Meloidogyne* spp. and BioYield$^{TM}$ is a biological inoculant containing *Paenibacillus macerans* and *B. amyloliquefaciens* to be incorporated into glass house planting mixes (Hallmann *et al.*, 2009). Commercial products containing *B. thuringiensis* such as Dipel and Turex, have been shown to reduce damage caused by root-knot nematodes (Radwan, 2007a).

## 2.5.2 Fluorescent Pseudomonads

The genus *Pseudomonas* belongs to the γ subclass of the proteobacteria and includes mostly fluorescent Pseudomonads as well as few non-fluorescent species. Among the wide array of rhizosphere bacteria, *Pseudomonas* spp. are referred to as plant growth-promoting rhizobacteria (PGPR) because of their intimate association with improved plant growth and health (Kloepper, 1993).

The effects of *Pseudomonas* in plant growth promotion have been observed in many crops (Lemanceau, 1992). The beneficial effects of these bacteria are positively correlated with its ability to promote plant growth and to protect the plant

against pathogenic microorganisms. Indole acetic acid (IAA) production by *Pseudomonas* and its role in the plant development is also evidenced (Patten and Glick., 2002).

*Pseudomonas fluorescens* can act as strong elicitors of plant defense reaction (M'Piga *et al.*, 1997). These bacteria are able to produce a wide range of antifungal metabolites, have catabolic versatility, and excellent root-colonizing capacity. Fluorescent *Pseudomonas* spp. are among the most effective rhizosphere bacteria in ameliorating diseases caused by soil-borne pathogens (Siddiqui and Shaukat 2004 ). *Pseudomonas fluorescens* which live in close proximity of plant roots, help in boosting the plant development and defence through various mechanisms is deleterious to the plant pathogens. The bacteria achieve this mainly by bio-stimulation, biocontrol, bio-fertilization and bio-remediation.

The biocontrol potential of fluorescent pseudomonads against root-knot nematode on tomato has been reported (Santhi and Subramanian, 1995). *P. aeruginosa* and *Paecilomyces lilacinus* used as single inoculation or combination significantly reduced infection of *M. javanica* and root infecting fungi on chillli. *P. aeruginosa* being more effective than *P. lilacinus* in reducing nematode infection (Perveen *et al.*, 1998). Combined use of *T. harzianum* and *P. aeruginosa* caused greatest reduction in gall formation (Siddiqui, 2000). Application of *P. fluorescens* at 10 g Kg$^{-1}$ seed was effective in reducing the menace of root knot nematode, *M. incognita* in tomato (Varma *et al.*, 1998). The effectiveness of pf (1) strain of *P. fluorescens* against *M. incognita* has been reported in tomato (Mani *et al.*, 1998) and brinjal (Sheela *et al.*, 1999). *Pseudomonas fluorescens* strain BICC602 suppresses root-knot nematode (*Meloidogyne incognita*) by eliciting defense mechanism leading to induced systemic resistance in cowpea (*Vigna unguiculata*) and tomato (*Solanum lycopersicum*) (Mukherjee and Sinha babu, 2012). Tomato plants treated with *P. lilacinus* had a high reduction (55%) in galling and nematode multiplication, while *P. putida* caused a 39% reduction in galling and nematode multiplication (Siddiqui and Akhtar, 2008).

Many strains of *Pseudomonas* can indirectly protect the plants by inducing systemic resistance against various pests and diseases (Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001; Zehnder *et al.*, 2001). *P. fluorescens* induced systemic resistance against root-knot nematode through accumulation of defense enzymes PO, PPO and PAL (Kavitha *et al.*, 2013). Secondary metabolites produced by *Pseudomonas* spp. such as 2, 4-diacetylphloroglucinol (DAPG), phenazines and hydrogen cyanide, inhibit soil-borne pathogens (Haas and Defago, 2005). DAPG negatively influence mitochondrial activity (Gleeson *et al.*, 2010) and also helps in the control of plant-parasitic nematodes (Cronin *et al.*, 1997; Siddiqui and Shaukat, 2003). *Meloidogyne arenaria* development in gound nut root treated with *P. fluorescens* was inhibited by reduced and poor development of giant cells (Kalairasan *et al.*, 2008). The culture filtrates of *Pseudomonas* spp. at 100 % concentration has resulted in 83.3 % *M. incognita* juvenile mortality after 48 hours of exposure (Rajkumar *et al.*, 2012). *P. fluorescens* is capable of inducing systemic resistance against *Meloidogyne incognita* in tomato by the accumulation of the defence enzymes like PO, PPO and PAL (Sankarimeena *et al.*, 2012).

## 2.6. FUNGAL BIOCONTROL AGENTS

Fungal bioagents such as *Paecilomyces lilacinus*, *Pochonia chlamydosporia* and *Trichoderma harzianum* were reported to improve plant growth as well as reduce the *M. incognita* reproduction in tomato (Joshi *et al.*, 2012). Application of VAM (*Glomus fasciculatum*) along with balanced fertilizer had significant effect in improving biometric characters and inhibition of *M. incognita* development in tomato (Sarangi *et al.*, 2014). For cotton, Saleh and Sikora (1984) reported that 38% mycorrhization by *Glomus fasciculatum* was required for control of *M. incognita*. Dual inoculation of AMF (*Funneliformis mosseae*) and *Pasturia penetrans* showed highest efficacy in reducing the final nematode densities in tomato. Higher reduction in galling and nematode multiplication was observed when *Pseudomonas putida* was

used with *Glomus intraradices* together with composted manure (Siddique and Akhtar, 2008).

Among the nematophagous fungi *Paecilomyces lilacinus* and *Pochonia chlamydosporia*, can parasitize both the egg and female stages of the nematode (Morgan–Jones *et al.*, 1982; Rodriguez–Kabana *et al.*, 1984).

Predacious fungi often referred to as nematode trapping fungi, are specialized forms of nematophagous, soil-borne fungi that form mycelium able to capture nematodes. Different fungal species produce different trapping structures. The most simple structures are fungal hyphae covered with adhesive secretions produced by *Stylopage* spp., followed by adhesive branches in the case of *Monacrosporium cionopagum* (Stirling, 1991).

Among the saprophagous fungi *Trichoderma* is a ubiquitous soil fungus that also colonizes the root surface and root cortex. *Trichoderma harzianum*, provide excellent control of root knot nematodes (Sharon *et al.*, 2001). Other species within the genus *Trichoderma* with antagonistic activity towards *Meloidogyne* include *T. viride*, *T. atroviridae* and *T. azperellum* (Sharon *et al.*, 2007).

The potential of endophytic fungi to reduce infestation by *Meloidogyne* spp. was first demonstrated for arbuscular mycorrhizal (AM) fungi in vegetable transplants (Sikora and Schonbeck, 1975). AM fungi compete with plant parasitic nematodes for nutrient sources and space (Diedhiou *et al.*, 2003).

Entamopathogenic nematodes have been found to reduce populations of root knot nematodes. A significant decrease in the number of galls and egg masses by *M. incognita* on tomato following soil application with *Steinernema feltiae* was reported (Lewis *et al.*, 2001).

## 2.6.1. *Piriformospora indica*

*Piriformospora indica*, a newly described root endophytic fungus has been reported to have beneficial effect on plant growth and productivity (Varma *et al.*, 1999; Farkya *et al.*, 2010). The fungus was isolated from the rhizosphere soils of the woody shrubs *Prosopis juliflora* and *Zizyphus nummularia* from the sandy desert soils of Rajasthan, India (Varma *et al.*, 2001). *P. indica* is widely distributed as a symptomless root endophyte, and it colonizes members of bryophytes, pteridophytes, gymnosperms and angiosperms. Molecular phylogenetic analyses have revealed that *P. indica* is a member of the basidiomycetous order Sebacinales (Basidiomycota: Agaricomycetes) (Qiang *et al.*, 2012; Weiss *et al.*, 2004).

The young mycelia of *P. indica* are white and almost hyaline, but inconspicuous zonations are observed in older cultures. The mycelia are mostly flat and submerged into the substratum. Chlamydospores are formed from thin-walled vesicles at the tips of the hyphae. The chlamydospores appear singly or in clusters and are distinctive because of their pear-shaped structure. *P. indica* grows best on modified Hill–Kaefer synthetic medium (Hill and Kaefer, 2001; Pham *et al.*, 2004). The fungus is easily cultivable, lacks host specificity and colonizes roots of many different plants, mostly in an endophytic fashion (Varma *et al.*, 2001).

*P. indica* is a wide-host root-colonizing endophytic fungus which allows the plants to grow under extreme physical and nutrient stresses. The fungus grows inter and intracellularly, forms pearshaped, auto fluorescent chlamydospores within the cortex of the colonized roots and in the rhizosphere zone, but it does not invade the endodermis and the aerial parts of the plants. The fungus promotes nutrient uptake, allows plants to survive under water, temperature and salt stresses, and confers systemic resistance to toxins, heavy metal ions, insects and pathogenic organisms (Das *et al.*, 2012). Further, it was shown to stimulate excessive production of

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biomass, early flowering and seed production. It is a potential microorganism imparting biological hardening of tissue culture-raised plants (Yadav *et al.*, 2010). Besides this direct beneficial interaction, autoclaved cell-wall extracts (CWE) as well as the application of culture filtrates (CF) containing fungal exudates were shown to promote plant growth (Vadassery *et al.*, 2009). However, there is only a limited knowledge about the chemical compositions of the CWE and CF.

*P. indica* has also been reported to increase plant stress tolerance towards abiotic stresses like drought, acidity, and heavy metals (Kumari *et al.*, 2004) and against biotic stresses such as plant pathogens (Waller *et al.*, 2008; Zuccaro *et al.*, 2009). In barley, *P. indica* triggers resistance against *Fusarium* head blight (*Fusarium graminearum*) (Deshmukh and Kogel, 2007) as well as against the leaf pathogen *Blumeria graminis* f. sp. *hordei* (Waller *et al.*, 2005). Thus, it is suggested that *P. indica* may induce systemic resistance in plants. The fungus interacts with fungal and viral pathogens and also improves the growth of tomato plants (Fukhro *et al.*, 2009)

*P. indica* colonization, as well as the application of fungal exudates and cell-wall extracts, significantly affects the vitality, infectivity, development, and reproduction of the cyst nematode *Heterodera schachtii* in *Arabidopsis thaliana* (Daneshkhah *et al.*, 2013).

In general, biological control of plant disease against a single pathogen is aided by a single biocontrol agent (Wilson and Backman, 1999). This may sometimes account for inconsistent performance because a single agent is not active in all soil environments or against all pathogens that attack the host plant. On the other hand, compatible combinations of biocontrol agents with different plant colonization patterns may be useful for management of different plant pathogens via different mechanisms of disease suppression (Akhtar and Siddiqui, 2008). Consortium of biocontrol agents with different growth requirements such

as optimum temperature, pH, and moisture conditions may colonize roots more aggressively, and act synergistically to improve the biocontrol potential (Siddiqui, 2006). Biocontrol agents with different mode of action can be dual inoculated in order to provide greater efficiency against plant pathogens on different crops than inoculation with a single agent (Guetsky *et al.*, 2002).

# Materials and Methods

# 3. MATERIALS AND METHODS

The study entitled "plant growth promotion and root-knot nematode management in tomato using *Piriformospora indica* and rhizobacteria" was undertaken in the Department of Agricultural Microbiology, College of Agriculture Vellayani during 2014-2016. Details of the materials used and methods employed for the study are furnished below:

## 3.1 MAINTENANCE OF BACTERIAL AND FUNGAL CULTURES.

The three species of *Bacillus viz., Bacillus pumilus, Bacillus subtilis and Bacillus amyloliquefaciens* were routinely cultivated on Nutrient broth (NB) or Nutrient Agar (NA) and stored on NA slants at refrigerated conditions. The Pseudomonad strains *Pseudomonas fluorescens* AMB 8 and *Pseudomonas fluorescens* PN026 used in the present study were cultured on King's medium B (KB) broth or Agar and maintained on KB agar slants. *Piriformospora indica*, the root endophytic fungus was grown on Potato Dextrose Broth (PDB) or PDA and preserved on PDA slants. All the microbial cultures were obtained from the Department of Agricultural Microbiology, College of Agriculture Vellayani.

## 3.2 MAINTANANCE OF NEMATODE CULTURE

Root-knot nematode, *Meloidogyne incognita* was cultured and maintained on tomato plants grown in pots filled with garden soil. Seedlings of the tomato variety Vellayani Vijay were obtained from the Department of Olericulture, College of Agriculture Vellayani. Tomato plants were artificially inoculated with initial culture of *M. incognita* at the rate of two nematodes per Kg of soil. Inoculated plants were maintained throughout the experiment for the collection of second stage juveniles ($J_2$) and egg masses, whenever necessary. Second stage juveniles were collected by hatching of the eggs collected from the galls.

## 3.3 COLLECTION OF EGG MASSES AND J₂ FROM CULTURE PLANTS

Infected tomato plants were carefully uprooted and roots were washed thoroughly in running tap water. Honeydew like brown egg masses adhered to the galls were hand-picked using forceps and collected in sterile distilled water. They were then surface sterilized in 0.5 per cent sodium hypochlorite for two minutes, followed by three washings with sterile water (Singh and Siddique, 2010). After surface sterilization, the egg masses were collected in sterile distilled water amended with rifampicin (100 $\mu$g/ml) (Pankaj *et al.*, 2011).

For collection of $J_2$ infective stages the eggs were allowed to hatch in sterile distilled water for two days using Modified Bearmann Funnel Technique (Southey, 1986). In brief, bottoms of Petri plates (10 cm dia) were poured with sterile water and above that a concave wire mesh covered with double layared tissue paper was placed carefully without breaking the tissue paper. The egg masses were spread evenly on the tissue paper. The edges of the tissue paper extending outside the wire mesh were folded in order to avoid the trickling of water drops from the edges which may carry nematodes. Petri plates were then completely filled with water, and the water level was maintained to 5 mm above the wire mesh. This was incubated at room temperature. After 24 to 48 hours the wire mesh along with filter paper was removed and the extracted nematodes in the Petri plates were collected and counted under a sterio zoom microscope.

## 3.4. *IN VITRO* ANTAGONISTIC ASSAY

### 3.4.1.1. Collection of Cell Free Extract (CFE) of *Bacillus* spp.

Three isolates of *Bacillus* such as *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus amyloliquifaciens* were used for the experiment. A loopful of bacterial cells from a single well isolated colony was inoculated into 100 ml of Nutrient broth in 250 ml conical flask and incubated at $28\pm 2^0$C for 24 hours at 100 rpm in

an incubator shaker. Bacterial cells were separated from Nutrient broth by centrifugation at 8000 rpm for 5 minutes and the supernatant was aseptically collected. CFE of each isolate was collected separately in sterile glass vials by filter sterilization using bacteriological filters of 0.2 μm size under aseptic conditions. The CFE were further diluted to S/2 (half strength) and S/4 (quarter strength) concentrations with appropriate amount of sterile water.

### 3.4.1.2 Collection of Cell Free Extract (CFE) of *Pseudomonas* spp.

*Pseudomonas fluorescens* AMB8 and *Pseudomonas fluorescens* PN026 were inoculated into 100 ml of King's B broth in 250 ml conical flask and incubated at $28 \pm 2^0C$ for 24 hours as described above. Collection of CFE and preparation of diluted CFE were done as mentioned in section 3.4.1.1.

### 3.4.1.3 Collection of Cell Free Extract (CFE) of *Piriformospora indica*

The fungus was grown on PDA plates for 7 days and a 5 mm diameter mycelial disc was cut out using a sterile cork borer and used for inoculating 100 ml of Potato Dextrose Broth (PDB) (pH 7) in 250 ml conical flask and incubated at $28 \pm 2^0C$ for 10 days with shaking at 100 rpm in an incubator shaker. The fungal mycelium was separated by centrifugation at 8000 rpm for 10 minutes. CFE was collected aseptically and filter sterilized using bacteriological filters of 0.2 μm size. The CFE was further diluted to S/2 and S/4 concentrations with appropriate amount of sterile distilled water.

### 3.4.2 *In vitro* screening of CFE of Rhizobacteria for *Meloidogyne incognita* egg hatching and $J_2$ mortality.

### 3.4.2.1. Effect of CFE of *Bacillus* on egg hatching of *M. incognita*

The cell free extracts of the *Bacillus* isolates were screened separately for egg hatching of root-knot nematode *M. incognita* under *in vitro* conditions. The

CFE of different concentration were transferred to sterile plastic Petri dishes of diameter 55 mm. Three surface sterilized egg masses of almost similar size were picked with sterilized forceps and placed in CFE concentrations of S, S/2, S/4 of each isolate separately, incubated at $28^0C$ and observed for egg hatching. For control, three egg masses were placed in 5 ml sterile distilled water. Each set was replicated six times and the experiment designed as CRD. Observations were taken at hourly intervals for the first four hours and then at 24, 48 and 72 hours of incubation.

### 3.4.2.2. Effect of CFE of *Pseudomonas* on egg hatching of *M. incognita*

The experiment was done as described in section 3.4.2.1 using the CFE of different concentration of the two fluorescent pseudomonads, *Pseudomonas fluorescens* PN026 and *Pseudomonas fluorescens* AMB8. There were six replications for the experiment. Observations were taken at hourly intervals for the first four hours and then at 24, 48 and 72 hours of incubation.

### 3.4.2.3. Effect of CFE of *P. indica* on egg hatching of *M. incognita*

The cell free extract of *P. indica* was screened for egg hatching of root-knot nematode *M. incognita* under *in vitro* conditions. From the CFE of different concentration, 5 ml each were transferred to sterile plastic Petri dishes of diameter 55 mm. Three surface sterilized egg masses of almost similar size were picked with sterilized forceps and placed in suspensions of S, S/2, S/4 and incubated at $28^0C$ and observed for egg hatching. For control, three egg masses were placed in 5 ml sterile distilled water. Each set was replicated six times and the experiment was performed as CRD. Observations were taken at hourly intervals for the first four hours and then at 24, 48 and 72 hours of incubation.

### 3.4.2.4 Effect of CFE of *Bacillus* on the mortality of *M. incognita* $J_2$

Diluted and undiluted CFE of the isolates were separately transferred to sterile plastic Petri dishes of diameter 55 mm. Hundred juveniles of *Meloidogyne incognita* were transferred to CFE of S, S/2, S/4 concentration from each isolate separately and incubated at $28^0C$. For control, 100 juveniles were placed in 5 ml sterile distilled water. Each set was replicated six times as CRD. Observations were taken at hourly intervals for the first four hours and at 24 hour of incubation.

### 3.4.2.5 Effect of CFE of *Pseudomonas* on the mortality of *M. incognita* $J_2$

The experiment was conducted as mentioned as in section 3.4.2.4. with CFE of different concentrations the two *P. fluorescens* strains.

### 3.4.2.6. Effect of CFE of *P. indica* on the mortality of *M. incognita* $J_2$

CFE of different strengths from *P. indica* were separately transferred to sterile plastic Petri dishes of diameter 55 mm. Hundred juveniles of *Meloidogyne incognita* were transferred to suspensions of S, S/2, S/4 of separately and incubated at $28^0C$. For control, 100 juveniles were placed in 5 ml sterile distilled water. Each set was replicated six times and designed as CRD. Observations were taken at hourly intervals for the first four hours and at 24 hour of incubation.

### 3.4.3 *In vitro* screening of rhizobacteria for egg hatching of *Meloidogyne incognita* and $J_2$ mortality.

*Bacillus* strains were cross streaked heavily on NA plates and incubated overnight at $28 \pm 2^0C$. Plates were then drenched with 10 ml each of sterile distilled water and scrapped with a sterile glass spreader, and the cell suspension was aseptically collected in glass vials. Similarly cell suspension of the *Pseudomonas* strains were obtained by growing the bacterial strain on KB agar medium. The OD

of the cell suspension was adjusted to 1.0 at 660 nm with sterile distillled water to make them uniform suspension of the cells. Suspensions were also diluted to S/2 (half strength) and S/4 (quarter strength) with sterile water.

### 3.4.3.1. Effect of rhizobacteria on egg hatching of *M. incognita*

The cell suspensions of the *Bacillus* isolates and *Pseudomonas* isolates were screened separately for the hatching of root-knot nematode *M. incognita* under *in vitro* conditions. Sterile petri plates of 55 mm diameter were separately filled with 5 ml of each dilution of *Bacillus* spp. and *Pseudomonas* spp. separately. Three surface sterilized healthy egg masses of nearly uniform size of *M. incognita* were transferred to each treatment. The egg masses placed in sterilized distilled water served as control and they were incubated at $28^0$ C. Percentage hatching was monitered for a period of up to 4 hour at hourly intervals and at 24, 48 and 72 hour intervals under a stereo binocular microscope. Each set was replicated six times and the experiment was designed as CRD.

### 3.4.3.2. Effect of rhizobacteria on the mortality of *M. incognita* $J_2$

The rhizobacterial isolates of *Pseudomonas* and *Bacillus* were screened for mortality of root-knot nematode *M. incognita* under *in vitro* conditions. Rhizobacterial cell suspensions of different dilutions were transferred to sterile Petri dishes of diameter 55 mm. Hundred juveniles were transferred to suspensions of S, S/2, S/4 of each isolate separately and incubated at $28^0$C. For control, 100 juveniles were placed in 5 ml sterile distilled water. Each set was replicated six times under CRD. Observations were taken at hourly intervals for the first four hours and after 24 hour of incubation.

### 3.4.4. Egg parasitism by *P. indica*

*P. indica* was grown on PDA plate for 10 days. Surface sterilized egg masses were placed on four sides of the mycelial growth and incubated for five

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days at $28^0$ C with three replications. Three control plates without fungus was also inoculated and incubated as above. After five days, egg masses were picked from the plates and stained using lactophenol cotton blue and observed under a light microscope.

## 3.5 COMPATIBILITY OF *Piriformospora indica* WITH RHIZOBACTERIA

Dual culture plate assay was done on PDA and coconut water agar (CWA) for assessing the *in vitro* interaction and compatibility of *P. indica* with all the five rhizobacterial strains. CWA was prepared by straining coconut water (pH adjusted to 7) and adding agar at the rate of two grams/100 ml. This was autoclaved at $121^0C$ (15 lbs) for 20 minutes. PDA and CWA plates were prepared and 8 mm diameter mycelial disc from *P. indica* previously grown on PDA plates for 7 days was placed at the centre of the plates and incubated at $28^0C$ for three days. Rhizobacteria to be tested were separately streaked as a band of 2 cm on the two sides of the plates inoculated with *P. indica* and the plates were incubated for 3 more days. Mycelial growth inhibition by the bacterial isolates was noted and those isolates showing no inhibition of growth were selected for further studies.

## 3.6. *IN VIVO* POT CULTURE EXPERIMENT

Pot culture experiment was conducted to check the plant growth promotion and root knot nematode infestation in tomato using *P. indica*–rhizobacterial combinations. Among the rhizobacteria, *B. pumilus* and *Pseudomonas fluorescens* AMB8 were found to be compatible with *P. indica* in the *in vitro* screening assay and therefore selected for *in vivo* test. The experiment comprised of 12 treatments consisting of single inoculation of rhizobacterial strains, *P. indica*, and combinations of *P. indica* and individual rhizobacteria with and without nematode inoculation. The experiment was conducted as CRD and replicated four times with three plants in each replication.

Treatment details of the experiments are given below

T1     - *B. pumilus* with nematode

T2     - *B. pumilus* without nematode

T3     - *P. fluorescens* AMB8 with nematode

T4     - *P. fluorescens* AMB8 without nematode

T5     - *Piriformospora indica* with nematode

T6     - *Piriformospora indica* without nematode

T7     - *B. pumilus* + *P. indica* with nematode

T8     - *B. pumilus* + *P. indica* without nematode

T9     - *Pseudomonas fluorescens* AMB8 + *P. indica* with nematode

T10    - *Pseudomonas fluorescens* AMB8 + *P. indica* without nematode

T11    - Nematode inoculated control

T12    - Absolute control

Seeds of tomato varietyVellayani Vijay were obtained from the Department of Olericulture, College of Agriculture, Vellayani. Planting medium for filling the protray cavities was prepared by mixing vermiculite and perlite in the ratio of 3:1 (v/v). After moistening sterilization of the mixture was done by performing autoclaving ($121^0C$) for one hour each for three consecutive days. Bacterial inoculants were prepared by inoculating single colony of both the bacteria on respective broths and 24 hour grown culture was used for seed bacterization after seeding into protray cavities. Bacterial cell suspension having cell concentration of $1*10^8$ cfu/ml @ 500 µl/protray cavity.

Fungal inoculation was done into the transplanting medium by incorporating *P. indica* mycelium at the rate of 1 % w/v. *P. indica* mycelial disc of 5 mm size was inoculated to 100 ml potato dextrose broth and incubated at $28^0C$ for 10 days. The mycelial growth was filtered out using a strainer. Mycelium was added to the sterile

planting medium at the rate of 1% (w/v), mixed properly and filled in protray cavities.

Seeds were surface sterilized with1 % sodium hypochlorite for five minutes followed by three washing with sterile water and blot dried with sterile blotting paper. Single seeds were planted in the protray cells and whenever bacterial inoculation was needed protray cells were drenched with 500 µl of the respective cultures. Plants were maintained in a glass house and watered twice daily with sterile water. At 14 days after sowing fertilization was done with 1 % NPK solution (19:19:19) at the rate of five ml per cavity.

### 3.6.2. Transplanting and inoculation with *Meloidogyne incognita* juveniles.

Seedlings were transplanted to plastic pots (30 cm diameter) filled with 1 Kg sterile planting medium of sand: soil: cowdung mix in the ratio of 2:1:1 on $21^{st}$day. Sterilization of the planting medium was done as mentioned in section 3.6.1. Second stage juveniles were added at the rate of two nematodes per gram of soil after 14 days of transplanting.

### 3.6.3 Biometric observations.

### 3.6.3.1. Observations of above ground parts

One month after transplanting, the plants were uprooted and biometric observations were taken. This included per plant shoot and root fresh weight (g), height of the plant (cm), number of leaves, number of flowers and number of fruits. Dry root and shoot weight of the plant samples were recorded after drying them in a drier at $50^{0}$ C for three days.

### 3.6.3.2. Observations of below ground parts

Observations on number of galls/ plant, number of egg mass/plant, number of eggs/egg mass, number of nematodes/g of root and initial and final nematode

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population in soil were made. Number of eggs/ egg mass was counted by randomly selecting three egg masses from each root system with three replications.

### 3.6.4. Root colonization by *P. indica*

Roots were collected from 30 day old plants treated with *P. indica* alone and the combination treatments with rhizobacteria. Both nematodes treated and untreated plants were subjected to root colonization assay. Roots were carefully separated from the plant, washed thoroughly with tap water to get rid of the planting medium. Roots were cut into small pieces of approximately of one cm length. Root bits were transferred to a beaker with five ml of 10% KOH and boiled for five minutes. KOH solution was drained out and three washing with tap water was given. Then the root bits were soaked in 2 % HCl for five minutes. They were then removed from the acid and transferred to lactophenol trypan blue (0.05%) for 10 minutes for staining. The stained root bits were then allowed to destain in lactophenol solution. After destaining they were placed on a glass slide and covered with cover slip with gentle pressing. The samples were viewed under a compound microscope and checked for presence of chlamydospores in each root bit. The percentage root colonization was found out by the following formula,

Percentage root colonization =<u>No. of root bits with chlamydospores</u> x100
Total no. o root bits observed

### 3.6.5. Staining of nematodes in root

Lactophenol method (Byrd *et al.*, 1983) was used to stain the nematodes in plant tissue. Roots of the test plant were thoroughly washed to remove the adhering soil particles and cut into one cm bits. Lactophenol was prepared by mixing liquid phenol (500 ml), lactic acid (500 ml), glycerin (100 ml) and distilled water (500 ml). Stock solution of acid- fuchsin was prepared by dissolving 3.5g acid fuschin in 250 ml of acetic acid and 750 ml of distilled water. Working solution of the stain was prepared by addition of one ml of the stock solution of the stain into 100 ml of

lactophenol solution. The stain was boiled in a beaker on a hot plate. The infected roots of each treatment were immersed in the boiling stain for one minute, rinsed with tap water, and then destained in lactophenol solution until the maximum contrast between the nematodes and the root tissue was obtained.

### 3.6.6. Nematode population in soil

Initial and final nematode population in soil was checked by Cobb's Sieving and Decanting method (Cobb, 1918). 100 CC of soil sample was collected from each treatment. The soil sample was passed through a course sieve to remove rocks, roots etc. The soil was placed in a plastic basin half filled with water and thoroughly mixed. It was allowed to stand until water almost stopped swirling. The soil suspension was passed through a 20 mesh sieve and supernatant collected in another plastic basin. The residue present in the first basin and sieve were discarded. The same procedure was followed for 100 and 200 mesh sieve. The supernatant from the 200 mesh sieve after settling was carefully transferred to 350 mesh sieve. The materials retained on the last mesh (350) were backwashed to a 250 ml beaker which contains $J_2$ of *Meloidogyne incognita*. Nematodes were extracted from the washate by Modified Bearmann Funnel Technique as mentioned in section 3.3.

### 3.7. MICROSCOPIC OBSERVATION OF GALL DEVELOPMENT

Separate set of plants that received treatments mentioned in section 3.6 were kept for analyzing histopathologic changes upon nematode infection and development. Developmental stages were analyzed in treated plants at five day intervals of nematode inoculation. Root samples were prepared and microscopic observations were carried out by Lactophenol method as mentioned in section 3.6.5.

## 3.8 STATISTICAL ANALYSIS

The data generated from the experiments were statistically analyzed using analysis of varience techniques (ANOVA) as applied to Completely Randomized Design (Panse and Sukhatme, 1985).

# Results

# 4. RESULTS

The experimental data collected from the investigation on "Plant growth-promotion and root knot nematode management in tomato by *Piriformospora indica* and rhizobacteria" were analyzed and the results presented in this chapter under the following headings.

## 4.1 *IN VITRO* STUDIES

### 4.1.1. Screening of cell free extracts (CFE) of rhizobacteria for nematicidal activity

#### 4.1.1.1. Influence of CFE of rhizobacteria on egg hatching of *Meloidogyne incognita*

The effect of CFE of five rhizobacterial strains on egg hatching of *M. incognita* are given in table 1 (Plate 1). Up to the first 4 hours of incubation egg hatching did not occur in any of the treatment. After 24 hours the full strength concentration of CFE of *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens* prevented egg hatching completely. The results represented in Table 1 indicate that highest hatching among treatments were observed with CFE of *P. fluorescens* PN 026 (0.66%) followed by that of *P. fluorescens* AMB8 (0.33%). Upon dilution of the CFE the percentage hatching was found to be increased for egg masses treated with *B. subtilis*. However the trend was not common for other rhizobacteria where there was decreased egg hatching when the diluted samples were used.

After 48 hours of incubation the highest hatching inhibition (8.33 per cent egg hatch) was observed for undiluted CFE *B. amyloliquefaciens* while in control 65.00 % hatching was observed. Half strength concentration of *P. fluorescens* PN 026 had the highest hatching inhibition (11.00 per cent egg hatch) followed by B. *amyloliquefaciens* (11.33 per cent egg hatch). In quarter strength concentration highest

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hatching inhibition was shown by *B. subtilis* (22.33 per cent egg hatch) indicating its less effect on preventing egg hatching.

After 72 hours of incubation, the highest hatching inhibition was observed for *P. fluorescens* AMB8 (22.33 per cent egg hatch) followed by *B. amyloliquefaciens* (22.66%) and lowest hatching inhibition was observed for *B. subtilis* (27 per cent egg hatch). In S/2 concentration CFE of *B. amyloliquefaciens* showed significantly superior hatching inhibition with 28 % hatching followed by *P. fluorescens* PN 026 (28.66%), *P. fluorescens* AMB8 (30.00%). Here also *B. subtilis* had the lowest hatching inhibition of 32.66 per cent egg hatch, among treatments while in control after 72 hours of incubation 99 % hatching was observed.

#### 4.1.1.2. Screening of CFE of rhizobacteria for mortality of *Meloidogyne incognita* juvenile

Observations were taken at one hour intervals up to 4 hours and after 24 hours of incubation. There was no mortality for any of the treatment up to 2 hours (Plate 2). After 3 hours of incubation highest mortality was shown by *B. amyloliquefaciens* (5.60%) followed by *B. pumilus* with 1.5 per cent. In half strength and quarter strength concentration also mortality percentage was more for *B. amyloliquefaciens*. Juvenile mortality was completely absent in CFE *P. fluorescens* PN 026, *P. fluorescens* AMB8, *B. subtilis*, and control. (Table. 2)

After 4 hour of incubation *B. amyloliquefaciens* exhibited the highest mortality in S concentration (6.66%) which was on par with *P. fluorescens* AMB8 (4.89%) and *B. subtilis* (4.66%). In S/2 concentration also highest mortality percentage was observed for *B. amyloliquefaciens*. In S/4 concentration *B. amyloliquefaciens* and *B. pumilus* showed same mortality. Upon diluting the CFE, mortality rate was found to be reduced. There was no mortality of $J_2$ for *P. fluorescens* PN 026 in all the dilutions as with the control.

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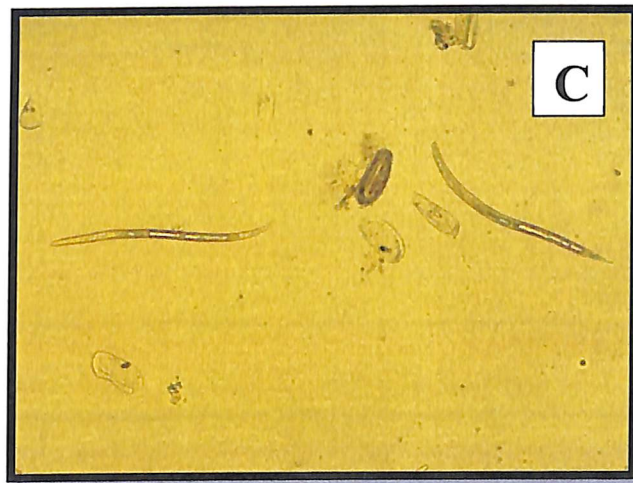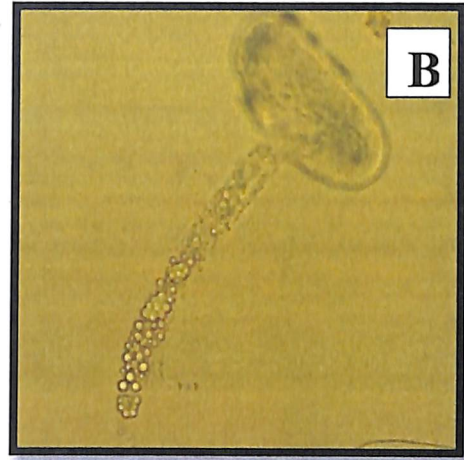
**Plate 1.** Eggs of *Meloidogyne incognita*.

A) Egg before hatch (100 X)
B) Hatching egg (100 X) C) Hatched juveniles (25 X)
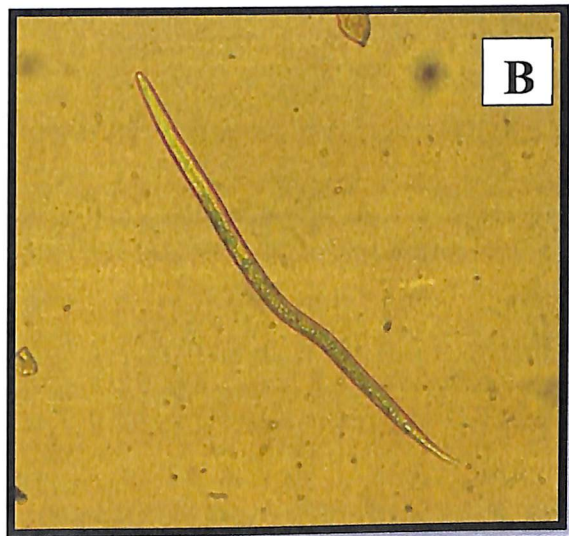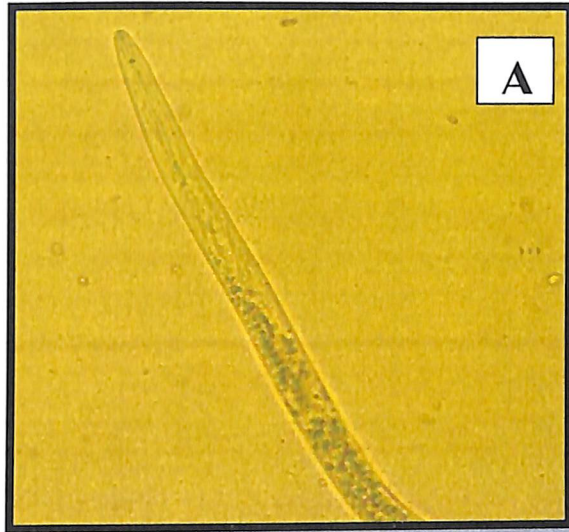
**Plate. 2**. *Meloidogyne incognita* J$_2$ after treatment with cell free extract.

(A-100 X, B- 25 X)

**Table 1.** *Meloidogyne incognita* egg hatching influenced by cell free extract (CFE) of rhizobacteria and *Piriformospora indica*.

| Treatment | Egg hatching (%)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | After 24 hrs. | | | After 48 hrs. | | | After 72 hrs. | | |
| | S | S/2 | S/4 | S | S/2 | S/4 | S | S/2 | S/4 |
| *P. fluorescens* PN 026 | 0.66 | 0.33 | 0.33 | 8.66 | 11.00 | 19.33 | 25.00 | 28.66 | 41.66 |
| *P. flourescens* AMB 8 | 0.33 | 0.00 | 0.00 | 11.33 | 12.00 | 21.00 | 22.33 | 30.00 | 48.33 |
| *B. pumilus* | 0.00 | 0.33 | 0.00 | 11.66 | 12.66 | 22.00 | 25.33 | 31.00 | 48.33 |
| *B. subtilis* | 0.00 | 0.33 | 1.33 | 12.66 | 13.00 | 22.33 | 27.00 | 32.66 | 52.33 |
| *B. amyloliquefaciens* | 0.00 | 0.00 | 0.00 | 8.33 | 11.33 | 20.00 | 22.66 | 28.00 | 45.66 |
| *P. indica* | 0.00 | 1.33 | 2.33 | 10.33 | 11.00 | 18.66 | 22.78 | 28.33 | 44.66 |
| Control | 34.33 | 34.33 | 34.33 | 65.00 | 65.00 | 65.00 | 99.00 | 99.00 | 99.00 |
| CD | 4.086 | 4.329 | 5.05 | 3.80 | 4.61 | 4.08 | 2.02 | 2.09 | 4.81 |

* Mean of six replications, S: Full strength CFE, S/2: Half strength CFE, S/4: Quarter strength CFE

**Table 2.** *Meloidogyne incognita* J$_2$ mortality influenced by cell free extract (CFE) of rhizobacteria and *Piriformospora indica*.

| Treatment | Mortality (%)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | After 3 hrs. | | | After 4 hrs. | | | After 24 hrs. | | |
| | S | S/2 | S/4 | S | S/2 | S/4 | S | S/2 | S/4 |
| *P. fluorescens* PN 026 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 68.21 | 59.38 | 45.87 |
| *P. flourescens* AMB 8 | 0.00 | 0.00 | 0.00 | 4.89 | 2.08 | 1.20 | 92.15 | 69.73 | 58.01 |
| *B. pumilus* | 1.50 | 0.76 | 0.00 | 3.74 | 1.23 | 2.00 | 90.62 | 60.00 | 52.80 |
| *B. subtilis* | 0.00 | 0.00 | 0.00 | 4.66 | 2.57 | 1.33 | 74.61 | 58.71 | 47.80 |
| *B. amyloliquefaciens* | 5.60 | 3.24 | 1.30 | 6.66 | 2.58 | 2.00 | 99.71 | 72.76 | 63.25 |
| *P. indica* | 11.00 | 9.33 | 6.60 | 18.00 | 15.00 | 9.89 | 98.00 | 71.16 | 60.21 |
| Control | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.33 | 1.33 | 1.33 |
| CD | 1.32 | 1.32 | 1.98 | 2.47 | 2.19 | 2.22 | 4.25 | 3.99 | 2.22 |

* Mean of six replications, S: Full strength CFE, S/2: Half strength CFE, S/4: Quarter strength CFE

After 24 hours of incubation of *M. incognita* juveniles in CFE of different bacteria the highest mortality was observed for *B. amyloliquefaciens* with 99.71 % in S, 72.76% in S/2, 63.25% in S/4 respectively. All the treatments with S concentration showed a mortality percentage of above 60%. The lowest mortality was observed for *P. fluorescens* PN 026 in all the three dilutions. After 24 hour of incubation mortality percentage in control plates was only 1.33%.

### 4.1.2. Screening of rhizobacteria for nematicidal activity

### 4.1.2.1. Screening of rhizobacteria for *Meloidogyne incognita* egg hatching

Data on the direct inhibition of the rhizobacterial suspensions *of B. pumilus*, *B. amyloliquefaciens*, *B. subtilis*, *P. fluorescens* PN 026, *P. fluorescens* AMB8 on egg hatching of *M. incognita* are presented in Table 3. During the first four hours of incubation there was no hatching in any of the treatments. After 24 hours of incubation a higher hatching inhibition was observed with *P. fluorescens* PN 026 which was followed by *B. amyloliquefaciens*. Reducing the concentration of bacterial cell population had positive effect on egg hatching in the case of all rhizobacterial isolates. All other treatments also showed low levels of hatching after 24 hours while in control 34.33 percent hatching was observed. In all the dilutions, the effect was found to be on par among the treatments except that in control.

After 48 hours of incubation the highest hatching inhibition was observed for *P. fluorescens* AMB8 with 22.33 % hatching in full strength concentration followed by 32.33 % and 43.33%, in S/2 and S/4 respectively. The minimum hatching inhibition was for *B. subtilis* with a 56.66 % hatching in S/4 concentration, which is near to the hatching per cent in control (65 %). Similar effect was also excerted by *B. pumilus* and *B. amyloliquefaciens*.

After 72 hour of incubation the hatching inhibition was more for *P. fluorescens* AMB8 (30 per cent egg hatch) in S concentration followed by undiluted cell

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suspension of *B. amyloliquefaciens* (30.66%). In half strength and quarter strength concentration also the lowest hatching was observed with *B. amyloliquefaciens* followed by *P. fluorescens* AMB8.

### 4.1.2.2. Screening of rhizobacteria on mortality of *Meloidogyne incognita* juvenile

There was no mortality of $J_2$ infective stages up to 4 hours of incubation in any of the treatment. After 24 hours of incubation highest mortality was observed for *B. amyloliquefaciens* (8.56 %) in full strength concentration and which was on par with *P. fluorescens* AMB8 (8.22 %), *B. subtilis* (8.30%), *B. pumilus* (8.16 %) (Table 4). Upon dilution of the cell suspension mortality was found to be reduced. In quarter strength dilution mortality by *P. fluorescens* PN 026 was significantly superior and *B. subtilis* and *B. amyloliquefaciens* was found to be reduced. In quarter strength dilution mortality by *P. flourescens* PN 026 was significantly superior.

### 4.1.3. Screening *P. indica* for nematicidal activity

### 4.1.3.1 Screening CFE of *P. indica* for nematicidal activity

CFE of *P. indica* was screened for egg hatching and mortality of *M. incognita* juveniles. After 24 hours of incubation very low level of egg hatching was observed in all the concentrations (Table 1). Incubation for 48 hours showed that hatching was increased to 10.33%, 11.0%, 18.66% in S, S/2, S/4. Dilution of the sample had a positive effect on egg hatching.

In the case of mortality of *M. incognita* influenced by the CFE of *P. indica*, the results indicated a positive impact. There was no mortality observed for the first and second hour of incubation. After third hour of incubation the highest mortality percentage of 11 was observed in the undiluted CFE (Table 2). Dilution of the samples had a negative effect on mortality. After 4 hour of incubation mortality was further found to be increased to 18.00%, 15.00% and 9.89% in S, S/2 S/4 concentrations

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**Table 3.** *Meloidogyne incognita* egg hatching influenced by rhizobacterial cell suspension.

| Treatment | Egg hatching (%)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | After 24 hrs. | | | After 48 hrs. | | | After 72 hrs. | | |
| | S | S/2 | S/4 | S | S/2 | S/4 | S | S/2 | S/4 |
| *P. fluorescens* PN 026 | 2.00 | 5.33 | 11.66 | 31.00 | 42.33 | 54.66 | 42.66 | 52.66 | 61.66 |
| *P. flourescens* AMB 8 | 4.33 | 7.66 | 12.66 | 22.33 | 32.33 | 43.33 | 30.00 | 47.33 | 57.66 |
| *B.pumilus* | 4.33 | 8.00 | 13.33 | 23.00 | 36.66 | 51.33 | 33.66 | 52.33 | 62.00 |
| *B.subtilis* | 5.66 | 10.00 | 14.00 | 36.33 | 42.66 | 56.66 | 50.00 | 61.66 | 64.33 |
| *B.amyloliquefacies* | 2.66 | 6.33 | 12.33 | 26.00 | 36.00 | 46.33 | 30.66 | 45.00 | 54.00 |
| Control | 34.33 | 34.33 | 34.33 | 65.00 | 65.00 | 65.00 | 99.00 | 99.00 | 99.00 |
| CD | 5.11 | 5.25 | 5.17 | 4.74 | 4.66 | 7.46 | 7.72 | 7.09 | 7.04 |

* Mean of six replications, S: Full strength cell suspension, S/2: Half strength cell suspension, S/4: Quarter strength cell suspension

**Table 4.** *Meloidogyne incognita* J$_2$ mortality influenced rhizobacterial cell suspension

| Treatment | Mortality of J$_2$ (%)* | | |
| --- | --- | --- | --- |
| | After 24 hrs. | | |
| | S | S/2 | S/4 |
| *P. fluorescens* PN 026 | 6.21 | 5.80 | 4.00 |
| *P. flourescens* AMB 8 | 8.22 | 7.35 | 2.00 |
| *B. pumilus* | 8.16 | 5.20 | 2.10 |
| *B. subtilis* | 8.30 | 6.94 | 3.22 |
| *B. amyloliquefaciens* | 8.56 | 3.70 | 2.97 |
| Control | 1.33 | 1.33 | 1.33 |
| CD | 1.50 | 1.51 | 1.10 |

* Mean of six replications, S: Full strength cell suspension, S/2: Half strength cell suspension, S/4: Quarter strength cell suspension

respectively. After 72 hour of incubation, CFE of *P. indica* recorded the highest mortality of 98.0% in full strength concentration.

### 4.1.3.2. Egg parasitization by the *P. indica*

Staining of the eggs treated with *P. indica* mycelium revealed that the mycelium of the fungus was not able to penetrate into the eggs even though the chlamydopores are adhered to the egg surface. However a few eggs were found to take up the stain. (Plate 3).

### 4.1.4. Compatibility of *P. indica* with rhizobacteria

The results of the dual culture plate assay done on PDA and CWA showed that *P. indica* growth was not inhibited by *B. pumilus* on both the media (Table 5; Plate 5) and *P. fluorescens* AMB8 on CWA. *P. fluorescens* PN026 produced a zone of inhibition of above 0.55 mm in both the media (Plate 7). Zone of inhibition of above 0.75 mm was observed with *P. fluorescens* AMB8 when tested on PDA while there was no inhibition on CWA (Plate 6). *B. subtilis* in both the media (Plate 8) and *B. amyloliquefaciens* on CWA (Plate 9) showed inhibition of *P. indica* mycelial growth though there was no clear production of inhibition zone that could be measured.

## 4.2. IN VIVO POT CULTURE EXPERIMENT

Results obtained from pot culture experiments for assessing the plant growth promotion and root knot nematode management in tomato using *P. indica* and compatible rhizobacterial combinations are furnished below in detail.

### 4.2.1. Root colonization by *P. indica*

Roots of both *M. incognita* inoculated and uninoculated plants were stained for assessing the colonization of *P. indica* (Plate 10). The percentage root colonization in nematode un-inoculated plants treated with *P. indica* alone was found to be 52.82%

**Plate 3.** Chlamydospores of *P.indica* attached to the egg of *Meloidogyne incognita* (A-25 X, B- 100 X)

**Plate 4.** Control plate.
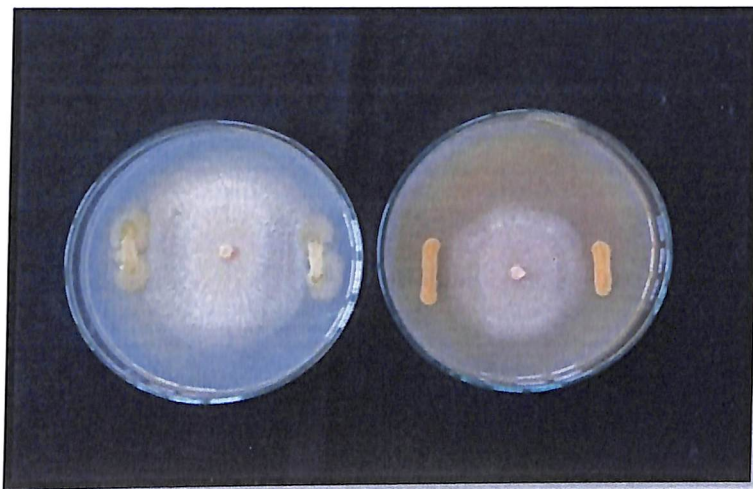


**Plate 5.** *In vitro* dual culture plate assay of *B. pumilus* against *P. indica* on potato dextrose agar and coconut water agar plates.
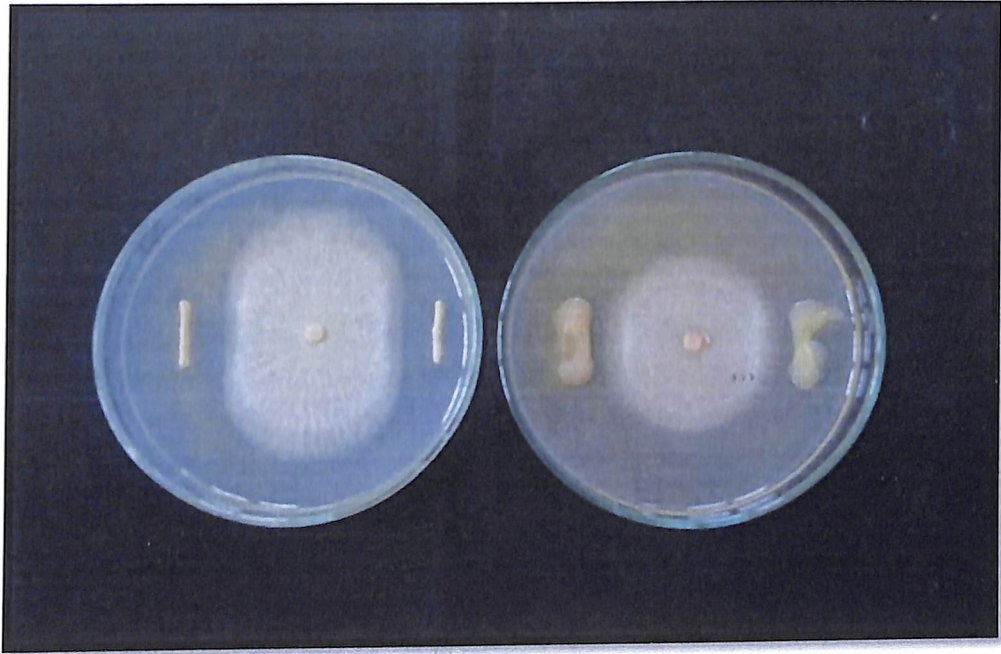
**Plate 6.** *In vitro* dual culture plate assay of *P. fluorescens* AMB 8 against *P. indica* on potato dextrose agar and coconut water agar plates.



**Plate 7.** *In vitro* dual culture plate assay of *P. fluorescens* PN 026 against *P. indica* on potato dextrose agar and coconut water agar plates.

**Plate 8**. *In vitro* dual culture plate assay of *B. subtilis* against *P.indica* on potato dextrose agar and coconut water agar plates.



**Plate 9.** *In vitro* dual culture plate assay of *B. amyloliquefaciens* against *P. indica* on potato dextrose agar and coconut water agar plates.
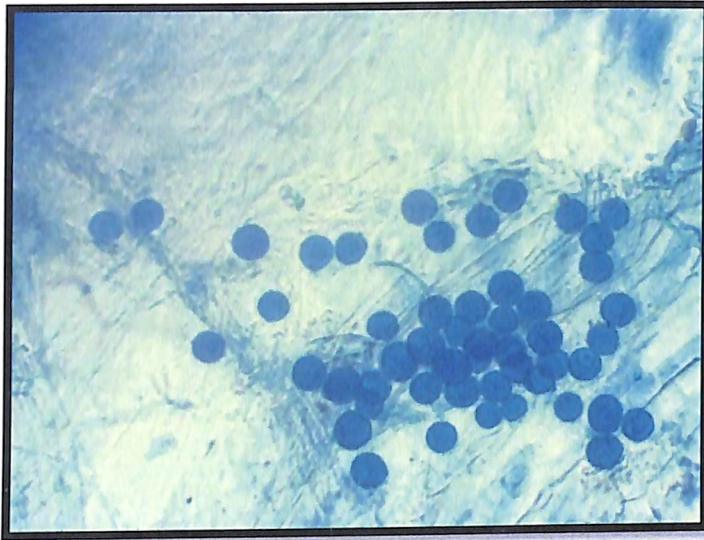
**Plate 10.** Light micrograph of chlamydospores of *P. indica* on tomato root surface.
(400 X)



**Plate 11.** Light micrograph of *P. indica* colonization in nematode uninoculated
tomato plant roots. (400 X)

**Table 5.** Compatibility of *P. indica* with rhizobacteria.

| Treatment | Test medium | |
|---|---|---|
| | PDA | CWA |
| *P. fluorescens* PN 026 | ++ | ++ |
| *P. fluorescens* AMB 8 | +++ | - ... |
| *B. subtilis* | ± | ± |
| *B. pumilus* | - | - |
| *B. amyloliquefaciens* | ++ | ± |

++    Inhibition zone above 0.5 mm

+++   Inhibition zone above 0.75 mm

-      No inhibition

±    Inhibition present no measurable zone of inhibition

(Plate 11). Combination treatments of *P. indica* and *B. pumilus* had colonization of 54.70% with the fungus (Plate 12). *P. indica* and *P. fluorescens* AMB8 combination displays a lesser colonization percentage of 52.23 (Plate 13).

Nematode inoculated plants were also tested for root colonization by *P. indica*. The results indicated that nematode penetration into the plants improves the root colonization by *P. indica*. In *P. indica* and *P. fluorescens* AMB8 treated combination the percentage root colonization by the fungus was 61.5% (Plate 15). *P. indica* alone also gave a good colonization of 60 % (Plate 14). However, the combination of *P. indica* and *B. pumilus* reduced the colonization of *P. indica* to 34% (Plate 16; Table 6).

## 4.2.2. Biometric characters of nematode inoculated plants.

Application of *P. indica*, combination of *P. indica* with *P. fluorescens* AMB8, and *P. indica* with *B. pumilus* had significant effect on the biometric characters such as shoot length, leaf number, flower number, fruit number, fresh and dry shoot weight, fresh and dry root weight, and yield of the treated tomato plants in the presence of root knot nematode (Table7).

There was significant effect on the shoot length of tomato treated with single inoculation and combinations of the selected organisms. The maximum mean height of 44.31 cm was observed for plants treated with *P. indica*. The combination of *P. indica* and rhizobacteria were found to be on par with the above value.

Plants treated with *P. indica* showed a maximum fresh shoot weight of 23.92 g/plant though the values for all other treatments other than control were on par statistically.

Treatments had significant effect on dry shoot weight of tomato seedlings. The maximum dry shoot weight of 8.82 g/plant was observed for plants treated with the combination, *P. indica* and *P. fluorescens* AMB8. Dry shoot weight by *P. indica* (8.08

**Plate 12.** Light micrograph of *P. indica* colonization in nematode uninoculated tomato plant roots treated with *P. indica* and *B. pumilus.* (400 X)



**Plate 13.** Light micrograph of *P. indica* colonization in nematode uninoculated tomato plant roots treated with *P. indica* and *P. fluorescence* AMB 8. (400 X)
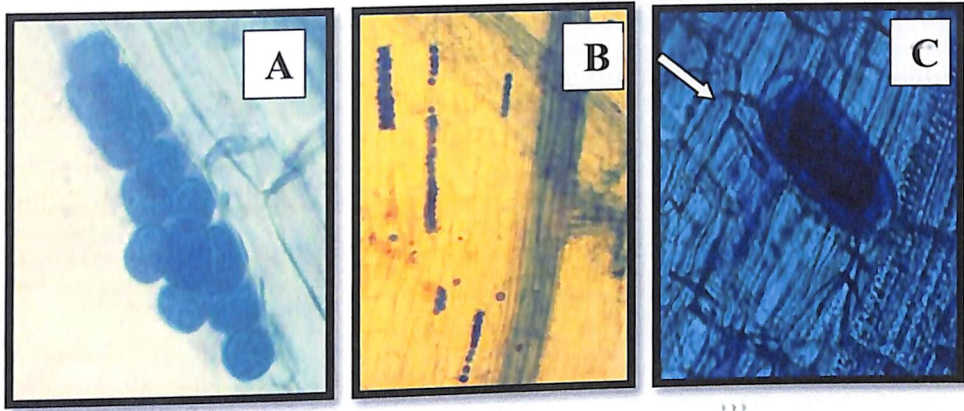
**Plate 14.** Light micrograph of *P. indica* colonization in nematode inoculated tomato plant roots (arrow head showing egg and chlamydospores). (A- 400 X, B and C-100X)
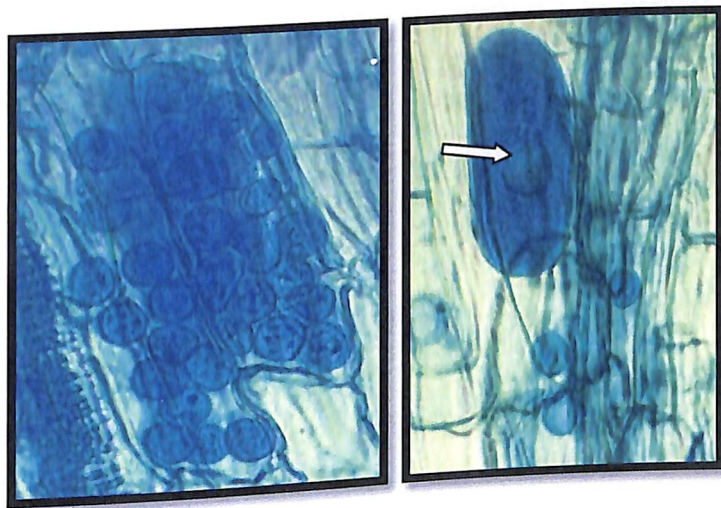


**Plate 15.** Light micrograph of *P. indica* colonization in nematode inoculated tomato plant roots treated with *P. indica* and *P. fluorescence* AMB 8. (Arrow head indicates chlamydospore adhered to egg). (400 X)
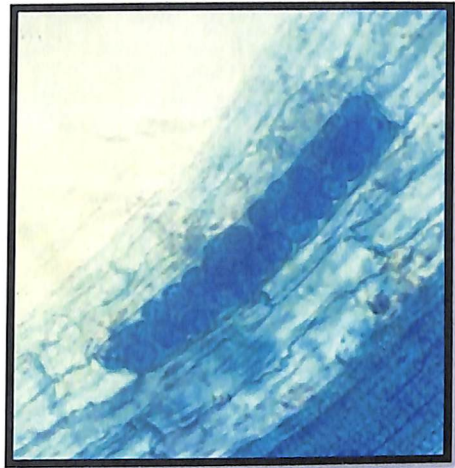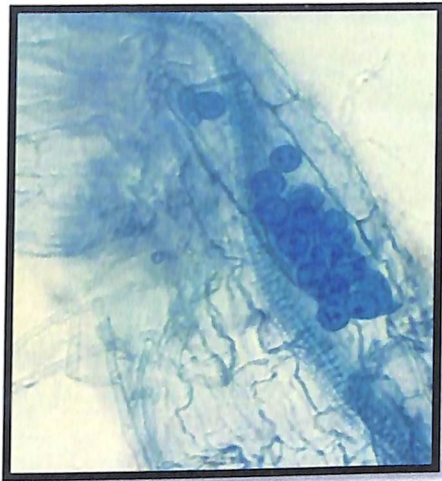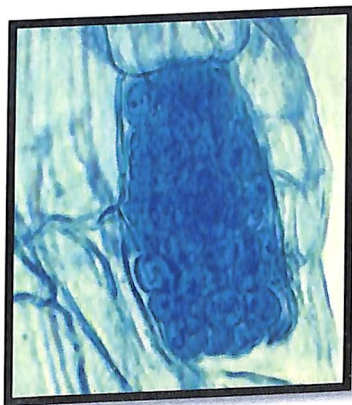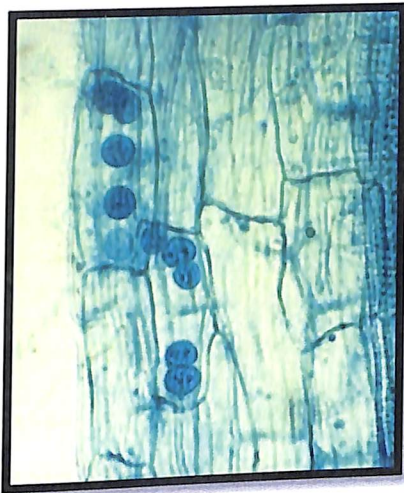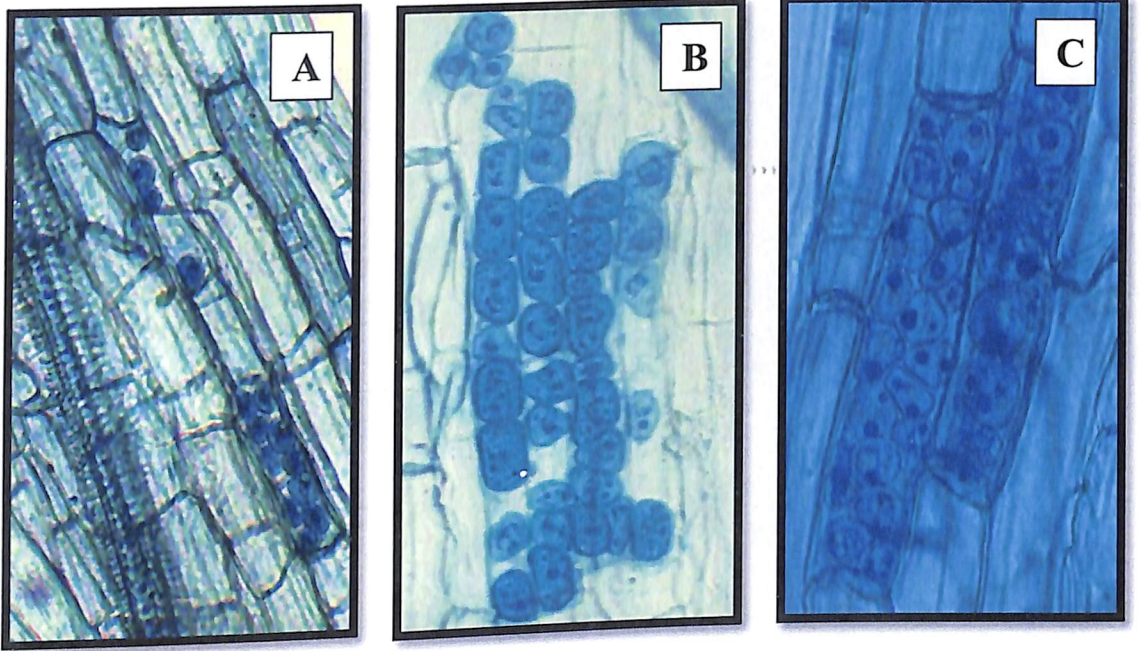
**Plate 16.** Light micrograph of *P. indica* colonization in nematode inoculated tomato plant roots treated with *P. indica* and *B. pumilus*.
(A-100X, B and C-400X)

g/plant) and combination of *P. indica* with *B. pumilus* (8.23 g/plant) treatments were found to be on par.

The maximum fresh root weight was observed for *P. indica* and *P. fluorescens* AMB8 treated plants (7.61 g/plant) which is on par with *P. indica* alone (6.33 g/plant) and combination of *P. indica* and *B. pumilus* (6.77 g/plant).

Dry root weight was found to be not significantly different among the treatments.

Number of leaves showed significant difference among the treatments and maximum mean number of leaves was observed in plants treated with *P. indica* and *P. fluorescens* AMB8 combination (25.91). This was followed by *P. indica* (24.08) and combination of *P. indica* and *B. pumilus* (23.08).

Highest flower number of 19.08 was observed for *P. indica* treated plants which was on par with that of *P. fluorescens* AMB8 (17.74), combination of *P. indica* and *B. pumilus* (17.08), *P. indica* and *P. fluorescens* AMB8 (17.33).

Treatments had significant effect on the number of fruits and the maximum mean number of fruits was observed in plants treated with *P. indica*.

Analysis of data implied that the treatments significantly influenced the yield of tomato. Highest yield of 94.11 g per plant was obtained in *P. indica* treated plants and which was significantly higher than the plants inoculated with nematodes with 59.29 g per plant.

### 4.2.3. Biometric characters of nematode un-inoculated plants

Biometric observation of nematode un-inoculated plants revealed that all the characters were showing higher values than that of nematode inoculated plants (Table 8).

In the case of shoot length, plants treated with *P. indica* (48.02 cm) were found to be superior to all other individual and combined treatments.

The combination treatment *P. indica* and *P. fluorescens* AMB8 was found to be having maximum fresh shoot weight of 25.48 g/plant. This was on par with *P. indica* (23.99 g /plant) and *P. indica* and *B. pumilus* (24.79 g/plant).

*P. indica* and *P. fluorescens* AMB8 had the maximum dry shoot weight of 9.09 g/plant.

Analysis of the data indicated that fresh root weight was also maximum for *P. indica* and *P. fluorescens* AMB8 treated plants (7.62 g/plant). This was on par with *P. indica* alone (6.81 g/plant) and *P. indica* and *B. pumilus* (6.68 g/plant).

Dry root weight was found to be non significant in all the treatments.

Maximum number of leaves was observed in plants treated with *P. indica* and *P. fluorescens* AMB8 combination (26.66). This was followed by *P. indica* alone (25.33), and *P. indica* and *B. pumilus* (25.08).

Highest number of flower (20.83) was noticed for plants treated with *P. indica*. This was on par with *P. indica* and *B. pumilus* (20.66), *P. fluorescens* AMB8 (18.49) combination of *P. indica* and *P. fluorescens* AMB8 (18.33).

Treatments showed significant effect on number of fruits and maximum mean number of fruits was observed in plants treated with *P. indica* (9.75).

The data presented in Table 8 revealed that the treatment had significant effect on yield of the plant. Highest yield was recorded in plants treated with *P. indica* with 118.05 g/plant where as in control plants it was only 72.63g/plant.

**Table 6.** Root colonization by *Piriformospora indica* in tomato.

| Presence of *Meloidogyne incognita* | Root colonization (%)* | | |
|---|---|---|---|
| | *P. indica* | *P. indica* and *P. fluorescens* AMB 8 | *P. indica* and *B. pumilus* |
| Un inoculated | 52.82 | 52.23 | 54.70 |
| Inoculated | 60 | 61.5 | 34 |

*Mean of three replications having five plants each

**Table 7.** Biometric characters of *Meloidogyne incognita* inoculated tomato plants treated with rhizobacteria and *Piriformospora indica* *

| Treatments | Shoot length /plant(cm) | Fresh shoot weight /plant(g) | Dry shoot weight /plant(g) | Fresh root weight /plant(g) | Dry root weight /plant(g) | Number of leaves /plant | Number of Flowers /plant | Number of Fruits /plant | Yield /plant(g) |
|---|---|---|---|---|---|---|---|---|---|
| B. pumilus | 36.88 | 18.03 | 7.16 | 4.98 | 0.48 | 20.58 | 12.83 | 7.91 | 69.70 |
| P. fluorescens AMB 8 | 35.64 | 18.95 | 7.18 | 5.03 | 0.54 | 16.99 | 17.74 | 8.41 | 80.13 |
| P. indica | 44.31 | 23.92 | 8.08 | 6.33 | 0.71 | 24.08 | 19.08 | 9.41 | 94.11 |
| P. indica and B. pumilus | 42.17 | 21.17 | 8.23 | 6.77 | 0.69 | 23.08 | 17.08 | 9.00 | 83.82 |
| P. indica and P. fluorescens AMB 8 | 42.73 | 23.64 | 8.82 | 7.61 | 0.84 | 25.91 | 17.33 | 9.16 | 89.74 |
| Control | 34.40 | 15.56 | 5.15 | 4.40 | 0.53 | 16.41 | 12.58 | 7.16 | 59.29 |
| CD | 6.11 | 5.90 | 1.14 | 1.58 | NS | 3.18 | 4.76 | 0.62 | 2.99 |

*Mean of four replications having three plants each

**Table 8.** *Biometric characters of Meloidogyne incognita uninoculated tomato plants treated with different rhizobacteria and P. indica\**

| Treatments | Shoot length /plant (cm) | Fresh shoot weight /plant(g) | Dry shoot weight /plant(g) | Fresh root weight /plant(g) | Dry root weight /plant(g) | Number of leaves /plant | Number of Flowers /plant | Number of Fruits /plant | Yield /plant(g) |
|---|---|---|---|---|---|---|---|---|---|
| *B. pumilus* | 39.25 | 19.18 | 7.405 | 5.11 | 0.50 | 21.41 | 18.24 | 8.66 | 93.77 |
| *P. fluorescens* AMB 8 | 41.96 | 20.23 | 7.640 | 5.27 | 0.56 | 19.41 | 18.49 | 9.16 | 97.58 |
| *P. indica* | 48.02 | 23.99 | 8.45 | 6.81 | 0.77 | 25.33 | 20.83 | 9.75 | 118.05 |
| *P. indica* and *B. pumilus* | 41.52 | 24.79 | 8.64 | 6.68 | 0.72 | 25.08 | 20.66 | 9.47 | 108.47 |
| *P. indica* and *P. fluorescens* AMB 8 | 43.02 | 25.48 | 9.09 | 7.62 | 0.72 | 26.66 | 18.33 | 9.49 | 116.07 |
| Control | 37.31 | 17.76 | 5.45 | 4.56 | 0.40 | 18.41 | 12.58 | 7.83 | 72.63 |
| CD | 6.47 | 5.23 | 1.80 | 1.71 | NS | 3.00 | 4.24 | 0.68 | 1.28 |

\*Mean of four replications having three plants each

## 4.2.4. Nematode reproduction in plant

Observation of roots infected with nematode indicated the nematode suppression capability of different treatments (Table 9).

Reduced number of galls/plant was observed for plants treated with *P. indica* (33.30). Percentage reduction of galls/plant over control was 76.58 in plants treated with the beneficial root endophyte. Combined application of *P. indica* and *P. fluorescens* AMB8 treatment recorded 50.31 galls/plant and this was on par to *P. indica.* (Plate 17 and 18).

*P. indica* treated plants (3.41) exhibited an eight times reduction in number of egg mass/plant than that of control (24.91).

Number of eggs/ egg mass was also found to be reduced two times than control (663.54) in *P. indica* treated plants (306.40) (Plate 19).

Assessment of number of nematodes present per gram of root revealed that *P. indica* treated plants were able to reduce the development of nematodes inside the root as well.

The initial nematode population present in soil was zero in all the treatments since sterilized planting medium was used for the experiment. Final nematode population in soil after termination of the experiment was also analyzed. In nematode inoculated control treatment the final soil nematode population in soil was 294.16/100 cc of soil. However, in *P.indica* treatment the final nematode population in soil was found to be reduced to 58.00/100 cc.
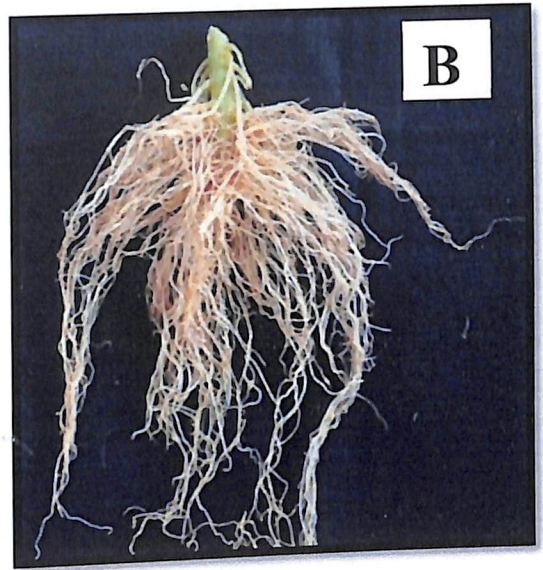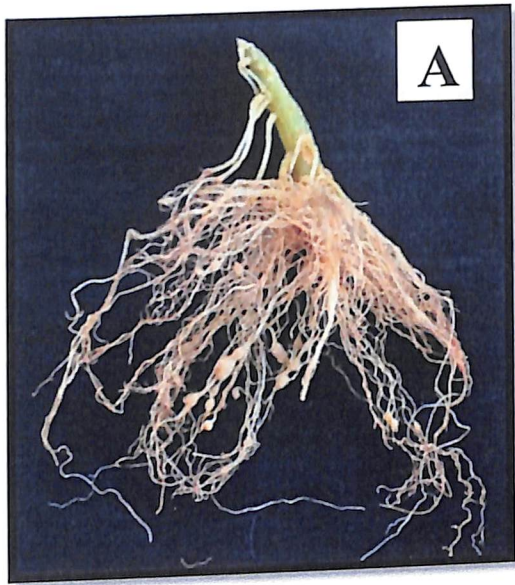
**Plate 17.** Gall development after ten days of nematode inoculation in A) control plants B)plants treated with *P. indica* and *P. fluorescens* AMB8 C) plants treated with *P. indica*
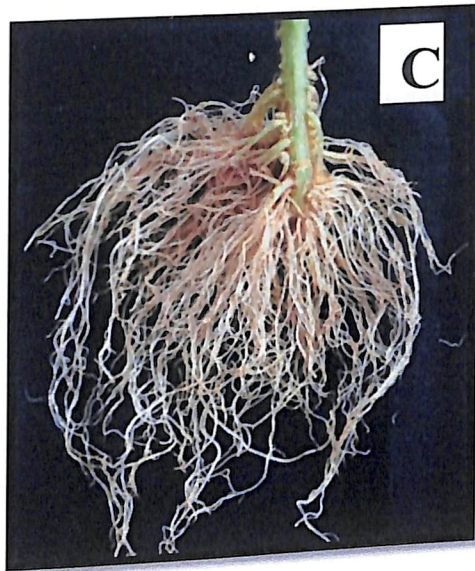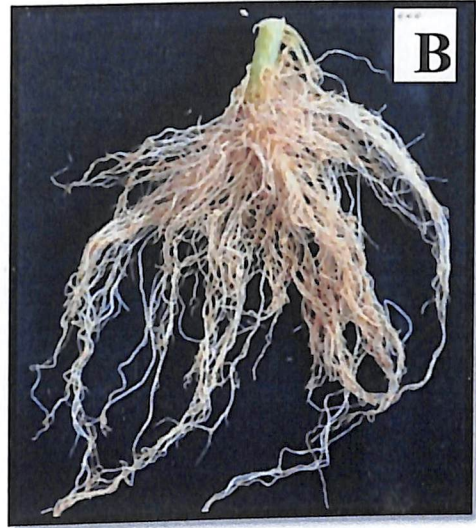
**Plate 18.** Gall development after ten days of nematode in plants: A) treated with *B. pumilus* B) treated with *P. fluorescens* AMB 8 C) treated with *P. indica* and *B. pumilus* treated plants.
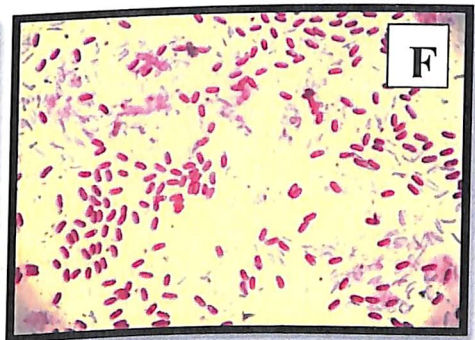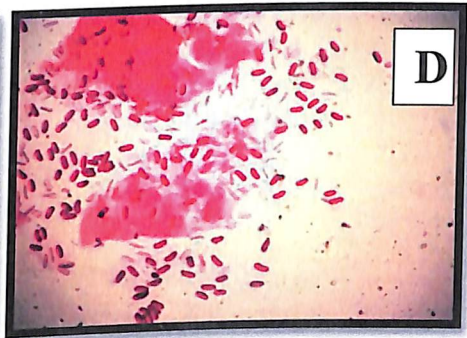
**Plate 19.** *Meloidogyne incognita* eggs obtained from the roots of tomato plants under different treatments A) *P. indica* B) *P. indica and B. pumilus* C) *P. fluorescens* AMB 8 D) *P. indicaand P. fluorescens* AMB 8 E) *B. pumilus* F) Control.

**Table 9.** Parameters of nematode infection and development

| Treatments | Number of galls/plant | Number of egg mass/plant | Number of eggs /egg mass | Number of nematodes/g of root | Final nematode population/100cc soil |
|---|---|---|---|---|---|
| *B. pumilus* | 82.58 | 7.66 | 479.36 | 170.49 | 186.99 |
| *P. fluorescens* AMB 8 | 91.66 | 7.33 | 424.88 | 165.16 | 135.33 |
| *P. indica* | 33.30 | 3.41 | 306.40 | 54.41 | 58.00 |
| *P. indica* and *B. pumilus* | 73.83 | 6.74 | 347.73 | 72.56 | 100.58 |
| *P. indica* and *P. fluorescens* AMB 8 | 50.31 | 6.58 | 430.19 | 165.16 | 81.41 |
| Control | 142.24 | 24.91 | 663.54 | 220.57 | 294.16 |
| CD | 29.64 | 3.82 | 41.17 | 12.95 | 18.62 |

*Mean of four replications having three plants each

### 4.2.5. Microscopic observation of developmental stages of *Meloidogyne incognita* in tomato plants

Separate set of plants maintained for studying the developmental stages were microscopically observed and photographs taken at regular intervals after sectioning and staining. The plant roots were stained in five days intervals after nematode inoculation. The developmental stages were found to show variation among the different treatments.

#### 4.2.5.1. Control plants

The penetration of *M. incognita* juveniles into the plant system started within five days of inoculation. In between five to ten days juveniles started feeding from the vascular tissue and gall development started. After fifteen days $J_2$ stages were destructively feeding from vascular area and transformed to peg tail ($J_3$) stage. Within 20 to 25 days of inoculation $J_3$ transformed into female. After 30 days of inoculation the life cycle was completed and mature females started laying eggs.

#### 4.2.5.2. *P. fluorescens* AMB8 treated plants

The developmental stages showed slight variation than the control plants. Penetration occurred between five to ten days of inoculation. $J_3$ developmental stages were observed within this period. In between ten to fifteen days $J_3$ to female transformation was observed. After 20 days of inoculation underdeveloped female with eggs were noticed and life cycle shortened to 20 days. Further development of female happened in 30 days.

#### 4.2.5.3. *B. pumilus* treated plants

Penetration of $J_2$ to the root was observed after ten days of inoculation. Within 15 days $J_2$ moved on to the vascular area and started feeding from there. This initiates the giant cell formation and gall development. After 20 days $J_2$ completely

transformed to $J_3$ (peg tail stage) and within 20 to 30 days $J_3$ completely transformed to female stage.

### 4.2.5.4. *P. indica* treated plants

In the case of *P. indica* treated plants root penetration by the juveniles was found to be restricted up to 15 days of inoculation. Nematode penetration and feeding of $J_2$ from vascular area started between 15 to 20 days after inoculation. Peg tail stages were visible within 25 days and transformation to female occurred within five days. Here the life cycle found to be shortened to 10 to 15 days.

### 4.2.5.5. *P. indica* and *P. fluerescens* AMB8 treated plants

Root penetration and feeding started within 15 days of inoculation. After 20 days the galls were formed. Peg tail stages were found in 25 and 30 days after inoculation. Developing females with eggs were found after 30 days. Life cycle was extended.

### 4.2.5.6. *P. indica* and *B. pumilus* treated plants

Root penetration was in between ten to fifteen days of nematode inoculation. Feeding resulted in gall development within 20 days and peg tail stages were also present. Transformation from $J_3$ to female was present in thirty days after inoculation. Here also life cycle found to be extended.
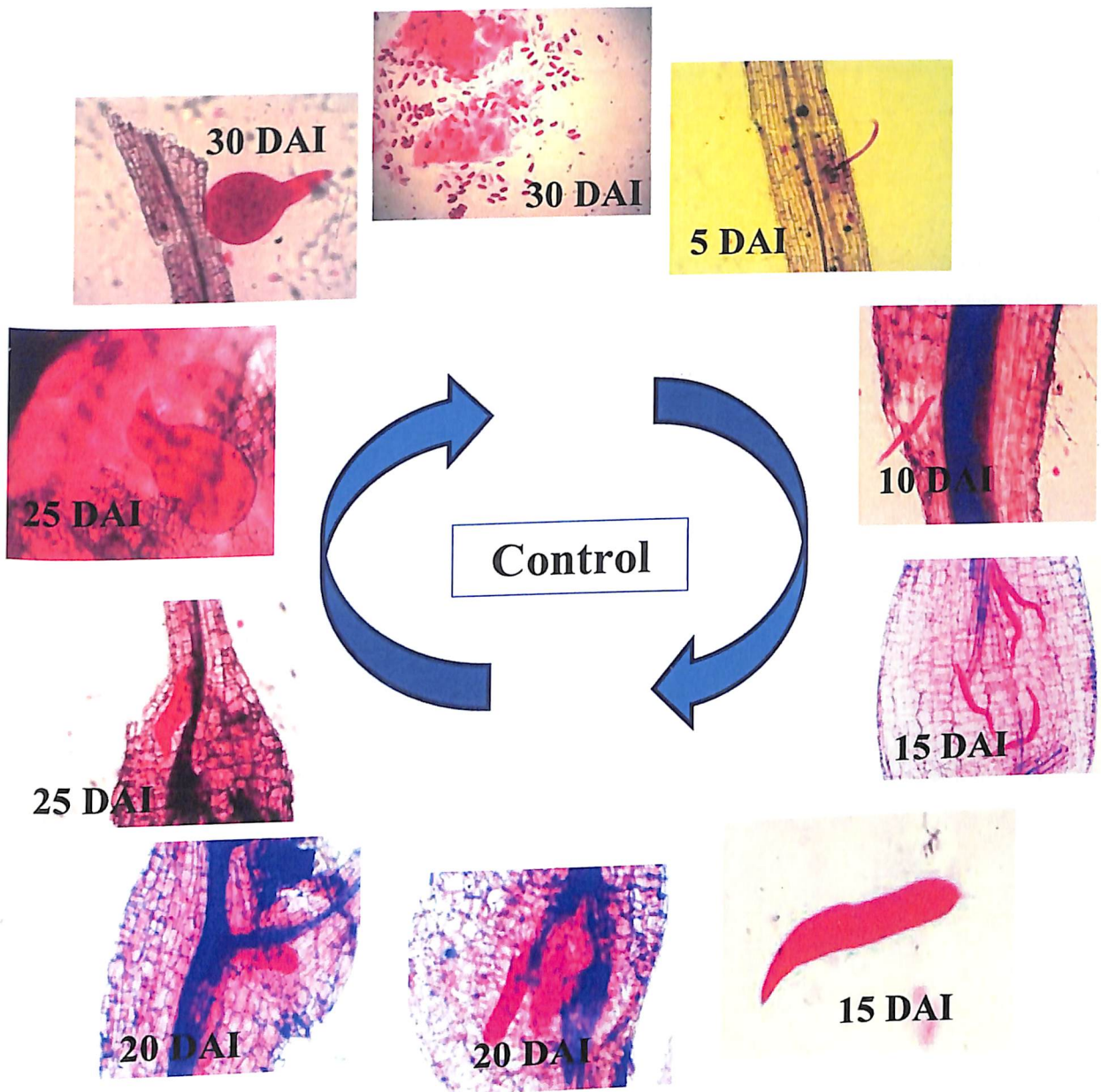
**Plate 20.** Developmental stages of *Meloidogyne incognita* in control tomato plants

**Plate 21.** Developmental stages of *Meloidogyne incognita* in tomato plants treated with *P. fluorescens* AMB 8.

**Plate 22.** Developmental stages of *Meloidogyne incognita* in tomato plants treated with *B. pumilus*

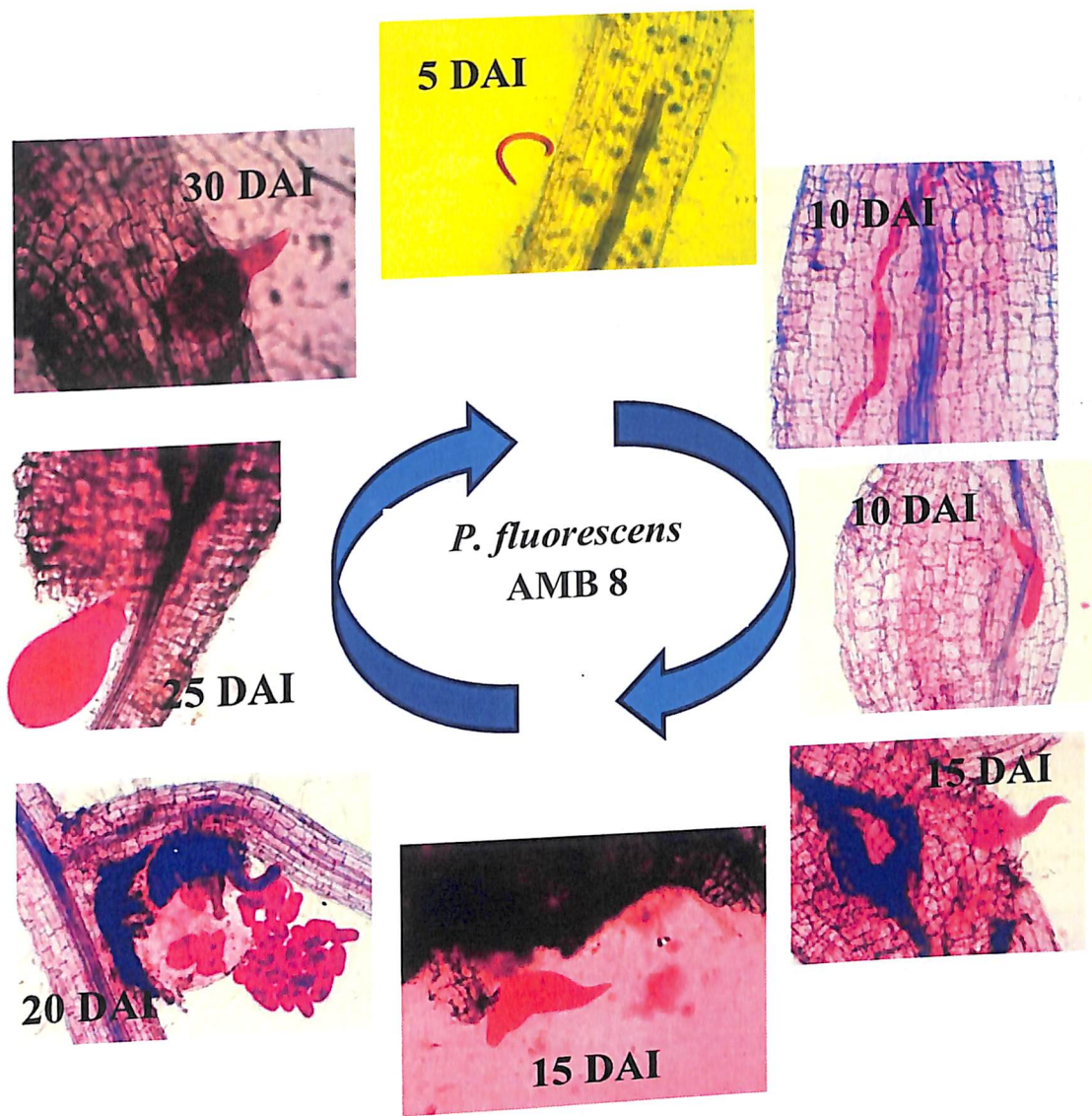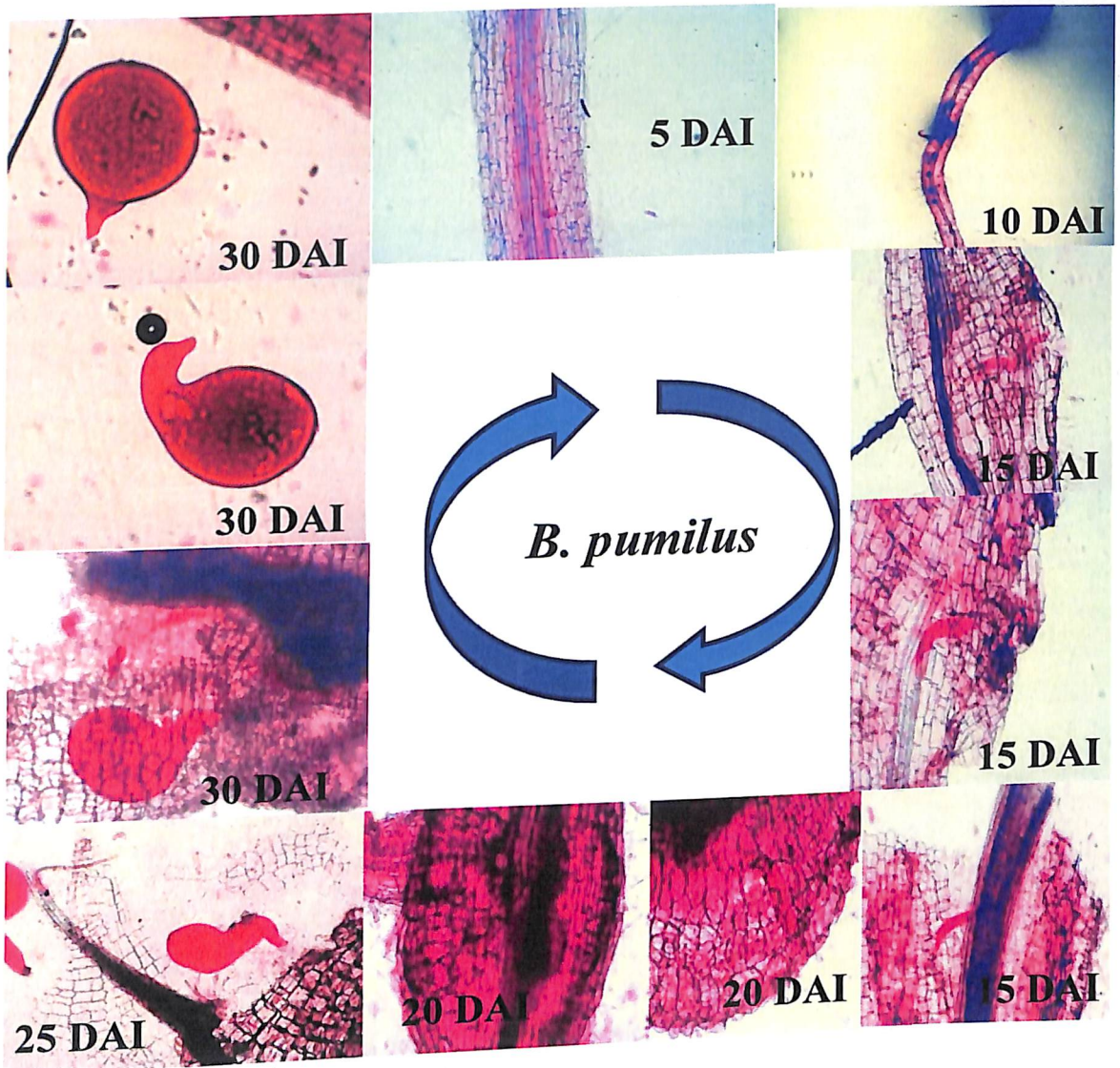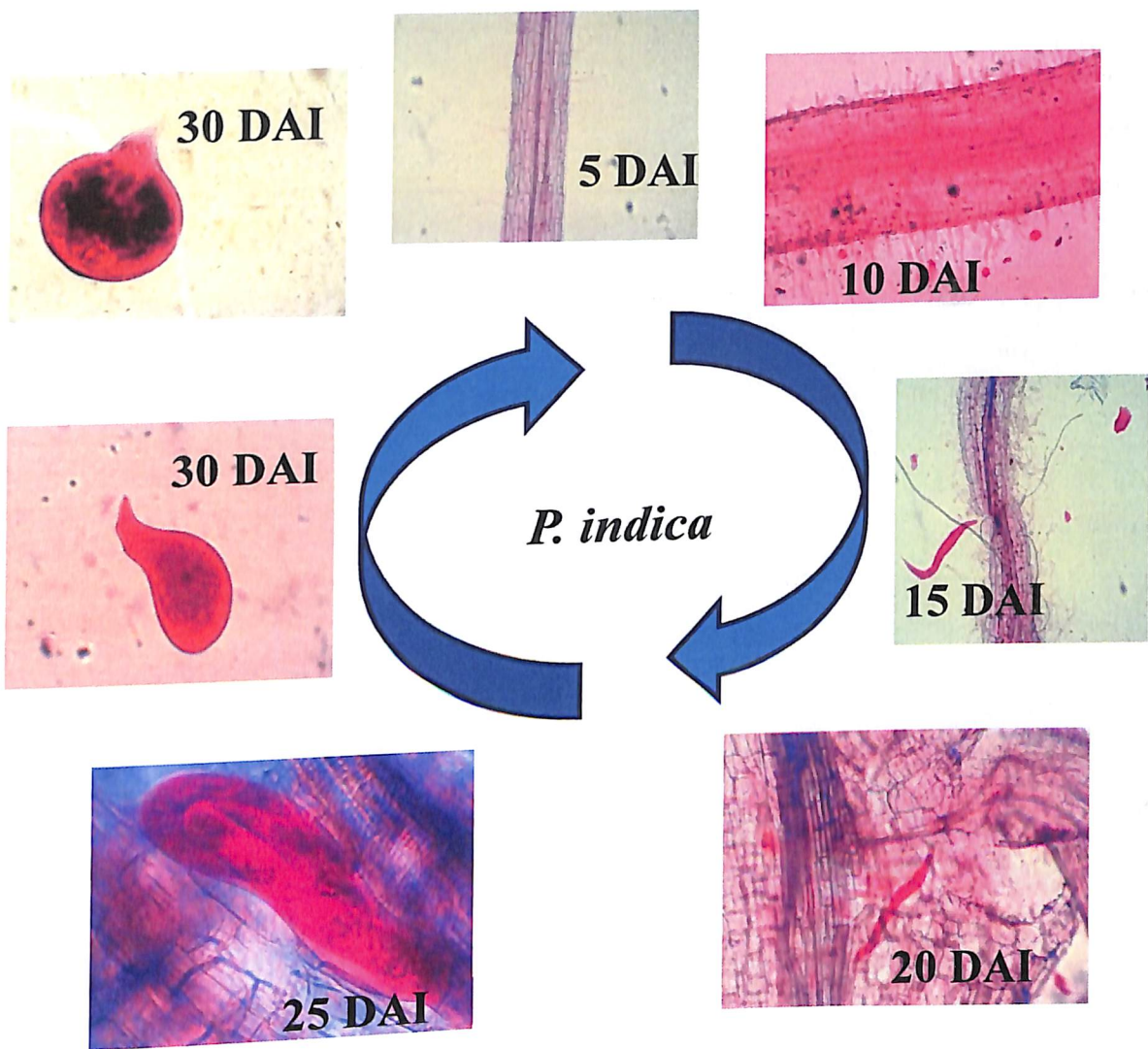**Plate 23.** Developmental stages of *Meloidogyne incognita* in tomato plants treated with
*P. indica.*
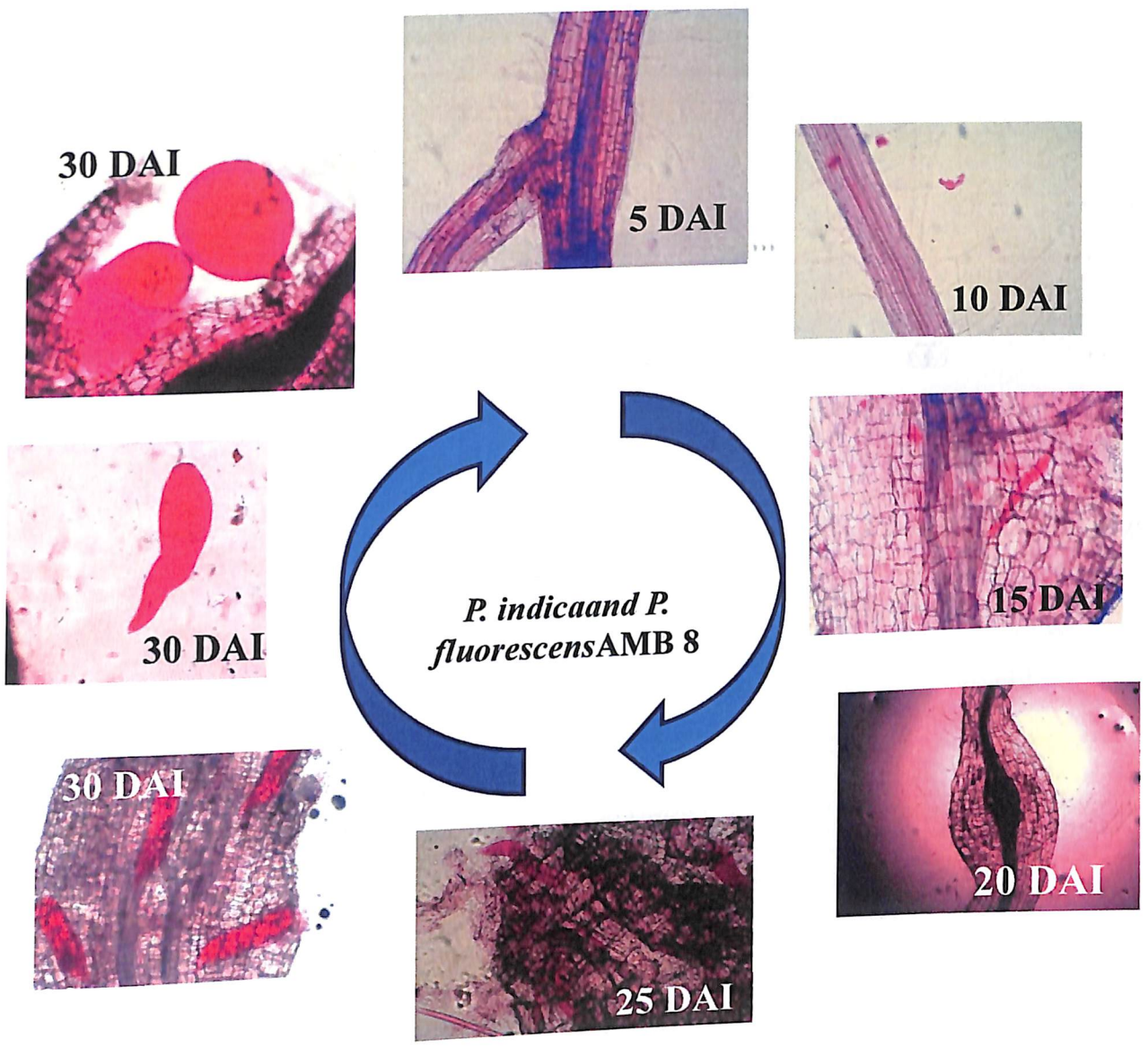
**Plate 24.** Developmental stages of *Meloidogyne incognita* in tomato plants treated with *P. indica* and *P. fluorescens* AMB 8.
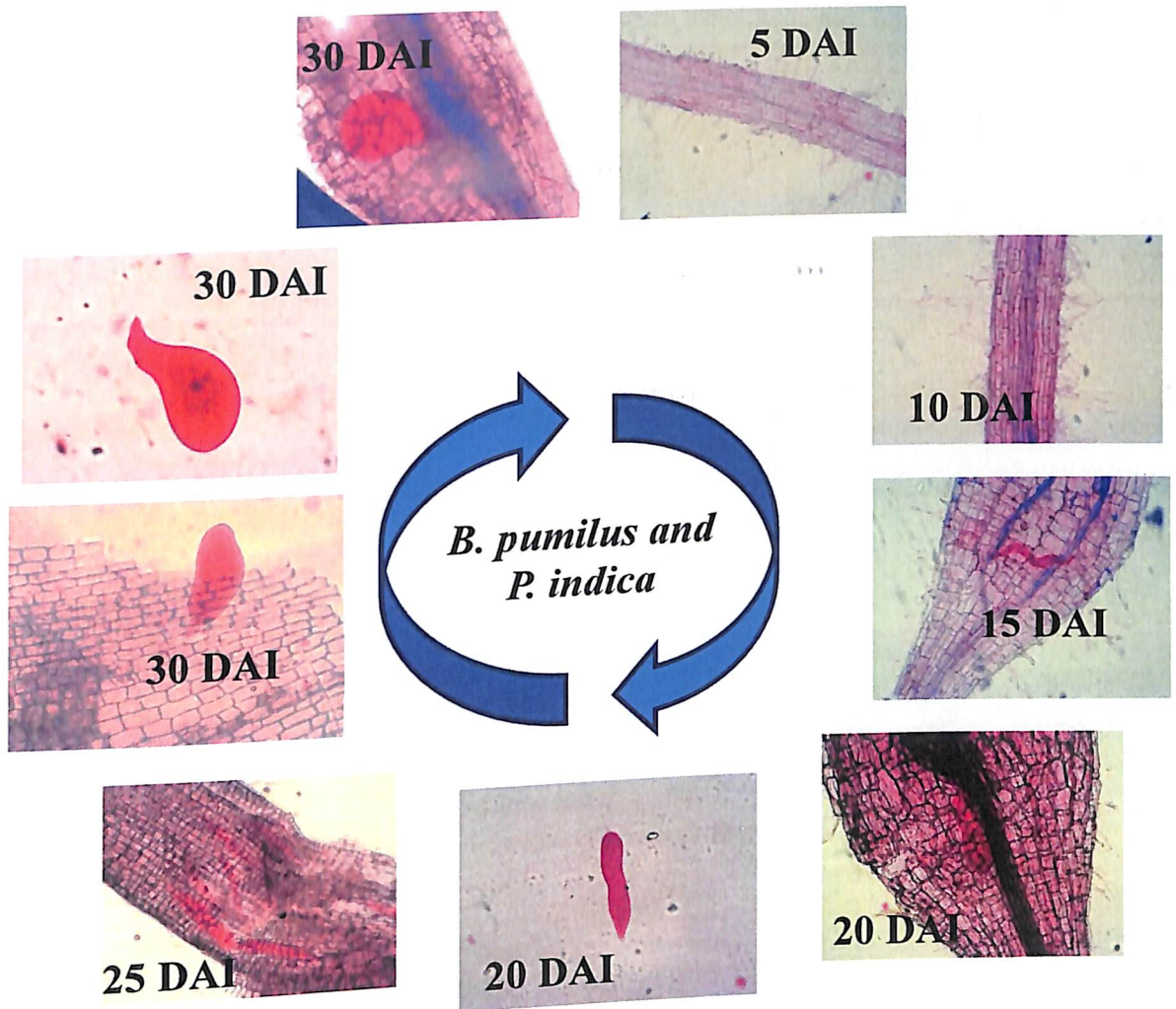
**Plate 25.** Developmental stages of *Meloidogyne incognita* in tomato plants treated with *B. pumilus* and *P. indica*.

*Discussion*

# 5. DISCUSSION

Root–knot nematodes are one of the devastating endoparasites of tomato since they feed from the vascular area of the plants and affect the normal physiological mechanisms. Research on microbial pathogens and antagonists of root-knot nematodes, as well as other economically important plant parasitic nematode species has progressed over several years and the developments process the isolation and identification of the organisms with biological control potential; ecological manipulation of the soil environment to improve antagonism, elucidation of mechanisms of parasitism and infection, and exploration for commercial product development. However, it is realized that after many years of extensive research the impact of biological control on root knot nematode management in the field is still marginal. The current investigation on *P. indica* and rhizobacteria both in *in vitro* and *in vivo* conditions has given promising results on biocontrol of *Meloidogyne incognita* in tomato.

The programme of work was divided into two parts *in vitro* and *in vivo* analysis. The *in vitro* screening methods were employed to assess the potential of the CFE of the biological agents against *M. incognita* juvenile mortality and egg hatching and also to detect the direct interaction of the rhizobacteria and the root endophyte, *P. indica* with the nematode pest. Previous reports reveal that bacterial and fungal culture filtrates containing different toxic metabolites and are harmful for the survival of nematode pest (Bansal *et al.*, 2005). The screening of rhizobacterial cell suspension and CFE of rhizobacteria and the root endophytic fungus *P. indica* revealed that they are having detrimental effects against the survival of *M. incognita* by inhibiting the egg hatching or by causing J2 mortality.

Egg hatching of *M. incognita* was inhibited by CFE of rhizobacteria and it was apparent that percentage hatching varied with exposure time as well as

dilution (Fig.1). Reducing the concentration of the CFE might have diluted the amount of toxic metabolites present exhibiting positive influence on egg hatching (Fig.2) with more hatching in most diluted sample (Fig.3). The results indicated that CFE of all the selected rhizobacteria influenced the mortality with a maximum mortality of 99.71 percentage for *B. amyloliquefaciens* upon 24 hours of incubation (Fig.4). However, dilution of the same had less effect (Fig 5 and 6). A wide variation in the mortality percentage of infective juveniles (J2) was noticed in all the treatments ranging from 99.71 to 68.21 in full strength concentration. The percentage mortality of juveniles increased along with increase in concentration of CFE and exposure time. Related results were reported in studies conducted by Pankaj *et al.* (2011) with *Bacillus* and *Pseuodomonas* strains. Delayed nematode egg hatch and reduced juvenile mobility of *Meloidogyne* spp. due to culture supernatants of *Pseudomonas* spp. (Sharma *et al.*, 1998) and *Bacillus* spp. (Padgham *et al.*, 2005) were also reported.

Screening of rhizobacterial cell suspension for egg hatching and juvenile mortality of *M. incognita* was helpful in finding out the direct antagonism. The data on Fig. 7 revealed that egg hatching was influenced by the bacterial cell suspension and varied with the exposure time. Figure 8 and 9 clearly describes that percentage hatching of *M. incognita* eggs increased on diluting the cell suspension. The results revealed that mortality was not much influenced by the rhizobacterial cell suspension (Fig. 10).

The present study also agreed with the findings of Siddique and Mahmood, (1999) that *M. incognita* egg hatching, juvenile mortality, and nematode reproduction was affected by the toxins produced in bacterial cell free extracts. Production of secondary metabolites such as phenazines, pyrolnitrin, tropolone, pyocyanin and 2,4-diacetylphloroglucinol was reported to be present in the cell free extracts of *P. fluorescens* by Becker *et al.*, (1988). Shanthi and Rajendran (2010) reported the presence of toxic metabolites in the culture filtrate of *P. fluorescens*, *Trichoderma*
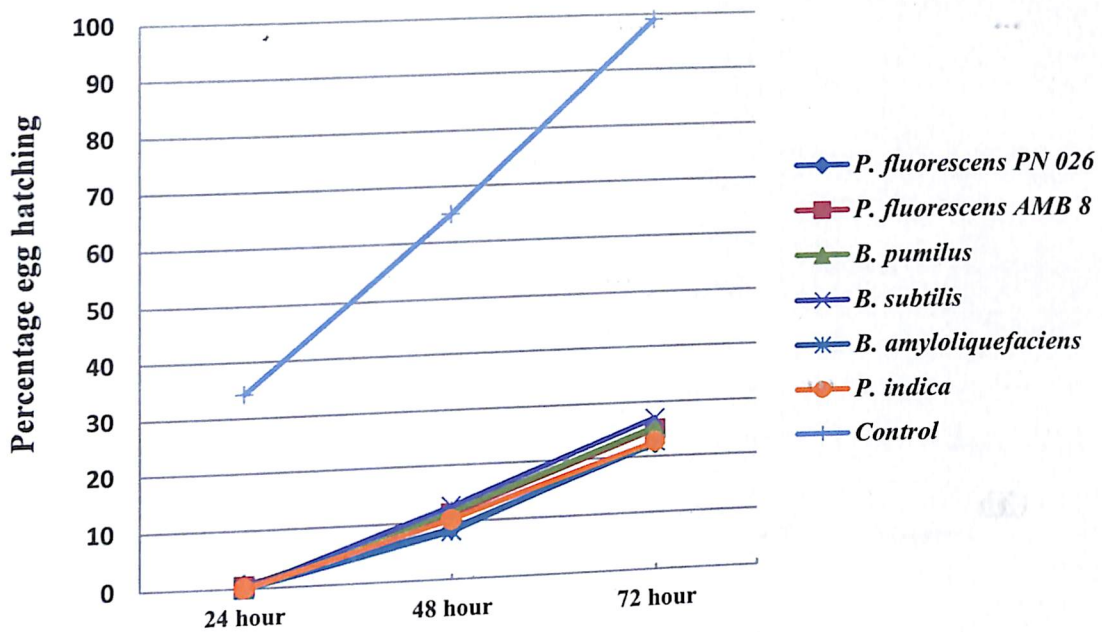
**Fig. 1.**Effect of full strength cell free extracts of rhizobacteria and *P. indica* on egg hatching of *Meloidogyne incognita.*
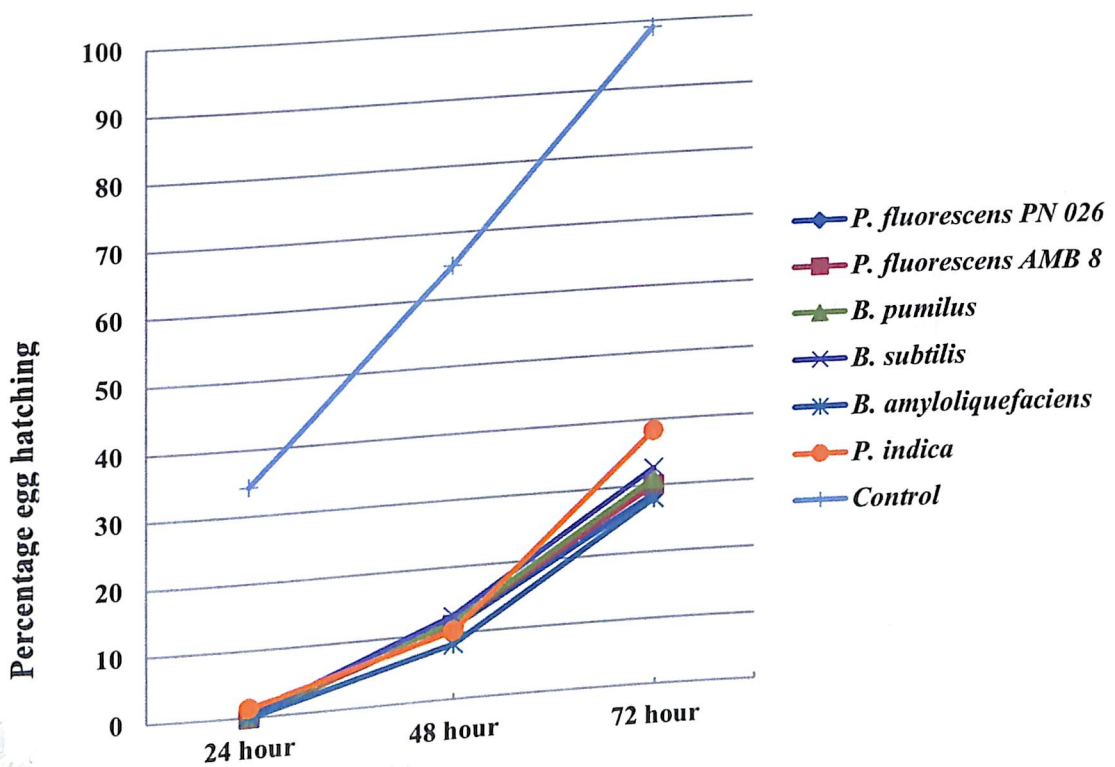


**Fig. 2.**Effect of half strength cell free extracts of rhizobacteria and *P. indica* on egg hatching of *Meloidogyne incognita.*
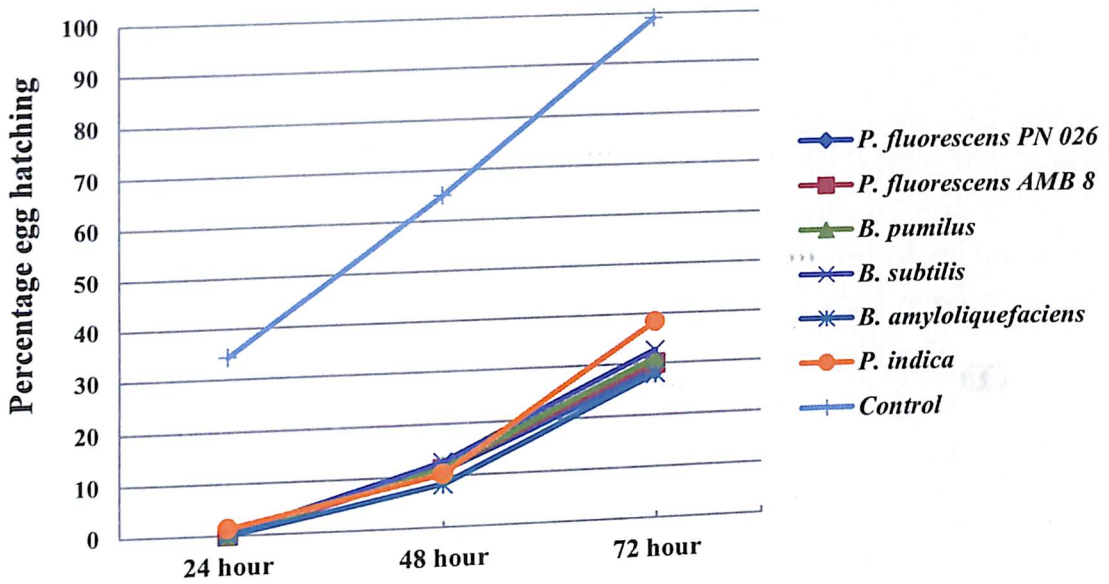
**Fig. 3.** Effect of quarter strength cell free extracts of rhizobacteria and *P. indica* on egg hatching of *Meloidogyne incognita*.
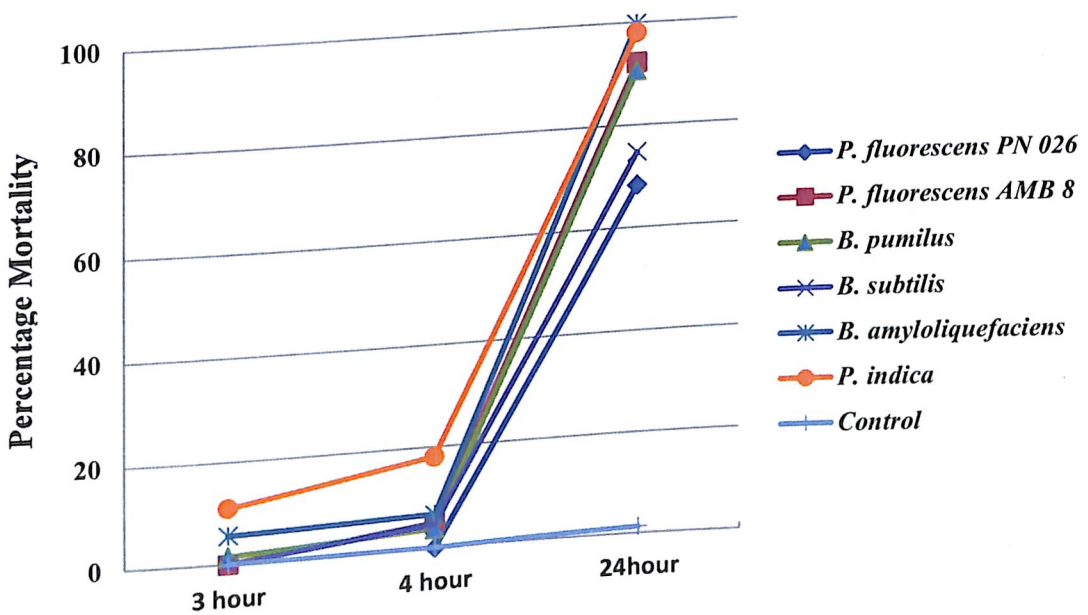


**Fig. 4.** Effect of full strength cell free extracts of rhizobacteria and *P. indica* on mortality of *Meloidogyne incognita*.
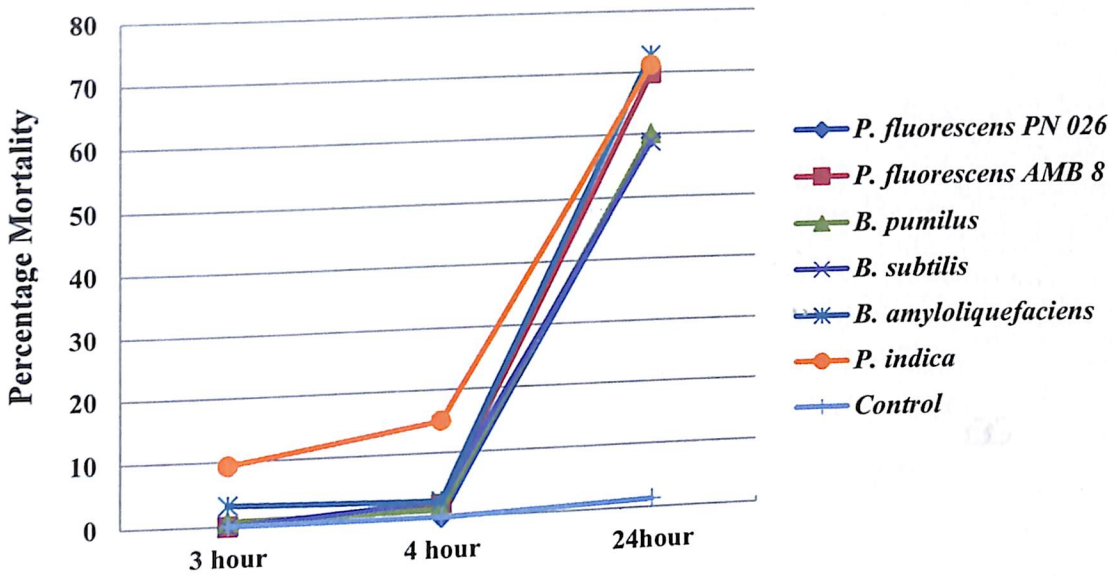
**Fig. 5.** Effect of half strength cell free extracts of rhizobacteria and *P. indica* on egg hatching of *Meloidogyne incognita*.
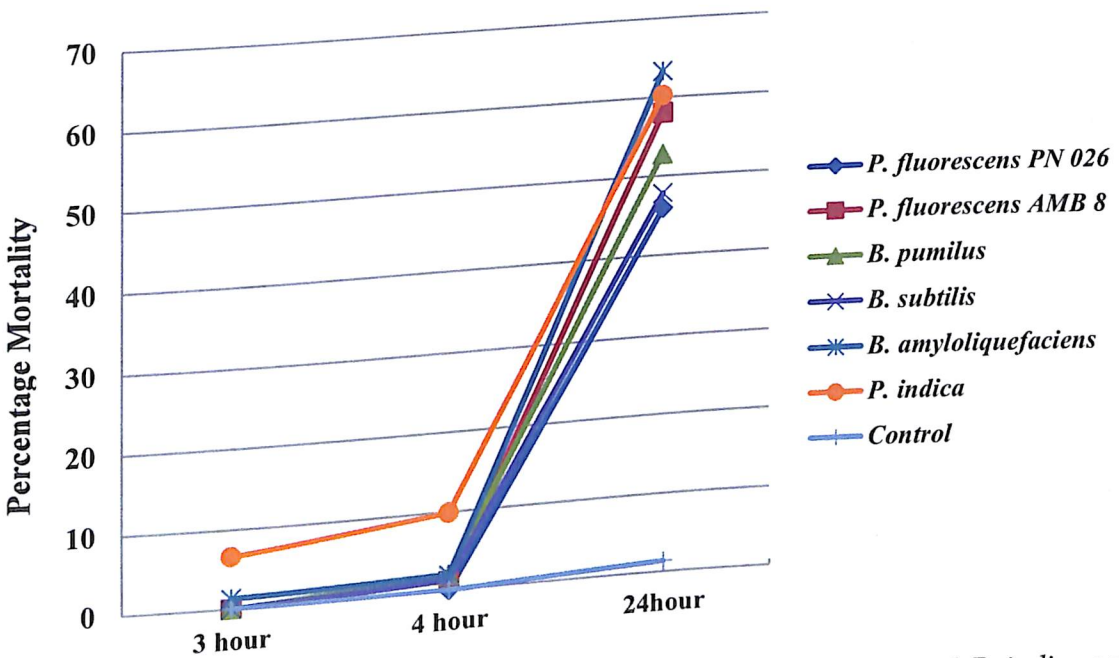


**Fig.6.** Effect of quarter strength cell free extracts of rhizobacteria and *P. indica* on egg hatching of *Meloidogyne incognita*.
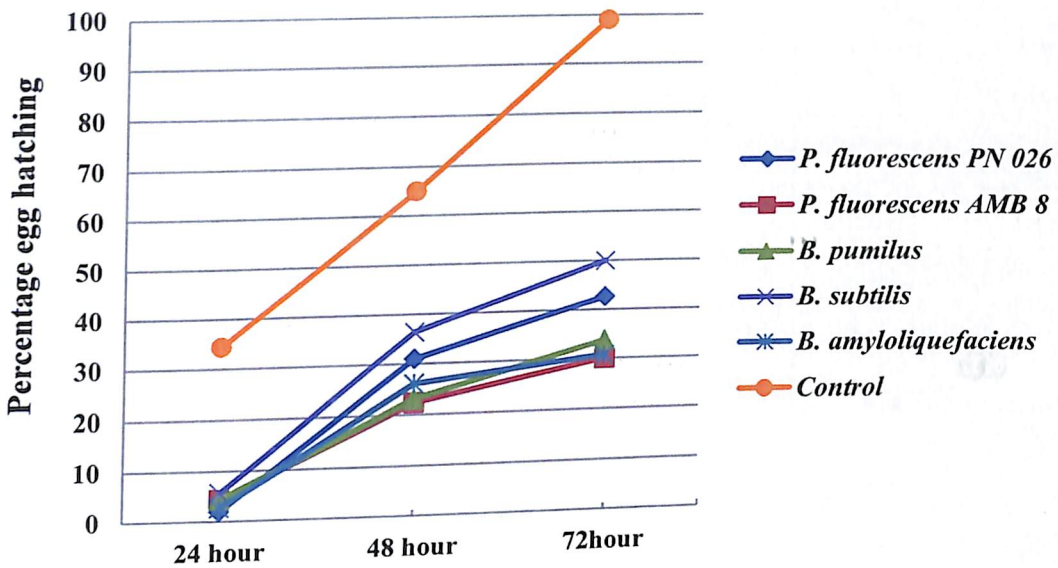
**Fig.7.** Effect of full strength rhizobacterial suspension on egg hatching of *Meloidogyne incognita.*
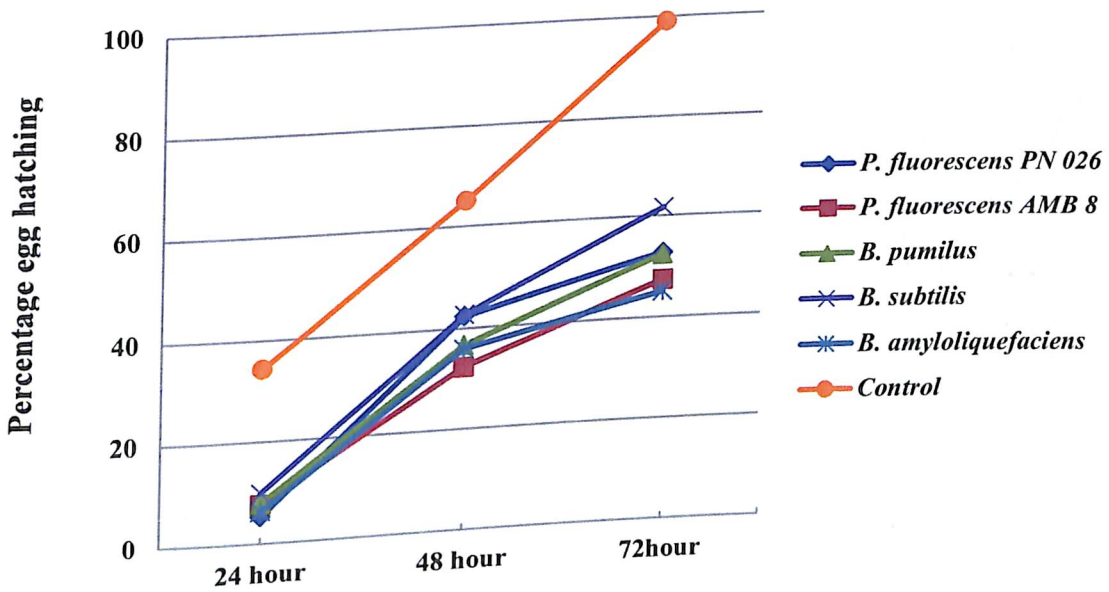


**Fig. 8.** Effect of half strength rhizobacterial suspension on egg hatching of *Meloidogyne incognita.*
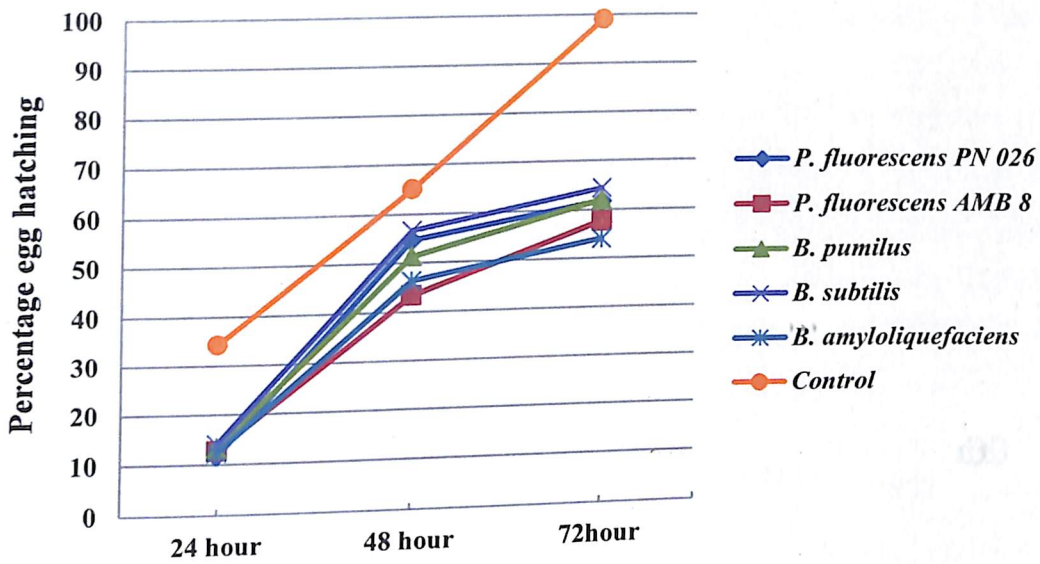
**Fig. 9.**Effect of quarter strength rhizobacterial suspension on egg hatching of *Meloidogyne incognita.*
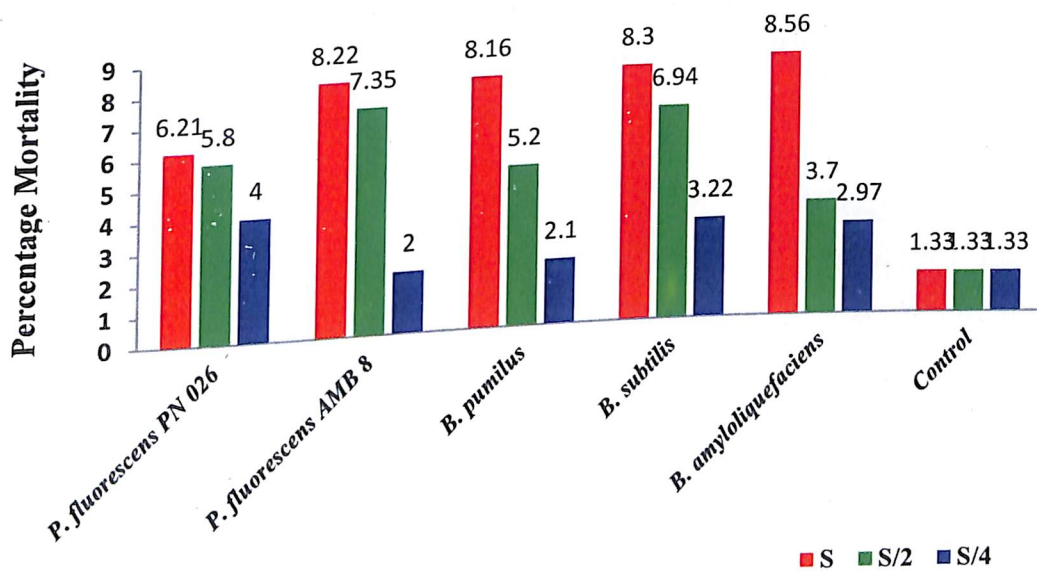


**Fig 10.**Effect of rhizobacterial suspension on mortality of J$_2$ of *Meloidogyne incognita.*

*viridae, B. subtilis* and *Paecilomyces lilacinus* that induced significant levels of mortality of banana lesion nematodes. The present work also facilitated the comparative analysis of the effect of CFE and rhizobactrial cell suspension on mortality and egg hatching of *M. incognita* at different concentrations. From the Fig. 11 it is evident that CFE of all the isolates caused high mortality and a reduced egg hatching. In cell suspension the juvenile death was very low. Though good inhibition of egg hatch was noticed (Fig. 12). The effect of diluting the CFE and cell suspension expressed in Figure 13 & 14 indicated less mortality and increased egg hatch than the undiluted suspensions. Dilution of rhizobacterial suspension improved hatching but had less influence on J2 mortality. In quarter strength, mortality percentage was much reduced and showing nearby values as that of percentage egg hatch (Fig. 15). An increase in egg hatching of *M. incognita* was noticed in quarter strength rhizobacterial suspension while effect on percentage mortality seems to be negligible (Fig. 16).

Fungal bioagents can directly parasitise nematodes and/ or secrete nematicidal metabolites that affect nematode viability (Nitao *et al.*, 1999). CFE of *P. indica* showed a higher mortality percentage and higher hatching inhibition. The trend of increasing efficiency with respect to exposure time and decreasing efficiency with dilution was noticed in case of *P. indica* also as in the case of CFE of rhizobacteria. This is in accordance with the findings of Mayer *et al.* (2004) that the nematicidal effect of fungal culture filtrates affecting *M. incognita* increased with increase in exposure time. Active compounds from culture filtrate of different endophytic fungi were described to have nematicidal or nematistatic potentials earlier (Mani and Sethi 1984 a, b ; Cayrol *et al.*, 1989; Cayrol and Djian, 1990; Hallman and Sikora, 1996; Chen *et al.*, 2007; Mayer *et al.*, 2000). Reports also suggest that activity of the antagonistic fungi involves nematicidal effect on adult nematodes, inhibition of egg hatching and juvenile development (Meyer *et al.*, 2004) and production of antagonistic compounds including phenolic metabolites (Benedict and Brady, 1972).

# PLANT GROWTH-PROMOTION AND ROOT KNOT NEMATODE MANAGEMENT IN TOMATO BY *Piriformospora indica* AND RHIZOBACTERIA

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2016
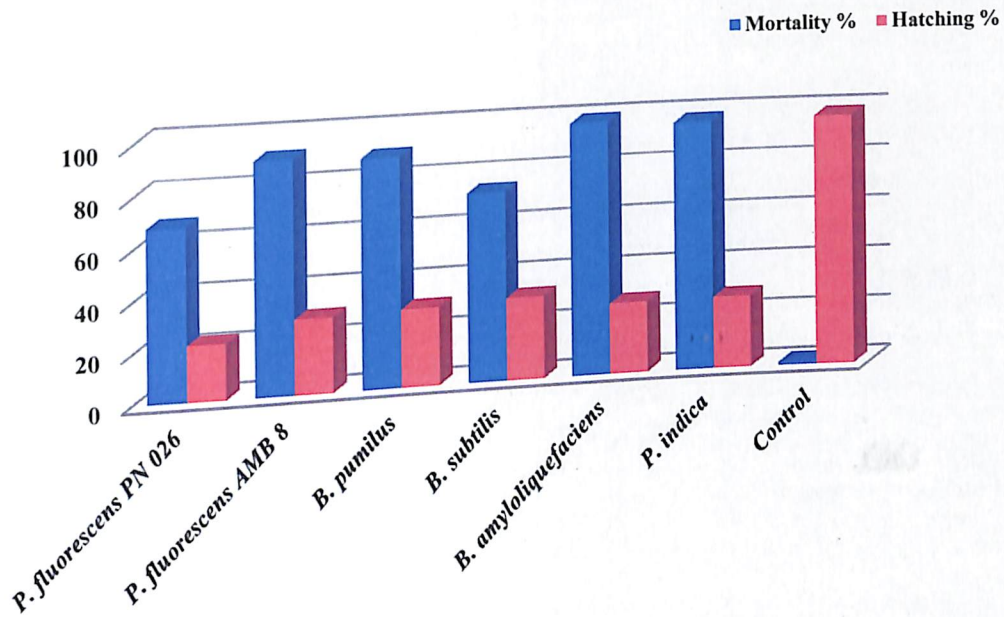
**Fig. 11.**Effect of cell free extract on J$_2$ mortality and egg hatching of *Meloidogyne incognita*at the final hour of incubation (S).
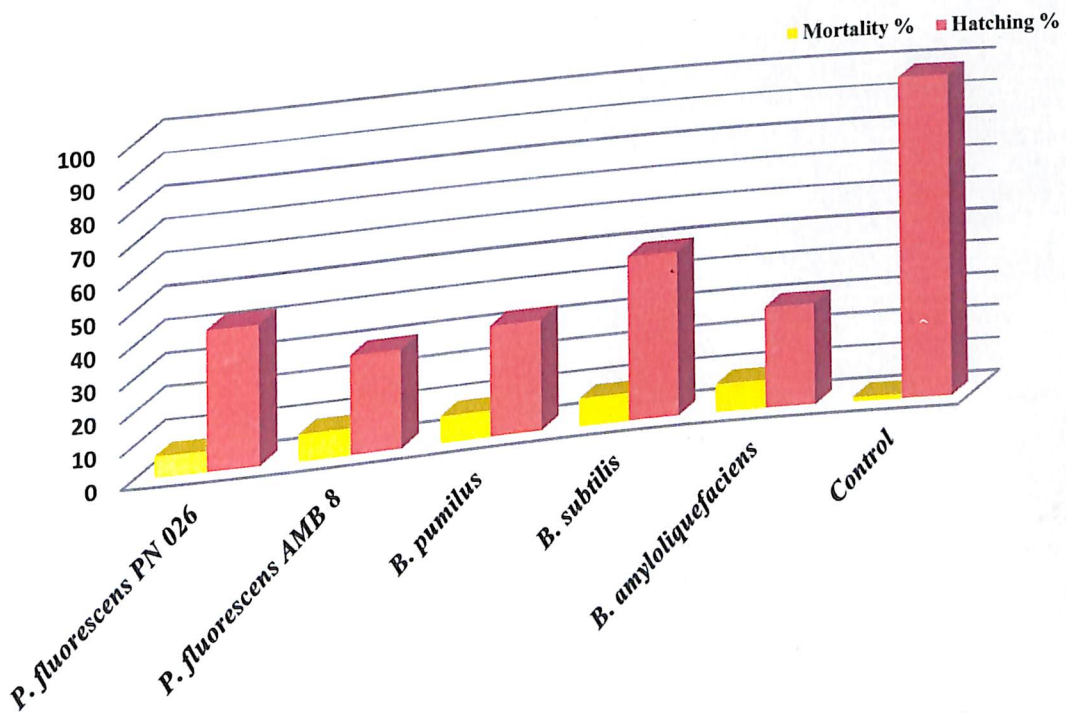


**Fig. 12.**Effect of Rhizobacteria on J$_2$ mortality and egg hatching of *Meloidogyne incognita* at the final hour of incubation (S).
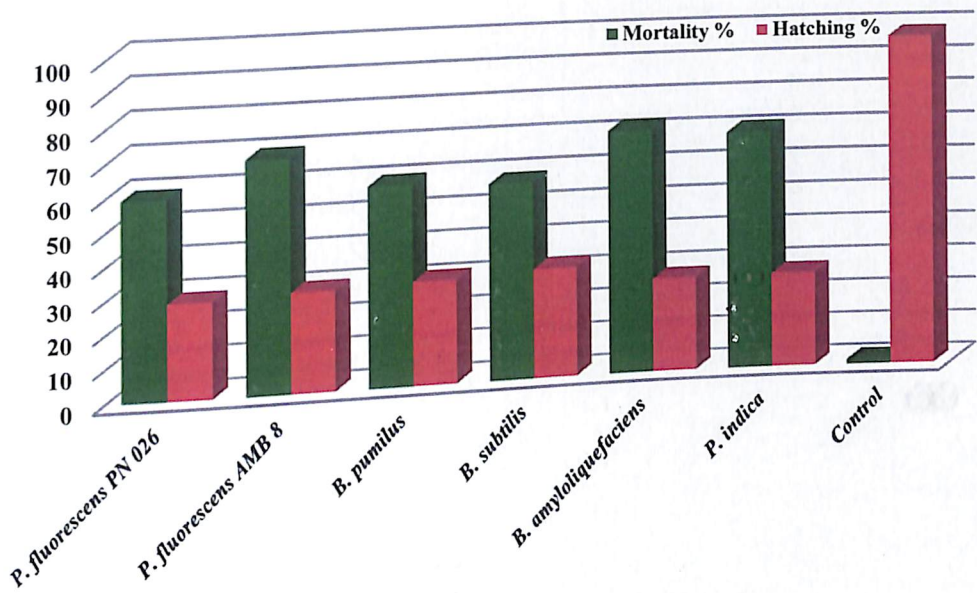
**Fig. 13.** Effect of cell free extract on $J_2$ mortality and egg hatching of *Meloidogyne incognita* at the final hour of incubation (S/2).
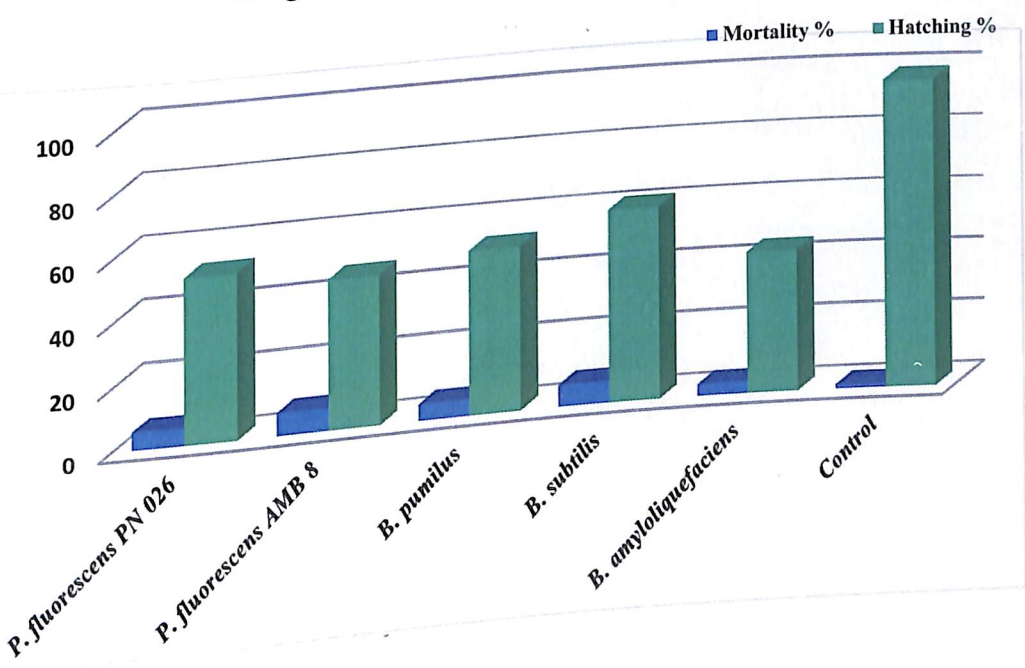


**Fig. 14.** Effect of rhizobacteria on $J_2$ mortality and egg hatching of *Meloidogyne incognita* at the final hour of incubation (S/2).
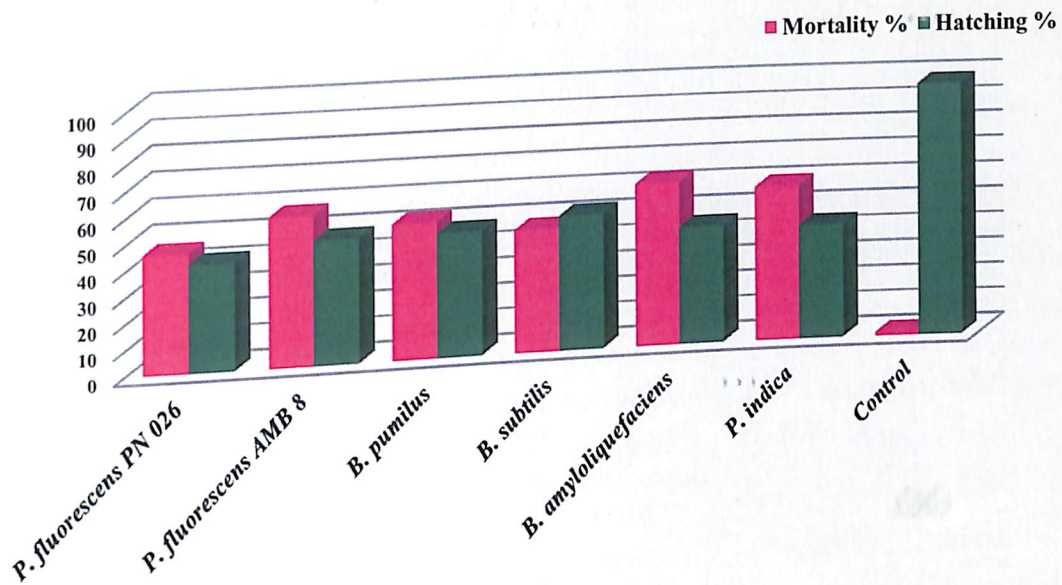
**Fig. 15.**Effect of cell free extract on $J_2$ mortality and egg hatching of *Meloidogyne incognita* at the final hour of incubation (S/4).
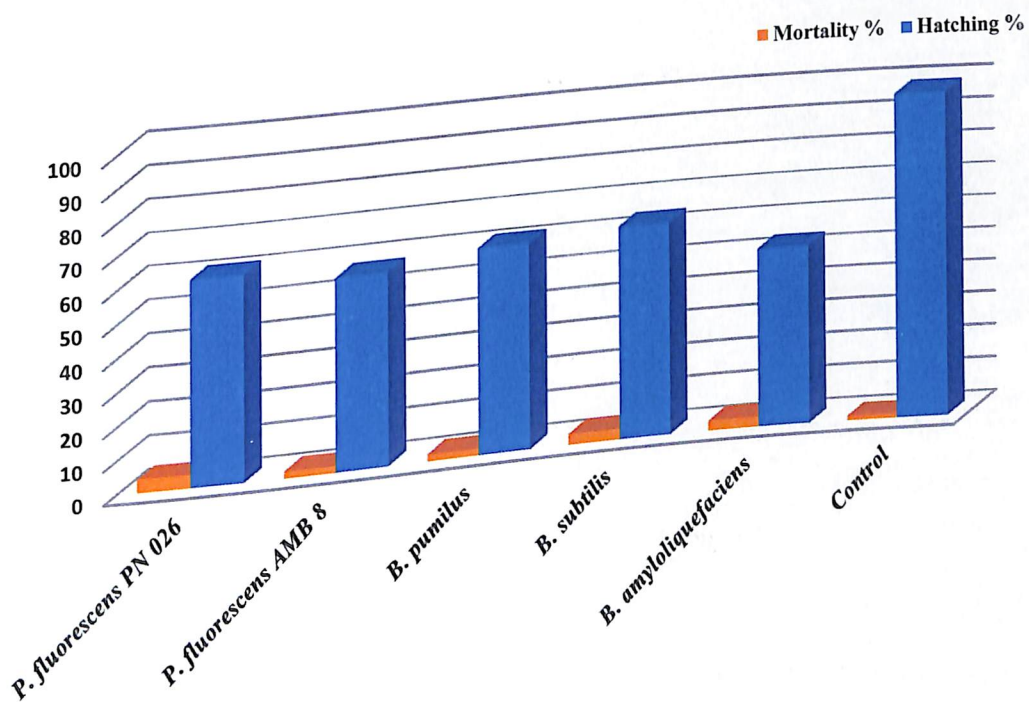


**Fig . 16**. Effect of rhizobacteria on $J_2$ mortality and egg hatching *of Meloidogyne incognita*at the final hour of incubation (S/4)

The effect of fungal culture filtrate was more pronounced with increase in exposure time with more inactivation of juvenile after 72 hours as compared to 24 hour. Similar results were obtained for Singh and Mathur (2009) with the CFE of *Acremonium*. The effect of culture filtrate and cell wall extract of *P. indica* in immobilizing *Heterodera schachtii* J2 within 24 hour of exposure has been already reported (Daneshkhah *et al.*, 2013).

Dual culture has been used to understand antagonism between different biological agents (Li *et al.*, 2002 and Aravind *et al.*, 2009). The endophytic root symbiont *P. indica* induced various reactions with rhizobacterial strains. Many had neutral response, but some displayed inhibitory to stimulatory reactions to *P. indica* when cocultivated on agar plates (Varma *et al.*, 2012). The present study established the compatibility of *P.indica* with rhizobacteria and it was found that *B. pumilus* a plant growth promoting rhizobacterial strain was compatible with *P. indica*. Dual culturing of the fungus and bacterial strains showed that *B. pumilus* had no antagonistic effect on the fungus both on PDA and CWA. Mycelial growth was highly inhibited by *B. amyloliquefaciens* and *B. subtilis*. The result was in correlation with the findings of Anith *et al.* (2015). Coconut water, a waste product from the coconut industry, was used as a medium for cultivation of the two biological agents. Reports reveal that coconut water free of microbial contamination and rich in nutrients like amino acids, vitamins and minerals and can be used as an efficient and cheap media for the multiplication of microorganisms (Survase *et al.*, 2007; Anith *et al.*, 2014). The present study was also in agreement with the result of Anith *et al.*, (2015) since coconut water agar facilitate co-cultivation of *P. indica* with *P. fluorescens* AMB8 and *B. pumilus* without inhibition. This ensures a cheap media alternative. The compatible combinations of *P. indica* with *P. fluorescens* AMB8 and *B. pumilus* were selected for further in vivo studies.

Staining root bits and microscopic observation revealed that treatments that included *P. indica* showed intense chlamydospore colonization in the cortical region

of the plant root. Microscopic observation revealed that in nematode non-infected roots *P. indica* hyphae grew along the root epidermis and invaded epidermal, cortical and endodermal cells intracellularly. A higher root colonization percentage of 54.70 was observed for *P. indica* and *B. pumilus* combination than *P. indica* alone and *P. indica* with *P. fluorescens* combination. This was in accordance with the findings of Anith *et al.* (2015).

Microscopic analysis of nematode infected root bits showed remarkable variation in root colonization than non-infected plants. Colonization by *P. indica* was found to be enhanced in all the treatments inoculated with nematodes. This might be due to the destruction of root cell by *M. incognita* which paved the easy passage of hyphae of the fungus and further proliferation inside the root. None of the root bits having presence of nematode feeding sites or intact nematodes exhibited chlamydospore colonization, though the noninfected root bits were colonized by *P. indica* (Plate 26). A higher root colonization was found in plants treated with combination of *P. indica* and *P. fluorescens* in the presence of nematode while it was least for the *P. indica* and *B. pumilus* combination (Fig. 17). The root colonization by *B. pumilus* might have prevented the colonization of *P. indica* thereby resulting in a lower colonization percentage. The pattern of chlamydospore formation in *P. indica* treated plants both in the presence and absence of nematode were same. Both the combination treatments in nematode inoculated and un-inoculated plants showed clustered colonization of chlamydospores.

The current work studied the potentials of the endophytic fungus *P. indica* as an antagonistic organism to battle the plant parasitic nematode *Meloidogyne incognita*. *M. incognita* J2 juvenile are the infective stages in tomato and many other plant species. Biocontrol agents preventing the penetration and multiplication of J2 are found to be successful as an effective management strategy. Inoculation of plants with *M. incognita* caused a significant reduction in plant growth compared to plants
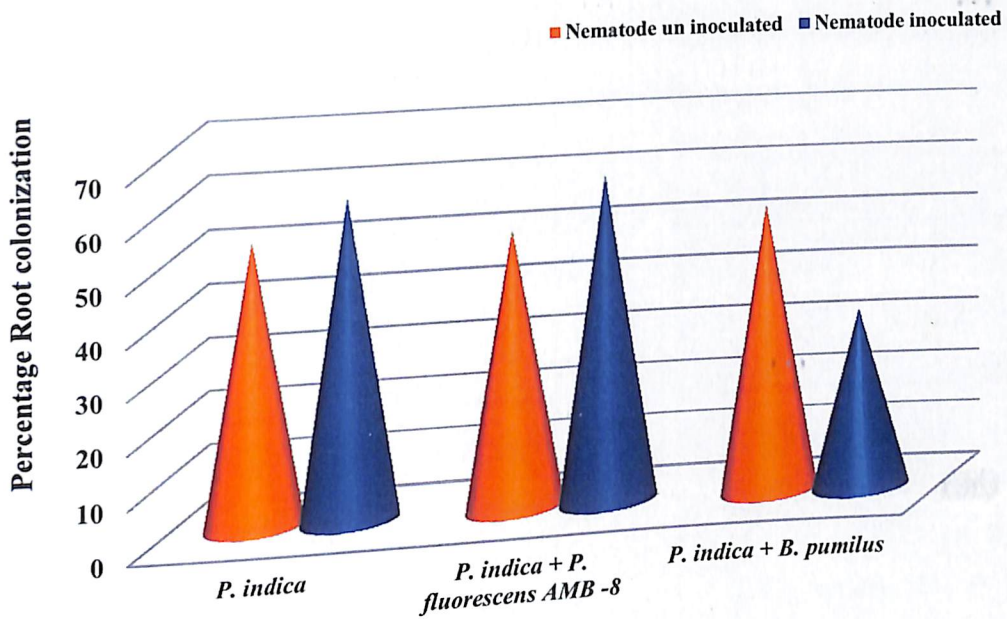
**Fig 17.** Root colonisation by *P. indica*.
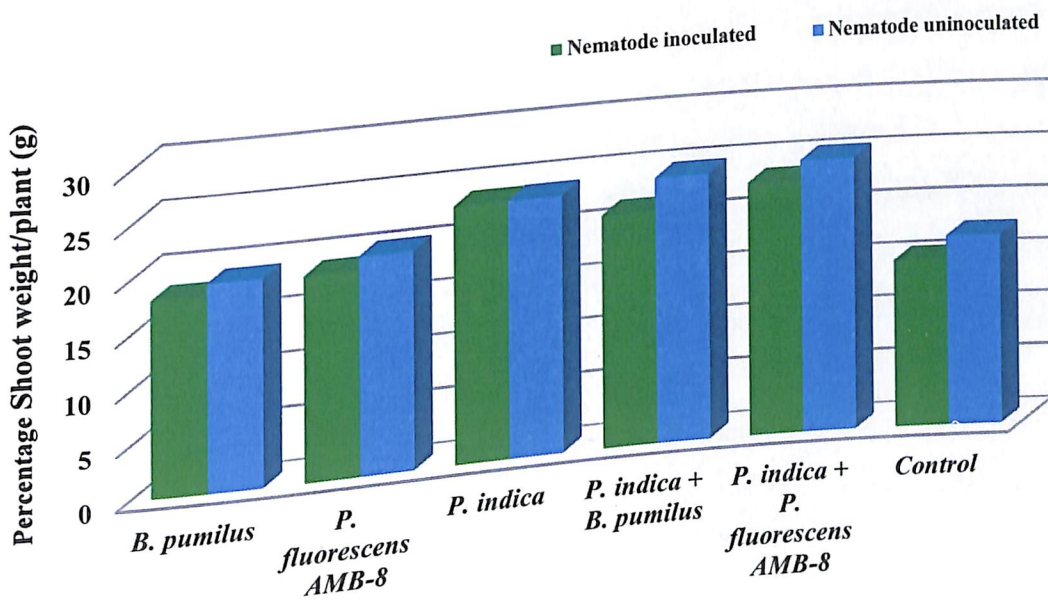
**Fig 18.** Fresh shoot weight/plant (g).

without nematode. From the results of the pot culture experiment it was clear that all the treatments had positive influence on the plant growth.

All the treatments caused a significant increase in the growth of plants in nematode inoculated plants than the control plants. *P. indica* treated plants showed a better performance even in the presence of nematode pest. Varma *et al.* (2012) reported that root colonization by *P. indica* results in an increase in plant growth, early flowering, higher seed yield and alteration in the secondary metabolites,. *P.indica* is shown to have enormous bioprotective potential against plant pathogens and pest of agricultural crops. This result might be supported by the presence of compounds of microbial origin that are reported to have toxic effect on plant parasitic nematodes including avermectins, valinomycins, volatile metabolites such as ammonia and various organic acids (Bansal and Bajaj, 2003). These antinemic compounds disrupt various biological activities of the nematode necessary for the successful plant- nematode interaction, such as embryogenesis, hatching (Neipp and Becker, 1999; Bansal and Bajaj, 2003), juvenile movement through soil, attraction to roots (Neipp and Becker, 1999), recognition of host and penetration in the roots (Becker et al., 1988; Neipp and Becker, 1999).

The combination treatments of *P. indica* and *P. fluorescens* AMB8 and *P. indica* alone were having good yield attributes in the absence of the nematode pathogen. Single inoculation of *P. indica*, combination of *P. indica* and *P. fluorescens* AMB 8 , *P.indica* and *B. pumilus* were better in all the parameters than the single inoculation of rhizobacteria both in the presence and absence of the nematode pest. This result are in accordance with the findings of Anith *et al* ( 2015) that co inoculation of *P. indica* and *B. pumilus* was more efficient than single inoculation of the bioagents for improving seedling growth in Tomato. However this trend was not followed for every parameter in the present study. Combination of beneficial organisms is advantageous than applying them individually. However *P. indica* treated plants were performing better in the presence of nematode pest.

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Negative influence of single inoculation of *P. striata* and *P. indica* in plant growth and yield was reported whereas combined inoculation was beneficial for plant growth (Meena *et al.*, 2010). The results showed that higher shoot length was observed for plants treated with *P. indica* in both nematode inoculated and uninoculated treatments with the maximum value for uninoculated plants. Analyzing the fresh shoot weight of nematode inoculated and uninoculated plants revealed that in the absence of nematode pathogen, *P. indica* and *P. fluorescens* AMB8 combination showed higher shoot weight compared to that in the presence of the nematode pest. Whereas *P. indica* treated uninoculated plants showed nearby value to that of nematode inoculated plants indicating the potential of the endophytic fungus in improving plant growth even in the presence of nematode (Fig 18). This proved the ability of the fungus in growth promotion during biotic stress. Higher dry shoot weight and fresh root weight were observed for combination of *P. indica* and *P. fluorescens* (Fig 19). This could be due to the synergestic plant growth promoting effect of *P. indica* and *P. fluorescens* AMB8 eventhough they were showing negative result in compatibility test. Also *P. indica* treated plants recorded a higher number of flowers, number of fuits, and higher yield in the case of nematode inoculated and un inoculated plants (Fig. 20). This *in vivo* study introduces *P. indica* as a plant growth promoter and *M. incognita* suppressor in tomato under controlled conditions.

Analysis of nematode reproduction in tomato plants revealed the potential of the root endophytic fungus *P. indica* in managing the penetration and development of *M. incognita* in plant and soil system. The combination of *P. indica* with *B. pumilus* and *P. fluorescens* also showed significant reduction in development than the single inoculation of the rhizobacteria. Significant reduction in gall development was observed in plants treated with *P. indica* with a more than four times reduction in galls than control (Fig. 21). All the treatments were able to reduce the gall development. The combination treatments also had significant effect than single inoculation. All the treatments were effective in reducing the number of egg mass per
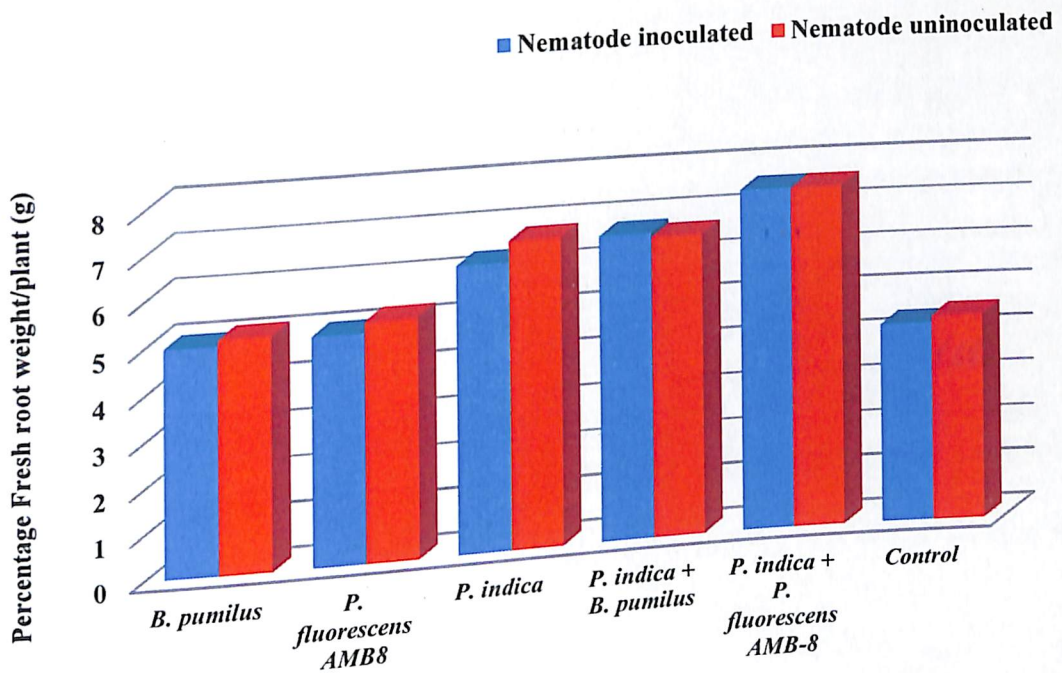
**Fig.19.** Fresh root weight (g).
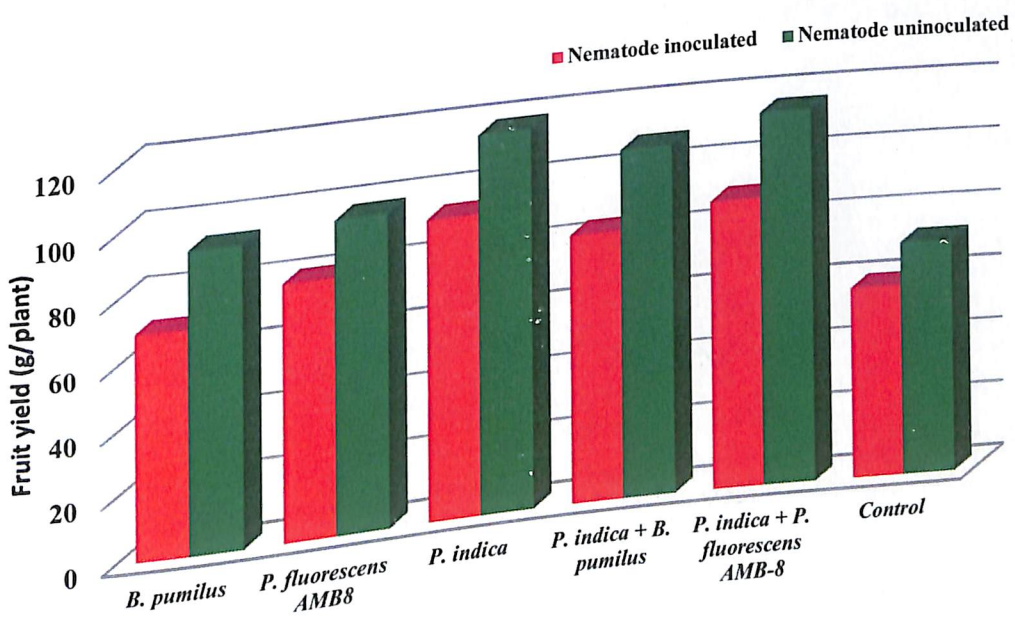
**Fig 20.** Yield/plant (g).
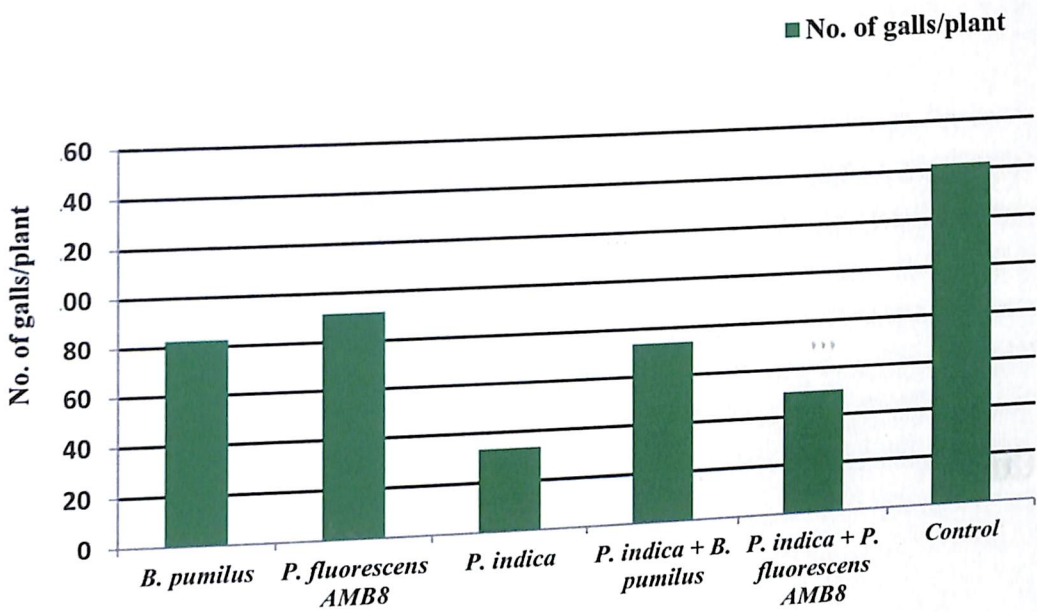
**Fig 21.** Number of galls/plant.
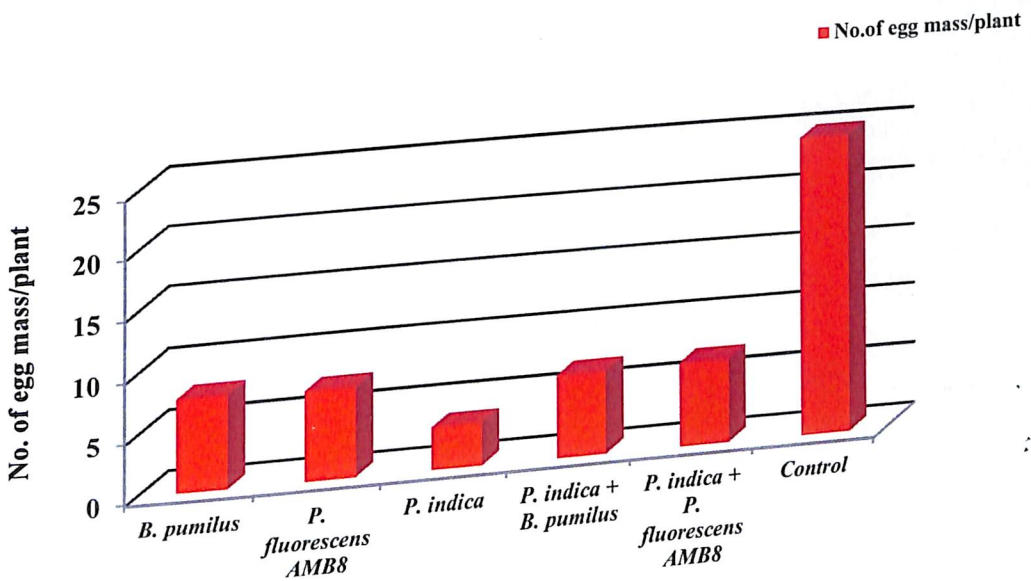
**Fig 22.** Number of egg mass/plant.
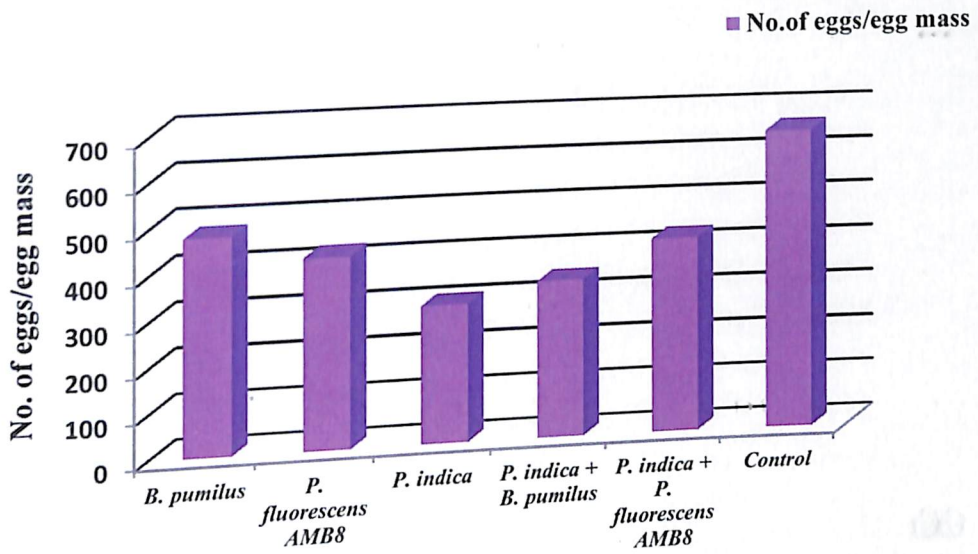
**Fig 23.** Number of eggs/egg mass.
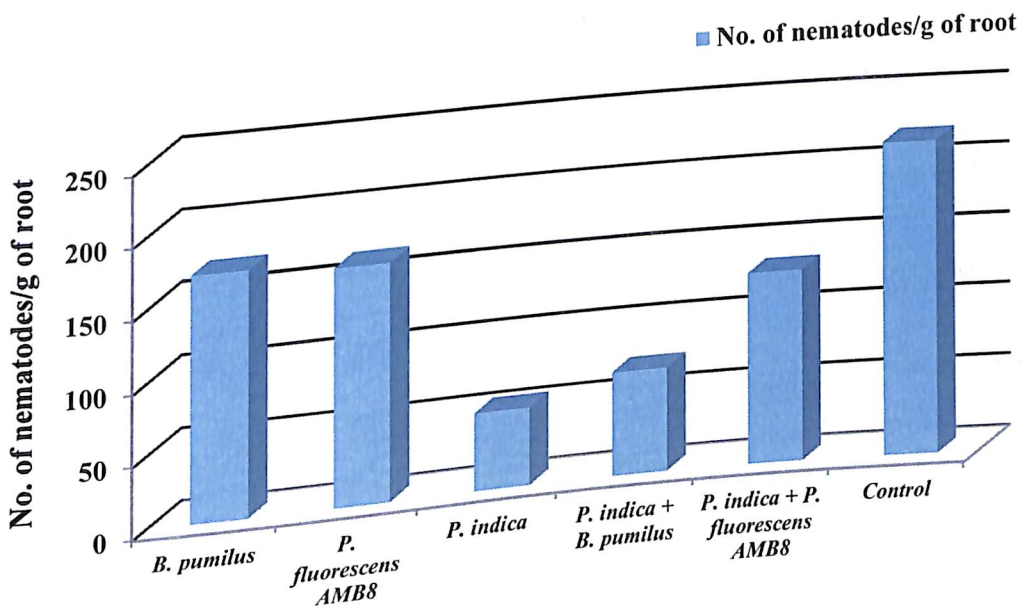
**Fig 24.** Number of nematodes/g of root.

plant. Eight times reduction in number of egg mass was noticed in *P. indica* treated plants (Fig. 22). *P. indica* also significantly reduced the number of eggs compared to other treatments and control (Fig 23). Nematode development inside the root tissue was also highly affected by the endophytic fungus (Fig. 24). Final nematode population showed about five times reduction than that of control (Fig 25.).This nematode antagonistic activity might be due to the production of secondary fungal metabolites and enzymes such as chitinase that are reported to act against plant-parasitic nematodes (Shinya *et al.*, 2008). Except for the direct nematicidal activity of fungus derived compounds, endophytic fungi are able to produce large amounts of toxic chemicals *in vitro* (Vu, 2005). Thus they may also secrete such compounds to the surrounding environment when growing *in planta*. Several studies have elucidated that fungal endophytes may alter chemical properties of root exudates or may stimulate plants to produce chemicals or hormones which repel or disturb nematode attraction (Shahasi *et al.*, 2006; Dababat and Sikora, 2007a; Lee *et al.*, 2009).

Apart from the infection, nematode development and reproduction were significantly affected by *P. indica* colonization. Reduced nematode infection might have lead to a decline in number of galls and development of adults. The result indicated that not only infection and migration of J2s but also later stages of nematode development were affected by *P. indica*. In the present study, a delayed penetration and development of *M. incognita* in tomato treated with *P. indica* was observed. However, since the plants were not grown in a split-root system, this response could not be correlated to the plant defence.

Developmental stages of the nematode inside the plant system showed clear variations according to the treatments. After 20 days of inoculation under developed females with eggs were observed in *P. fuorescens* treated plants. This result is in accordance with the findings of Kalairasan *et al.* (2008) that *P. fluorescens* treated root sections showed limited occurance of hypertrophic cells with immature female
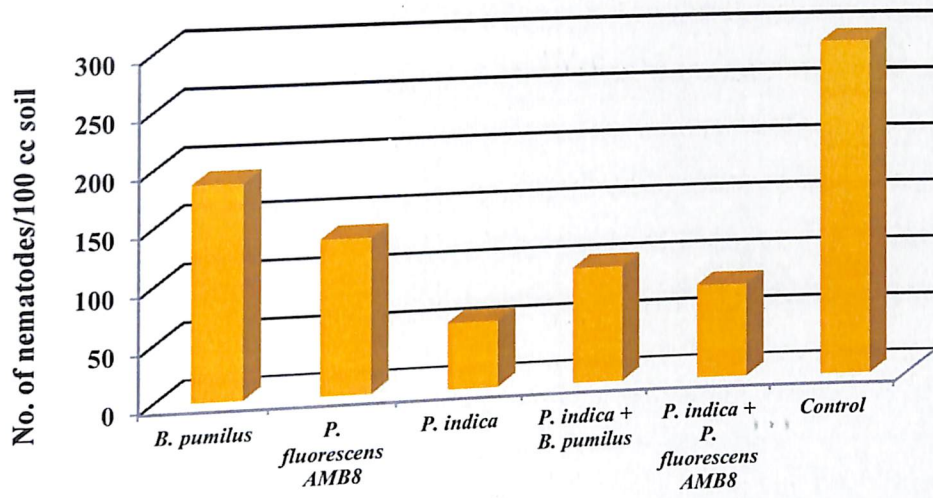
**Fig 25.** Final nematode population in soil.

nematodes and adults trapped in cortical region in ground nut. Other studies also reported that suppression of nematode multiplication by *P. fluorescens* was due to its capability of altering root exudates which could alter nematode behavior and reduced nematode population in root system as reported by Oostendrop and Sikora, (1989). The defence enzymes cause biochemical and physiological changes which are directly inhibitory to nematodes (Paul and Kumar, 2003). Jonathan *et al.* (2009) reported the inhibiting effect of consortium of *P. fluorescens* and *B. subtilis* against root-knot nematode, *M. incognita* in tomato.

B. pumilus treated plants exhibited a normal lifecycle. *Bacillus* isolates were reported to cause improved growth of tomato and reduction in galling. Singh and Siddique, (2010) reported that *Bacillus* spp. cause a greater increase in plant growth and better reduction in nematode multiplication. The combination treatments found to extend the life cycle of *M. incognita*. However the nematode multiplication rate and galling was less compared to single inoculation of the rhizobacteria. Earlier reports by Siddique and Mahmood (1999) reported the capability of *Bacillus* spp. inhibiting the pathogen by diffusible or volatile products, induction of resistence in plants and aggressive root colonization and stimulation of plant growth.

The results revealed that root endophytic fungus *P. indica* is an effective biocontrol agent against *M. incognita* and is a plant growth promoter under biotic stress conditions in tomato.

# Summary

# 6. SUMMARY

Biological management of diseases and pest is a need based strategy for the subsistence of sustainability in crop production. Moreover sustainable development is the masterful balance of meeting our own needs without jeopardizing future generation's ability to do the same. *Meloidogyne incognita* is one of the major parasites of tomato causing dramatic yield loss across world. Most of the chemical nematicides failed to maintain the farming practices without compromising the environmental purity. Here comes the importance of biological agents, which potentially improve plant growth and suppresses the biotic stress and work in harmony with the other beneficial flora and fauna of the soil system. Many biocontrol organisms such as plant growth promoting rhizobacteria and antagonistic fungi have been tested for their ability to control *M. incognita* and stimulate plant growth both in *in vitro* and *in vivo* conditions

The present study envisages the capacity of the root colonizing endophytic fungus *Piriformospora indica* and its interaction with other beneficial rhizobacteria in improving plant growth and controlling nematode infection. The fungus has been reported to have plant growth promotional effect by eliciting defence response in plants, improve the uptake of nutrients like phosphate and nitrate by different mechanisms, plays a role in management of many diseases and pest, and tolerate abiotic stresses like drought, low temperature and salt stress. The present study evaluated the effect of this fungus and rhizobacteria in managing *M. incognita* and improving plant growth in *in vitro* and *in vivo* situations. The salient results of the study are summarized below.

Rhizobactria and their cell free extracts were screened for juvenile mortality and egg hatching of M. incognita. CFE of all the rhizobacteria was found to influence the *M. incognita* juvenile mortality and egg hatching. The effects varied with the

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exposure time and concentration of the CFE used. After 24 hours of incubation *B. amyloliquefaciens* showed the highest mortality among the tested ones. The least was for *P. fluorescens* PN026 in full strength concentration. *P. fluorescens* AMB 8 was with the minimum highest hatching inhibition (22.33 %) followed by B. amyloliquefaciens (22.66%) after 72 hours of incubation in full strength concentration. *M. incognita* juvenile mortality was less influenced by rhizobacterial cell suspension and among the treatments highest mortality was registered by *B. amyloliquefaciens* with only 8.56 percentage juvenile death, though the percentage egg hatch was affected by the rhizobacterial cell suspension. After 72 hours of incubation lowest hatching was observed for *P. fluorescens* AMB 8 (30.00%) and *B. amyloliquefaciens* (30.66%). From the *in vitro* screening it was evident that maximum juvenile death was recorded by *B. amyloliquefaciens* for both CFE and rhizobacterial suspension. Among the treatments, egg hatching was inhibited mostly by *P. fluorescens* AMB 8 and *B. amyloliquefaciens* for both CFE and rhizobacterial cell suspension.

Analysis of the effect of *P. indica* CFE on *M. incognita* juvenile mortality and hatching revealed that it had antagonism against egg hatching and affected the juvenile mortality and the values were very near to the best rhizobacterial treatment in both the cases. There was no direct penetration of the fungus and therefore no egg parasitism was observed. However a few eggs were found to take the stain and chlamydospores were adhered to the eggs.

Dual culture plate assay conducted for understanding the *in vitro* interaction revealed that *B. pumilus* (in CWA and PDA) and *P. fluorescens* AMB 8 (in CWA) showed no antagonism against *P. indica*. These compatible rhizobacteria were selected for the *in vivo* pot culture experiment.

All the plants treated with *P. indica* were colonized by the fungus and the presence of chlamydospore in cortical region of the plant root was observed by light

microscopy. Above 50 percentage colonization was observed for plants treated with *P. indica* and its combination with the selected rhizobacteria where maximum colonization accounted for plants treated with combination of *B. pumilus* and *P. indica* in nematode uninoculated plants. In the presence of nematode percentage root colonization was found to be increased in all the treatments and highest root colonization was observed for combination of *P. indica* and *P. fluorescens* AMB8 treated plants.

*In vivo* pot culture experiments revealed that the results varied with the nematode inoculated and un-inoculated plants for the same treatment. All the biometric parameters were with higher values in the absence of the nematode pathogen. However in the presence of nematode, the treatment *P. indica* alone was found to produce better yield attributes. Since the endophytic fungus gives good yield in the presence of nematode pathogen the study confirms the capability of the fungus for growth promotion and stress tolerance.

Study of developmental stages of *M. incognita* in tomato plants revealed that *P. indica* treated plants were not infected by the nematode upto fifteen days of inoculation. The life cycle was found to be shortened to ten to fifteen days. In *P. fluorescens* AMB8 treated plants underdeveloped females with eggs were noticed within twenty days of inoculation. Combination of *P. indica* with *B. pumilus* and *P. fluorescens* AMB8, revealed that the life cycle was found to be extended beyond thirty days with a few developing and mature females after thirty days of inoculation.

The present study introduces *P. indica* as a potential antagonist against the root knot nematode *M. incognita* and a biocontrol agent against the nematode infection and development in tomato. Future identification of fungal derived compounds and exact composition of CFE might be helpful in commercial agriculture for developing formulated products. Understanding the genetic mechanisms of upregulation of genes and protiens involved in nematode inhibition will enlighten the knowledge on basis of biocontrol mechanism by the endophyte.

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# Appendices

# 8. APPENDIX-I

## 8.1 MEDIA COMPOSITION

### 8.1.1 Nutrient agar

| | |
|---|---|
| Glucose | -5.0 g |
| Peptone | -5.0g |
| Beef extract | -3.0g |
| NaCl | -5.0g |
| Agar | -20.0g |
| Distilled water | -1000 ml |
| pH | -7 |

### 8.1.2 King's B agar

| | |
|---|---|
| Protease peptone | -20.00g |
| Dipotassium hydrogen phosphate | -1.500g |
| Magnesium sulphate heptahydrate | -1.500g |
| Agar | -20g |
| Glycerol | -15 ml |
| Distilled water | -1000 ml |
| pH | -7 |

### 8.1.3 Coconut Water Agar

| | |
|---|---|
| Coconut water | -1000 ml |
| Agar | -20g |
| pH | -7 |

### 8.1.4 Potato Dextrose Agar

| | |
|---|---|
| Potato | -200g |
| Dextrose | -20g |
| Agar | -20g |
| Distilled water | -1000 ml |
| pH | -7 |

"PLANT GROWTH-PROMOTION AND ROOT KNOT
NEMATODE MANAGEMENT IN TOMATO BY
*Piriformospora indica* AND RHIZOBACTERIA."

by

SHILPA VARKEY

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ABSTRACT

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# ABSTRACT

The study entitled "Plant growth-promotion and root knot nematode management in tomato by *Piriformospora indica* and rhizobacteria" was conducted at the Department of Agricultural Microbiology College of Agriculture, Vellayani during the period 2014-2016 with the objective to assess the potential of the root endophytic fungus *Piriformospora indica* and plant growth-promoting rhizobacteria in improving plant growth and suppressing root knot nematode infestation in tomato.

The experiment was conducted as *in vitro* and *in vivo* studies involving interaction assay between the biological agents and the nematode pest, and *in vivo* pot culture studies. Egg hatching and $J_2$ mortality as influenced by treatment with rhizobacteria and their cell free extracts (CFE) were analyzed. Cell free extracts of rhizobacteria significantly reduced egg hatching and increased the mortality of *Meloidogyne incognita* juveniles. After 72 hours, the minimum egg hatching was with CFE of *Pseudomonas fluorescens* AMB8 (22.33 %) followed by *B. amyloliquefaciens* (22.66 %) and the maximum was with *B. subtilis* (27%). Juvenile mortality was highly influenced by the cell free extracts of all the isolates. Higher rate of mortality was observed with undiluted CFE than the diluted ones. Mortality percentage ranged from 68.21% to 99.71% with highest accounted for CFE of *B. amyloliquefaciens*. Treatment with undiluted CFE of *P. indica* resulted in mortality of the $J_2$ by 98% and egg hatching was reduced to 23.33%.

All the rhizobacterial strains ($1\times10^8$ bacterial cell/ml) negatively influenced the egg hatching (from 30 to 50%) as compared to the control (99 % hatching). Upon 72 hours of incubation, there was only 30 % egg hatching in the case of egg masses treated with *Pseudomonas fluorescens* AMB8 and in the case of *Bacillus amyloliquefaciens* it was 30.66 per cent. However, reducing the cell concentration in

the test suspension of all the rhizobacterial strains had positive effect on egg hatching. Treatments with cell suspension of all rhizobacterial strainsshowed significantly low mortality of J₂ juveniles, with highest mortality for *B. amyloliquefaciens* (8.56 %) indicating less influence by the rhizobacteria as such.

*Piriformospora indica* showed no egg parasitism. Compatibility between *Piriformospora indica* and rhizobacteria was assessed by dual culture plate assay under *in vitro* condition and it was found that *B. pumilus* and *Pseudomonas fluorescens* AMB8 were compatible with the fungus though the test medium differed. The compatible bacteria as single inoculation and combination with *P. indica* were used for *in vivo* analysis.

Pot culture experiments using sterile planting medium with single inoculation of the selected rhizobacteria, *Piriformospora indica*, and their combinations in the presence or absence of nematode were carried out. All treatments with *P. indica*showed root colonization by the fungus.In the presence of nematode, percentage root colonization was found to be increased and the highest was for combination of *P. indica* and *P. fluorescens* AMB8. Higher biometric characteristics were observed with nematode untreated plants for all parameters. In the nematode inoculated treatments, application of combination of *P. indica* and *P. fluorescens* AMB8 had positive effect on leaf number, fruit number, shoot weight and root weight when compared to the control. Treatment with *P. indica* resulted in formation of less galls/plant (33.30), egg mass/root system (3.41), eggs/egg mass (306.405), number of nematodes/g of root (54.415) and final nematode population/100cc soil (58) as compared to the control plants, where the values were 142.24, 24.91, 663.54, 220.57, 294.16 respectively.

The study established the biocontrol potential of *P. indica* against root knot nematode in tomato. Application of *P. indica* in the protray seedling production ensures better performance in terms of plant growth and suppression of root knot nematode infection in the main field.