BIOLOGICAL CONTROL OF PLANT PARASITIC NEMATODES OF SPICES

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By

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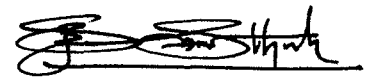
2003

DECLARATION

I, Santhosh J. Eapen, hereby declare that the thesis entitled 'Biological control of plant parasitic nematodes of spices' submitted for the award of Doctor of Philosophy under the Faculty of Science, University of Calicut, is an authentic record of the work done by me under the guidance of Dr. M. A. Haq, Professor, Department of Zoology, University of Calicut. No part of this thesis has been submitted earlier to any University for the award of any degree or diploma.



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CERTIFICATE

This is to certify that the thesis entitled 'Biological Control of Plant Parasitic Nematodes of Spices', submitted by Mr. Santhosh J. Eapen, for the award of degree of Doctor of Philosophy in Zoology under the Faculty of Science of the University of Calicut, is a *bona fide* record of work carried out by him under my supervision and guidance. No part of this thesis has been submitted earlier for the award of any other degree, diploma or other similar title or recognition.

He has successfully completed the qualifying examination prescribed by the University of Calicut as part of the Ph.D. programme.

31.3.03

12 March 2003

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Chapter 1

Introduction

Spices, mostly grown in tropical and sub-tropical countries, are aromatic and pungent vegetable substances that add zest, flavour, or excitement to foods or beverages. The word 'spice' is derived from the Latin word *species*, meaning specific kind, goods or merchandise. Spices are aromatic dried roots, rhizomes, bark, buds, seeds, berries or other parts of plants. They get their characteristic odour from volatile constituents present in the plant materials. Exotic and aromatic spices give us pleasure in our enjoyment of food. They are invaluable in folk medicines and modern medications. Spices are used for seasoning and preserving food, besides their use in confectionaries, medicines, perfumes and cosmetics. Spices have played a major influence in our lives and in the economic development of many countries for centuries.

India is the 'home of spices' with an array of spices originated and cultivated in different parts of the country. It is a major producer and exporter of spices and spice products viz. black pepper (*Piper nigrum* L.), cardamom (*Elettaria cardamomum* Maton), ginger (*Zingiber officinale* Rosc.), turmeric (*Curcuma longa* L.), chillies (*Capsicum* spp.) and several seed and tree spices. At present about 2.50 million ha of area is under spice cultivation in India producing about 3.02 million MT of spices annually. The country exported about 0.23 million MT of various spices valued at Rs. 1,612 crore during 2000-01. Spices play a crucial role in the agricultural economy of India by contributing about 19.37% of the total export earnings of all agricultural commodities. Spices face a variety of production constraints. The menace due to innumerable pests and diseases causing enormous loss in quantity and quality of spices is a very serious constraint.

Among the various pests and diseases of spices, the damage caused by plant parasitic nematodes is quite serious. Plant-parasitic nematodes are important pathogens on most

food, horticultural and fiber crops and without appropriate controls will cause loss of yield and quality. Approximate yield losses due to plant parasitic nematodes have been estimated to be $ 100 billion worldwide each year. Crops such as banana, citrus, coconut, coffee, cotton, fruits, peanut, pineapple, potato, rice, soybean, tobacco, vegetables, ornamentals and turf all over the world are severely affected by these microscopic organisms. The extent of nematode damage to a particular crop is influenced by several factors of which the host plant and variety used, nematode species involved, level of infestation and environmental impact are important.

The symptoms of nematode attack on plants are observed both on shoot and/or roots. Root lesions, galling, abnormal branching and cessation of root growth are common nematode-induced symptoms on root systems. Such roots cannot efficiently absorb from the soil and translocate water and nutrients. Consequently, symptoms of nutrient deficiency such as yellowing of foliage, wilting and stunting may appear. Nematodes rarely kill plants outright, but cause a gradual decline in the crop. They often check crop productivity by predisposing plants to attack by fungi and bacteria and by serving as vectors of a number of plant viruses. Nematodes are also known to break down the resistance to fungal and bacterial pathogens in many crops.

Plant parasitic nematodes belonging to 30 genera and 54 species are reported in black pepper, all over the world. Out of these, only 14 genera of plant parasitic nematodes are generally associated with black pepper in Kerala and Karnataka. However, among these only very few species like *Meloidogyne incognita, Radopholus similis, Trophotylenchulus piperis, Rotylenchulus reniformis* and *Helicotylenchus* spp. are predominant in India. The slow decline disease which is widely prevalent in black pepper gardens is mainly caused due to infestation by *R. similis* and

Meloidogyne spp. (Plate 1. A). In cardamom, though plant parasitic nematodes belonging to 20 genera are reported, the most important and more widely distributed are *R. reniformis*, *R. similis, Pratylenchus coffeae, Meloidogyne* spp. and *Helicotylenchus* spp. Similarly, plant parasitic nematodes belonging to several genera are reported on ginger, turmeric, and seed spices like coriander, fennel, fenugreek, celery, cumin and dill. But root-knot nematodes attack all these crops and are of much economic importance than others. Therefore, in a tropical country like India, where spices are generally cultivated, only root-knot nematodes (*Meloidogyne* spp.), burrowing nematodes (*R. similis*) and lesion nematodes (*Pratylenchus* spp.) can be considered as the most important plant parasitic nematodes (Plate 1).

In spices, root-knot nematodes represent an important fraction as they infest almost all crops in this category. Several species of root-knot nematodes viz. *M. incognita, M. javanica, M. arenaria, M. hapla* and *M. piperi* are reported in many spice crops. Among them, *M. incognita* is the most commonly and widely distributed species in most of the spices and causes 37 to 46% reduction in growth and yield of black pepper. These plants show foliar yellowing with interveinal chlorosis. They cause typical galls in black pepper roots (Plate 1.C). Apart from black pepper, they pose serious problems in cardamom nurseries and plantations (Plate 1.D). *M. incognita* is widely distributed in ginger fields and causes 46.4% to 74% reduction in rhizome weight. Significant reductions in growth and yield are noticed in turmeric plants infested with root-knot nematodes (Plate 1.E). It is also well known that they cause severe yield losses in seed spices such as coriander, cumin and fennel.

Burrowing nematodes (*R. similis*) and lesion nematodes (*Pratylenchus* spp.) are two other important and destructive root parasites of several spice crops (Plate 1.B). Both are

Plate 1

Nematode Problems in Major Spices

Plate 1

A. Symptoms of slow decline disease in a black pepper vine

B. Lesions, rotting and loss of feeder roots due to infestation by *Radopholus similis* in a black pepper cutting

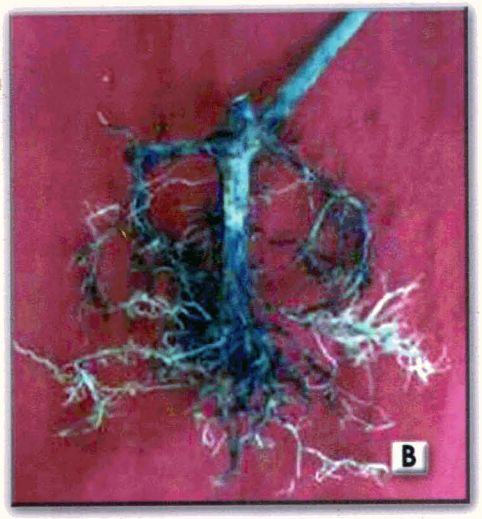
C. Galling and rotting on roots of a black pepper cutting due to infestation by *Meloidogyne incognita*

D. Galling and abnormal branching of roots of cardamom seedlings infested by *Meloidogyne incognita*

E. Turmeric roots depicting galls caused by root-knot nematode (*Meloidogyne incognita*) infestation

F. Dry rot in ginger rhizomes induced by feeding of *Pratylenchus coffeae*


A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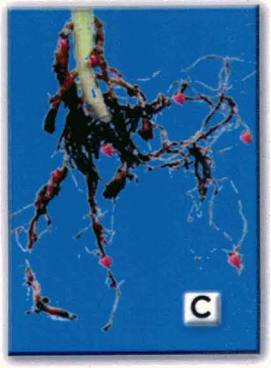
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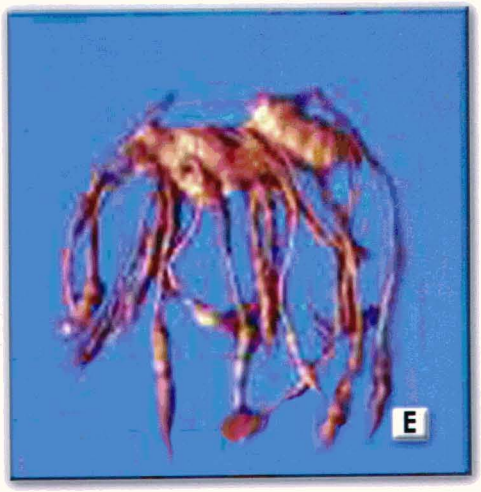
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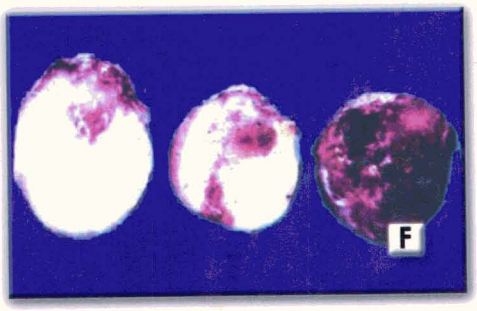
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migratory endoparasites and occur throughout tropical and warm temperate regions, mainly because of man-made dispersal through infested plant materials. They have a wide host range and cause extensive damage to root cortical tissues, but contamination by microorganisms leads to root decay. *R. similis* causes very serious problems in black pepper, ginger and turmeric. The lesion nematodes cause severe damage to roots and rhizomes of ginger and turmeric (Plate 1.F). Spread and distribution of the nematodes are mostly through infested seed rhizomes.

Nematode management is difficult and complicated as no known control method eradicates them in an established garden or plantation. Therefore, the main objective of nematode management is to bring down the nematode population to below economic threshold or non-injurious level in plantations, while eradication should be the goal in nurseries. Efficient management requires the careful integration of several practices. Important components of a nematode management program are physical control, use of resistant cultivars, cropping sequences, chemical control, sanitation and biological control.

Excellent reduction in populations of plant parasitic nematodes can be achieved through use of various heat treatments like steam, hot water and sunlight. Soil solarization has been successfully employed in many crops including some spices, as a pre-sowing treatment to bring down the initial nematode population in a field. Fallowing and flooding are also being used to manage nematode problems in some crops. But all these techniques are employed only under special conditions and are not reliable for economic control of nematodes when used in isolation.

The use of resistant or tolerant cultivars is an economically attractive and effective means of nematode management. Resistant varieties have been developed for only a few crops and these must be used judiciously to reduce the selection pressure for races that are able to break down the nematode resistance. Molecular approaches towards gene transfer and genetic engineering are also successful in a few crops. In spices too resistant lines have been developed against root-knot nematodes through conventional means in some crops. The black pepper variety 'Pournami', 'Mahima', a ginger selection and several turmeric lines are examples for root-knot nematode resistant lines developed in the recent past. But these resistant lines may succumb to nematodes as the nematode population varies from place to place. In the case of *R. similis* and *Pratylenchus* spp. the breeding programmes are not yet successful.

Crop rotation or planting crops in special sequences is an old but effective management practice to reduce nematode populations in the soil. This is effective, provided crops that are resistant or non-hosts for the target nematode are rotated with susceptible crops. In spite of its effectiveness, crop rotation has serious limitations. Rotation alone is limited by logistics like requirements of specific crops, economic feasibility and the host range of the nematode species. The damage inflicted on a crop depends on the degree of resistance in the crops used in the rotation. Further, a non-host or a resistant crop, effective against a specific nematode, may be susceptible to another nematode present in the same field. In the case of perennials like black pepper and cardamom, crop rotation is not feasible.

Chemical nematicides have been the primary management tool for over fifty years. The use of chemicals is very popular and highly effective, if used properly. Chemicals are effective but the application rates needed to kill nematodes in soil are quite high and they are

highly expensive. Complete or over dependence on chemicals is dangerous and no more advisable. Many of these chemicals are proven to be carcinogens, build up residues in food plants, infiltrate into ground water, develop resistance in pest populations, affect non-target organisms, cause secondary pest outbreaks and resurgence of target pests. Moreover, escalating costs of producing and marketing pesticides and the depletion of resources to produce them make them feasible only in highly intensive agriculture ecosystems. Several of these toxic chemicals have been banned and are no more available in the market. Pesticide manufacturers have set nematicide research and development as low priority due to the costs associated with registration and the high risks associated with product development. Considering the fact that 'clean' spices with 'zero' level of pesticides are preferred in world market, the chemical control option is given the lowest priority.

Sanitation covers a wide range of cultural practices such as weed control, crop-residue destruction, use of selective crops as mixed or companion crops, use of nematode-free planting materials etc. Though not very powerful, these tactics, when used along with other techniques, can bring down the nematode incidence significantly.

Unfortunately, in spices, still chemical nematicides have been the primary option for spice growers for managing nematodes. There is an urgent need for the development of a non-chemical and eco-friendly control option. Such work is necessary to develop environmentally sound agricultural systems that minimize chemical use while maintaining high production standards. The tropical soils are rich in biodiversity of beneficial microbes and the biocontrol potential of the resident microbial fauna and flora is under exploited. Suppression of plant-parasitic nematodes with nematode predators, parasites or disease agents is a desirable alternative to chemicals. This emerging area is called biological control. In a nematological

perspective, biological control is the reduction of nematode populations, which is accomplished through the action of living organisms other than nematode-resistant host plants, which occur naturally or through the manipulation of the environment or the introduction of antagonists. The mechanisms involved in biological control of nematodes are mainly hyper-parasitism, predation, competition and antibiosis.

Hundreds of organisms, which parasitize or prey on nematodes, are reported and they belong to diverse taxa including nematode trapping or endoparasitic fungi, predatory nematodes, arthropods (e.g. mites and collembola), bacterial parasites, and predatory protozoa. Deploying and managing biocontrol agents will likely become increasingly important components of integrated pest management programs and sustainable agricultural systems.

As in spices, root-knot nematodes are widely distributed and globally important and therefore intensive efforts are being taken to manage them through biocontrol agents. Root-knot nematodes are sedentary endoparasites and remain deep within the root tissues. The endoparasitic habit has proved to be a major impediment to the development of successful biological control. Besides, the eggs are laid in egg masses, embedded in gelatinous matrices, which harden and shrink to protect eggs from desiccation and other microbial pathogens. The second stage juveniles (J2) are generally seen in soil and their population fluctuates according to soil edaphic factors. By invading roots the nematode juveniles can escape from microbial parasites. Therefore, instead of the active juveniles, the eggs that are generally located on the root surface and that take at least ten days to develop and hatch, can be the targets. The hydrated matrix of mucopolysaccharide and protein acts as an additional nutrient source for some parasites and may facilitate parasitism. Being migratory

endoparasites, burrowing and lesion nematodes are not ideal target nematodes for most of the biocontrol agents. Therefore very little success has been reported on the biological management of these two nematodes.

Biological control is yet to emerge as a viable option to manage nematodes affecting spices. There is tremendous scope to exploit the rich microbial diversity of tropical soils to develop efficient biological control programmes for controlling nematodes. Most of the spices had their origin in Indian peninsula. Therefore it is quite natural that specific pests of these crops might have evolved in this region. Antagonists specific to these pests too should be present in the same biological niche. New biological control agents have to be found out by surveying areas where the target pest is indigenous and in soils where known pests appear to cause little damage. Moreover several spices like black pepper and cardamom are perennial crops and chances of locating efficient antagonists are high in such tropical agro ecosystems. Therefore this study was undertaken to identify and develop potential biological control agents for the management of plant parasitic nematodes, especially root-knot nematodes, affecting spice crops.

Chapter 2

Review of Literature

Studies on biological control of plant parasitic nematodes of spices are still in infancy. In general biological control of nematodes is less developed in India and it is more so in the case of spices. Very few reports are available in literature in which biological control is used as the primary and successful means for managing nematodes of any spice crop. Isolated efforts in this direction have been compiled recently (Eapen & Ramana, 1997). Various aspects of biological control of plant parasitic nematodes have been reviewed by several workers (Mankau, 1981; Jatala, 1986; Kerry, 1987, 1990; Jairajpuri *et al.*, 1990; Stirling, 1991). Therefore, in this review, an attempt is made to mention about various bacterial and fungal antagonists of plant parasitic nematodes, their modes of action and their biocontrol potential under in vitro and in vivo conditions and to assess their current status with special emphasis on root-knot, burrowing and lesion nematodes, the major nematode pests of spice crops.

A. Nematode Antagonists

a. Bacteria

Several bacteria were reported to have inhibitory effects on nematodes, but species belonging to *Pasteuria* are the only obligate parasites of nematodes (Poinar & Hansen, 1986; Sayre & Starr, 1988). The first report of an organism resembling *Pasteuria* sp., infecting a nematode was on *Dorylaimus bulbiferus* (Cobb, 1906). Later a parasite from *Pratylenchus pratensis* was described as a protozoan namely *Duboscqia penetrans* (Thorne, 1940). *D. penetrans* obtained from infected females of *Meloidogyne* spp. was subsequently related to bacteria rather than protozoa through electron microscopic studies and was named as *Bacillus penetrans* (Mankau, 1975a, 1975b). Because of the resemblance of *B. penetrans* with *Pasteuria ramose*, a parasite of water fleas, it was renamed as *P. penetrans* (Sayre & Starr, 1985). Soon it became apparent that the bacterium constituted an assemblage of numerous pathotypes and those that parasitized primarily root-knot nematodes were called *P. penetrans*. A second species, which parasitized the root-lesion nematode *P. brachyurus*, was named *P. thornei* (Starr & Sayre, 1988). Another isolate that multiplied on cyst nematodes was called *P. nishizawae* (Sayre *et al.* 1991a, 1991b).

Members of the *P. penetrans* group were found to infest 323 nematode species in about 116 genera from 80 countries (Sturhan, 1988; Chen & Dickson, 1998). Some isolates of *Pasteuria* spp. displayed cross-generic host ranges and varying biological characteristics (Bhattacharya & Swarup, 1988). On the contrary, spores isolated from *Meloidogyne* spp. did not attach to any other genera of plant parasitic, animal parasitic or free-living nematodes (Gives *et al.*, 1999b).

Several root-colonizing bacteria were reported to inhibit the development of plant parasitic nematodes (Zavaleta-Mejia & vanGundy, 1982; Becker *et al.*, 1988; Siddiqui & Husain, 1991b). These included several coryneform and Gram-negative bacteria like *Serratia* (Zavaleta-Mejia & vanGundy, 1989), *Pseudomonas* spp. (Oostendorp & Sikora, 1989, 1990; Spiegel *et al.*, 1991; Kloepper *et al.*, 1992a; Kluepfel *et al.*, 1993), *Bacillus* spp. (Becker *et al.*, 1988), *Enterobacter cloacae* (Duponnois *et al.*, 1999), and many others from egg masses of *M. incognita* (El-Sherif *et al.*, 1995). A novel *Streptomyces* sp. was isolated from nematode suppressive soils in Costa Rica (Dicklow *et al.*, 1993).

Second stage juveniles of *M. javanica* were disorientated in the presence of the bacterial symbiont, *P. oryzihabitans* of the entomopathogenic nematode, *Steinernema abbasi* (Samaliev *et al.*, 2000).

b. Fungi

Several reviews have been published exclusively on the fungal antagonists of nematodes (Barron, 1977; Kerry, 1984; Gray, 1987, 1988; Jansson & Nordbring-Hertz, 1988; Morgan-Jones & Rodriguez-Kabana, 1988). Interest in biological control of nematodes was elicited as soon as plant parasitic nematodes were recognized as pests. Fungi belonging to widely divergent orders and families, from Phycomycetes to Basidomycetes, were reported as antagonists of nematodes (Mankau, 1980). The existence of fungi that trap and prey on nematodes was noticed quite early (Zopf, 1888).

Almost after one decade, the use of predacious or nematode-trapping fungi to control root-knot nematodes of pineapple was first attempted in Hawaii (Linford, 1937; Linford *et al.*, 1938), which stimulated interest among several workers in nematode-trapping fungi. Several reviews were published accounting apparently, over 100 species of these fungi that attack nematodes and the major genera were *Arthrobotrys, Dactylaria, Dactylella,* and *Monacrosporium* (Duddington, 1954, 1962; Christie, 1960; Sayre, 1971; Barron, 1977; Mankau & McKenry, 1976; Mankau, 1980; Esser, 1983; Gray, 1987, 1988; Persson & Jansson, 1999). Majority of nematode-trapping species were reported to be fairly ubiquitous, but their population levels in soil were quite low, as they exist in soil as mycelium rather than as spores (Gray, 1987, 1988). Another group was fungal endoparasites of vermiform nematodes belonging to Chitridiomycete (*Catenaria anguillulae*), Oomycetes (*Myzocytium humicola*), Zygomycete (*Meristacrum asterospermum*), Deuteromycete (*Harposporium anguillulae*), and Basidiomycete (*Nematoctonus* sp.) (Jatala, 1986).

Two obligate parasites of females of cyst nematodes viz. *C. auxiliaries* (Tribe, 1979) and *Nematophthora gynophila* (Kerry & Crump, 1980) were isolated. Several isolates of *Hirsutella rhossiliensis* were screened and most of them showed parasitization of soybean cyst nematode (Liu & Chen, 2001). A large number of species have been recorded as facultative parasites of root-knot nematode females and eggs (Stirling & Mankau, 1978a; Godoy *et al.*, 1982; Morgan-Jones & Rodriguez-Kabana, 1988; Stirling, 1991). The first report on the natural occurrence of *Verticillium chlamydosporium* was from England (Kerry, 1974). Natural parasitism of *Meloidogyne* females and eggs by this fungus has been reported from peanut and soybean fields in Alabama, USA (Morgan Jones *et al.*, 1981b, 1984c; Godoy *et al.*, 1983a). It was also reported from the tropical and subtropical soils in California, USA (Gaspard *et al.*, 1990), in Australia (Stirling & West, 1991) and from black pepper gardens in Kerala (Sreeja *et al.*, 1996). Many species of *Verticillium* have been recorded from eggs of *Meloidogyne* (Godoy *et al.*, 1982; Morgan Jones & Rodriguez-Kabana, 1988). Based on the ecological requirements and

other characteristics, *V. chlamydosporium* sensu lato has been separated into two distinctive types, *V. chlamydosporium* sensu stricto and *V. suchlasporium* (Gams, 1988).

The genus *Paecilomyces*, a close relative of *Penicillium,* was first observed to be associated with nematode eggs in 1976 (Lysek, 1976). Later *P. lilacinus* was found parasitizing eggs of *M. incognita* in Peru (Jatala *et al.*, 1979), in USA (Morgan-Jones *et al.*, 1984c) in Australia (Stirling & West, 1991) and in India too (Khan & Goswami, 2000b). It was also obtained from *M. arenaria* in Alabama, USA (Godoy *et al.*, 1983a). Two other species, *P. marquandii* from 'chinampa' soils (Marban-Mendoza *et al.*, 1992) and *P. fumosoroseus* from Cuba (Cuadra *et al.*, 2000), were equally suppressive of root-knot nematodes.

D. oviparasitica has been the first parasite of *Meloidogyne* eggs to be described (Stirling & Mankau, 1978a, 1978b). It had a limited distribution in the peach orchards and vineyards of California (Stirling *et al.*, 1979) and in a single citrus orchard (Gaspard & Mankau, 1986). Some isolates of *Trichoderma* spp., widely used as antagonists of many fungal diseases in several crops, were found to have good potential as a nematophagous fungus (Saifullah & Thomas, 1996; Spiegel & Chet, 1998; Sharon *et al.*, 2001). *Cylindrocarpon destructans* was frequently found in association with nematode eggs (Freitas *et al.*, 1995). A hyphomycete similar to the genus *Scytalidium* was isolated from black-coloured egg masses of *M. javanica* (Oka *et al.*, 1997). *Beauveria bassiana*, a known insect pathogen, parasitized eggs of *M. incognita* (Saikia, 1998). Recently *Phoma glomerata* and *Curvularia* species were reported to be parasitic on juveniles of *M. javanica* (Varaprasad *et al.*, 1998). *Aspergillus niger, A. terreus, Cladosporium oxysporum, Fusarium oxysporum* and *F. dimerum* (*Microdochium dimerum*) were observed inhibitory to nematodes (Goswami *et al.*, 1998a, 1998b). Among these, *C. oxysporum* parasitized root-knot nematode larvae and eggs.

Destruction of nematode eggs by *Fusarium* species was noted quite early (Lysek, 1963). *F. oxysporum* was isolated from egg masses of root-knot nematodes by several others too

(Godoy *et al.*, 1983a; Odour-Owino & Waundo, 1996). A survey for fungi associated with root-knot nematode egg masses in India showed the highest frequency of occurrence of *F. oxysporum* followed by *F. solani*, *F. pallidoroseum* and *F. moniliforme* (Goswami & Umarao, 1996). Several species of fungi were isolated from parasitized eggs and females of *M. javanica* in Pakistan (Erma & Shahzad, 1998).

B. Mode of Action

Nematode antagonism of various bioagents was studied intensively using relatively simple systems in laboratory bioassays. Nematode trapping fungi used novel predacious organs like adhesive hyphal networks, adhesive knobs, adhesive branches, or constricting rings to capture vermiform nematodes (Sayre, 1971; Stirling, 1991). However, they had no effect on the eggs and endoparasitic stages of root-knot nematodes (Linford & Yap, 1939). An adhesive substance was seen on the internal surfaces of the trapping organs, mainly at the points of contact between predator and prey, including bacteria (B'-chir, 1984; Belder *et al.*, 1996). But inefficient species producing adhesive network traps did not produce traps spontaneously and existed in soil largely as saprophytes (Gray, 1988). More number of traps was produced when these fungi grew from parasitized nematodes (Jaffee *et al.*, 1992). The nematode-destroying capability was found to be dependant on lectins (Rosenzweig *et al.*, 1985; Tunlid *et al.* 1992), extracellular proteases (Tunlid & Jansson, 1991; Persson & Friman, 1993; Tunlid *et al.*, 1994; Bedelu *et al.*, 1998) or toxins produced by these fungi (Balan & Gerber, 1972). Some fungi produced substances that attracted nematodes, as was evident in *A. oligospora* during infection of nematodes, in the form of electron dense microbodies proliferating from the trophic hyphae (Balan & Gerber, 1972; Field & Webster, 1977; Jansson & Nordbring-Hertz, 1979; Veenhuis *et al.*, 1989a, 1989b; Dijksterhuis *et al.*, 1993). Predatory nematodes and mites also employed this mode of action to feed on their nematode preys (Mankau, 1980; Stirling, 1991). However allelopathy has been demonstrated in the

suppression of plant parasitic nematodes by inundative applications of entomopathogenic nematodes (Grewal *et al.*, 1999).

The motile, flagellated zoospores of endoparasitic fungi belonging to Chitridiomycetes and Oomycetes located their nematode hosts through chemotaxis (Jansson & Thiman, 1992). Adhesive polymers helped the fungus to adhere to the nematode cuticle (Tunlid *et al.*, 1991). These zoospores encysted near an orifice in nematode's body and infected the nematode by the germ tubes passing through these orifices or by direct penetration of nematode cuticle. Further the vegetative thalli inside nematode's body differentiated into zoosporangia and zoospores were produced (Tribe, 1977; Gray, 1988). *C. anguillulae* invaded eggs of *M. incognita* at an early stage of embryonic development and not those eggs with juveniles ready to hatch or those that had hatched (Wyss *et al.*, 1992).

The small adhesive conidia or spores of *H. rhossiliensis*, *Drechmeria coniospora* (syn. *Meria coniospora*), *Nematoctonus* etc adhered to nematode's cuticle and penetrated the cuticle by a germ tube and the hyphae that formed inside the nematode rapidly filled the body cavity and broke through the cuticle, bearing conidia (Saikawa, 1982; Dowsett *et al.*, 1982; Jaffee & Zehr, 1982; Bernard & Arroyo, 1990). Nematoxic compounds secreted by the germinating spores caused rapid immobilization and death of nematodes (Giuma & Cooke, 1971; Giuma *et al.*, 1973). Despite their obligate nature, there was little evidence of extreme host specificity among these fungi (Minter & Brady, 1980; Jansson *et al.*, 1985a, 1987). Their spores contained limited food reserves and therefore they could not be cultured *in vitro* (Jaffee & Zehr, 1982; Stirling & Kerry, 1983; Jansson *et al.*, 1985a; Cayrol *et al.*, 1986; Tedford & Jaffee, 1995).

Among the bacterial antagonists of nematodes, *P. penetrans* has been reported as the only obligate parasite of nematodes with a life cycle remarkably well-adapted to parasitism of certain plant nematodes (Mankau & Imbriani, 1975; Mankau & Prasad, 1977; Sayre &

Wergin, 1977; Sayre, 1980; Stirling & Watchel, 1980; Mankau, 1981; Serracin *et al.*, 1997). Different mode of action of *P. penetrans* suggested by various workers included reduction in the number of J2 in soil (Davies *et al.*, 1988; Chen, Z.X. *et al.*, 1997), J2 penetrating roots (Brown & Smart, 1985; Davies *et al.*, 1988; Sekhar & Gill, 1990b), number of females in roots (Davies *et al.*, 1991) and number of eggs on roots (Bird & Brisbane, 1988; Ahmed & Gowen, 1991; Weibelzahl-Fulton *et al.*, 1996; Chen, Z.X. *et al.*, 1997). The spores attached to free-living and parasitic second stage juveniles but not to males and females of root-knot nematodes (Stirling *et al.*, 1986). Proteins, carbohydrates, glycoproteins, protein antigens and a fibronectin were reported as some of the molecules on the surface of either nematode or bacterial endospores that were involved in the attachment of the endospores to the nematode (Davies *et al.*, 1992; Davies & Danks, 1993; Davies, 1994; Davies *et al.*, 1996; Spiegel *et al.*, 1996; Chen *et al.*, 1997).

Among the opportunistic or facultative parasites, *V. chlamydosporium* parasitized both eggs and females of root-knot nematodes (Morgan-Jones *et al.*, 1981; Freire & Bridge, 1985). It formed a branched mycelial network in close contact with the eggshell prior to infection and the lateral branches formed specialized perforation organs or appressoria (Morgan-Jones *et al.*, 1983; Lopez-Llorca & Duncan, 1988; Segers *et al.*, 1996; Saifullah & Thomas, 1997). The fungus caused disintegration of the vitelline layer of the egg shell and partial dissolution of the chitin and lipid layers due to the activity of exoenzymes produced by the fungus (Morgan-Jones *et al.*, 1983; Meyer *et al.*, 1990; Segers *et al.*, 1996).

P. lilacinus parasitized *M. incognita* females by penetration through the anal or vulval openings (Jatala *et al.*, 1979; Freire & Bridge, 1985). The individual hyphae of *P. lilacinus* penetrated the nematode egg by mechanical and/or enzymatic activities (Morgan-Jones *et al.*, 1984a). Occasionally a specialized structure, an appressorium, was associated, and the lipid and chitin layers of nematode eggs were disrupted (Dunn *et al.*, 1982). Eggs of all stages,

including those containing unhatched juveniles were infected by *P. lilacinus* that totally destroyed the egg content (Zaki & Bhatti, 1990b; Holland *et al.*, 1999). *P. lilacinus* colonized roots of plants and was able to maintain close contact with plant parasitic nematodes (Cabanillas *et al.*, 1988; Hewlett *et al.*, 1988; Cabanillas & Barker, 1989). *P. lilacinus* was reported to colonize fungi, insects and even human and animal tissue (Stirling, 1991).

D. oviparasitica produced appressoria on the surface of eggs for mechanical penetration and chitinase for enzyme degradation of egg shells (Stirling & Mankau, 1979). It occasionally parasitized *Meloidogyne* females too (Mankau, 1981). *M. lysipagum* and *M. ellipsosporum*, two nematode trapping fungi, invaded egg masses and preyed on juveniles as they hatch (Esser, 1983; Mankau & Wu, 1985).

In addition to their predacious or parasitic ability, many of the above organisms produced antibiotics or metabolites that disrupted or altered the physiology of nematodes and thereby harmful to plant-parasitic nematodes (Olthof & Estey, 1963; Jansson *et al.*, 1997). Culture filtrates of *Alternaria* sp. (Siddiqui & Husain, 1991a), *A. oligospora* (Walia & Swarup, 1985), *Aspergillus* spp. (Dahiya & Singh, 1985; Vaishnav *et al.*, 1985; Siddiqui & Husain, 1991a; Goswami *et al.*, 1998a, 1998b), *Cunninghamella elegans* (Galper *et al.*, 1991), *D. brochopaga* (Walia & Swarup, 1985), *Fusarium* spp. (Mani & Sethi, 1984; Mani *et al.*, 1986; Siddiqui & Husain, 1991a; Goswami *et al.*, 1998a, 1998b; Nitao *et al.*, 1999; Chen *et al.*, 2000), *Paecilomyces* spp. (Jatala *et al.*, 1985; Cayrol *et al.*, 1989; Caroppo *et al.*, 1990; Siddiqui & Husain, 1991a; Zaki, 1994; Chen *et al.*, 2000), *Rhizoctonia solani* (Singh *et al.*, 1983; Ali, 1989; Sharma & Saxena, 1992), *Sclerotium rolfsii* (Ali, 1989), *Trichoderma* spp. (Singh *et al.*, 1983; Sharma & Saxena, 1992; Pathak & Kumar, 1995; Sankaranarayanan *et al.*, 1997) and *V. chlamydosporium* (Saifullah, 1996) were reported to be toxic to root-knot nematodes. Root dip treatment with culture filtrates of several fungi reduced *Meloidogyne* larval penetration, suppressed nematode reproduction and gall formation on tomato (Khan *et al.*, 1984; Khan & Saxena, 1997).

Culture filtrates of *A. niger, C. tuberculata* and *P. coryophilum,* when used as seed treatments in chickpea, reduced root galling and root rot (Siddiqui & Mahmood, 1995). Culture filtrates from *T. virens* and *Burkholderia cepacia* contained extracellular factors that inhibited egg hatch and second-stage juvenile (J2) mobility of *M. incognita* (Meyer *et al.*, 2000).

Several extracellular proteases have been purified and characterized from nematode antagonists like *A. oligospora* (Tunlid & Jansson, 1991; Ahman *et al.*, 1996), *V. suchlasporium* (Lopez-Llorca, 1990; Lopez-Llorca & Robertson, 1992), *V. chlamydosporium* (Segers *et al.*, 1994, 1996), *P. lilacinus* (Bonants *et al.*, 1995) and *T. harzianum* (Sharon *et al.*, 2001). A novel collagenolytic/proteolytic enzyme isolated from *B. cereus* damaged nematode cuticle by digesting the collagens (Sela *et al.*, 1998). Chitinolytic activity was detected in several species of egg parasitizing nematophagous fungi such as *V. suchlasporium*, *V. chlamydosporium*, *P. lilacinus*, *D. oviparasitica* (Stirling & Mankau, 1979; Godoy *et al.*, 1983b; Dackman *et al*, 1989; Bidochka *et al.*, 1999; Davila *et al.*, 1999) and the bacterium *S. saraceticus* (Lee *et al.*, 1996).

A wide range of nematicidal compounds was purified from crude culture fluids of bacteria and fungi and mycelial extracts. These included several secondary metabolites like toxins, antibiotics and simple compounds like ammonia. Volatile metabolites like ammonia, resulting from general bacterial metabolic activity of *Serratia marcescens*, several *Bacillus* spp. and other soil bacteria, were toxic to nematodes (Becker *et al.*, 1988; Zavaleta-Meija & vanGundy, 1989; Oka *et al.*, 1993; Carneiro *et al.*, 1998). Similarly acetic acid from *P. lilacinus* (Djian *et al.*, 1991), linoleic acid from mycelial extracts of *Arthrobotrys* spp. (Stadler *et al.*, 1993a), milbemycins and dianemycin type polyethers from a strain of *S. hygroscopius* (Whaley *et al.*, 1990), aromatic nitro compounds and griseulin from *Streptomyces* spp. (Nair *et al.*, 1995), phomalactone from *V. chlamydosporium* (Khambay *et al.*, 2000) and trichothecenes from *F. equiseti* (Nitao *et al.*, 1999, 2001) also had nematicidal activity. Antibiotics that had very good antimicrobial activity were isolated from *Arthrobotrys* spp. (Stadler *et al.*, 1993b), *A. niger* (Zuckerman *et al.*, 1994),

P.lilacinus (Isogai *et al.*, 1980, 1981), *F. oxysporum* (Hallmann & Sikora, 1996). Several nematicidal alkaloids like peniprequinolone were isolated from *Penicillium* cf. *simplicissimum* (Kusano *et al.*, 2000). A number of toxins viz. Aspyrone from *A. melleus* (Kimura *et al.*, 1996), Omphalotin A produced by *Omphalotus olearius* (Mayer *et al.*, 1999), T2-toxin, a mycotoxin from *Fusarium* sp. (Ciancio, 1995), avermectins isolated from *S. avermitilis* (Garabedian & van Gundy, 1983), thermostable exotoxins of *B. thuringiensis* (Borgonie *et al.*, 1996a, 1996b; Carneiro *et al.*, 1998) exhibited high nematicidal activity under *in vitro* and *in vivo* conditions. Fungi that produce nematode toxins were reported within wood-decomposing *Pleurotus* and other genera (Thorn & Barron, 1984; Barron & Thorn, 1987). Nematoxic compounds have been reported from other non-traditional nematophagous fungi too (Anke *et al.*, 1995).

Several rhizosphere-inhabiting organisms called plant growth promoting rhizobacteria (PGPR) significantly influenced plant growth and yield (Schippers *et al.*, 1987; Schroth & Becker, 1990). Induced resistance as another mechanism of biocontrol agents to suppress fungal diseases was first reported in cucumber (Gessler & Kuc, 1982). Two types of induced resistance in plants; systemic acquired resistance (SAR) involving salicyclic acid pathway and the production of pathogenesis-related proteins (pr-proteins) and induced systemic resistance (ISR) were demonstrated (Kloepper *et al.*, 1992b; Hasky-Gunther *et al.*, 1998; Owen *et al.*, 1998; Kempster *et al.*, 2001). Lipopolysacharides of *R. etli* acted as the inducing agent of systemic resistance in potato roots. Similarly, a few bacterial strains called 'Nematophagous Fungus Helper Bacteria' (NHB) enhanced the activity of *A. oligospora* against root-knot nematodes, which resulted in better control of the nematode and improved plant growth (Duponnois *et al.*, 1998). Two other species of bacteria, *E. cloacae* and *P. mendocina*, increased the attachment of the endospores of *P. penetrans* and significantly increased its reproduction in plant roots (Duponnois *et al.*, 1999).

C. Biocontrol Potential

Various workers assessed the biocontrol potential of different antagonists of nematodes under natural conditions. Pot and field experiments on the control of *Meloidogyne* had shown that *Arthrobotrys* spp. when applied to infested soil before planting effectively controlled nematodes of pineapple (Linford & Yap, 1939), sugar beet (Duddington *et al.*, 1956), oats (Hams & Wilkin, 1961; Duddington *et al.*, 1961), tomato (Mankau, 1961; Mankau & Wu, 1985;; Cayrol & Frankowski, 1979; Pelagatti *et al.*, 1986; Vouyoukalou, 1993; Dias & Ferraz, 1994; Duponnois *et al.*, 1995, 1996; Stirling & Smith, 1998), pea (Hams & Wilkin, 1961), grapes and oranges (Tarjan, 1961), maize (Al-Hazmi *et al.*, 1982a; Rhoades, 1985), okra (Rhoades, 1985); melons (Ali, 1990), kiwifruit (Cayrol *et al.*, 1991) and red pepper (Mi *et al.*, 1993). *M. ellipsosporium* and *M. lysipagum* were tested for the control of *M. incognita* on tomatoes and showed fungal invasion in egg masses, parasitism on juveniles and significant reduction in plant damage (Esser, 1983; Mankau & Wu, 1985; Gaspard & Mankau, 1987). Suppression of *M. javanica* by *M. ellipsosporum* increased gradually and was nearly 100% on 120th day. But suppression by *M. cionopagum* increased rapidly to 100% within 10 days and then declined sharply (Jaffee & Muldoon, 1995b). Root-knot nematodes were generally more susceptible to the nematode-trapping fungi, *M. ellipsosporum, M. cionopagum, A. haptotyla* and *A. thaumasia* that used adhesive traps (Jaffee & Muldoon, 1995a; Jaffee, 1998). Different granular formulations of *A. dactyloides* and *D. candida* prepared by mixing the fungal biomass harvested from liquid culture with carrier substances kaolin and vermiculite suppressed *M. javanica* juveniles by more than 90% and reduced the number of galls by 57 - 96% (Stirling & Smith, 1998; Stirling *et al.*, 1998b). Application of *A. conoides* and *A. musiformis,* colonised on wheat grains significantly reduced the number of eggs and total population of *M. exigua* of coffee compared to the spore suspension application (Campos & Campos, 1997). In another study, *Duddingtonia flagrans* was found to be the most effective trapping fungus (Gives *et al.*, 1999a). A new biological nematicide using *Arthrobotrys* sp. protected the roots of potted micropropagated banana (Musa AAB) plants (Castellanos-Lopez *et al.*, 2000).

D. coniospora reduced galling due to root-knot nematodes in tomato and alfalfa (Jansson *et al.*, 1985b; Townshend *et al.*, 1989). Though *H. rhossiliensis* did not reduce *M. javanica* on tomato in a microplot study (Tedford *et al.*, 1993), addition of dried pellets of *H. rhossiliensis* to soil suppressed invasion of roots by nematodes (Lackey *et al.*, 1993; Jaffee & Muldoon, 1997). Because of the difficulty to mass-produce, only few attempts were made to use endoparasitic fungi for nematode control (Giuma & Cooke, 1974; Zehr, 1985; Townshend *et al.*, 1989; Tedford *et al.*, 1993).

V. chlamydosporium reduced galling by root-knot nematodes substantially when it was introduced into field soil (Godoy *et al.*, 1983a; Rodriguez-Kabana *et al.*, 1984). It significantly reduced the number of eggs and total population of root-knot nematodes of coffee (Campos & Campos, 1997) and tomato (Leij *et al.*, 1992a, 1993b; Mousa *et al.*, 1995). *V. lecanii* controlled *M. incognita* in a greater degree when combined with neem leaf extracts (Nagesh & Reddy, 1995; Rao *et al.*, 1997b). Damage caused by *Meloidogyne* species on a range of crops has been reduced after treatment with *P. lilacinus* (Lay *et al.*, 1982; Godoy *et al.*, 1983a; Jatala, 1985; Croshier *et al.*, 1985; Davide & Zorilla, 1985; Shahzad & Ghaffar, 1987; Ibrahim *et al.*, 1987; Hewlett *et al.*, 1988; Cabanillas & Barker, 1989; Zaki & Bhatti, 1990a; Nagesh & Reddy, 1995; Nagesh *et al.*, 1997; Khan & Goswami, 2000a; Jonathan *et al.*, 2000). *P. lilacinus* also reduced root-knot nematodes in some spice crops like black pepper (Ramana, 1994; Sosamma & Koshy, 1997), fenugreek (Sharma & Trivedi, 1989) and *Capsicum* (Pandey & Trivedi, 1992). The efficacy of *P. lilacinus* was more when applied together with *P. penetrans* (Dube & Smart, 1987; Maheswari & Mani, 1988) or mycorrhizal fungi (Rao *et al.*, 1998a) or along with chopped leaves of castor (Walia *et al.*, 1999). *P. lilacinus* was also effective against some migratory endoparasites. It reduced *Pratylenchus* sp. in corn (Gapasin, 1995) and in potato (Davide & Zorilla, 1995). *P. lilacinus* helped to manage R. *similis* on banana (Generalao & Davide, 1995; Esnard *et al.*, 1998).

There were a number of reports on the successful use of *P. lilacinus* to suppress nematodes under field conditions (Lay *et al.*, 1982; Godoy *et al.*, 1983a; Rodriguez-Kabana, 1984; Shahzad & Ghaffar, 1987; Hewlett *et al.*, 1988; Cabanillas & Barker, 1989; Noe & Sasser, 1995; Campos & Campos, 1997; Nagesh *et al.*, 1997). It showed good promise to be an efficient biocontrol agent against root-knot nematode but lacked consistency. *V. chlamydosporium*, when added to field soil, reduced nematode population in several field tests (Godoy *et al.*, 1983a; Kerry *et al.*, 1984; Rodriguez-Kabana *et al.*, 1984; Irving & Kerry, 1986; Leij & Kerry, 1989, 1991; Stirling & Smith, 1998).

Non-pathogenic forms of endophytic fungus *F. oxysporum* reduced *M. incognita* populations on tomato (Hallmann & Sikora, 1994; 1996). *T. harzianum* and *T. koningii* suppressed *M. arenaria* on maize (Windham *et al.*, 1989), *M. incognita* on egg-plant (Rao *et al.*, 1998b). In Israel, *T. harzianum* preparations improved growth of nematode-infected plants and decreased the root galling index and number of eggs per gram of root (Spiegel & Chet, 1998). Inhibition of *Pratylenchus* spp. in ginger was best with pine needle organic amendment in combination with *T. harzianum* seed treatment and soil application (Sharma & Dohroo, 1997). *Gliocladium roseum* and *G. catenulatum* reduced galling due to root-knot nematodes in squash (Rodriguez-Kabana *et al.*, 1984), *F. solani* and *R. solani* in groundnut (Sakhuja & Sethi, 1986), *C. elegans* and *B. bassiana* in tomato (Galper *et al.*, 1991; Saikia, 1998). *D. oviparasitica* (McKenry & Kretsch, 1987), *A. niger* (Zuckerman *et al.*, 1994) and *T. harzianum* (Sharma & Dohroo, 1997; Rao *et al.*, 1998b) were some of the saprophytic fungi evaluated for nematode control under field conditions. DiTera, a new biological nematicide derived from a nematode parasitic isolate of *Myrothecium* sp., was evaluated on various crops under field conditions (Warrior *et al.*, 1999). In China, root-knot nematode disease of groundnut was significantly reduced by the application of *Pleurotus ostreatus* (Xiang & Feng, 2001).

The suppressive potential of the hyperparasite, *P. penetrans*, had been tested in many crops, but mostly under greenhouse conditions (Chen & Dickson, 1998). It exerted various degrees of nematode biocontrol under greenhouse and field conditions (Williams, 1967; Mankau, 1975a, 1981; Sayre, 1980; Stirling & Wachtel, 1980; Stirling, 1984; Brown & Nordmeyer 1985; Leij *et al.*, 1992a; Chen, Z.X. *et al.*, 1996). *P. penetrans* suppressed populations of *Meloidogyne* spp. in black pepper too (Ratnasoma *et al.*, 1991; Sosamma & Koshy, 1997). The combination of *P. penetrans* and *G. mosseae* resulted in higher parasitism of female nematodes and significant increase in the growth of tomato seedlings (Rao *et al.*, 2000). Though difficult to mass culture, ample number of field experiments was conducted to confirm its potential as a nematode biocontrol agent, especially against root-knot nematodes (Stirling, 1984; Brown & Nordmeyer, 1985; Dube & Smart, 1987; Raj & Mani, 1988).

B. subtilis when compared with culture filtrates of several fungi gave better reduction of *M. incognita* and improved the growth of chickpea (Siddiqui & Mahamood, 1995). Several strains of *Bacillus* spp. were proved to possess nematicidal activity against root-knot nematodes *in vitro* and greenhouse bioassays (Sheela *et al.*, 1993; Carneiro *et al.*, 1998). A nematicidal strain of *B. thuringiensis* was found reducing the number of galls and increased the yield of tomatoes, pepper and strawberry (Zuckerman *et al.*, 1993). On barley, the extent of control of *M. incognita* obtained with *B. thuringiensis* var. *thuringiensis* and *B. thuringiensis* var. *israelensis* ranged from 53 to 66% (Sharma, 1994). In Costa Rica, a novel *Streptomyces* sp. isolated from nematode suppressive soils suppressed *M. incognita, R. reniformis* and *P. penetrans* in greenhouse and field trials (Dicklow *et al.*, 1993). *P. fluorescens* (Oostendorp & Sikora, 1989), an avirulent strain of *P. solanacearum* (McLaughlin *et al.*, 1990) and *B. thuringiensis* (Zuckerman *et al.*, 1993) were some of the candidate strains that have been field tested for nematode control. Efficacy of *P. aeruginosa* alone or in combination with *P. lilacinus* controlled root-knot nematodes and root-infecting fungi of tomato under laboratory and field conditions (Siddiqui *et al.*, 2000). Several of these rhizobacteria when used as seed dressing or as soil drench significantly

suppressed root rot and root-knot infections in mung bean (Khan & Kounsar, 2000; Siddiqui *et al.*, 2001b).

A strain of *P. chlororaphis* was found to significantly reduce *P. penetrans* in roots of strawberries (Hackenberg *et al.*, 2000). In greenhouse and field microplot experiments, *B. megaterium* reduced the population densities of *M. chitwoodi* and *P. neglectus* up to 50% on potato plants (Al-Rehiayani *et al.*, 1999). Different strains of *P. fluorescens* significantly reduced *R. similis* invasion in banana by 50% in greenhouse experiments (Aalten *et al.*, 1998) and *M. incognita* infecting tomato (Santhi & Sivakumar, 1995). In banana, the best plant responses (height, leaf numbers, healthy root weight), lowest numbers of *R. similis* and highest numbers of free-living nematodes were observed for treatments with *S. costaricanus* sp. nov. and *P. marquandii* containing wheat as a component (Esnard *et al.*, 1998). Field experiments showed *B. thuringiensis* var. *kurstaki* H-3 to be highly effective against high populations of *R. similis* in banana plantations (Mena *et al.*, 1997). Several endophytic fungi isolated from banana caused reduction in the number of *R. similis* when tested in pot experiments (Pocasangre *et al.*, 2000).

In pot studies, predacious nematodes too effectively controlled *M. javanica* on subabool seedlings (Azmi, 1991) and *P. penetrans* on maize (Azmi, 1995; Fauzia *et al.*, 1998). Application of entomopathogenic nematode, *S. carpocapsae,* around banana roots resulted in a reduced invasion by *R. similis* in greenhouse experiments (Aalten & Gowen, 1998). *S. carpocapsae* and *S. riobravis*, provided 86 – 100% control of nematodes attacking turf grass (Grewal *et al.*, 1997). *S. feltiae* reduced *M. incognita* galling, egg production per plant and egg hatch while not affecting individual female egg production in tomato (Lewis *et al.*, 2001).

D. Ecology of Biocontrol Agents

a. Temperature

Arthrobotrys was active in the soil between at temperature 5 and 30°C, with an optimum at 25°C and at temperature above 35°C the fungus was destroyed (Cayrol & Frankowski, 1980;

Cayrol, 1981; Al-Hazmi *et al.*, 1982b). The optimal growth of two strains of *A. oligospora* from Senegal occurred at 25 - 30°C but salinity inhibited their development (Duponnois *et al.*, 1995). The formation of an adhesive hypha in *A. oligospora* was less temperature and nutrition dependant than the development of the morphologically more complex adhesive networks (Belder & Jansen, 1994a, 1994b). *D. oviparasitica* grew most rapidly between 24 - 27°C but more eggs were parasitized at lower temperatures (Stirling, 1979). Isolates of *V. chlamydosporium* varied in their temperature responses (Kerry, 1981). An isolate of *V. chlamydosporium* was most abundant in soil at 22°C, the optimal temperature for sporulation, while the optimal growth of this isolate was at 32°C (Leij *et al.*, 1992b). *V. suchalosporium* grew better and was parasitic at low temperatures than *V. chlamydosporium* (Dackman & Baath, 1989). The optimal temperature for growth and spore production of *P. lilacinus* was 25 to 30°C (Jatala, 1986) and that of *C. anguillulae* was at 24 - 28°C (Sayre & Keeley, 1969). Maximum growth of *P. lilacinus* occurred at 24 - 30°C and least growth was at 12 and 36°C (Cabanillas *et al.*, 1989b).

A survey in Hawaiian Islands showed that the occurrence of *Pasteuria* spp. was more abundant in lowlands (<900 m in elevation in areas with a mean annual temperature >21°C) than in subalpine and alpine regions (>900 m in elevation) and they were not found in areas with a mean annual temperature below 10°C (Ko *et al.*, 1995). The endospores remained viable for a period of one to six years but their infectivity was affected by storage (Mani, 1988; Espanol *et al.*, 1997; Giannakou *et al.*, 1997; Sekhar, 1998). Endospores were resistant to high temperature and sonication (Williams *et al.*, 1989). Endospore attachment to J2 increased with increasing temperature upto ca. 30°C (Hatz & Dickson, 1992; Singh & Dhawan, 1990; Stirling *et al.*, 1990). Endospore germination, pathogenesis and numbers of endospores per female or root system were all favoured by higher temperature (Chen & Dickson 1997a, 1997b; Hatz & Dickson, 1992; Orui, 1997; Serracin *et al.*, 1997; Stirling, 1981). But high temperature treatment decreased the

incidence of *P. penetrans* infection of J2 and females (Freitas *et al.*, 1997; Giannakou *et al.*, 1997). The optimal growth temperature of *P. penetrans* was between 28°C and 35°C (Sekhar & Gill, 1990a; Hatz & Dickson, 1992; Serracin *et al.*, 1997) and the minimum temperature for its development was 17°C (Chen & Dickson, 1997a).

b. Soil moisture

V. chlamydosporium was less affected by soil moisture (Stirling & Kerry, 1983). Though endospores were resistant to desiccation (Williams *et al.*, 1989), not much is known about the effect of soil moisture on endospore attachment and development of *P. penetrans*. Increasing soil moisture decreased the number of *P. penetrans*-infected females per root system (Davies *et al.*, 1991). High water potentials reduced the attachment of *H. rhossiliensis* spores to juveniles of *M. javanica* (Tedford *et al.*, 1992). Moisture levels of 60 and 70% were favourable for the establishment of *G. mosseae* on black gram (Sanakaranarayanan & Sundarababu, 2001).

c. Soil pH

Species of *Arthrobotrys* varied in their growth and trapping ability at different levels of pH (Mankau, 1964). *Arthrobotrys* was effective only in soils with near neutral pH (Pelagatti & Caroppo, 1987; Pelagatti & Piccolo, 1990) while *Monacropsorium* strains preferred acid pH (Peloille *et al.*, 1984). Sporulation of *Nematoctonus concurrens* was more sensitive to pH (Giuma & Cooke, 1974) and infection by the zoospores of *C. anguillulae* was reduced in acid conditions (Sayre & Keeley, 1969). *P.lilacinus* was found adaptable to a wide range of soil pH and the optimum pH was 5.9 (Jatala, 1986). In *P. penetrans* generally endospore attachment was greater at a higher pH (Davies *et al.*, 1988; Orui, 1997). The maximum spore population and maximum mycorrhizal colonization were obtained in the pH range of 6-7 (Sanakaranarayanan & Sundarababu, 2001).

d. Soil texture and organic content

C. auxiliaris was found in clayey soils in Australia whereas *V. chlamydosporium* was little affected by soil texture (Stirling & Kerry, 1983). But *V. chlamydosporium* multiplied well in peaty sand (Leij *et al.*, 1993b). Sandy soil favored endospore attachment and thereby abundance of *P. penetrans* (Mateille *et al.*, 1995; Oostendorp *et al.*, 1990; Singh & Dhawan, 1992; Singh, M. *et al.*, 1998) and *H. rhossiliensis* (Tedford *et al.*, 1992)

Nematode-trapping fungi established well where organic matter was high and competed saprophytically with the microbes involved in the decomposition of such materials, or preyed on free-living nematodes proliferating in these nutrient sources (Jaffee *et al.*, 1998). It was proved that poor conditions for saprophytic growth were not a prerequisite for predacious mode of feeding (Belder & Jansen, 1994c). Research on *Arthrobotrys* (used for *Meloidogyne* control) showed that organic amendments (>0.8%) were good for the development of the fungus (Cayrol & Frankowski, 1980; Cayrol, 1981; Al-Hazmi *et al.*, 1982b). The best conditions for the growth of *A. irregularis* and for its nematicidal effectiveness were >2% organic substance (Pelagatti & Caroppo, 1987; Pelagatti & Piccolo, 1990). Soils amended with plant residues enhanced the growth and parasitic activity of *V. chlamydosporium* (Owino *et al.*, 1993).

e. Pesticides

The fumigant nematicide, dichloropropane-dichloropropene (DD), killed nematode trapping fungi whereas ethylene dibromide (EDB) temporarily reduced their numbers and dibromochloropropane (DBCP) had no effect (Mankau, 1968; Mitsui, 1972). Application of different fungicides did not cause toxicity to *Arthrobotrys* in the soil when applied at usual rates to aerial parts. *P. lilacinus* was apparently compatible with many fungicides and nematicides (Villanueva & Davide, 1984). However, nematicides like ethoprophos, fenamiphos, aldicarb, oxamyl and carbofuran were toxic to *P. lilacinus* (Eguiguren-Carrion, 1995). Fungicides mancozeb and ipridione markedly suppressed the radial growth of both *P.*

lilacinus and *V. chlamydosporium* (Mertens & Stirling, 1993) while *V. lecanii* was tolerant to benomyl (Meyer, 1992). Herbicide ethofumesate totally inhibited strains of *Monacrosporium* (Peloille *et al.*, 1984). Carbofuran was compatible and had no adverse effect on the growth of *P. penetrans* (Singh & Dhawan, 1998).

E. Scaling Up and Formulation

M. ellipsosporum, M. thaumasia, A. oligospora, A. musiformis and *D. candida* were multiplied on sugarcane bagasse (Linford & Yap, 1939). Various fungi belonging to this group were grown on oat hulls (Tarjan, 1961), wheat or rye (Mankau & Wu, 1985), rice bran (Machado & Campos, 1997), compost blocks (Duponnois *et al.*, 1996) and chopped water lily leaves (Davide & Zorilla, 1995).

P.lilacinus was grown on oat kernels (Godoy *et al.*, 1983a; Rodriguez-Kabana *et al.*, 1984), on wheat-grain substrate (Dube & Smart, 1987; Ibrahim *et al.*, 1987; Hewlett *et al.*, 1988; Cabanillas & Barker, 1989), on rice bran (Machado & Campos, 1997), on rice seeds (Rodriguez-Kabana & Morgan-Jones, 1986), on castor leaves (Zaki & Bhatti, 1990a), on chopped water lily leaves (Davide & Zorilla, 1985), on several leaf extracts and residues (Siddiqui & Mahamood, 1994). Several oil cakes and agricultural waste materials were found to support rapid growth of *P. lilacinus* (Sharma & Trivedi, 1987). The supplementation of castor (*Ricinus communis*) and neem (*Azadirachta indica*) oil cakes with nitrogen, phosphorus and potassium in the form of inorganic fertilizers, had an additive effect on the mycelial growth and sporulation of *P. lilacinus* (Nagesh *et al.*, 2001a). Botanicals, plant extracts and oil cake suspensions too supported better propagule density of *P. lilacinus* (Rao & Reddy, 1994; Nagesh & Reddy, 1995; Nagesh *et al.*, 1997; Rao *et al.*, 1997a; 1997b). These suspensions were used as bare root dip treatment for controlling nematodes in aubergine (Rao *et al.*, 1999). When different whole grains were evaluated as substrates for multiplying *P. lilacinus*, maximum sporulation was observed on rice followed by gram (Zaki & Bhatti, 1991). Wood

charcoal powder was used as a carrier for *P. lilacinus* (Bansal *et al.*, 1992). The addition of neem extracts mixed with spores of *P. lilacinus* to the soil was found effective (Rao & Reddy, 1994). *P. lilacinus* grown on substrates like ipil-ipil, rice hulls or banana leaves and bracts was better than the direct use of spores and mycelial suspension applied as soil drenches (Generalao & Davide, 1995).

V. chlamydosporium was grown on oat kernels (Godoy *et al.*, 1983a; Rodriguez-Kabana *et al.*, 1984; Kerry *et al.*, 1984), several coarse grains and their bran (Machado & Campos, 1997; Sankaranaryanan *et al.*, 2001). It could be established in soil using inocula on sand/bran, powdered grain or as aqueous suspension of chlamydospores (Leij & Kerry, 1991). Establishment of *V. chlamydosporium* in soil was significantly greater if the fungus was introduced as hyphal fragments and chlamydospores, without a food base (Leij & Kerry, 1991). Neem followed by karanj (*Pongamia pinnata*) were the best substrates for *V. lecanii.* Supplementing oil cakes with N, P and K in the form of inorganic fertilizers, had a synergistic effect on the growth, behaviour and sporulation of *V. lecanii* (Nagesh & Reddy, 1997).

Multiplication and spore production of *F. oxysporum* and *F. solani* were higher on chicken manure than on wheat grains and remained viable for more than 6 months (Abu-Laban & Saleh, 1992). Drenching of spores of *A. niger* multiplied on oat meal controlled root-knot nematodes on tomato (Zuckerman *et al.*, 1994). Chopped water lily leaves were used as a substrate for growing *G. roseum* (Davide & Zorilla, 1995). Aqueous extracts of castor and pongamia supported maximum growth and spore production of *T. harzianum* (Rao *et al.*, 1998b).

Addition of *N. concurrens* and *N. haptocladus* as conidia reduced nematode population (Giuma & Cooke, 1974). Spores of *H. rhossiliensis* when added to soil controlled nematode populations (Eayre *et al.*, 1983, 1987; Zehr, 1985) but lacked competitive saprophytic ability (Jaffee & Zehr, 1985). Actively growing mycelia rather than spores helped in attachment of

spores to nematodes (McInnis & Jaffee, 1989). *D. coniospora* was applied as a coating to alfalfa and tomato seeds or as a dip or spray to tomato transplants (Townshend *et al.*, 1989). Calcium alginate pellets of hyphae of *H. rhossiliensis, M. cionopagum* and *M. ellipsosporum* were repellent to *M. incognita* juveniles (Robinson & Jaffee, 1996). Cracked maize grain was proved to be the best substrate for multiplying *Monacrosporium* spp. (Pria & Ferraz, 1996).

P. penetrans was multiplied on its nematode host on greenhouse-grown plants (Stirling & Wachtel, 1980; Sharma & Stirling, 1991), in a hydroponic cultivation system (Serracin *et al.*, 1994) and an in vitro system of culturing the nematode and the parasite in excised or transformed root cultures (Verdejo & Jaffee, 1988; Verdejo & Mankau, 1986). Endospores per female increased to 0.031 million per 100 degree-days increment based on a developmental threshold of 17°C and negatively correlated to the nitrogen level in the soil (Chen & Dickson, 1997b). Various media have been evaluated for their ability to support the growth of different isolates of *Pasteuria* but with limited success (Bishop & Ellar, 1991; Reise *et al.*, 1988; Williams *et al.*, 1989). A patent was obtained (Previc & Cox, 1992) for a system for multiplying *P. penetrans* by adding explanted tissue from *Ascaris suum* to an enriched medium.

The best medium for growing *B. thuringiensis* var. *thuringiensis* consisted of acid hydrolysates of wheat or rice bran together with solid FYM (Rai & Rana, 1979). Spraying of rhizobacteria in suspension with 0.2% methylcellulose increased the adhesion of bacteria on seed pieces and their efficiency compared to water suspensions (Racke & Sikora, 1992). Modern fermentation technology involving liquid or solid media and formulation in a suitable matrix like diatomaceous earth granules, lignite spillage granules, vermiculite, mineral carriers and alginate-clay pellets, was used on a limited scale for mass production of antagonists of nematodes (Tarjan, 1961; Al-Hazmi *et al.*, 1982b; Cabanillas *et al.* 1989a; Coosemans, 1990; Lackey *et al.*, 1993; Stirling & Mani, 1995; Shahzad *et al.*, 1996). Vegetative colonies of *H.*

rhossiliensis were grown in shake culture and their hyphae were sporulated *in vitro* in moist chambers and pelletized in 1% alginate and dried (Lackey *et al.*, 1992). Except a few cases of liquid and granular formulation of *A. amerospora* (Rhoades, 1985), *V. chlamydosporium* (Stirling *et al.*, 1998a) and *P. lilacinus* (Cabanillas *et al.*, 1989a) large-scale fermentation had never been for nematode antagonistic fungi. Fermentation conditions like the type and quantity of ingredients played a crucial role on the nematicidal activity of *P. fluorescens* (Weidenborner & Kuntz, 1993). Two commercial formulations of *Arthrobotrys*, Royal 300 and Royal 350, were developed in France (Cayrol & Frankowski, 1979). 'DiTera' is another commercial formulation from a nematophagus fungus *Myrothecium* sp. (Warrior *et al.*, 1999).

Chapter 3

Materials and Methods

A. Collection and Maintenance of Test Organisms

Though a large number of organisms are reported as antagonists of nematodes, fungi and bacteria are most commonly used for control of plant parasitic nematodes. They can be easily cultured, mass multiplied and applied to soil for suppression of root-knot and burrowing nematodes, the two target nematode pests of spices.

a. Sampling

Soil and root samples were collected from the rhizosphere of randomly selected and apparently healthy black pepper, cardamom and ginger plants from Kerala, Karnataka and Tamil Nadu (Table 1). Maximum number of samples was collected from Kerala (108) followed by Karnataka (44) and Tamil Nadu (16). Samples were taken from the rhizosphere of black pepper (41 nos.) and ginger (67 nos.) in Kerala, while from Karnataka they were collected from black pepper (29 nos.) and cardamom (15 nos.) rhizosphere. Out of the 168 samples, 86 samples were collected from black pepper, 67 from ginger and the remaining 15 were from cardamom. Sampling was confined to black pepper rhizosphere (16 nos.) only in Tamil Nadu. Fields from a wide range of agro-ecological conditions were chosen to obtain a wide range of nematode antagonists that are adapted to diverse climatic conditions. The top 3-5 cm of soil and litter layer were removed and about 250 cc of soil and 10 g of feeder roots were collected up to a depth of 30 cm in polythene bags. These samples were used for isolation of various antagonists.

b. Isolation

Fungi and bacteria from rhizosphere soil were isolated by the standard dilution plate method (Waksman, 1922). Fungi were isolated directly from root-knot nematode egg masses or individual eggs or females. For this, roots were washed in tap water and the egg masses were collected after staining with Phloxin B (Holbrook *et al.*, 1983), rinsed in tap water

Table 1. Sampling sites and number of samples collected from major spice crops in three states of South India.

Sampling site	No of samples collected			
	Black pepper	Cardamom	Ginger	Total
Kerala				
Idukki	8	-	8	16
Kannur	3	-	7	10
Kasaragod	-	-	6	6
Kozhikode	21	-	21	42
Wyanad	9	-	25	34
Karnataka				
Dakshina kannada	5	-	-	5
Kodagu	20	9	-	29
Uttara Kannada	4	6	-	10
Tamil Nadu				
Nilgiris	6	-	-	6
Valparai	10	-	-	10
TOTAL	86	15	67	168

followed by sterile distilled water (SDW). They were then transferred to 2% water agar (WA) plates. After 2 days, egg masses showing fungal growth were aseptically transferred to potato-dextrose agar (PDA) containing streptomycin sulphate (50 $\mu g\ l^{-1}$).

For isolating fungi that parasitize root-knot nematode females, fresh root galls were selected, rinsed in tap water and SDW. Intact females with egg masses were extracted by carefully tearing the galls using sterile forceps and blades. The females were washed in SDW and were placed on a sterile cover slip (24 mm square) kept in a petri plate (9cm-diam.) containing 2% WA. The females were crushed on the cover slips and incubated at 28°C for 4 days (Crump, 1987). The fungi growing out were transferred to PDA. Similarly a suspension of nematode eggs was prepared by dispersing the egg masses of root-knot nematodes in SDW (Hussey & Barker, 1973). One ml of the suspension containing about 100 eggs was poured into a petri plate containing 2% WA and was incubated at 28°C for 4 days. The fungi growing from the eggs were aseptically transferred to new WA plates and again incubated for 4 days (Godoy *et al.*, 1983a).

Bacteria were isolated both from soil and roots. Small segments of feeder roots were separated from the soil, placed in sterile distilled water and shaken at 250 rpm for 15 minutes. The water suspension was serially diluted in SDW and plated on Kings B Medium amended with cycloheximide (100 $\mu g\ ml^{-1}$) or on fluorescent pseudomonads selective medium (Kluepfel *et al.*, 1993).

For isolating the endosporite, *Pasteuria* sp. from suppressive soil, 100 g of field soil was placed on two layers of facial tissue paper supported by a wire mesh. This was placed in a petri dish containing water (Whitehead & Hemming, 1965). The nematodes already present in the soil were extracted for 4 days at room temperature and discarded. Root-knot nematode juveniles (ca. 500 J2) were spread out evenly over this soil placed on filters. By

sieving through a 25 μm aperture sieve, the J2 were collected after 48-hour incubation. These juveniles were observed at 100x magnification for any attachment of endospores of *Pasteuria* (Stirling & White, 1982).

Root-knot nematodes (*M. incognita* (Kofoid & White) Chitwood) were generally isolated from roots showing galling due to root-knot nematode infestation. Initially the eggs of *M. incognita* were extracted with a sodium hypochlorite solution (Hussey & Barker, 1973). Second-stage juveniles were allowed to hatch in a modified Baermann funnel (Pitcher & Flegg, 1968).

c. Culturing

Single fungal colonies isolated from nematode eggs, females or soil were transferred and cultured on PDA medium in 9 cm-diam petri dishes for 10 days at 28°C. The purified fungal isolates were then maintained on potato-dextrose-agar (PDA) slants until used. The discrete bacterial colonies obtained by plating on Kings B medium were selected, purified and then transferred to nutrient agar (NA) after 1 or 2 days.

For culturing *P. penetrans*, the endospore-encumbered J2 were inoculated on tomato plants, grown in 15 cm-diam pots containing steam-pasteurized soil. These tomato plants were maintained in a green house with routine management practices. After 40-60 days, endospore-filled females, conspicuous by an opaque and white appearance when illuminated from above under a stereomicroscope, were handpicked after excising them from roots. These females were ground in sterile water using a glass grinder. A 50 ml nematode suspension containing 1000 J2 ml^{-1} was exposed to 10 ml of endospores in 250 ml conical flasks. The flasks were shaken on a mechanical shaker for 24h and were incubated in shallow dishes at 28°C until 80% of J2 was encumbered with 6-12 endospores. These J2 were further used for multiplying the bacterium or as inoculam in experiments with *Pasteuria.*

The root-knot nematode *M. incognita*, originally collected from black pepper, was routinely maintained on tomato (*Lycopersicon esculentum* cv. Local) in greenhouse conditions. The burrowing nematode was also extracted from black pepper roots, cultured on carrot discs placed on 2% water agar (O'Bannon & Taylor, 1968) and were maintained at 28 ± 1°C.

B. *In vitro* Bioassays

All the isolated microorganisms were screened in vitro for studying their antagonistic effect, if any, on nematodes. For this, a relatively simple screening on water-agar plates was employed. The test fungi were evaluated for their ability to parasitize various life stages of the nematode viz. eggs, juveniles, females and egg masses. Simultaneously, to detect the involvement of any toxic metabolites on nematode inhibition, their effect on egg hatching was also studied. The effect of fungal culture filtrates was assessed only in a few cases. For bacteria, only the egg hatch test was conducted while no in vitro bio assays were done for *Pasteuria* spp. as they were originally isolated from soil after determining the ability to parasitize the nematode J2.

a. Test organisms

The fungi used in this study included 73 freshly collected isolates and 76 isolates obtained from other sources. All these isolates were obtained from nematode eggs/egg masses, females or nematode-suppressive soils around the rhizosphere of spice plants. Altogether there were five isolates of *Verticillium* spp., eight isolates of *Paecilomyces* spp., 88 isolates of *Trichoderma* spp. and 48 isolates of various other fungi.

The bacterial isolates included 46 strains of newly isolated bacteria from the rhizosphere of spice plants. Forty-two strains of *P. fluorescens* Migula, the Plant Growth Promoting Rhizobacterium (PGPR), were obtained from the repository of the Plant Pathology section. Three *Pasteuria* isolates were collected from the rhizosphere of ginger and another one was

obtained from the Nematology Department of Central Plantation Crops Research Institute, Regional Station, Kayangulam.

An isolate of root-knot nematode (*M. incognita*), originally collected from black pepper, was used in all bioassay studies.

b. Egg parasitization

The root-knot nematode eggs were collected as mentioned earlier, washed 5 times in SDW and their numbers were adjusted to about 200 ml^{-1} suspension. A 4 mm-diameter plug of each fungal isolate, grown on PDA, was transferred colony-side-up to the centre of a 80 x 15 mm petri plate containing 2 % WA and was incubated at 28°C until the whole agar surface was covered with the fungal mycelium. An egg suspension (0.5 ml) containing ca.100 eggs was then spread over each fungal covered WA surface. Control plates had received the same quantity of eggs, but a non-colonized PDA plug. The plates were arranged in a randomized design with 3 replications and were incubated in dark for 2 weeks at 28°C. After the incubation period, 20 eggs per plate were stained with a drop of 0.05% cotton blue in lactophenol and were examined at random using a research microscope at 100x magnification. The proportion of eggs parasitized by each fungus was determined.

c. Juvenile parasitization

The suspension of second stage juveniles of root-knot nematodes containing c.100 J2 was also tested following the above procedure, to record any juvenile parasitization by the test fungi. Effect of various bacteria on juveniles of root-knot nematodes was studied separately by following similar methods. Ten µl of nematode suspension (25 J2) and 20 µl of bacterial cell suspension ($2x10^6$ cfu) were added to 170 µl of 25 mM potassium phosphate buffer (Cronin *et al.*, 1997) in the wells of a 96-well titration plate. The plates were incubated at 25°C for 48 hours and the ratio of total to motionless juveniles was determined microscopically by sampling juveniles with a micropipette.

d. Female parasitization

Female root-knot nematodes were dissected out from infected tomato roots dipped in 0.5% sodium hypochlorite (NaOCl) solution for one minute, washed three times in SDW and once in streptomycin sulphate. Five each surface-sterilized females were then placed on sterile WA plates that had been inoculated with 4 mm diameter PDA plug of the respective fungal isolate. Control plates had females treated as above and placed on WA plates inoculated with PDA plugs without any fungus. The plates were replicated thrice and kept in a randomized design for two weeks at 28°C. The number of females parasitized by the fungi was counted.

e. Egg hatch

Three freshly extracted root-knot nematode egg masses were surface sterilized and placed on sterile WA plates that had been inoculated with the test fungus, as described above. Control plates had no fungus. After 14 days, the egg masses were crushed in a droplet of 0.01% NaOCl solution to dissolve the gelatinous matrix. The number of eggs and juveniles present in each egg mass was counted at 100x magnification. These eggs and juveniles were suspended in 1ml of SDW and the suspension was poured on a facial tissue paper nested on a 2 cm diameter sieve. This sieve was placed in a water-filled petri dish (40 x 15 mm) and the juveniles were allowed to hatch and migrate into the water. They were incubated at 28°C for 48 hours and the number of juveniles hatched was determined using the formula.

$$\% \text{ hatch} = (Jf * 100) / (Ei + Ji)$$

where Ji = the number of J2 observed initially

Ei = the number of intact eggs observed initially

Jf = the number of J2 hatched

The ability of different bacterial isolates to influence the hatching of root-knot nematode eggs was investigated by an in vitro assay, slightly modified from the above. In this method,

the root-knot nematode egg masses were incubated in bacterial suspension (2×10^5 to 2×10^9 cfu) for 48 hours and the egg hatch was estimated directly using an inverted microscope.

f. Effect of fungal culture filtrates

Metabolites of 22 fungi, which showed very good suppression of the egg hatching process of nematodes, in spite of the absence of any parasitism on females or eggs, were screened for the nematicidal activity. For this, these fungi were cultured on Czapek-Dox medium (pH 6.4) by inoculating with a spore suspension (10^7 spores ml^{-1}) of the respective fungus. After incubation for 14 days at 28°C, the medium was filtered first through sterilized Whatman No.2 filter paper, which removed the mycelium. To avoid spore contamination, the filtrate was subsequently passed through 0.22 µm millipore filter. The filtrate thus collected was tested, pure or diluted, in SDW (1/2 and 1/4). The tests were done in 96-well titration plates. Pure or diluted test filtrate (100 µl) was added in each well, to which about 25 J2 were added in 20 µl water. Each treatment was replicated three times. Toxicity was estimated based on the number of juveniles paralysed or died. There were two controls for comparison; one in pure distilled water and the other in the uninoculated sterilized culture medium, corrected to the same pH as that of tested culture filtrates.

C. Greenhouse and Field Evaluation

Antagonistic activity in simple agar tests cannot be extrapolated to the natural soil environment. The levels of activity that are observed on agar plates have never been observed under natural conditions. Therefore, it is essential to carry out additional testing in natural soil to understand whether an organism has attributes like host specificity, rhizosphere competence, competitive saprophytic ability and ability to thrive under extreme environments. For this the promising antagonists were further tested in greenhouse and field to confirm their practical utility.

a. Greenhouse studies

A series of greenhouse experiments were conducted using various antagonists of nematodes, found to be promising in the laboratory bioassays, to study their interaction with the test plant and nematodes. For this, the candidate organisms were multiplied on the respective substrate or media and were used to inoculate the test plants. The dosage was determined based on the spore load in unit weight of the substrate. The number of spores g^{-1} that colonized a substrate was estimated by suspending 1 g of the substrate in 9 ml sterile water followed by further dilution to allow direct counting using a haemocytometer. The test plants were uprooted and observations on biomass production, nematode multiplication and fungal colonization were recorded.

For estimating the nematode population, plant parasitic nematodes were extracted from soil samples by sieving and a modified Baermann's funnel method (Pitcher & Flegg, 1968). The roots were washed, stained with Phloxin – B and were rated for galling or egg mass production using a 0-5 scale (Taylor & Sasser, 1978). The nematode population in roots was estimated by staining with acid-fuchsin, blending and sampling the suspension to count nematodes (Byrd *et al.*, 1983). The treatment details, test plant, and the observations recorded in each experiment are as follows.

Several species of *Trichoderma,* a fungus widely present in soil, are known to be good biocontrol agents of fungal pathogens. A few are reported as antagonists to nematodes too (Saifullah & Thomas, 1996; Spiegel & Chet, 1998). Eleven promising *Trichoderma* isolates were screened in two separate experiments in greenhouse pot trials. One month-old cardamom seedlings (Malabar type) of uniform growth, raised in steam-sterilised soil, were transplanted to plastic pots containing 10,000 cc sterile or non-sterile soil. Four seedlings were planted in each pot. The soil was inoculated with five *Trichoderma*_isolates viz. C.20, C.21, C.22, C.23 and C.24 ($2.9 - 3.6 \times 10^7$ cfu g^{-1}) multiplied on decomposed coffee husk

(@40g pot^{-1}). Nematode-inoculated and uninoculated checks were included as controls. Thus the experiment was a factorial experiment of 2 x 7 treatments with 4 replications in CRD. After one week, aqueous suspensions of *M. incognita* eggs and juveniles (1000 numbers pot^{-1}) were incorporated. The plants were uprooted gently, washed and weighed individually after six months. The roots were rated for galling or egg mass production and the nematode population in roots was estimated by methods described earlier. Random samples of egg masses, roots and soil were collected to reisolate the fungus and to compare with the original isolates.

In another experiment six isolates of *Trichoderma* spp. (P.26, T.2, T.5, T.10, T.12 and T.14) were tested for their nematode antagonism using 6 m-old black pepper rooted cuttings (cv. Panniyur 1). The cuttings were raised in autoclaved soil to avoid contamination. At the time of transplanting, *Trichoderma* isolates, multiplied on decomposed coffee husk (approximately 10^5 cfu g^{-1}), were added @ 40g $plant^{-1}$ to sterilized potting mixture (sand : soil : FYM – 1:1:1). The factorial experiment had 2 x 7 treatments (six isolates of *Trichoderma* spp. and a check) in RBD with 5 replications. Nematodes were inoculated @ 500 J2 $plant^{-1}$ after 2 weeks of transplanting. The plants were maintained in the greenhouse for one year and the growth of the plants, mycofloral changes in the soil and nematode multiplication were monitored. For estimating the microbial load in soil, one gram of soil sample from the rhizosphere was used to prepare a 10-fold dilution series in sterile water and population densities of fungi and bacteria were determined by plating out 0.1 ml aliquots of the appropriate soil dilution to either PDA or nutrient agar. The number of colony forming units (cfu) was counted.

The egg parasitic hyphomycete, *V. chlamydosporium* (Vc3), was used in another pot trial to study its effect on root–knot nematodes infesting cardamom. Polythene bags containing about 5 kg of autoclaved soil were planted with one-year-old cardamom (cv. Malabar - Cl.37)

seedlings. Nematodes and the fungus were inoculated alone or in combination and in various sequence viz. nematodes first followed by the fungus (after 60 days), fungus first followed by the nematodes (after 60 days) or both simultaneously. There were 8 treatments with 5 replications and the design of the experiment was RBD. The nematodes were inoculated @ 2000 J2 plant^{-1} while *V. chlamydosporium*, multiplied on rice grains, was added @ 20 g plant^{-1}. The experiment was continued for 10 months. On completion, observations on nematode infestations and plant growth characters viz. number of tillers, total biomass and root weight were recorded as described above.

P. lilacinus (Pl.1) was tested for its nematicidal properties in another experiment using cardamom as the test plant. Cardamom seedlings (Malabar type – Cl.37) were raised in steam-sterilised soil. After one month, seedlings of uniform growth were transplanted to plastic pots containing 10 l sterile or non-sterile soil, as the case was. Four seedlings were planted in each pot. The soil was inoculated with two levels (5 g and 10 g) of *P. lilacinus* multiplied on decomposed coffee husk (2.4 – 2.6 x 10^5 cfu g^{-1}). Another set of plants received same quantities of coffee husk free of any fungal colonization. Nematode-inoculated and uninoculated checks were included as controls. Thus there were six treatments with four replications each in CRD. After one week, aqueous suspensions of *M. incognita* eggs and juveniles (1000 numbers pot^{-1}) were incorporated and maintained in the greenhouse. The plants were uprooted gently, washed and weighed individually after nine months. The roots were washed, rated for galling or egg mass production and the nematode population in roots was estimated by methods described earlier. Fungal colonization was cross-checked by reisolating the fungus from egg masses removed at random in *P.lilacinus* specific medium (Gaspard *et al.*, 1990).

Forty-two isolates of *P. flourescens* (obtained from the Plant Pathology repository) were evaluated in different batches for studying their efficiency to suppress root-knot nematodes.

In the first batch, four isolates (Is.1, 22, 40 and 44) of *P. flourescens* were evaluated using one month-old tomato seedlings as test plants in a 2 x 5 factorial experiment. In the second batch, another seven isolates (Is.4, 7, 10, 12, 26, 29 & 34) of *P. fluorescens* were screened using black pepper OP seedlings (Panniyur 1) in a 2 x 8 factorial experiment. Subsequently, 23 isolates of *P. fluorescens* were evaluated on black pepper OP seedlings (Panniyur I). In the last two-factorial experiments, eight isolates (AP2, 8, 13, 22, 38, 40, 47 and 50) were evaluated using black pepper cuttings (Panniyur I) instead of OP seedlings. Six one month-old test plants were grown in 10 cm-dia plastic pots containing a mixture of sand : soil : FYM (1:1:1). The bacterial isolates were cultured on King's B Agar medium for two days and suspended in sterile water. Except in the last experiment, the aqueous bacterial suspensions (1.8 - 2.6 x 10^7 cfu ml^{-1}) were applied in the root zone of test plants. Instead of soil application, the black pepper cuttings were dipped in the bacterial suspension for one hour and then planted in soil to study the effect of early bacterization. A set of plants not treated with any of the bacteria served as control in all the above experiments. Three plants out of the six from each treatment were inoculated 24 h later (except in the last experiment where nematodes were inoculated only after rooting of cuttings) with a 5 ml aqueous suspension of *M. incognita* juveniles (@500 J2 pot^{-1}). All treatments had 3 replications and the designs were RBD for all experiments. Observations on height of the plant, total biomass, root weight and root galling were recorded 60 days after nematode inoculation (90 days in the third experiment to study the longevity of effects due to bacterial colonization). The plants were maintained in the greenhouse and final observations like height of the plant and total biomass were recorded on completion of the experiment. Nematode population level was assayed as stated above.

The bacterial endoparasite, *P. penetrans* (Pp1), was tested for its ability to suppress root-knot nematodes of cardamom. Cardamom (cv. Malabar - Clone 37) seedlings of one-month age were planted in 1000 cc polythene bags containing sterilized potting media comprising a

mixture of sand : soil : FYM (1:1:1). There were four levels of bacterial infestation and two levels of population densities of *M. incognita* (0 and 500 J2 plant^{-1}). The 2 x 4 factorial experiment was replicated 8 times in a randomized block design. Four levels of *P. penetrans* were established by combining 0, 25, 50 and 100% of endospore-encumbered J2 with uninfested J2 for each nematode density. The nematodes with the desired percentages of *P. penetrans* infestations were suspended individually in 300 ml water and sprinkled uniformly over the soil in each pot. The experiment was concluded after 10 months and observations on growth, nematode population and endospore attachment on juveniles were recorded. Enumeration of endospores of the bacterial parasite, *Pasteuria* was achieved by counting the number of endospores attached to juveniles. The endospores present in nematode females were quantified by mortar disruption method (Chen, Z.X. *et al.*, 1996).

b. Field testing

Trichoderma spp., *P. lilacinus*, *V. chlamydosporium* and the bacterial hyperparasite, *P. penetrans* which showed excellent anti-nematode properties in both laboratory and greenhouse tests, were evaluated in the field. For this purpose, three separate experiments were conducted at different locations.

The experiment was conducted in a cardamom nursery at Appangala, Kodagu, Karnataka and cardamom seeds of a high yielding Malabar type (Cl.37), were sown at the recommended rate. *V. chlamydosporium* (Vc3) was multiplied on rice grains while endospore-encumbered J2 of root-knot nematodes were used as inoculum for *P. penetrans* (Pp1). After germination of seeds, 20 g of fungus-colonised rice grains were mixed with sand (1:4%) and incorporated in the soil. The J2 attached with endospores of *P. penetrans* were added to the soil at 2000 J2 bed^{-1} after suspending them in 500 ml of water. Half of the beds were inoculated with root-knot nematodes (2000 J2 bed^{-1}) at the time of germination while the other half was left as such. Each treatment was replicated four times in a RBD fashion. The experiment was

concluded after 10 months and five plants were removed at random from each bed for recording final observations like height of the plant, number of tillers and nematode population. Root-knot nematode females were excised from the roots to reisolate the fungus/bacterium by methods described above.

Three trials were laid out in two nematode-infested cardamom nurseries at Appangala and Biligiri in Kodagu District, Karnataka. Soil beds (5 x 1 m) were prepared in these nurseries and half of the beds were solarized for 45 days by spreading a 300-gauge transparent polythene sheet over the nursery beds. The other half was left as such. Besides solarization the other treatments were i) a mixture of *Trichoderma* populations (C. 20, C. 21, C.22, C. 23 and C. 24) alone, ii) *P. lilacinus* (Pl. 1) alone, iii) *Trichoderma* spp. + *P. lilacinus* and iv) control (without any biocontrol agent). Cardamom seeds of Clone 37 were sown at the recommended rate. Three isolates of *T. harzianum* (C20, C22 and C23) and one *T. viride* isolate (C21) were multiplied together on sterilized neem oil cake and decomposed coffee husk (1:1 w/w) mixture. *P. lilacinus* (Pl1) was multiplied on rice grains. Fifteen-day-old biocontrol inocula of both fungi were mixed well with the nursery soil thrice - first before sowing (50 g sq. m^{-1}), followed by second inoculation at rhizome formation (75 g sq. m^{-1}) and finally at tillering stage (100 g sq. m^{-1}). Control beds received same quantity of the carrier substances without the fungi. Each treatment was replicated 4 times in a completely randomized design. Germination, growth of seedlings (height of the seedling, no. of tillers, no. of primary roots and biomass) and incidence of diseases were monitored periodically. After 9 months, the total number of saleable seedlings, having a standard number of tillers and girth at collar region, in each bed was counted. Further 20 seedlings were selected at random from each bed and their biomass (fresh wt. seedling^{-1}) and number of nematodes per gram root were recorded as explained earlier.

A root-knot nematode infested (mean population – 1615.97 per g of root) 5-year-old Panniyur V black pepper garden at Mullankolli, Wyanad District, Kerala was selected for this study in 1999. Each vine in the garden was indexed for yellowing and wilting (common symptoms of nematode infestation) using a 0-5 scale. The experimental site was divided into plots consisting of 60-70 vines. The treatments were i). *T. harzianum* (C. 22), ii). *V. chlamydosporium* (Vc. 3), iii). *P. penetrans* (Pp. 1), iv). Phorate @ 30 g vine^{-1} + 0.3% potassium phosphonate @ 3-5 l vine^{-1}, v). Phorate @ 30 g vine^{-1} and vi). check. Each treatment was replicated three times in a randomized block design. *T. harzianum* and *V. chlamydosporium* were multiplied on sorghum while *Pasteuria* was multiplied by the method described by Stirling & Wachtel (1980). The plants were treated with the biocontrol agents twice in a year, during May-June and after monsoon during October-November for four consecutive years. The nematode population in soil and roots were estimated during the post monsoon period using methods stated earlier. The nematodes were checked for bacterial/fungal colonization by methods already described. The mortality and yellowing of vines were recorded at the time of application of biocontrol agents and pesticides. The yield of experimental vines in each plot was recorded for three years.

D. Standardization of Optimum Conditions

The physical and chemical environment of the soil affects nematodes as well as their antagonists. The ecological parameters studied were soil temperature, pH of the soil and also the effect of agrochemicals like pesticides.

a. Temperature

The ability of promising biocontrol agents namely, *T. harzianum* (C.22), *P. lilacinus* (Pl.2) and *V. chlamydosporium* (Vc.3) was tested at various temperatures to determine the optimum temperature requirement for each fungus. One-day-old cultures (4 mm disc) of these fungi were inoculated on to PDA in petri plates and incubated at 15, 20, 25, 30 and 35°C. There

were 3 replications for each temperature and each fungal strain. The radial growth of the fungus was measured at 24 h intervals for 168 h except for *T. harzianum* for which the growth was recorded for 72 h only.

b. pH

A series of PDA media with pH ranging from 3 – 9 were prepared in conical flasks by adding 1N hydrochloric acid or IN sodium hydroxide. The autoclaved medium was poured into sterile petri plates 4 mm PDA discs of one day-old cultures of two isolates of *T. harzianum* (C.22 and P.26) and one isolate of *Fusarium* sp. (F.47) were transferred aseptically to the center of the medium with a specific pH. There were seven treatments with three replications. These plates were sealed with parafilm and incubated at 28°C. The growth rate of the test fungus was compared over the pH range by measuring the colony diameter at 24 h intervals.

c. Pesticides

The compatibility of the fungal biocontrol agents with agrochemicals was studied by poisoned food technique. Effects of two each commonly used fungicides and insecticides in spice crops viz. potassium phosphonate, metalaxyl, phorate and chlorpyriphos were tested against three of the antagonistic fungi viz. *T. harzianum* (C22), *V. chlamydosporium* (Vc3) and *P. lilacinus* (Pl2). Each pesticide was separately added to PDA so as to get concentrations ranging from 5 ppm to 48 ppm and thoroughly mixed by stirring. The amended medium was poured into four 9 cm-diam petri dishes and was inoculated with a 4 mm-diam plug of each fungus. Suitable checks were maintained on PDA without pesticides. These plates were incubated at 28°C and radial growth was measured every 24 h and compared with the control.

E. Studies on Mass Multiplication

The fungal antagonists are generally multiplied on solid or liquid substrates for field application. In a laboratory experiment, organic substances like neem oil cake, farmyard manure, vermi-compost, sorghum, rice grains, saw dust, coir dust and several leaf powders were evaluated for their ability to support growth and multiplication of fungi like *P. lilacinus* (Pl2), *V. chlamydosporium* (Vc3) and *T. harzianum* (C22). These substances were washed on a 53 μm aperture sieve, oven-dried, mixed thoroughly and moistened with water at the ratio of 1:1 (w/w). Tissue culture bottles (250 ml) were partially filled with 75 cc of each substrate and were autoclaved at 1.05 kg cm^{-2} for 15 min. When cooled, each flask was inoculated with a 4 mm plug of the respective actively growing fungi in separate experiments. The flasks were incubated at 28°C for 21 days. For the first few days, the flasks were shaken to disperse the fungus throughout the media. They were then left undisturbed for facilitating sporulation. The number of spores and cfu g^{-1} of the carrier substance were estimated for each fungus at 7, 14 and 21 days of incubation (except for *V. chlamydosporium*).

F. Statistical Analysis

Angular or square-root transformation of the percentage data was done before analysis and logarithmic transformation was used for nematode densities and numbers of eggs/juveniles per root system. Means given in the results were back-transformed values. ANOVA or two-way analysis of variance was used for data analysis (Gomez & Gomez, 1984). Means were separated by Least Significant Difference (LSD) or Duncan's Multiple Range Test (DMRT).

Chapter 4

Observations

A. Isolation and Identification of Biocontrol Agents

The rhizosphere samples collected from various spices yielded a large number of fungi and bacteria. Those isolates with distinct features and characteristics in the colony morphology were only selected and maintained for further studies (Plate 2 & 3). Isolates from a locality having similar features were discarded to avoid any duplication. A few species of mites were also extracted from some of these soil samples. Thus 57 bacteria and 73 fungi were short-listed for further studies (Table 2). Out of the 73 fungi, 61 isolates were saprophytes, some of which were previously reported as facultative parasites of nematodes. Majority of them (17 isolates) belonged to the genus *Trichoderma*, while 7 isolates were of *Aspergillus* spp., 6 each of *Paecilomyces* spp. and *Pencillium* spp., 5 isolates of *Fusarium* spp., and 2 isolates of *Verticillium* spp (Plate 4 & 5). Taxonomic identity of 22 isolates could not be established as there was no sporulation.

Among the 57 bacteria isolated, 17 isolates belonged to different species of *Bacillus* while 12 were *P. fluorescens* isolates. Three isolates of *P. penetrans* were obtained from ginger samples collected from Kasaragod District. About 50 per cent of the bacterial isolates could not be identified even up to the generic level as they lacked easily distinguishable taxonomic features.

B. Mode of Action of Biocontrol Agents

The pathogenicity of fungi to eggs of root-knot nematode, *M. incognita* on water agar varied among species and isolates of various fungi (Plate 6 & 7). Sixty-seven out of the 110 isolates screened were not parasitic on females of root-knot nematodes. Significant parasitism on adult females was observed only in three isolates viz. *P. lilacinus* (Pl. 1), *T. harzianum* (Plate 6.F) and *V. lecanii* (Vl) (Table 3).

Table 2. Bacteria and fungi isolated from the rhizosphere of spices.

Antagonist	Black pepper	Cardamom	Ginger	TOTAL
BACTERIA				
Bacillus spp.	5	-	12	17
Pseudomonas flourescens	8	-	4	12
Pasteuria sp.	-	-	3	3
Unidentified	12	-	13	25
FUNGI				
Aspergillus sp.	1	-	-	1
A. fumigatus	1	-	1	2
A. nidulans	-	-	1	1
A. restrictus	-	-	1	1
A. tamarii	-	-	1	1
A.ustus	-	-	1	1
Aurobasidium sp.	1	-	-	1
Cephalosporium sp.	1	-	-	1
Drechslera sp.	1	-	1	2
Fusarium sp.	-	-	4	4
F.oxysporum	-	-	1	1
Humicola sp.	-	-	2	2
Paecilomyces sp.	1	-	1	2
P. carneus	-	-	1	1
P. lilacinus	-	1	2	3
Penicillium sp.	1	-	2	3
P. citrinum	-	-	1	1
P. fumiculosm	-	-	1	1
P.digitatum	-	-	1	1
Scopulariopsis sp.	-	-	1	1
Scolecobasidium sp.	-	-	1	1
Trichoderma sp.	7	-	3	10
T. harzianum	-	3	1	4
T. virens	-	1	-	1
T. viride	-	1	1	2
Verticillium chlamydosporium	1	-	-	1
V. lecanii	-	1	-	1
Unidentified	17	-	5	22
Total	57	7	66	130

Plate 2

Colony Morphology of Fungal Antagonists of Plant Parasitic Nematodes

Plate 2

A. *Trichoderma harzianum* – C.22

B. *Trichoderma* sp. – Is.16

C. *Trichoderma virens* – Gv. 13

D. *Penicillium* sp. – Is. 17

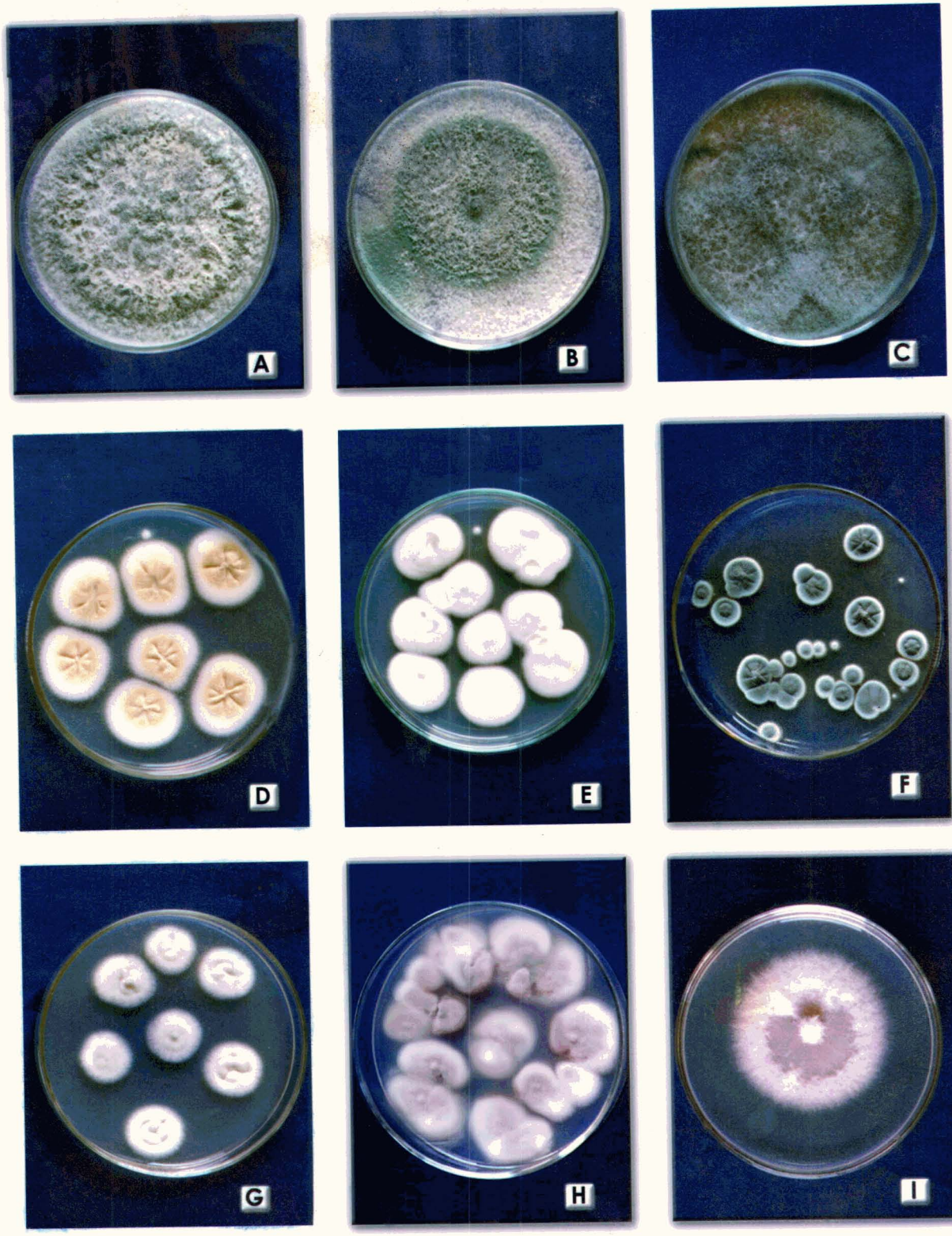
E. *Humicola* sp. – Is. 19

F. *Paecilomyces* sp. – Is. 20

G. *Penicillium digitatum* – Is. 23

H. *Scopulariopsis* sp. – Is. 14

I. *Fusarium oxysporum* – Is. 11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B
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Plate 3

Colony Morphology of Fungal Antagonists of Plant Parasitic Nematodes (cont'd)

Plate 3

A. *Verticillium lecanii* – Vl

B. *Verticillium chlamydosporium* – Vc. 2

C. *Scolecobasidium* sp. – Is. 15

D. *Aspergillus* sp. – Pat. 7

E. *Aspergillus tamarii* – Is. 2

F. *Aspergillus fumigatus* – F. 6

G. *Aspergillus ustus* – Is. 21

H. *Aspergillus fumigatus* – Pat. 4

I. *Aspergillus restrictus* – Is. 7

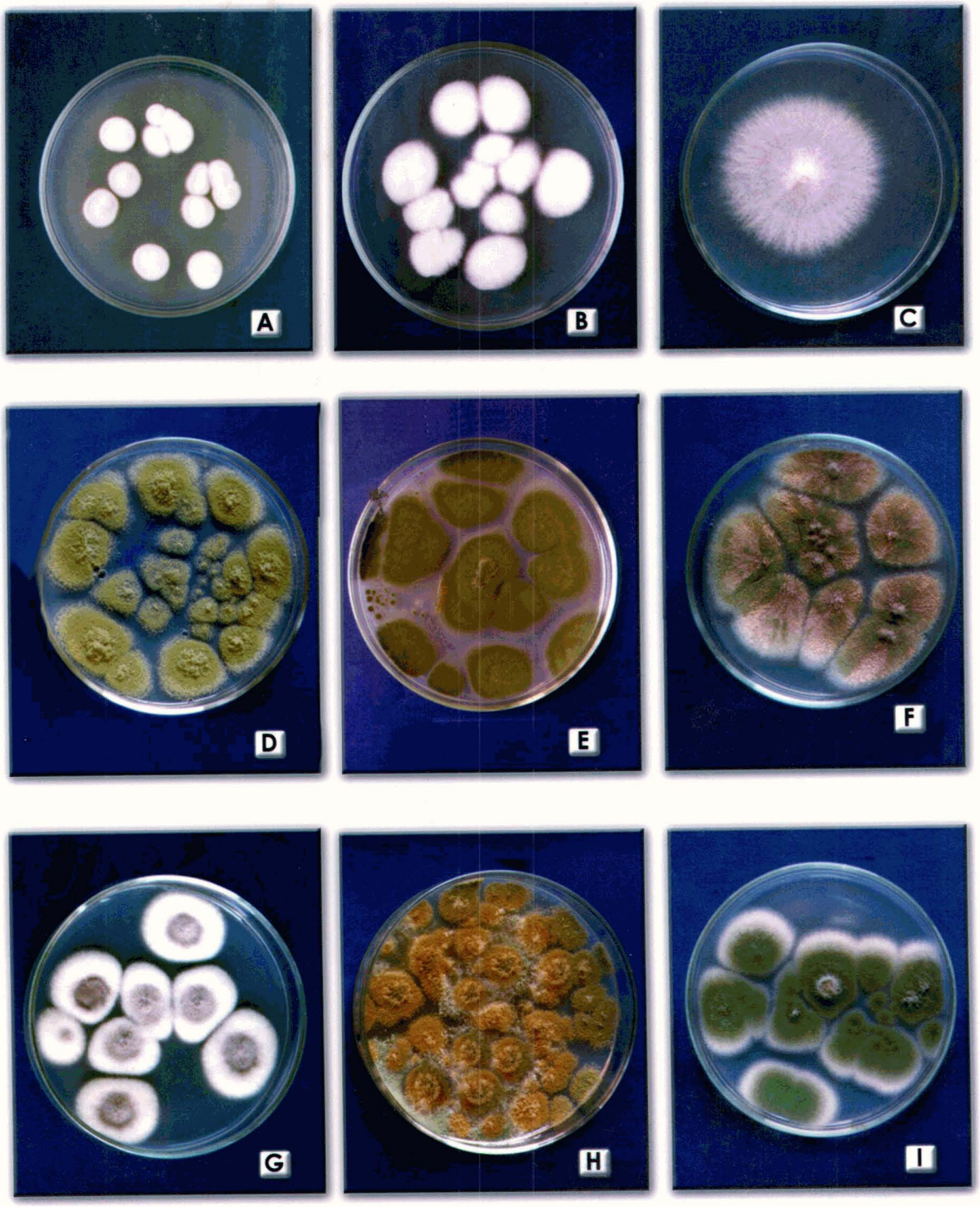
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Plate 4

Structure of Hyphae of Fungal Antagonists of Plant Parasitic Nematodes

Plate 4

A. *Penicillium digitatum* – Is. 23

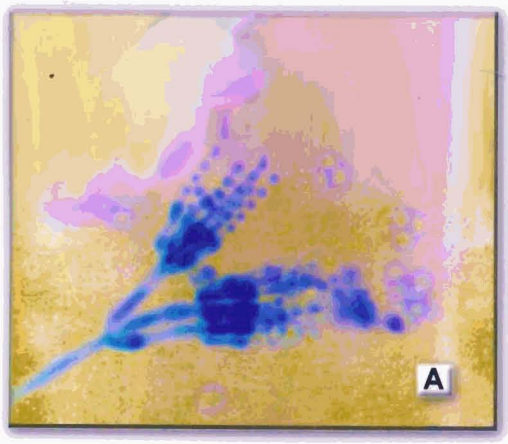
B. *Verticillium lecanii* – Vl

C. *Trichoderma virens* – Gv. 21

D. *Trichoderma virens* – Gv. 13

E. *Trichoderma harzianum* – C.22

F. *Verticillium chlamydosporium* – Vc. 1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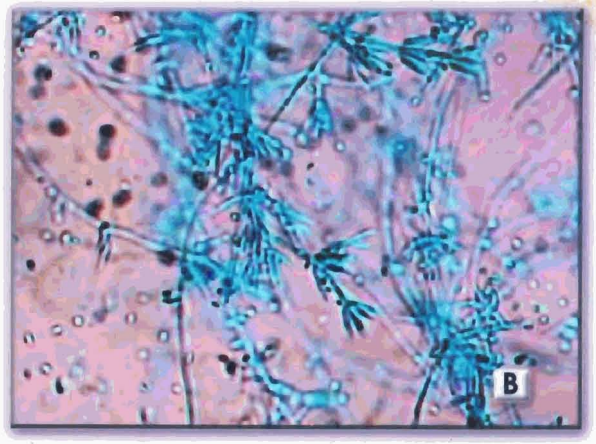
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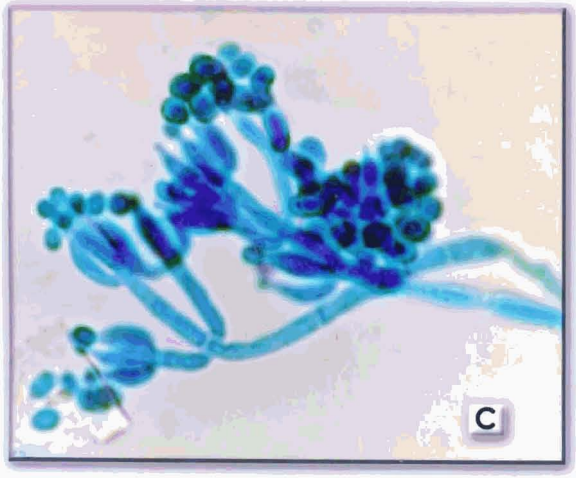
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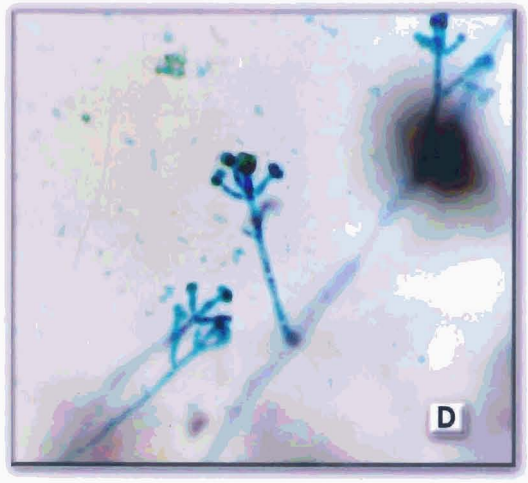
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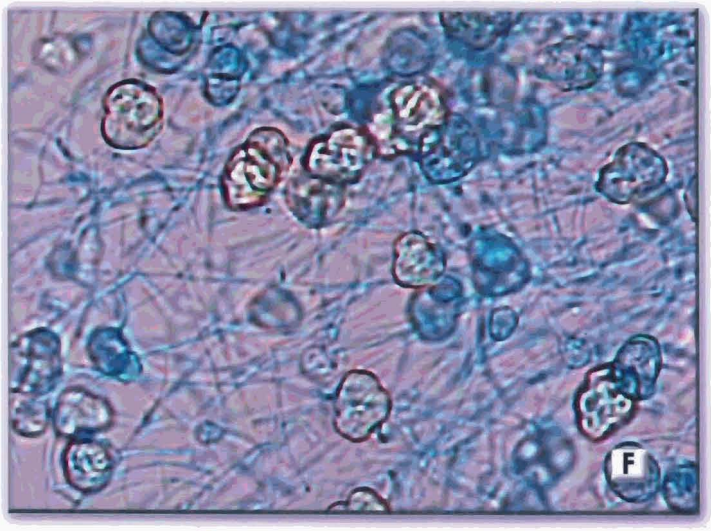
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Plate 5

Structure of Hyphae of Fungal Antagonists of Plant Parasitic Nematodes (cont'd)

Plate 5

A. *Aspergillus ustus* – Is. 21

B. *Aspergillus* sp. – Pat. 7

C. *Aspergillus tamarii* – Is. 2

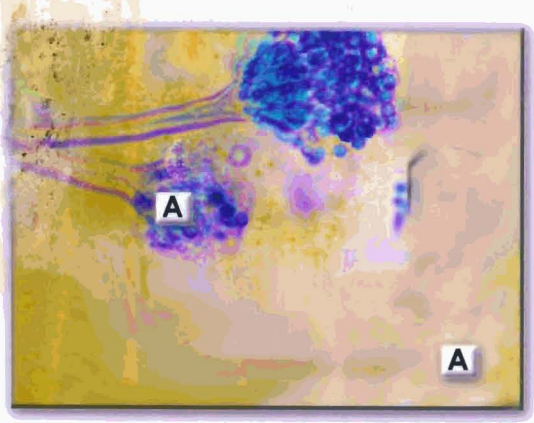
D. *Paecilomyces lilacinus* – Is. 27

E. *Scolecobasidium* sp. – Is. 15

F. *Scopulariopsis* sp. – Is. 14

G. *Paecilomyces lilacinus* – Pl. 1

H. *Paecilomyces lilacinus* – Pl. 2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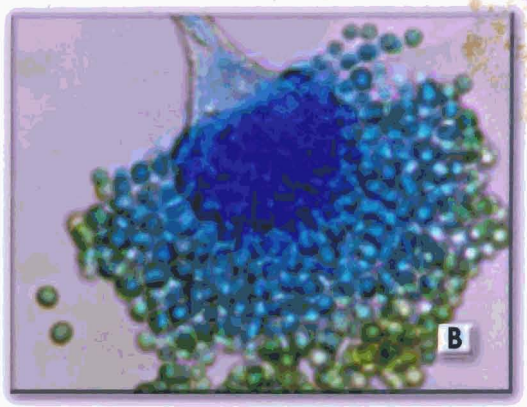
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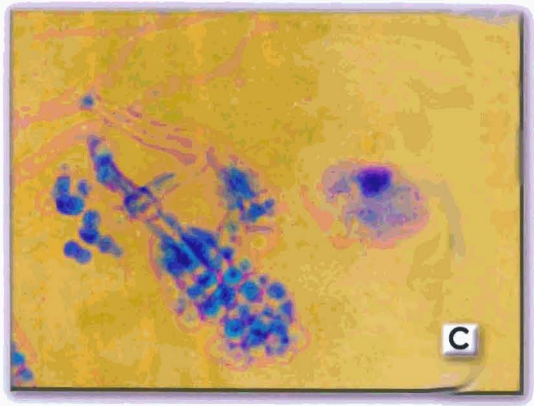
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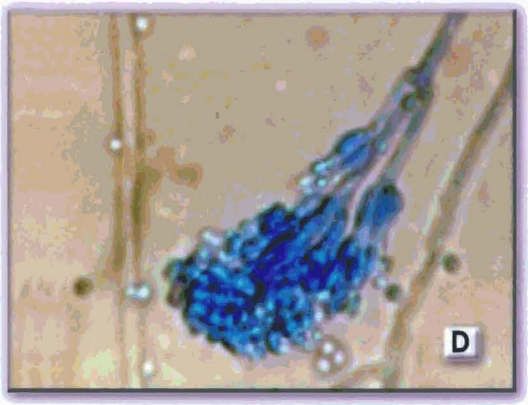
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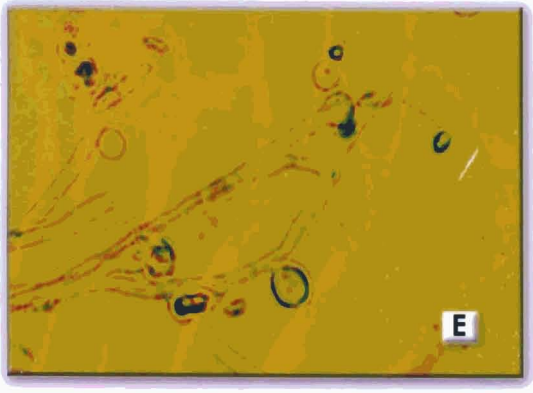
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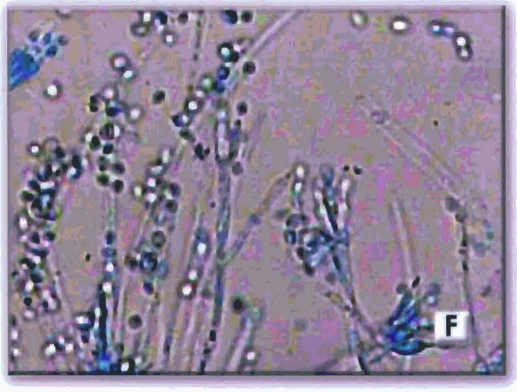
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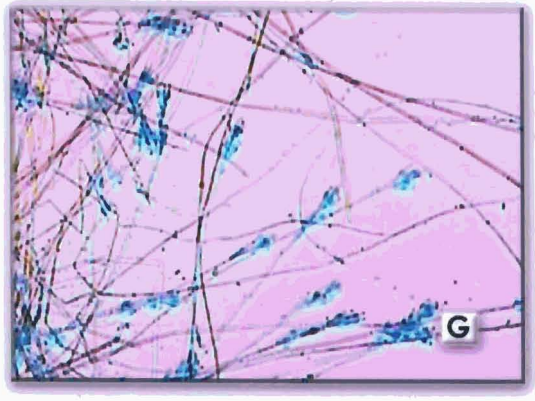
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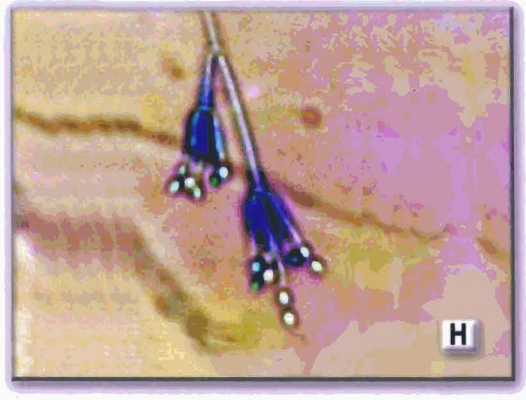
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Out of the 149 fungal isolates screened, 115 isolates colonized the gelatinous matrix of root-knot nematode egg masses (77.18%). Majority of them (73.8%), though colonized the egg masses, did not show high parasitism on nematode eggs. Only four isolates showed remarkable egg parasitism (>25%) and they were two each isolates of *V. chlamydosporium* (F.30 and Vc.3) (Plate 7.C) and *P. lilacinus* (Pl.1 and Pl.2) (Plate 7.F). Another 33 isolates exhibited moderate egg parasitism (Table 4). These included fungi belonging to the genera *Aspergillus* (F.6, F.34, F.37, F.40, F.45 and F.46), *Fusarium* (F.41 and F.47), *Trichoderma* (F.3, F.5, F.52, F.60, F.71, Gv.21, Th.12, Th.22a, Th.29/11, Th.32, Th.44, Th.45, Thm.7b and Thm.22b) (Plate 6), *Drechslera* (F.32), *Humicola* (F.43), *Scopulariopsis* (F.50) (Plate 7.D), *Verticillium* (Vl) and four unidentified strains of fungi (F.2, F.15, F.21and F.62). Most of these isolates were parasitic on root-knot nematode females too. But none of the 149 isolates of fungi was parasitic on root-knot nematode juveniles.

In the bioassay for suppression of hatching, except two isolates (F.48 and F.57), all the fungi screened showed various degrees of adverse effect on the egg hatch process (Table 5). But only 11 isolates had shown >90% suppression in hatching. Fifty-nine isolates showed 50 - 90 % inhibition in egg hatch. The most promising isolates in this study were one isolate each from *Aspergillus* (F.45), *Fusarium* (F.47) and *Penicillium* (F.59); three each isolates of *Trichoderma* (F.3, F.52 and F.60) and *Verticillium* (F.30, Vc.3 and Vl); and two isolates of fungi that could not be identified (F.28 and F.62). Considerable variability was observed among isolates with regard to inhibition of egg hatch (0-100%), parasitism on eggs or females (0 - 42.32 and 0 - 32.82%, respectively).

The results clearly indicated that the promising fungal antagonists have different modes of action (Table 6). A few of them like *P. lilacinus*, *Trichoderma* spp., *Verticillium* spp. possessed multiple modes of action. *V. chlamydosporium, V. lecanii, P. lilacinus* and few

Table 3. Parasitism on root-knot nematode females by different isolates of fungi.

Intensity of parasitism (%)	Number of isolates	Isolate No.
0	67	Av.7, C.21, F.1, F.4, F.10, F.11, F.12, F.17, F.22, F.23, F.24, F.25, F.26, F.29, F.32, F.34, F.35, F.37, F.38, F.39, F.40, F.41, F.42, F.43, F.44, F.45, F.46, F.47, F.48, F.49, F.50, F.51, F.54, F.55, F.56, F.59, F.60, F.61, F.64, F.65, F.67, F.68, F.69, F.70, F.71, Gv.10, Gv.12, Gv.13, Gv.30, Is.37, P.26, T.5, T.7, T.8, T.14, Th.5, Th.10, Th.18a, Th.20a, Th.30, Th.39, Th.42, Thm.13a, Thm.16a, Thm.16b, Thm.17b, Tk.1
0.1 – 10.0	3	F.9, Pk.3, Tv.1
10.1 – 25.0	37	Av.22, C.20, C.23, C.24, F.2, F.3, F.5, F.6, F.13, F.15, F.16, F.20, F.21, F.28, F.30, F.52, F.62, Gv.17, Gv.21, Gv.24, Is.7, Pl.2, T.2, Th.8, Th.9, Th.12, Th.22a, Th.26, Th.29/11, Th.32, Th.44, Th.45, Thm.7b, Thm.15b, Thm.22b, Vc.2, Vc.3
25.1- 50.0	3	Pl.1, C.22, Vl

Table 4. Parasitic efficiency of various fungi on eggs of root-knot nematodes.

Intensity of parasitism (%)	Number of isolates	Isolate No.
0	110	Av.7, Av.22, C.21, C.24, F.1, F.4, F.7, F.8, F.9, F.10, F.11, F.12, F.13, F.14, F.16, F.17, F.18, F.19, F.20, F.22, F.23, F.24, F.25, F.26, F.27, F.28, F.29, F.31, F.33, F.35, F.36, F.38, F.39, F.42, F.44, F.48, F.51, F.53, F.54, F.55, F.56, F.57, F.58, F.59, F.63, F.64, F.65, F.66, F.67, F.68, F.69, F.70, F.72, F.73, Gv.10, Gv.12, Gv.13, Gv.17, Gv.19, Gv.20, Gv.24, Gv.27, Gv.30, Is.7, Is.37, Lb.4, P.26, Pk.3, T.2, T.5, T.7, T.8, T.10, T.14, T.12, Th.5, Th.8, Th.9, Th.10, Th.13, Th.18a, Th.20, Th.20a, Th.21, Th.25, Th.26, Th.29, Th.30, Th.39, Th.42, Thm.6a, Thm.6b, Thm.7a, Thm.8, Thm.10, Thm.13a, Thm.15a, Thm.15b, Thm.16a, Thm.16b, Thm.17a, Thm.17b, Thm.18b, Thm.23a, Thm.23b, Thm.25, Tk.1, Tp.10, Tv.1, Vc.2
0.1– 10.0	2	F.49, F.61
10.1 – 25.0	33	C.20, C.22, C.23, F.2, F.3, F.5, F.6, F.15, F.21, F.32, F.34, F.37, F.40, F.41, F.43, F.45, F.46, F.47, F.50, F.52, F.60, F.62, F.71, Gv.21, Th.12, Th.22a, Th.29/11, Th.32, Th.44, Th.45, Thm.7b, Thm.22b, Vl
25.1- 50.0	4	F.30, Pl.1, Pl.2, Vc.3

Table 5. Suppression in hatching of root-knot nematode eggs by various fungi.

Suppression of hatch (%)	Number of isolates	Isolate No.
0	2	F.48, F.57
0.1 – 25.0	54	F.1, F.7, F.8, F.9, F.10, F.11, F.12, F.14, F.18, F.19, F.27, F.31, F.33, F.36, F.44, F.53, F.58, F.63, F.66, F.67, F.69, F.72, F.73, Gv.19, Gv.20, Gv.27, Lb.4, Pk.3, T.7, T.10, T.12, Th.5, Th.10, Th.13, Th.20, Th.21, Th.25, Th.29, Thm.6a, Thm.6b, Thm.7a, Thm.8, Thm.10, Thm.13a, Thm.15a, Thm.16a, Thm.16b, Thm.17a, Thm.17b, Thm.18b, Thm.23a, Thm.23b, Thm.25, Tp.10
25.1 – 50.0	25	Av.22, F.2, F.4, F.13, F.34, F.35, F.38, F.54, F.64, F.68, F.70, Gv.10, Gv.12, Gv.13, Gv.24, Is.37, T.5, T.8, T.14, Th.18a, Th.20a, Th.30, Th.39, Tk.1, Tv.1
50.1 – 90.0	57	Av.7, C.20, C.21, C.22, C.23, C.24, F.5, F.6, F.15, F.16, F.17, F.20, F.21, F.22, F.23, F.24, F.25, F.26, F.29, F.32, F.37, F.39, F.40, F.41, F.42, F.43, F.46, F.49, F.50, F.51, F.55, F.56, F.61, F.65, F.71, Gv.17, Gv.21, Gv.30, Is.7, P.26, Pl.1, Pl.2, T.2, Th.8, Th.9, Th.12, Th.22a, Th.26, Th.29/11, Th.32, Th.42, Th.44, Th.45, Thm.7b, Thm.15b, Thm.22b, Vc.2
90.1 – 100.0	11	F.3, F.28, F.30, F.45, F.47, F.52, F.59, F.60, F.62, Vc.3, Vl

Plate 6

Parasitization of Root-knot Nematode Eggs by Egg Parasitic Fungi

Plate 6

A. *Trichoderma virens* – Gv. 21

B. *Trichoderma* sp. – Pat. 5

C. *Trichoderma virens* – Gv. 13

D. *Trichoderma virens* – Gv. 13

E. *Penicillium digitatum* – Is. 23

F. *Trichoderma harzianum* – C.22

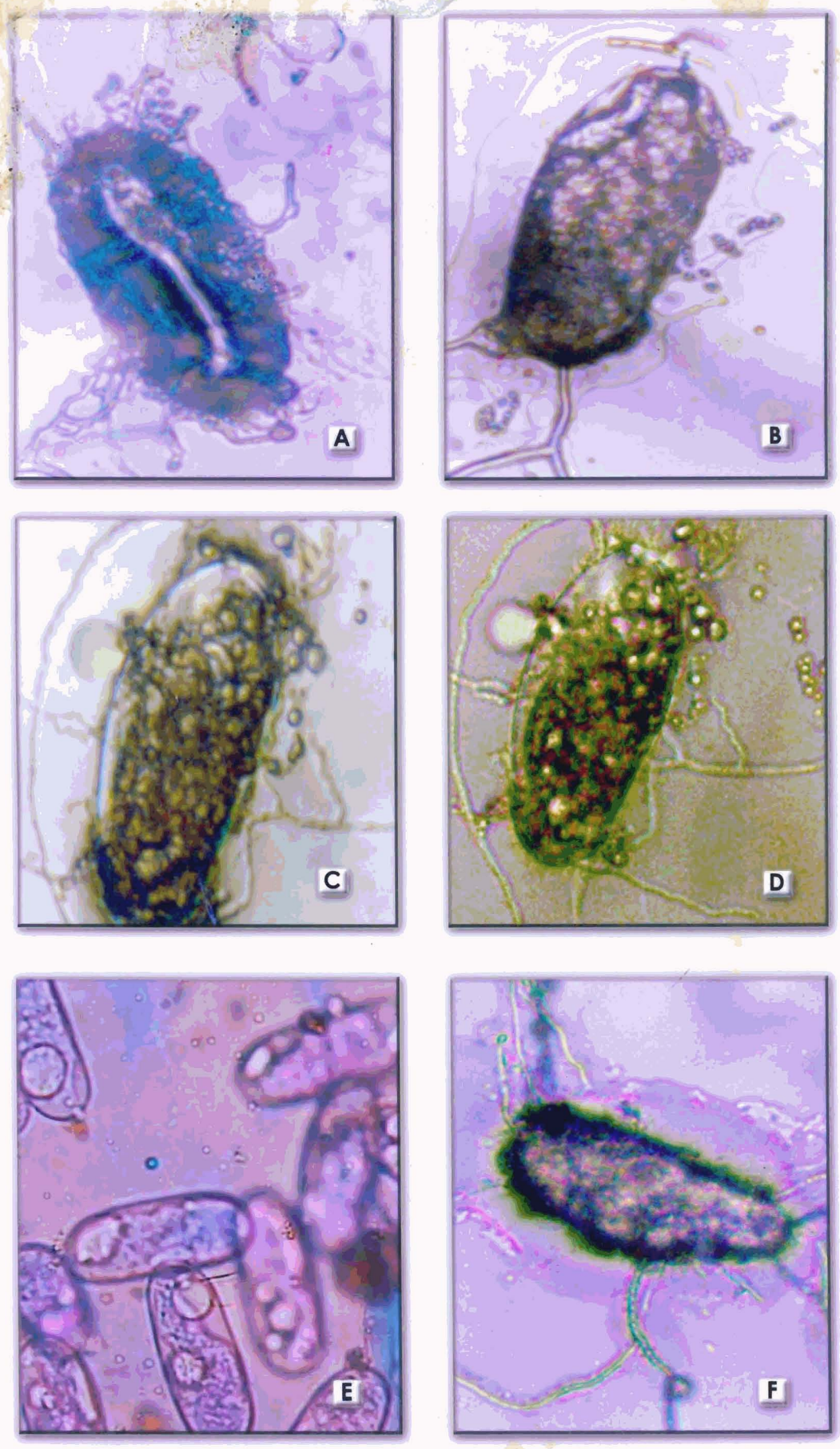

A
B
C
D
E
F

Plate 7

Parasitization of Root-knot Nematode Eggs by Egg Parasitic Fungi (cont'd)

Plate 7

A. *Fusarium oxysporum* – Is. 11

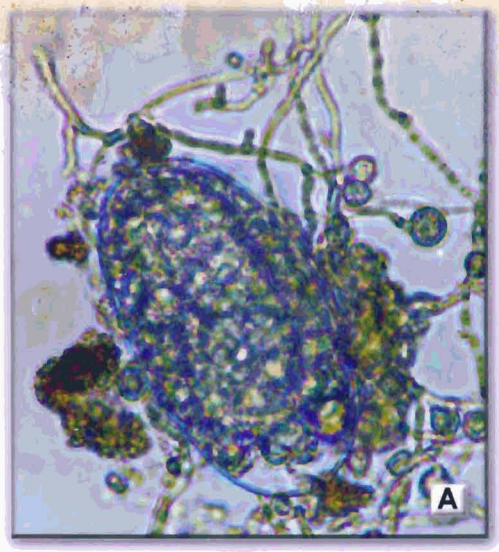
B. *Scolecobasidium* sp. – Is. 15

C. *Verticillium chlamydosporium* – Vc. 3

D. *Scopulariopsis* sp. – Is. 14

E. *Aspergillus tamarii* – Is. 2

F. *Paecilomyces lilacinus* – Pl. 2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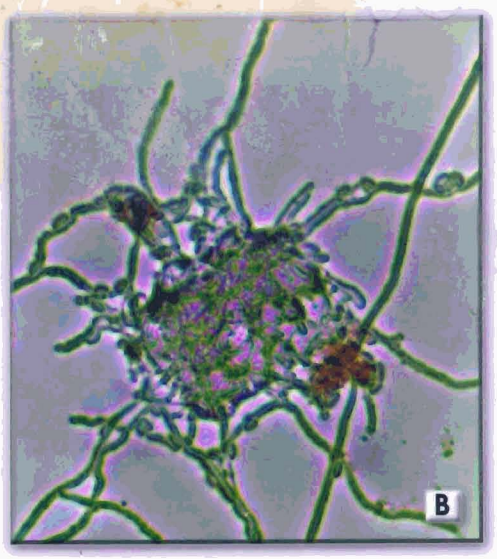
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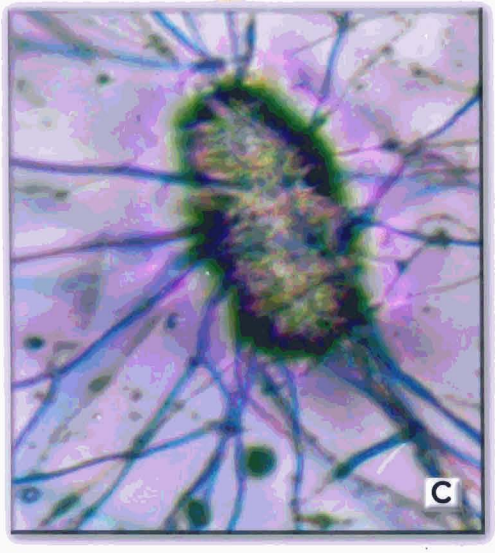
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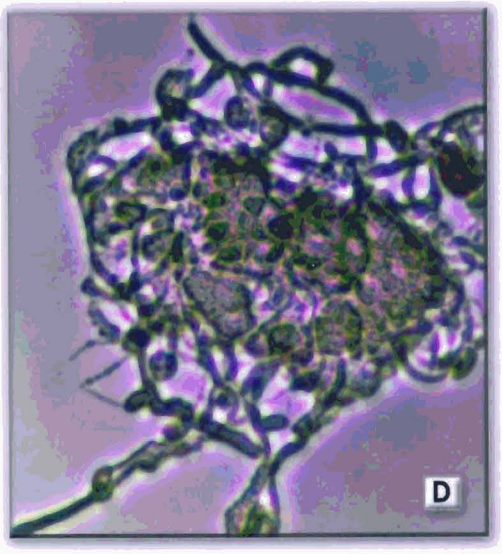
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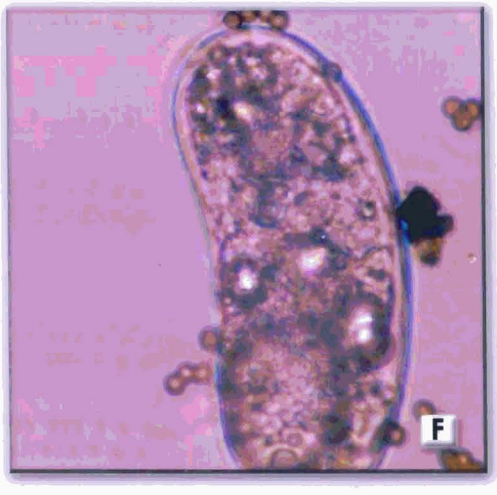
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Table 6. Comparison of nematode suppression mechanisms of promising fungal antagonists.

Fungal antagonist	Isolate No.	Parasitism on		Hatching suppression[b] (%)
		Eggs [a] (%)	Females [a] (%)	
Aspergillus restrictus	F.45	20.82	0.00	91.80
Fusarium oxysporum	F.47	21.22	0.00	95.71
Paecilomyces lilacinus	Pl.1	42.32	28.12	78.12
	Pl.2	36.12	24.22	72.06
Penicillium digitatum	F.59	0.00	0.00	100.00
Trichoderma sp.	F.52	14.66	10.21	97.20
	F.60	16.38	0.00	93.56
T. harzianum	C.22	24.26	32.82	88.89
T. viride	F.3	10.26	15.38	91.03
Verticillium chlamydosporium	F.30	35.63	18.36	95.45
	Vc.3	26.42	19.48	91.24
V. lecanii	Vl	24.36	26.48	91.89
Unidentified	F.28	0.00	12.36	90.64
	F.62	19.36	12.48	95.45

Values are mean of three replications. Percentage data transformed to arc sine for analysis and converted to original means. Table compiles results of different screening experiments carried out separately. Hence, no direct comparisons among isolates were attempted. However, results allow classification of each isolate based on its ability to suppress nematodes.

a. Proportion of eggs / females parasitized by the candidate fungus.

b. Hatching suppression = (1 - T/C) x 100, where T is the mean of J2 hatching in the treatment and C is the mean of J2 hatching in the control.

isolates of *Trichoderma* spp. invaded the root-knot nematode eggs (Plate 6 & 7). Immature eggs in early embryonic development stage were more susceptible to these fungi. Those eggs containing second stage juveniles were seldom parasitized. Infected eggs were pale, yellowish-brown and in most cases, no trace of larvae could be detected. They were stained easily due to the increased permeability of the eggshell. The embryonic development was arrested by the fungal invasion. Fungal mycelium radiated profusely from eggs in the advanced stage of infection. In some cases high vacuolation was observed within the infected eggs (Plate 6.E).

Culture filtrates of selected fungi definitely had a crucial role to play in the suppression of nematodes (Table 7). The mean mortality of second stage juveniles varied from 16.63% - 47.78%. Among the 6 fungal isolates which were good parasites of eggs and females, 4 isolates (Pl.1, Pl.2, Vc.3 and C.22) produced culture filtrates highly toxic to nematode juveniles, indicating a dual mode of action. Similarly, culture filtrates of some isolates (F.5, F.56, Th.22a, Th.32 and Vc.2), which were poor colonizers of either root-knot nematode females or eggs, caused high mortality of J2.

The results of *in vitro* screenings using 57 bacterial isolates are given in Table 8. There was no sign of egg parasitism by any one of the bacterial strains. Except 8 strains (B_3, B_5, B_6, B_7, B_{11}, B_{27}, B_{29} and B_{42}), majority of them were poor inhibitors of the egg hatch. Similarly, except a few isolates that caused 100% immobility of root-knot nematode larvae, most of them did not have much adverse effect on the juveniles.

C. Biocontrol Potential of Antagonists

a. Greenhouse studies

Among the five isolates of *Trichoderma* evaluated, isolates C.22 and C.23 were found superior in suppressing root-knot nematode populations than the other isolates. C.22

Table 7. Toxicity of culture filtrates of some antagonistic fungi on root-knot nematode juveniles.

Isolate No.	Mortality of J2 at different dilutions of fungal culture filtrate (%)			
	25%	50%	100%	Mean
C.22	33.13 ± 4.04	35.38 ± 3.13	55.61 ± 1.73	41.48 ± 2.74
F.3	25.27 ± 1.87	32.42 ±1.00	36.89 ± 2.25	31.75 ± 0.81
F.5	26.35 ± 0.70	32.82 ± 1.34	45.47 ± 5.56	35.22 ± 2.27
F.6	20.20 ± 4.54	23.15 ± 2.13	28.03 ± 2.74	24.07 ± 0.76
F.28	31.46 ± 2.60	35.01 ± 2.81	38.89 ± 2.07	35.20 ± 1.01
F.30	25.30 ± 1.63	29.82 ± 2.58	31.81 ± 0.77	29.08 ± 1.07
F.45	23.35 ± 2.36	25.34 ± 1.56	25.48 ± 1.90	24.76 ± 1.24
F.47	28.15 ± 2.55	31.83 ± 0.83	34.51 ± 1.49	31.57 ± 1.14
F.52i	32.54 ± 1.56	36.40 ± 2.20	41.03 ± 4.71	36.73 ± 1.47
F.56	33.13 ± 1.67	37.63 ± 1.17	49.20 ± 5.57	40.07 ± 2.62
F.59	26.09 ± 0.98	29.61 ± 2.39	29.51 ± 1.13	28.44 ± 0.94
F.60	30.56 ± 4.09	34.10 ± 1.81	39.47 ± 0.89	34.83 ± 1.40
F.61	24.70 ± 3.06	33.68 ± 2.83	37.70 ± 3.22	32.34 ± 0.71
F.62	27.00 ± 2.28	32.04 ± 0.60	38.12 ± 2.18	32.57 ± 0.32
P.26	25.07 ± 0.90	29.12 ±1.71	31.66 ± 0.73	28.71 ± 0.58
Pl.1	38.14 ± 2.01	40.97 ± 0.80	52.07 ± 0.77	43.73 ± 0.38
Pl.2	35.06 ± 2.04	42.43 ± 3.54	44.87 ± 3.18	40.85 ± 0.48
Th.22a	31.30 ± 2.56	33.59 ± 1.73	37.21 ± 2.93	34.08 ± 2.22
Th.32	29.96 ± 2.37	30.50 ± 3.47	33.77 ± 2.33	31.44 ± 2.67
Vc.2	32.77 ± 1.26	35.39 ±1.72	39.43 ± 0.98	35.91 ± 1.13
Vc.3	31.54 ± 2.09	33.38 ± 1.02	44.42 ± 1.49	36.60 ± 1.43
Vl	31.60 ± 1.40	34.00 ± 0.46	37.64 ± 0.50	34.46 ± 0.36

Values are mean ± S.D. of three replications after arc sine transformation.

Table 8. Effect of bacterial isolates on hatching of *Meloidogyne incognita* eggs and the mobility of hatched juveniles under *in vitro* conditions.

Bacterial isolate	Egg hatch*	Juvenile immobility**
B1	0.82 ± 0.20	0.05 ± 0.11
B2	0.77 ± 0.21	0.26 ± 0.36
B3	0.12 ± 0.16	1.00 ± 0.00
B4	0.94 ± 0.11	0.02 ± 0.06
B5	0.13 ± 0.08	1.00 ± 0.00
B6	0.18 ± 0.21	1.00 ± 0.00
B7	0.10 ± 0.07	1.00 ± 0.00
B8	0.96 ± 0.08	0.02 ± 0.06
B9	0.95 ± 0.04	0.00 ± 0.05
B10	0.92 ± 0.12	0.05 ± 0.00
B11	0.08 ± 0.22	1.00 ± 0.00
B12	0.87 ± 0.23	0.05 ± 0.20
B13	0.89 ± 0.12	0.01 ± 0.39
B14	0.89 ± 0.16	0.04 ± 0.19
B15	0.88 ± 0.07	0.12 ± 0.07
B16	0.62 ± 0.31	0.23 ± 0.42
B17	0.78 ± 0.07	0.12 ± 0.25
B18	0.84 ± 0.14	0.01 ± 0.27
B19	0.90 ± 0.12	0.06 ± 0.26
B20	0.92 ± 0.18	0.14 ± 0.02
B21	0.92 ± 0.28	0.08 ± 0.38
B22	0.91 ± 0.22	0.14 ± 0.11
B23	0.88 ± 0.09	0.01 ± 0.12
B24	0.77 ± 0.24	0.14 ± 0.26
B25	0.38 ± 0.18	0.40 ± 0.22
B26	0.93 ± 0.12	0.10 ± 0.12
B27	0.12 ± 0.14	0.86 ± 0.24
B28	0.28 ± 0.23	0.61 ± 0.67
B29	0.12 ± 0.07	1.00 ± 0.00
B30	0.68 ± 0.22	0.20 ± 0.18
B31	0.66 ± 0.18	0.28 ± 0.20

(continued)

Table 8. Continued

Bacterial isolate	Egg hatch*	Juvenile immobility**
B32	0.32 ± 0.21	0.10 ± 0.18
B33	0.78 ± 0.18	0.42 ± 0.20
B34	0.61 ± 0.05	0.22 ± 0.38
B35	0.62 ± 0.24	0.20 ± 0.36
B36	0.65 ± 0.19	0.26 ± 0.09
B37	0.87 ± 0.21	0.22 ± 0.10
B38	0.76 ± 0.17	0.26 ± 0.19
B39	0.84 ± 0.15	0.13 ± 0.06
B40	0.61 ± 0.22	0.23 ± 0.18
B41	0.32 ± 0.08	0.54 ± 0.00
B42	0.12 ± 0.05	0.85 ± 0.27
B43	0.89 ± 0.31	0.56 ± 0.27
B44	0.92 ± 0.24	0.09 ± 0.37
B45	0.79 ± 0.21	0.33 ± 0.34
B46	0.77 ± 0.23	0.18 ± 0.20
B47	0.81 ± 0.14	0.40 ± 0.66
B48	0.84 ± 0.16	0.41 ± 0.13
B49	0.82 ± 0.28	0.24 ± 0.43
B50	0.76 ± 0.18	0.05 ± 0.18
B51	0.68 ± 0.22	0.19 ± 0.43
B52	0.91 ± 0.12	0.18 ± 0.11
B53	0.65 ± 0.16	0.28 ± 0.14
B54	0.62 ± 0.21	0.32 ± 0.15
B55	0.70 ± 0.20	0.29 ± 0.06
B56	0.74 ± 0.14	0.22 ± 0.18
B57	0.80 ± 0.26	0.18 ± 0.08

Values are mean ± S.D. of three replications. The Table compiles results from all screening experiments. Due to variations in bacterial concentrations in the experiments, no direct comparison among strains were made.

*Mean proportion of eggs hatched or juvenile immobilized adjusted as a fraction of control hatch in respective experiments.

**Mean number of juveniles immobilized adjusted as a fraction of total nematodes taken.

performed better in sterile soil while C.23 was more effective in native (non-sterile) soil. The suppression was more prominent in native soil than in sterilized soil (Table 9a). They reduced nematode populations in cardamom roots by 31.50 to 86.80 per cent in sterile soil and by 31.58 to 82.14 per cent in non-sterile soil. In general the nematode multiplication was poor in non-sterile soil, even in control pots. Except C.23, none of the *Trichoderma* isolates was able to improve significantly the growth of cardamom plants in sterile soil (Table 9b and Plate 8). However, when plants in sterile soil were infested with root-knot nematodes, all the *Trichoderma* isolates were ineffective in either improving the growth or suppressing the nematodes. Though not significant, most of the isolates induced some degree of growth promotion. C.23 in sterile soil caused significant growth promotion in cardamom plants. *Trichoderma* isolates (C.20, C.21 and C.23) alone and all of them together were able to offset the nematode damage in cardamom plants (Table 9b). The study clearly proved that by combining different isolates of *Trichoderma* the growth of cardamom seedlings can be sustained even when they were challenged by nematodes (Plate 8.F).

Among the six isolates of *Trichoderma* screened against nematodes affecting black pepper plants, increased growth response was not observed with any of the isolate (Fig. 1). Slight improvement in growth was noticed only with T.12 isolate. All the growth parameters viz. height of the plant, total biomass and number of nodes plant^{-1} were on par with those of the check plants which received neither the nematodes nor the fungi. Nematode inoculation significantly reduced the growth of black pepper cuttings in all respects. However, T.10, T.12 and to a limited extent T.5 were able to protect the black pepper cuttings against the nematode induced damages. The numbers of colony forming units of *Trichoderma* as well as the total fungi were very high in all treatments excluding

Table 9. Interaction of *Trichoderma* isolates with *Meloidogyne incognita* infesting cardamom seedlings under greenhouse conditions.

a. Effect on root-knot nematodes

Treatment	No. of egg masses		No. of nematodes per g root	
	Sterile soil	Non sterile soil	Sterile soil	Non sterile soil
Trichoderma C.20	10.45 abcd	4.18 a	285.42 abc	163.44 ab
T. viride C.21	16.45 abc	7.85 a	671.98 a	104.93 ab
T. harzianum C.22	7.20 bcd	8.52 a	86.70 bcd	118.58 ab
T. harizianum C.23	9.99 abcd	4.98 a	259.62 abc	42.66 b
T. virens C.24	15.70 abc	9.35 a	449.82 ab	318.15 a
C.20 to C.24 together	19.02 ab	6.13 a	372.25 ab	54.98 ab
M. incognita	23.45 a	13.54 a	656.66 a	238.88 ab

Mean values followed by same alphabet in a column are statistically not significant.
Data were log transformed for analysis and back transformed to original means.

b. Effect on total biomass of seedlings

Treatment	Total biomass (fresh wt - g seedling^{-1})					
	Sterile soil			Non sterile soil		
	N-	N+	Diff.	N-	N+	Diff.
Trichoderma sp C.20	23.01 ab	15.17 a	7.84*	22.58 bcd	17.02 b	5.56*
T.viride C.21	21.08 ab	17.37 a	3.71	24.62 abc	18.25 ab	6.37*
T.harzianum C.22	22.12 ab	17.92 a	4.20	19.04 d	15.21 bc	3.83
T.harizianum C.23	25.00 a	16.50 a	8.50*	26.75 abc	22.85 a	3.90
T.virens C.24	22.86 ab	18.96 a	3.9	21.46 bcd	15.08 bc	6.38*
C.20 to C.24 together	19.20 b	17.08 a	2.12	29.12 a	20.54 ab	8.58*
Control	19.16 b	15.33 a	3.83	20.29 cd	11.33 c	9.06*

Means followed by the same alphabet in a column do not differ significantly. * Significant difference between means in the pair; N+ Nematode inoculated and N- uninoculated.

Plate 8

Effect of *Trichoderma* spp. on Growth of Cardamom Seedlings and Suppression of Root-Knot Nematodes in Native and Sterilized Soil

Plate 8

(From left in pictures A - F) *Trichoderma* isolate + root-knot nematodes in non-sterile soil; *Trichoderma* isolate alone in non-sterile soil; *Trichoderma* isolate + root-knot nematodes in sterile soil; *Trichoderma* isolate alone in sterile soil.

(In pictures G & H) Left – Absolute control; Right – Root-knot nematodes alone.

A. *Trichoderma harzianum* – C. 20

B. *Trichoderma viride* – C. 21

C. *Trichoderma harzianum* – C. 22

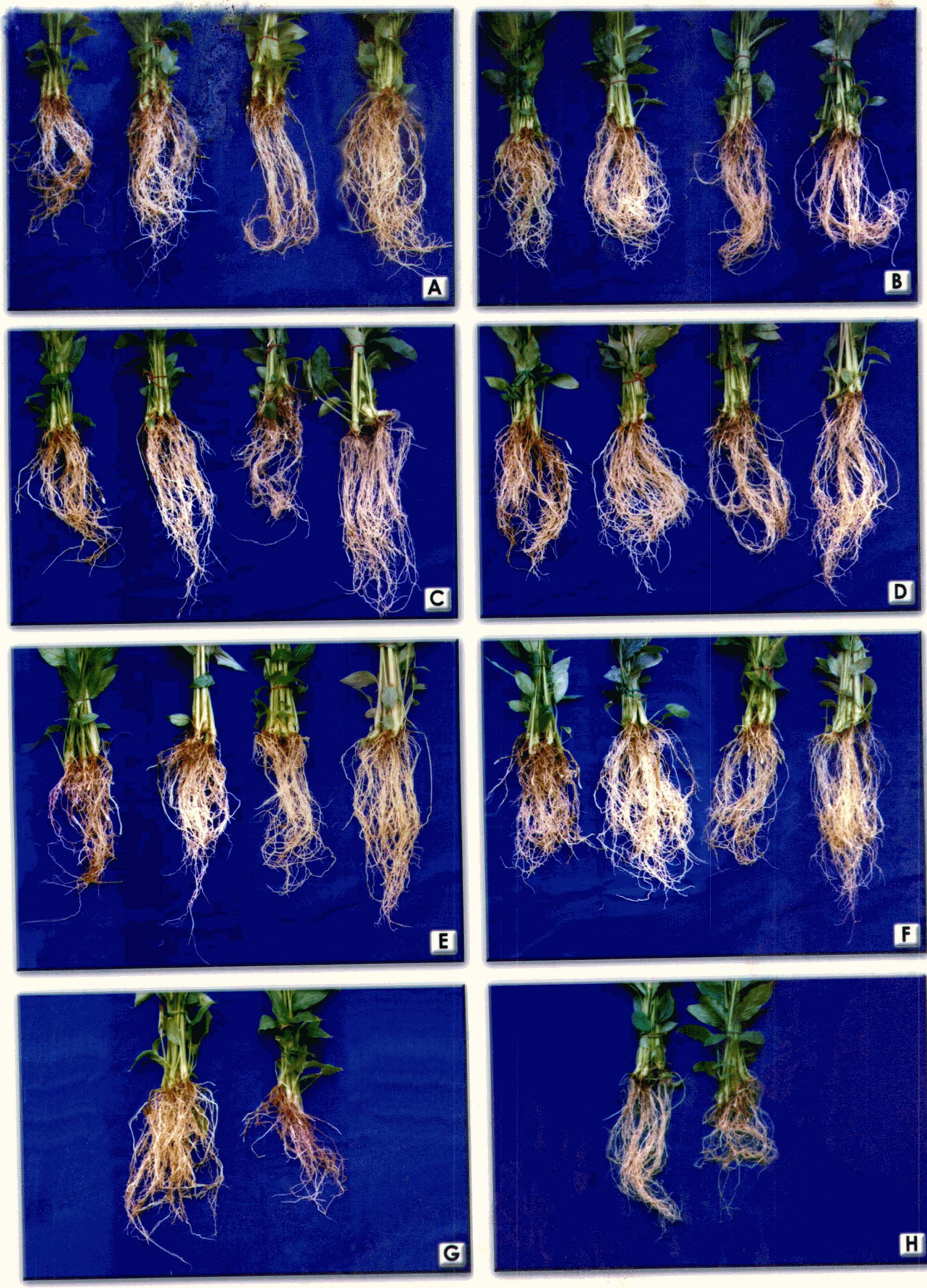
D. *Trichoderma harzianum* – C. 23

E. *Trichoderma virens* – C. 24

F. C. 20 + C. 21 + C. 22 + C. 23 + C. 24 together

G. Control plants in non-sterile soil

H. Control plants in sterile soil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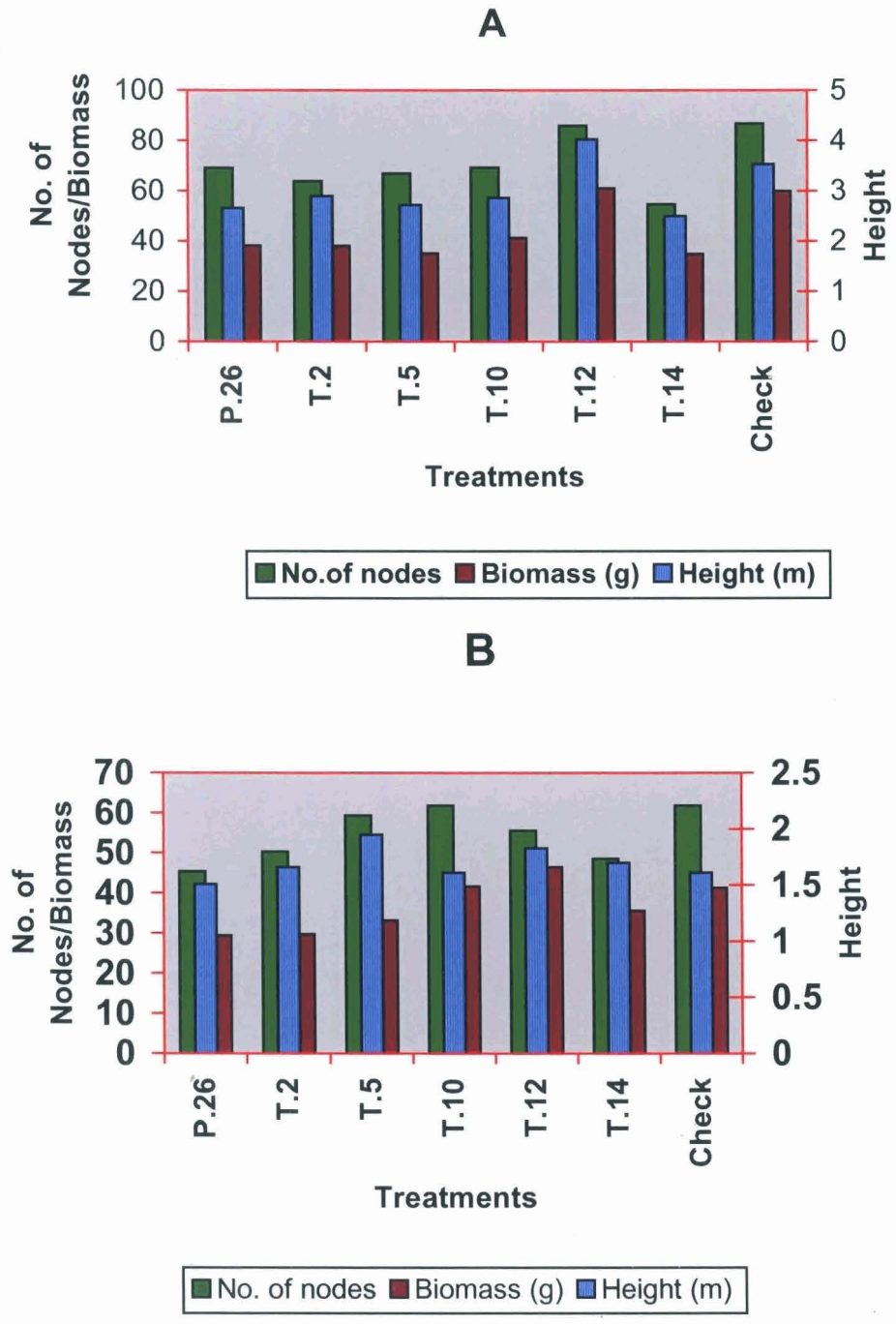

Fig. 1. Effect of six isolates of *Trichoderma* spp. on growth of black pepper rooted cuttings. A. Root-knot nematode inoculated B. Nematode-free plants.

the control, the maximum being in P.26 (1.3 x 10^5 cfu g^{-1} soil) and T.12 (2.81 x 10^5 cfu g^{-1} soil). But the fungal count came down drastically 12 months after the initial application.

Results of the experiment on application of *V. chlamydosporium* in various sequences to cardamom seedlings are given in Table 10 (Plate 9). Statistically significant differences in any of the growth parameters were not observed in all the treatments. Nevertheless application of *V. chlamydosporium*, irrespective of its sequence, increased the height of the cardamom plants by 20.44% - 43.08%, the total biomass by 24.20% - 25.40%, and the root weight by 5.24% - 36.82%. The maximum increase in height and root biomass was noticed when *V. chlamydosporium* was inoculated first (Plate 9.E). Inoculation of *V. chlamydosporium* subsequent to nematode inoculation could not reduce the root-knot nematode population and the plant growth was comparatively poor too (Plate 9.F). Therefore, it can be concluded that application of *V. chlamydosporium* prior to nematode inoculation or along with nematodes was more effective in improving the growth of cardamom seedlings. In the initial phase of the experiment (two months after inoculation), the nematode multiplication was substantially reduced with the application of the fungus either simultaneously or prior to nematode inoculation. But the final nematode populations in all the treatments were on par indicating the poor rhizosphere competence of this fungus (Table 10).

The higher dosage of *P. lilacinus* (10 g $plant^{-1}$) significantly reduced nematode population in cardamom roots compared to the lower dosage (Table 11). But the nematode control obtained was not reflected in the growth of cardamom plants. On the contrary, though the impact of the lower dosage of *P. lilacinus* on nematode population as well as root galling was very less, the treated plants showed maximum increase in height (165.89%), total biomass (21.64%) and root weight (81.69%). The carrier substance used (decomposed coffee husk), at both levels moderately reduced the nematode population

Plate 9

Effect of Sequential Inoculation of *Verticillium chlamydosporium* and Root-Knot Nematodes on Growth of Cardamom Seedlings and Suppression of Nematodes

Plate 9

A. Absolute control

B. *Verticillium chlamydosporium* alone

C. *Meloidogyne incognita* alone

D. *V.chlamydosporium* + *M.incognita* simultaneously

E. *V.chlamydosporium* first followed by *M.incognita*

F. *M.incognita* first followed by *V.chlamydosporium*

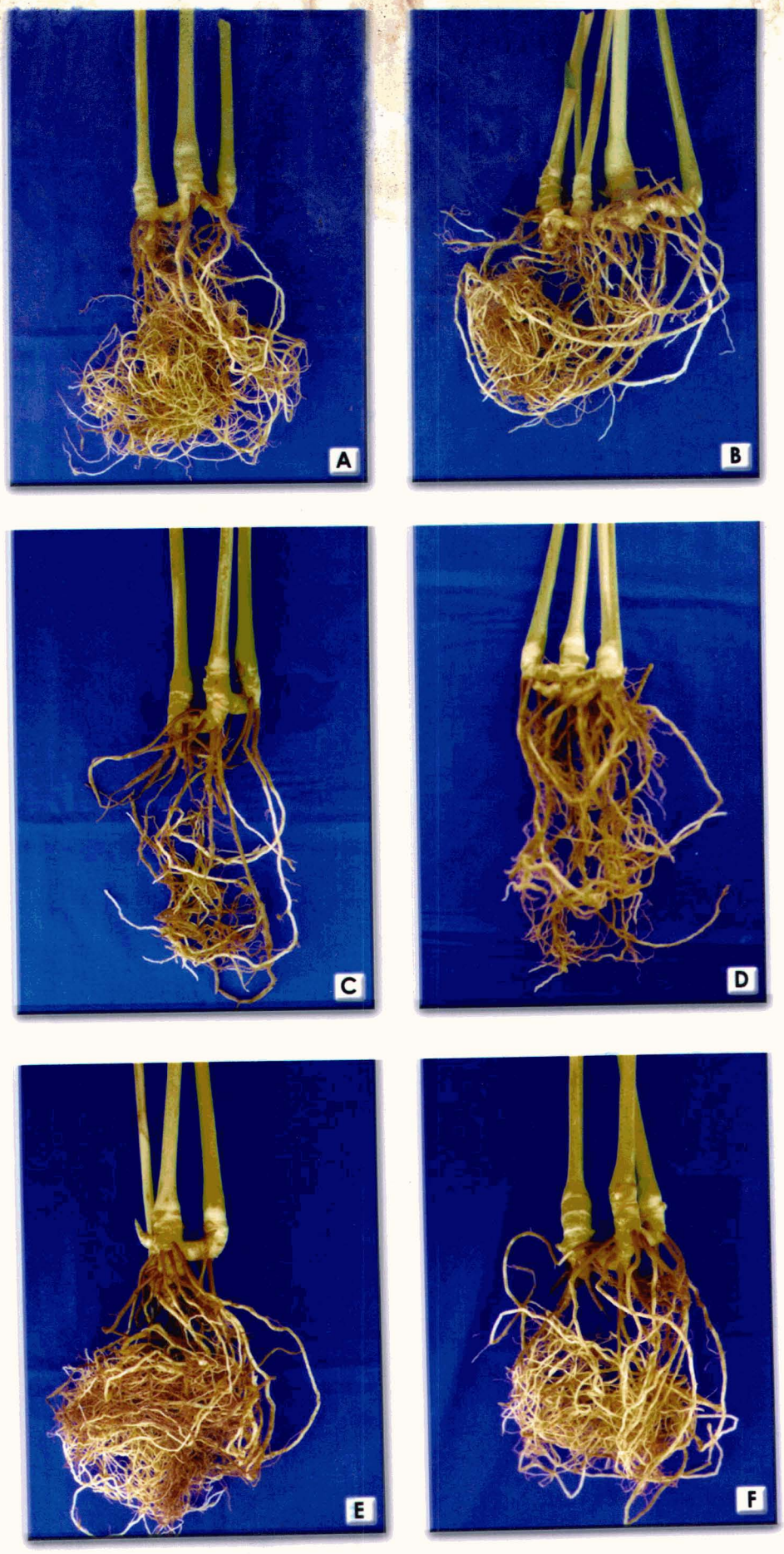
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Table 10. Effect of *Verticillium chlamydosporium* on growth of cardamom seedlings and on root-knot nematodes.

Treatment	No. of tillers	Biomass (g)	Root wt. (g)	Nematode g^{-1} root	
				After 2m	Final
Check	3.18 a	210.00 ab	31.67 abc	-	-
V.chlamydosprium	4.55 a	204.00 ab	30.00 bc	-	-
M.incognita	4.22 a	133.33 b	38.33 ab	968.82 a	447.75 a
Vc + Mi	4.39 a	263.33 a	33.33 abc	242.08 b	561.05 a
Vc > Mi	4.54 a	261.00 a	43.33 a	162.12 b	1207.81 a
Mi > Vc	3.83 a	210.00 ab	23.33 c	364.32 a	500.03 a

Means in a column followed by the same letter are not significantly different. Data are means of five replications. Vc - *V. chlamydosporium*, Mi - *M. incognita*

Table 11. Effect of *Paeciliomyces lilacinus* on growth and nematode multiplication in cardamom seedlings.

Treatment	No. of tillers	Biomass (g)	Root		No. of galls	Nematodes g^{-1} root (Pf)
			Weight (g)	Length (cm)		
Pl (5g) + Mi	3.43 a	78.25 a	29.67 a	40.50 b	159.12 ab	2456.19 ab
Pl (10g) + Mi	2.81 a	63.33 ab	19.67 b	36.00 bc	120.95 b	1528.57 b
CH (5g) + Mi	2.64 a	77.00 a	26.00 a	61.50 a	83.75 b	2361.48 ab
CH (10 g) + Mi	2.93 a	45.00 b	10.00 c	21.67 c	94.41 b	3891.45 a
Mi alone	2.12 ab	53.33 ab	11.33 c	38.50 b	329.10 a	4798.33 a
Check	1.29 b	64.33 ab	16.33 b	43.67 b	0.00 c	0.00 c

Data are means of four replications. Means followed by the same letter in a column are not significantly different. Pl – *P. lilacinus*, CH - Coffee husk, Mi - *M. incognita*

Table 12. Evaluation of four isolates of fluorescent pseudomonads for control of root-knot nematodes infesting tomato.

Bacterial isolate	Root wt (g)			No. of egg masses	Nematodes g^{-1} root (Pf)
	N-	N+	Mean		
Is. 1	2.55 bc	2.61 abc	2.58 bc	3.33 b	1005.93 c
Is. 22	2.23 c	1.82 c	2.02 c	0.50 bc	2268.86 b
Is. 40	2.62 abc	3.69 a	3.15 ab	28.71 a	2534.13 b
Is. 44	1.98 c	2.78 abc	2.38 bc	0.0 c	2375.84 b
Control	3.77 a	3.19 ab	3.48 a	30.33 a	5558.04 a

Data are means of three replications. Means followed by the same alphabets are not significantly different. N- without nematodes, N+ with nematodes.

and thereby the root galling due to nematode infestation. Besides, the results also indicated that higher level of coffee husk was toxic to the young cardamom seedlings. The data on root length was erratic.

Among the four *P. fluorescens* isolates screened on tomato, Is. 44 followed by Is. 22 and Is. 1 gave maximum reduction in number of egg masses (Plate 10). All the 4 isolates significantly reduced nematode population level in roots (Table 12). Excellent root proliferation was observed when plants were inoculated with *P. fluorescens* Is.40 (Plate 10.G). The results of screening seven isolates of *P. fluorescence* on black pepper OP seedlings are given in Tables 13 and 14 (Plate 11). None of the isolates had any significant influence on any of the plant growth characters or in reducing the plant damage due to root-knot nematode infestation. However, Is. 2, 7, 10, 11, 14, 17, 30, 31, 35, 36 and 49 increased the total biomass of black pepper plants. The increase in growth ranged from 26.7 to 55.6% even when the plants were affected by root-knot nematodes. But some strains (Is. 12, 14, 23 and 28) caused a detectable decrease on gall index that ranged between 30.0-39.9%. Dipping black pepper cuttings in *P. fluorescence* suspension also did not influence the rooting of cuttings. But early inoculation of rhizobacteria gave good protection to the cuttings against root-knot nematode infection (Fig. 2). In this method too different isolates of fluorescent pseudomonads reduced the root galling due to root-knot nematodes by 31.58-42.10%. The damage caused by root-knot nematodes, though statistically not significant, was quite high in the control treatments as well as in treatments where Is. 7, 29 and 34 were used.

Results of the trial on evaluating *P. penetrans* clearly indicated that plants inoculated with increasing levels of *P. penetrans* correspondingly decreased the root galling and root-knot nematode population in cardamom plants (Fig. 3). However, the juvenile populations in soil varied widely. The endospore attachment on J_2 at the end of the experiment was generally low and ranged from 0.57 to 3.1 endospores J_2^{-1}. Nevertheless, *P. penetrans* at various levels

Plate 10

Suppression of Root-Knot Nematodes Infesting Tomato on Treating with *Pseudomonas fluorescens*

Plate 10

A. Absolute control (no nematode and no bacteria)

B. Root-knot nematodes (RKN) alone

C. *Pseudomonas fluorescens* – Is. 1 alone

D. *Pseudomonas fluorescens* – Is. 1 + RKN

E. *Pseudomonas fluorescens* – Is. 22 alone

F. *Pseudomonas fluorescens* – Is. 22 + RKN

G. *Pseudomonas fluorescens* – Is. 40 alone

H. *Pseudomonas fluorescens* – Is. 40 + RKN

I. *Pseudomonas fluorescens* – Is. 44 alone

J. *Pseudomonas fluorescens* – Is. 44 + RKN

K. Root-knot nematodes (RKN) alone – closer view

L. Absolute control – closer view


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Plate 11

Evaluation of Isolates of *Pseudomonas fluorescens* for Suppression of Root-Knot Nematodes and Growth Promotion in Black Pepper Seedlings

Plate 11

(From left in pictures A - F) Absolute control; Root-knot nematodes (RKN) alone; *Pseudomonas fluorescens* alone; *Pseudomonas fluorescens* + RKN

A. *Pseudomonas fluorescens* – Ps. 7

B. *Pseudomonas fluorescens* – Ps. 10

C. *Pseudomonas fluorescens* – Ps. 12

D. *Pseudomonas fluorescens* – Ps. 26

E. *Pseudomonas fluorescens* – Ps. 29

F. *Pseudomonas fluorescens* – Ps. 34


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Table 13. Effect of seven isolates of fluorescent pseudomonads on growth and root galling in black pepper seedlings infested with root-knot nematodes.

Bacterial isolate	Height (cm)			Biomass (g)			Root wt. (g)			Gall index
	N-	N+	Diff.	N-	N+	Diff.	N-	N+	Diff.	
Is.4	27.83 b	17.23 d	10.60	14.74 c	8.82 a	5.92	3.57 bcd	2.68 a	0.89	3.67 a
Is. 7	32.67 b	44.67 a	12.00	24.82 a	11.07 a	13.75*	6.91 a	2.54 a	4.37*	3.00 a
Is. 10	29.00 b	37.83 ab	8.83	14.46 c	16.16 a	1.70	1.70 d	4.16 a	2.46	3.00 a
Is. 12	29.73 b	24.67 bcd	5.06	12.78 c	15.30 a	2.52	2.93 cd	2.73 a	0.20	1.00 b
Is. 26	71.00 a	40.67 a	30.33*	16.51 bc	13.47 a	3.04	5.32 abc	4.34 a	0.98	3.00 a
Is. 29	27.00 b	24.67 bcd	2.33	14.33 c	8.08 a	6.25	6.99 a	2.32 a	4.67*	3.33 a
Is. 34	38.50 b	22.50 cd	16.00*	24.25 ab	12.06 a	11.65*	6.05 ab	4.37 a	1.68	4.00 a
Control	41.33 b	30.17 abcd	11.16	18.35 abc	11.75 a	6.43	4.93 abc	3.77 a	1.16	4.33 a

Data are means of three replications. Means followed by the same alphabets are not significantly different. N- without nematodes, N+ with nematode and * significant difference in the pair of means of the row.

Table 14. Growth and galling of black pepper seedlings inoculated with 24 different isolates of *Pseudomonas fluorescens.*

Bacterial isolate	Height (cm)			Biomass (g)			Gall index
	N-	N+	Diff.	N-	N+	Diff.	
2	65.00	76.67	11.67	34.33	48.33	14.00	4.67
6	77.00	59.33	-17.67	41.67	35.00	-6.67	3.00
9	76.33	52.67	-23.66	32.33	26.67	-5.66	3.67
11	41.67	77.67	36.00	21.67	36.67	15.00	3.67
13	84.00	77.00	-7.00	40.00	33.33	-6.67	2.33
14	71.33	73.00	1.67	21.33	43.33	22.00	1.67
16	84.00	32.67	-51.33*	42.33	16.67	-25.66	2.00
17	30.00	42.00	12.00	15.00	20.67	5.67	2.00
19	50.33	48.67	-1.66	26.67	24.33	-2.66	4.33
20	92.33	94.33	2.00	50.00	43.33	-6.67	2.33
21	53.67	41.67	-12.00	25.00	18.33	-6.67	2.33
23	53.33	43.33	-10.00	25.00	27.33	2.33	1.33
27	107.00	92.67	-14.33	58.33	53.33	-5.00	2.33
28	100.70	72.33	-28.37	41.67	46.67	5.00	1.33
30	95.33	107.00	11.67	48.33	54.33	6.00	4.33
31	35.67	83.00	47.33*	22.33	38.00	15.67	2.00
33	86.00	76.67	-9.33	53.33	40.00	-13.33	3.33
35	72.33	94.00	21.67	40.33	46.67	6.34	2.00
36	78.67	85.33	6.66	35.00	45.00	10.00	4.00
37	80.00	54.00	-26.00	35.33	37.33	2.00	4.33
43	68.67	72.00	3.33	41.67	45.00	3.33	3.33
49	91.00	109.70	18.70	40.00	46.33	6.33	2.33
51	107.70	60.67	-47.03*	47.00	35.33	-11.67	2.00
Check	116.00	61.33	-54.67*	51.00	30.00	-21.00	3.33
$LSD_{0.05}$	36.6	36.6	-	N.S.	N.S.	-	2.1

* Means in a row are significantly different, N- without nematodes, N+ with nematode and N.S. not significant

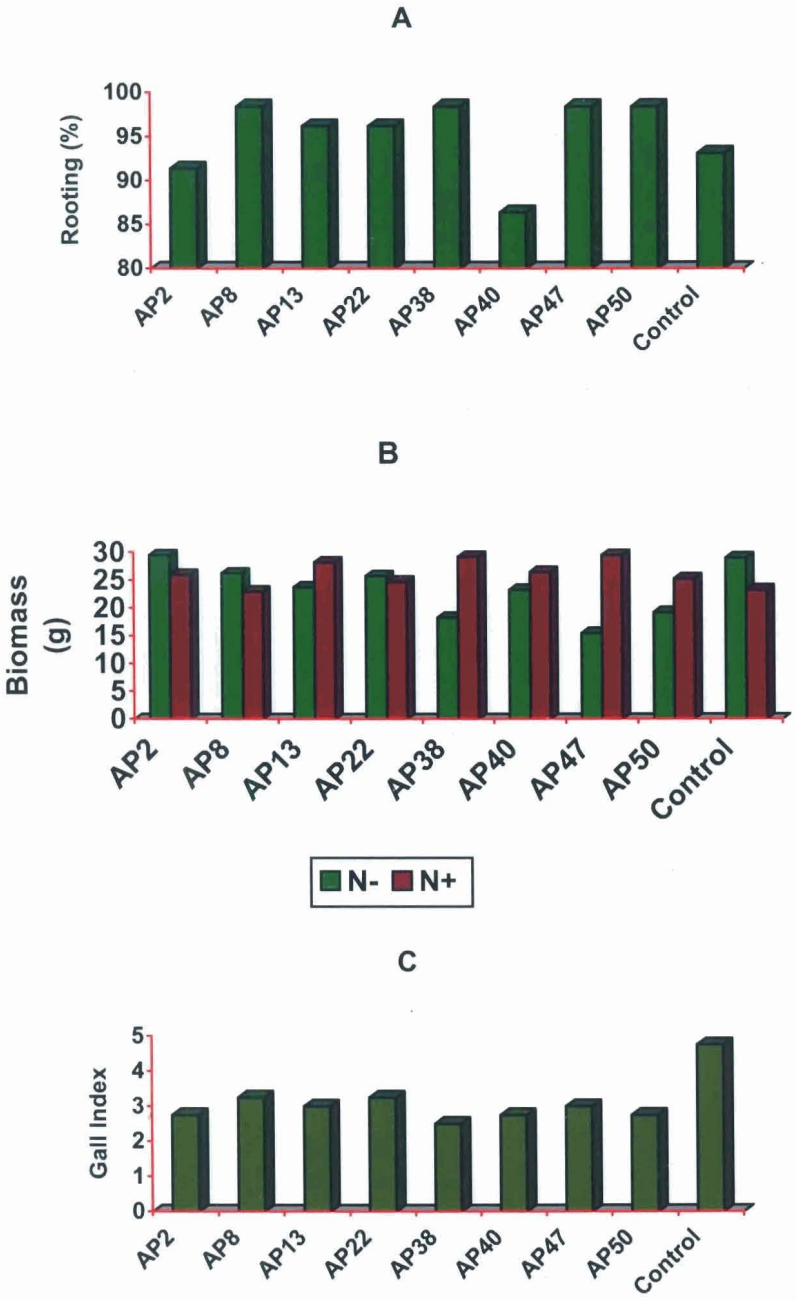


Fig. 2. Effect of eight fluorescent pseudomonads on growth and root-knot nematode infestation in black pepper rooted cuttings. A. Rooting, B. Biomass and C. Root Galling

improved the growth of the plants by suppressing the nematode multiplication. Various stages in the life cycle of the bacterium could be identified on examining the body contents of infected nematodes (Plate 12). The data also showed that infestation by root-knot nematodes, if ignored, could appreciably decrease the growth and biomass production of cardamom seedlings.

b. Field evaluation

The egg parasitic fungus *V. chlamydosporium* and the obligate bacterial parasite *P. penetrans* were able to check the root-knot nematode multiplication in cardamom (Table 15). Significant reduction in nematode population was observed in plots where *V. chlamydosporium* was applied. Although there was no significant improvement in the growth of cardamom seedlings with either of the biocontrol agents, the maximum growth improvement was obtained with *V. chlamydosporium* treatment. Mild infestation by root-knot nematodes was observed in plants treated with *P. penetrans* alone. In the present study both the bioagents could be successfully reisolated from the soil, even after 10 months.

The growth of cardamom seedlings over a period of nine months in nursery beds, where various biocontrol agents were incorporated, is given in Tables 16 (Plate 13). No significant improvement was noticed in the germination of cardamom seeds with any of the treatments and hence the data were not given. All the biocontrol agents influenced tillering in seedlings grown in non-solarized beds, and the improvement was statistically significant when the two bioagents were applied together. On the contrary, no drastic improvement was noticed in the tillering of plants grown in solarized beds. At the same time, there was a significant increase in the total biomass of individual cardamom seedlings treated with *P. lilacinus* (Plate 13.B). But both *Trichoderma* and *P. lilacinus*, either

Plate 12

Pasteuria penetrans, the Bacterial Antagonist of Root-Knot Nematodes

Plate 12

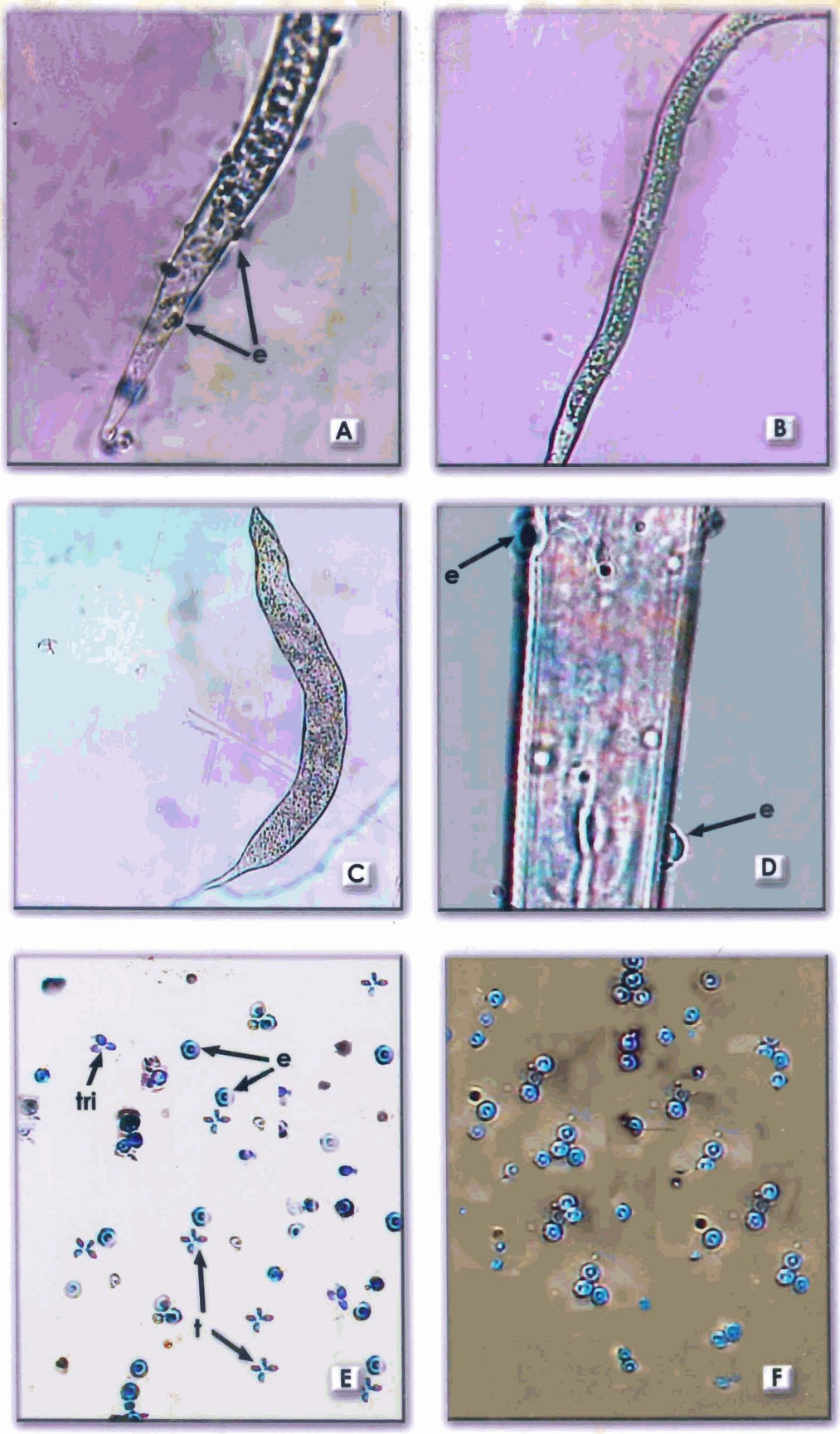
A. & B. A root-knot nematode second-stage larva infested with endospores (e) of *Pasteuria penetrans*

C. A pre adult of root-knot nematode filled with endospores of *Pasteuria penetrans*

D. Endospores (e) of *Pasteuria penetrans* attached to root-knot nematode

E. Diads, triplets (tri) and tetrads (t), various stages in the development of *Pasteuria penetrans*

F. Mature endospores of *Pasteuria penetrans*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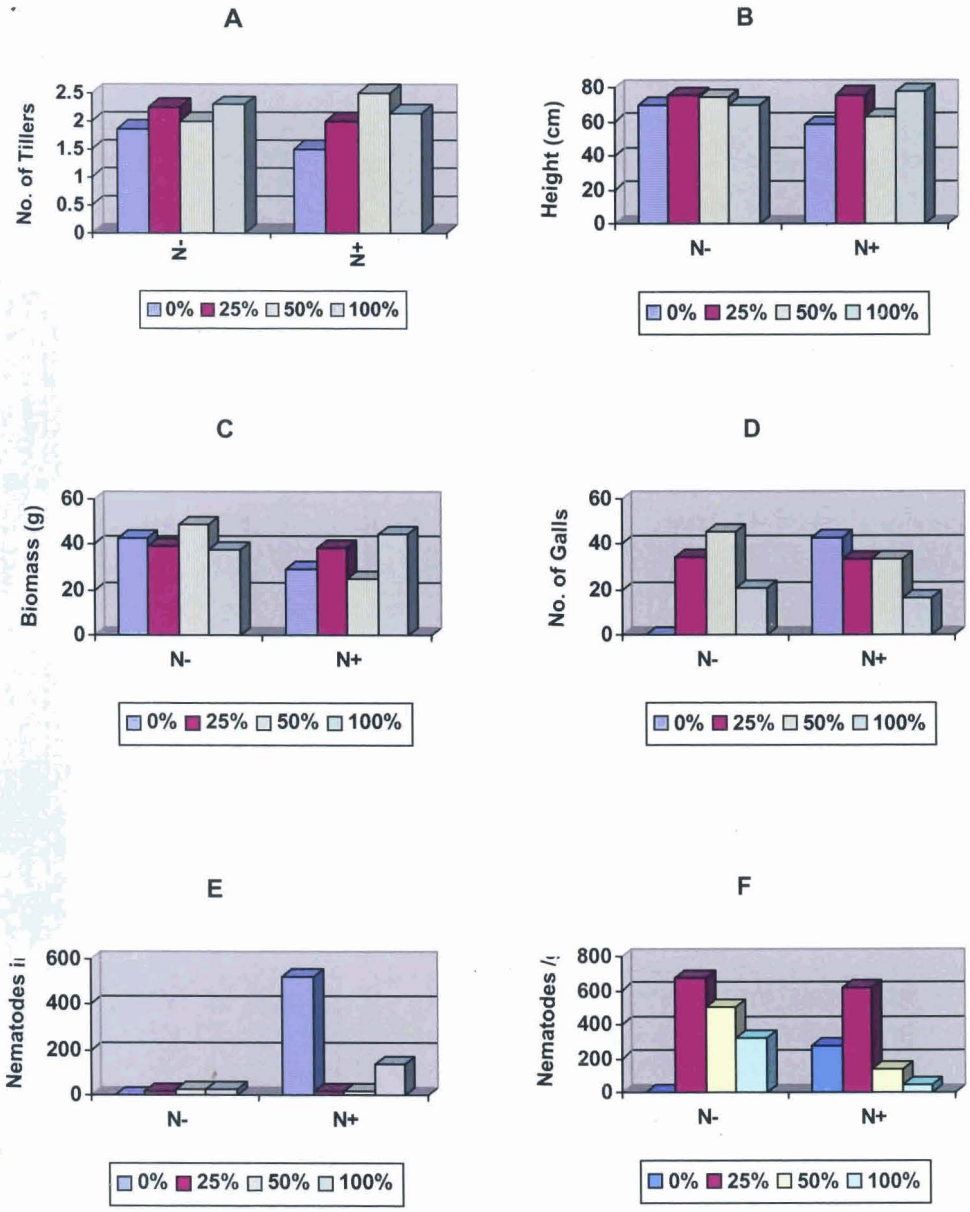

Fig. 3. Effect of *Pasteuria penetrans* on *Meloidogyne incognita* and growth of cardamom plants A. No. of tillers per plant, B. Height of the plant, C. Biomass of the plant, D. Root galling, E. Nematode population in soil and F. Nematode population in roots.

Table 15. Evaluation of *Verticillium chlamydosporium* and *Pasteuria penetrans* in cardamom nurseries at Appangala, Karnataka.

Treatment	No of tillers		Height (cm)		Nematodes g^{-1} root	
	N-	N+	N-	N+	N-	N+
V. chlamydosporium	3.15 a	2.60 a	64.80 a	60.65 a	0 a	47.75 b
P. penetrans	2.80 a	2.35 a	70.50 a	50.12 a	16.67 a	116.75 a
Control	2.65 a	2.40 a	67.60 a	55.90 a	0 a	323.33 a

Data are means of four replications. Means in a column followed by the same letter are not significantly different. N- without nematodes, N+ nematode inoculated.

Table 16. Evaluation of *Trichoderma* spp. and *Paeciliomyces lilacinus* in cardamom nurseries at Appangala, Karnataka.

a. Effect on quality of seedlings

Treatment	No. of standard seedlings bed^{-1} (%)	Rhizome rot incidence (%)	Root-knot nematodes - final *
Trichoderma spp.	63.16 a	19.83 a	163.06 ab
P.lilacinus	60.94 a	22.75 a	100.62 bc
Trichoderma + *P. lilacinus*	66.43 a	21.48 a	51.17 c
Control	51.94 b	42.47 b	390.74 b

Means followed by the same letter are not significantly different at 5% level. Data are means of four replications combined over three trails. * Root-knot nematodes in one gram of root.

b. Effect on growth of seedlings

Treatment	No of tillers			Biomass (g seedling^{-1})		
	S-	S+	Diff.	S-	S+	Diff.
Trichoderma spp	2.80 ab	3.50 bc	0.7*	36.1 ac	49.9 bc	13.8*
P. lilacinus	2.70 ab	4.00 a	1.3*	39.8 ac	68.5 a	28.8*
Trichoderma + *P. lilacinus*	3.00 b	3.60 ac	0.6*	44.9 ac	58.5 ac	13.6*
Control	2.60 a	3.8 a	1.2*	31.3 a	55.3 b	24.1*

Data are means of four replications combined over three trials. Means followed by the same letter in a column are not significantly different at 5% level. S- No solarization, S+ solarized and * significant difference between the pair of means in a row.

alone or together, significantly improved the number of quality seedlings (Plate 13. A&C). The rhizome rot incidence was drastically reduced wherever any of these bioagents was applied. The nematode population was also suppressed in all the treatments compared to that in control plots. The reduction in nematode population was statistically significant only with the combined application of the above two fungal antagonists. The study also revealed that soil solarization alone had considerably improved the growth and vigour of cardamom seedlings. The number of tillers increased from 2.59 to 3.80 while the biomass increased from 31.25 g to 55.3 g. In general biocontrol agents performed better in solarized soil than in non-solarized beds.

In black pepper the health status of vines was generally superior in plots treated with biocontrol agents and chemicals (Table 17). The mean incidence of foliar yellowing in the plot at the start of the experiment was 72.92% (Plate 14). The yellowing started reducing within one year in all the plots, irrespective of the treatments. The reduction in yellowing was statistically significant only in the case of biocontrol agents. The phorate-treated vines had the least incidence of yellowing after 4 years followed by *V. chlamydosporium* treated vines (15.25% and 20.50%, respectively). However, statistically significant reduction in the pooled mean (yellowing) was observed only wherever biocontrol agents were applied. The highest mean yield (5.14 kg vine^{-1}) was obtained in *V.chlamydosporium* treated plots followed by combined application of phorate and potassium phosphonate (4.20 kg vine^{-1}), which were significantly higher than that of control plots (Table 17, Plate 8). The root-knot nematode population decreased in all treatments compared to the initial population (Fig.4), but the reduction was not statistically significant in any of the treatment. However, the lowest mean population of root-knot nematodes in black pepper roots was observed in phorate + potassium phosphonate treated plants followed by *P. penetrans* treated vines. The reduction in

Plate 13

Field Evaluation of *Trichoderma harzianum* and *Paecilomyces lilacinus* for Control of Root-knot Nematodes in Solarized Cardamom Nurseries

Plate 13

A. Cardamom plants treated with *Trichoderma* spp. consortium (C. 20, C. 21, C.22, C. 23 and C. 24)

B. *Paecilomyces lilacinus* (Pl. 1) treated cardamom plants

C. Combined application of *Trichoderma* spp. and *Paecilomyces lilacinus*

D. Control plot where soil solarization was adopted as a pre-sowing measure


A
B
C
D

Table 17. Crop stand and yield of black pepper in a biocontrol field trial at Pulpally, Kerala (Mean of three replications)

Treatment	Vines showing yellowing (%)					Healthy vines (%)					Yield (kg-green)		
	1998	1999	2000	2001	Mean	1998	1999	2000	2001	Mean	2000	2001	Mean
Trichoderma harzianum	46.67 (43.09)	31.69 (34.26)	17.30 (24.60)	36.93 (36.91)	32.44 (34.72)	53.33 (40.91)	68.31 (55.74)	72.54 (58.42)	63.06 (53.08)	64.68 (53.53)	3.46	3.94	3.70
Verticillium chlamydosporium	58.18 (49.71)	33.69 (35.48)	24.82 (29.88)	20.50 (26.92)	33.72 (35.50)	41.82 (40.29)	55.32 (48.06)	40.18 (39.34)	79.47 (63.08)	54.69 (47.69)	4.66	5.62	5.14
Pasteuria penetrans	59.41 (50.43)	47.98 (43.84)	37.27 (37.63)	22.82 (28.34)	41.42 (40.06)	40.18 (39.34)	50.63 (45.36)	59.49 (50.47)	77.18 (61.66)	57.32 (49.21)	3.43	3.18	3.30
Phorate + Potassium phosphonate	83.81 (66.27)	66.29 (54.51)	50.51 (45.29)	34.44 (35.90)	59.53 (50.49)	13.27 (21.36)	31.09 (33.89)	44.87 (42.05)	65.56 (54.10)	37.65 (37.85)	3.04	5.36	4.20
Phorate	86.40 (68.36)	67.43 (55.20)	32.86 (34.98)	15.25 (21.60)	50.06 (45.03)	10.92 (19.30)	32.57 (34.80)	67.42 (55.01)	84.75 (68.40)	48.91 (44.38)	2.81	4.76	3.79
Control	92.41 (74.01)	66.95 (54.91)	41.04 (39.84)	32.39 (34.67)	60.15 (50.86)	7.59 (15.99)	32.56 (34.79)	54.88 (47.80)	67.61 (55.38)	38.74 (38.49)	2.09	3.63	2.86
Mean	72.92 (58.64)	52.38 (46.37)	33.51 (35.37)	26.10 (30.72)	-	25.81 (30.53)	44.96 (42.11)	56.70 (48.85)	73.91 (59.28)	-	3.25	4.42	-
LSD0.05	Years (Y)– 8.22; Treatments (T) – 9.93 Y x T – N.S.					Y – 9.19; T – 9.06 Y x T – N.S.					Y – 1.0 2; T – 1.16 Y x T – N.S.		

Figures in parentheses are arc sine transformed values.

Plate 14

Field Evaluation of *Trichoderma harzianum*, *Verticillium chlamydosporium* and *Pasteuria penetrans* in a Root-Knot Nematode Infested Black Pepper Garden in Pulpally, Wyanad, Kerala

Plate 14

A. Black pepper vines treated with *Trichoderma harzianum* (C. 22)

B. Vines treated with *Verticillium chlamydosporium* (Vc. 3)

C. *Pasteuria penetrans* (Pp. 1) applied black pepper plot


A


B


C

Plate 15

Field Evaluation of *Trichoderma harzianum*, *Verticillium chlamydosporium* and *Pasteuria penetrans* in a Root-Knot Nematode Infested Black Pepper Garden in Pulpally, Wyanad, Kerala (Cont'd)

Plate 15

A. Plot where potassium phosphonate and phorate were applied

B. Phorate treated black pepper vines

C. Control plot (no pesticides and no biocontrol agents)


A


B


C

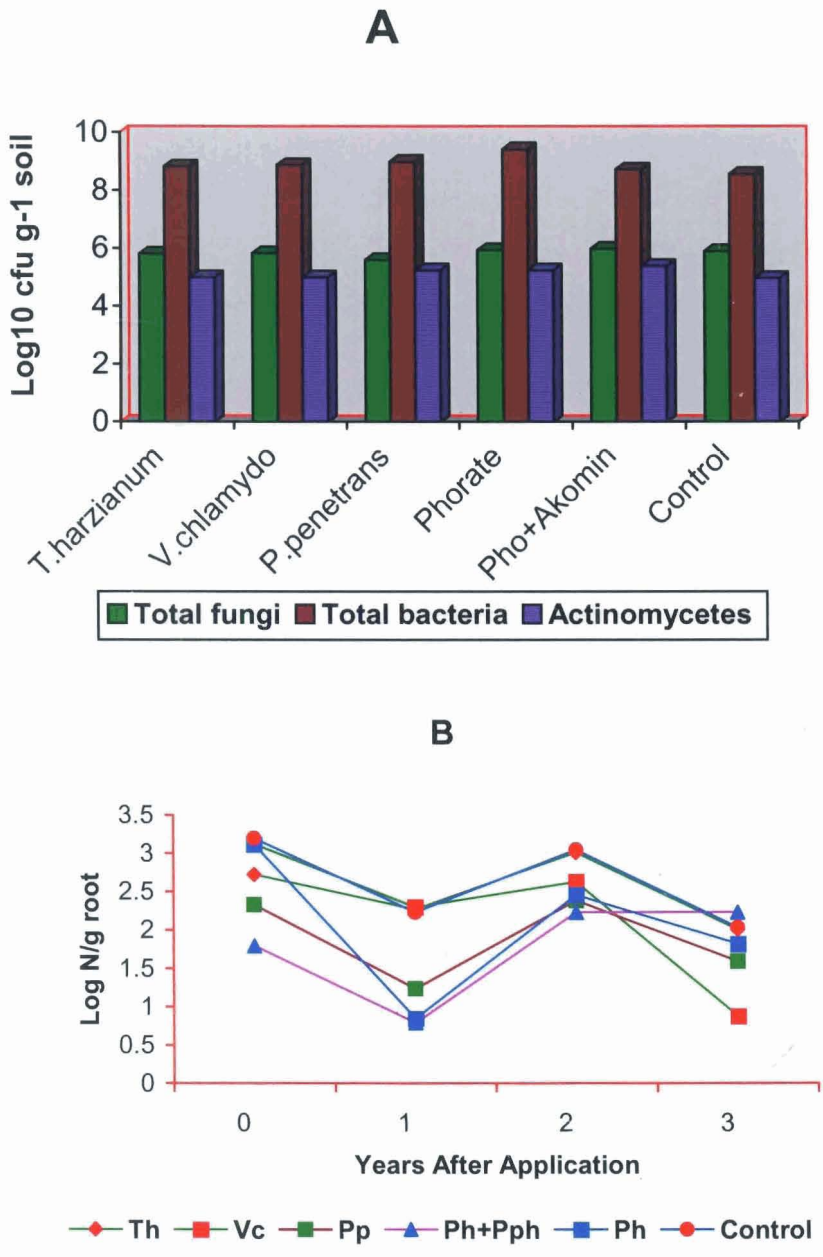

Fig. 4. Effect of biological control agents on nematodes and soil microbes of the field trial at Pulpally, Kerala.

a. Mean number of colony forming units of fungi, bacteria and actinomycetes in the soil

b. Mean root-knot nematode population in roots of black pepper plants

nematode population in alternate years very well illustrated the density-dependant multiplication of root-knot nematodes. After 4 years of field evaluation, the nematode level was the lowest in *V. chlamydosporium* treated plots. The microbial load in these plots also varied widely (Fig. 5).

D. Effect of Ecological Factors on Biocontrol Agents

a. Temperature

The results of the experiments to study the optimum temperature requirements of promising fungi are given in Table 18. The maximum radial growth (3.63 cm) of *T. harzianum* (C.22) was observed after 3 days after inoculation (DAI) at 30 °C followed by 3.41 cm at 25 °C, and both were on par (Table 18). Beyond 30 °C, the growth was drastically reduced. Similarly below 25°C too, the growth of this fungal isolate was quite slow. The daily increments in growth were 0.97 cm, 1.31 cm, 1.50 cm, 1.50 cm and 0.40 cm at 15°C, 20°C, 25°C, 30°C and 35°C, respectively. On the other hand, *P. lilacinus* was a slow growing fungus and the maximum growth of 2.04 cm was recorded after 7 DAI at 25°C. Above and below this temperature, the growth of the fungus was considerably reduced. The rate of growth was the lowest in the case of *V. chlamydosporium*. The radial growths recorded clearly indicated that the optimum temperature of this fungus was between 25°C and 30°C. The growth of all the 3 fungi was severely affected at temperature >30°C.

b. pH

The results showed that maximum growth of *Trichoderma* (C.22) was at pH 4 and for *Trichoderma* (P.26) was at pH 5 (Fig. 5). C.22 showed adaptation to a wide range of pH (4 - 7) while P.26 showed adaptability to pH ranging from 4 to 6. The fungus *Fusarium* has shown more adaptability to higher pH (>6). Similarly maximum growth of *Fusarium* sp. (F.47) was at pH 6. Thus the result indicated that the optimum pH required for growth varied with species of fungus (biocontrol agent) and also with different isolates of the same fungus.

Table 18. Effect of temperature on the growth of promising biocontrol agents.

Temp	Radial growth (cm) at different intervals					
	24 h	48 h	72 h	144 h	168 h	Mean
			Trichoderma harzianum (C.22)			
15°C	0.47 h	1.74 e	2.90 c	-	-	1.70
20°C	0.89 g	2.15 d	3.92 b	-	-	2.32
25°C	1.67 e	4.05 b	4.50 a	-	-	3.41
30°C	1.91 de	4.47 a	4.50 a	-	-	3.63
35°C	0.35 h	0.73 g	1.20 f	-	-	0.76
Mean	1.06	2.63	3.40	-	-	-
L.S.D.0.05: Interval = 0.11 cm Temperature = 0.14 cm						
			Paecilomyces lilacinus (Pl. 2)			
15°C	0.31 f	0.35 f	0.74 cdef	0.97 cd	1.16 bc	0.70
20°C	0.34 f	0.50 ef	0.70 def	0.76 cdef	1.52 b	0.76
25°C	0.37 f	0.67 def	0.85 cde	1.42 b	2.04 a	1.07
30°C	0.45 f	0.73 cdef	0.97 cd	0.67 def	1.94 a	0.95
35°C	0.31 f	0.31 f	0.33 f	0.36 f	0.39 f	0.34
Mean	0.35	0.51	0.72	0.84	1.41	-
L.S.D.0.05: Interval = 0.18 cm Temperature = 0.17 cm						
			Verticillium chlamydosporium (Vc.3)			
15°C	0.30 i	0.37 i	0.47 h	0.65 fg	0.71 f	0.50
20°C	0.31 i	0.37 i	0.52 h	0.74 e	0.82 d	0.55
25°C	0.35 i	0.61 g	0.67 efg	1.31 b	1.52 a	0.89
30°C	0.33 i	0.48 h	0.66 fg	1.10 c	1.30 b	0.77
35 °C	0.30 i	0.31 i	0.31 i	0.32 i	0.33 i	0.31
Mean	0.32	0.43	0.52	0.82	0.94	-
L.S.D.0.05: Interval = 0.02 cm Temperature = 0.03 cm						

Average of three replications. In a row (or column), means followed by a common letter are not significantly different at 5% level.

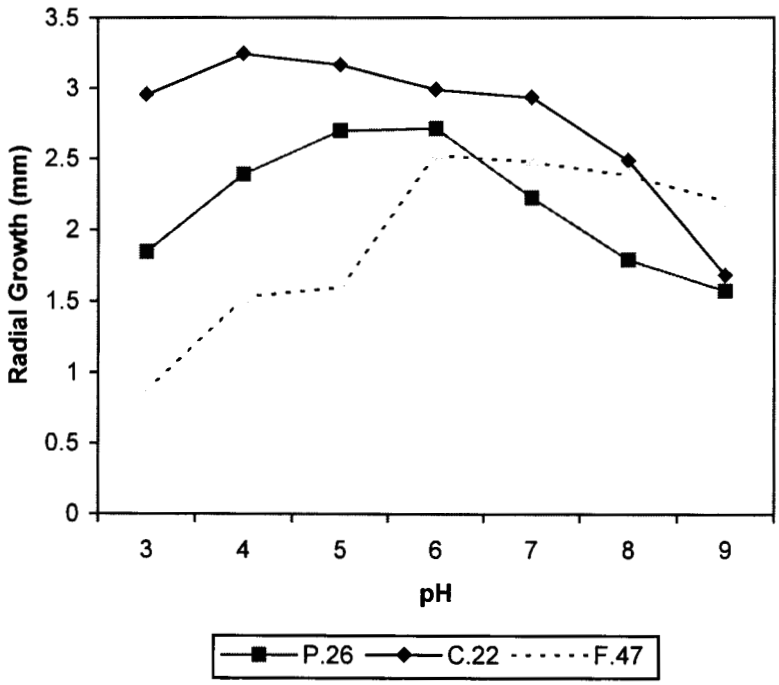

Fig. 5. Effect of pH on growth of three opportunistic fungi that are antagonistic to root-knot nematodes

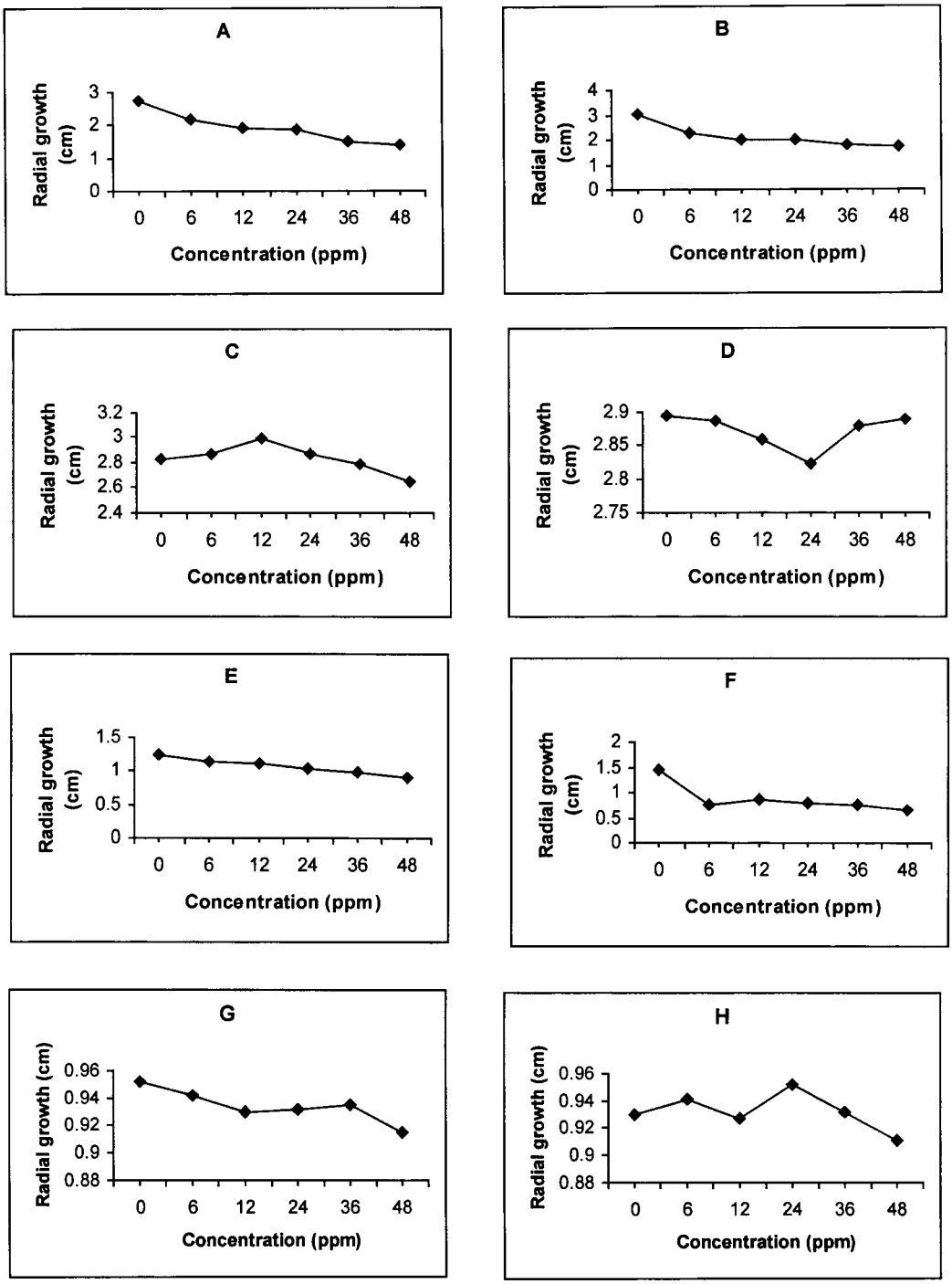

Fig. 6. Effect of pesticides on *Trichoderma harzianum* (A-D) and *Paecilomyces lilacinus* (E-H). A & F – Metalaxyl-mancozeb, B & E – Chlorpyriphos, C & G – Potassium phosphonate and D & H – Phorate.

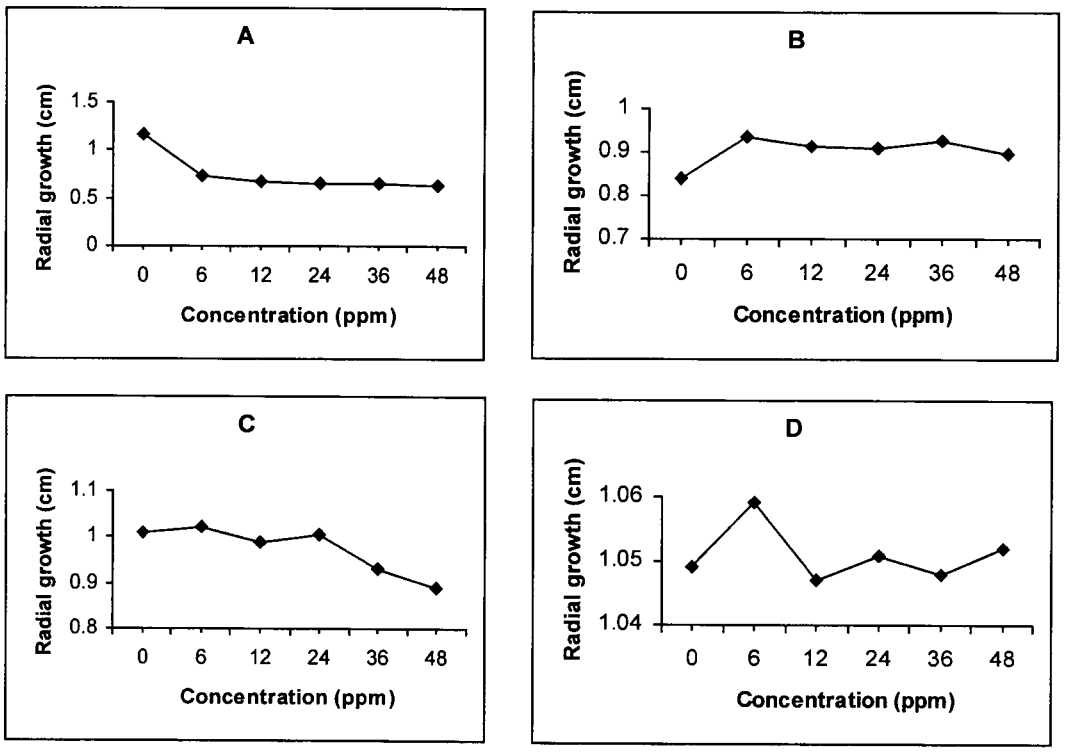

Fig. 7. Effect of pesticides on *Verticillium chlamydosporium* (A-D) A– Metalaxyl-mancozeb, B – Potassium phosphonate, C – Chlorpyriphos and D – Phorate

c. Pesticides

The compatibility of agro chemicals viz. metalaxyl, potassium phosphonate, phorate and chlorpyriphos with three fungal isolates viz. *Trichoderma* (Is.33), *Verticillium* (Is.34) and *Paeciliomyces* (Is.36) are given in Fig. 6 and Fig. 7. Among the 4 pesticides studied for their compatibility with biocontrol agents, metalaxyl-mancozeb, at all concentrations, was detrimental to all the 3 fungi. Its toxicity increased with the increase in concentration. Though not inhibiting at the recommended dosage, chlorpyriphos also had a negative linear response on all the 3 fungi studied. Potassium phosphonate was compatible with *T. harzianum* and *V. chlamydosporium*. But it had some adverse effect on *P. lilacinus*. The nematicide phorate was a safe chemical to all the above fungi, as it had no adverse effect on their growth at the recommended dosage.

E. Mass Multiplication of Fungal Antagonists

Among the three fungal isolates evaluated, *T. harzianum* and *P. lilacinus* multiplied readily on several solid substrates like sorghum grains, decomposed coffee husk and leaf powders. Moderate to abundant growth was observed for *T.harzianum* on sorghum grains, decomposed coffee husk and leaf powder (Fig. 8a). But *P. lilacinus* grew abundantly on rice grains and ginger leaf powder (Fig. 8b). Mycelial growth of *V. chlamydosporium* was very slow irrespective of the substrate used. Compared to the other two fungi, *V. chlamydosporium* multiplied poorly on most of the substrates tried. The study showed that substrates like farm yard manure, vermicompost, coir dust and saw dust were poor substrates for any of these fungi. Neem oil cake supported good multiplication of *V. chlamydosporium* but was not so good for *P. lilacinus* and *T. harzianum*. There was no correlation between sporulation and multiplication for most of the carrier substances.

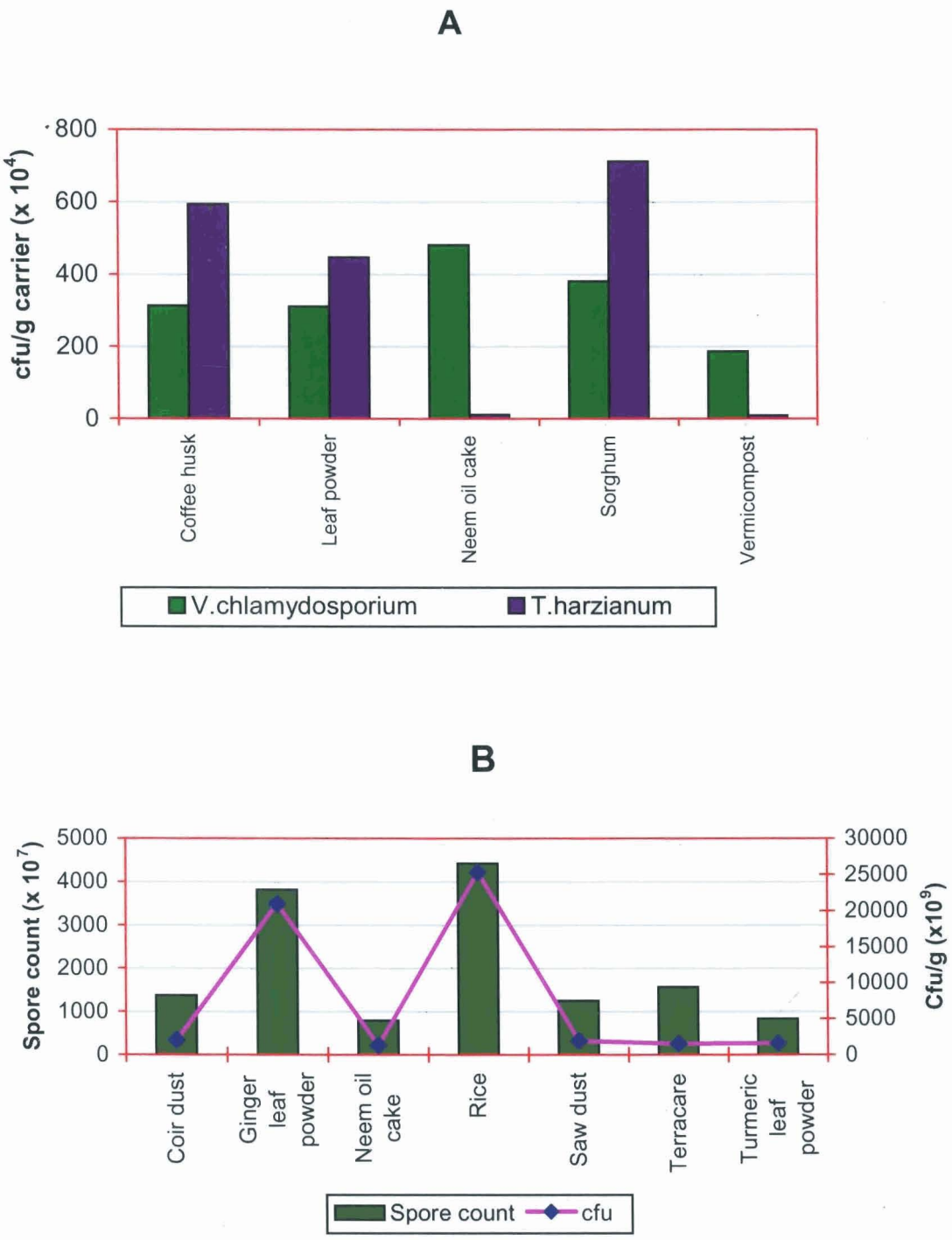

Fig. 8. Evaluation of solid substrates for mass multiplication of biocontrol agents.

A. *Trichoderma harzianum* & *Verticillium chlamydosporium* B. *Paecilomyces lilacinus*

Chapter 5

Discussion

India cultivates over 50 different varieties of spices. Out of the total production of around 2.7 million tonnes, about 0.25 million tonnes (8-10 per cent) are exported to more than 150 countries. The Indian share of the world trade in spices is 45-50 per cent by volume (25 per cent in value terms). The export earnings have increased from Rs. 1231 crores in 1996-97 to Rs. 1758 crores in 1998-99 and Rs. 1861 crores in 1999-2000. The area under spice cultivation is expanding by their introduction to non-traditional areas. Already ravaged by a number of pests and diseases, this initiative can further aggravate the problems. Most of these pests and diseases are at present managed by pesticides. Plant parasitic nematodes too are not an exception. The global demand for organically produced foods is growing rapidly in developed countries like Europe, USA, Japan and Australia, which are the major importers of spices. Under this context, the present study is of high relevance as its outcome can be an alternative to the dangerous use of pesticides. Biological control capitalizes on the numerous microbes that are antagonistic to plant parasitic nematodes and the interactions of soil nematodes with these microorganisms in soil. However, in spices biological control is yet to become an acceptable alternative for pesticides because of inadequate efforts in this direction. A number of biotic and abiotic factors that play a very crucial role, right from the isolation of the antagonist, its mass multiplication to adaptability in the natural field conditions further complicates the success of any solitary efforts in this highly relevant area.

The random survey enabled to collect 168 samples from the rhizosphere of various samples and out of these 57 bacteria and 73 fungi were isolated. Most of the fungi isolated from these samples belonged to the group of opportunistic fungi, which are predominantly saprophytes. A few predatory species like *Cephalosporium* were also obtained. This was mainly

due to the fact that the isolation was done mainly from egg masses and rhizosphere soil. It permitted isolation of either a range of different species or a number of strains of a species in pure culture with comparative ease. As nematodes are more abundant in rhizosphere than in the bulk soil, their obligate parasites will probably also be more numerous in the rhizosphere. Recent studies have shown that egg masses of root-knot nematodes are more densely populated microbial niche than the rhizosphere (Papert & Kok, 2000; Kok *et al.*, 2001). But isolation of fungi and bacteria from single eggs will be more precise (Kim & Riggs, 1994; Odour-Owino & Waudo, 1996). This strategy will yield only egg parasitic forms and hence not followed in this study.

In the present study, the most commonly isolated fungus was *Trichoderma* spp. Moreover, majority of isolates obtained from other sources too belonged to this genus. Other fungi of common occurrence were *Aspergillus*, *Penicillium*, *Paecilomyces* and *Fusarium*. Earlier studies in spice agro ecosystems showed the prevalence of these fungi in the rhizosphere soil of spices like black pepper and cardamom (Sankaran, 1981). That is why rhizosphere is considered as the first line of defense for roots against attack by soil-borne pathogens (Weller, 1988). Fungi like *A. niger*, *F. oxysporum* and *P. lilacinus* were frequently isolated from egg masses of root-knot nematodes by other workers too (Goswami *et al.*, 1998a). Some fungi, such as *V. chlamydosporium*, are largely confined to the rhizosphere. In the present study no attempt was made to quantify the incidence of these fungi in samples. Therefore the diversity index of the microbial fauna associated with nematodes could not be estimated, which indicates the diversity in fungal community and the dominance of species (Chen & Chen, 2002).

Many of the bacteria obtained in this study could not be identified even up to the genus level by the common procedures. However, isolates of *Bacillus* spp. and *P. fluorescens*, two

groups of rhizobacteria that are known for their nematotoxic properties, could be distinguished from the rest. Similar results have been reported with other rhizobacteria and parasitic nematodes (Becker *et al.*, 1988; Oostendorp & Sikora, 1989; Racke & Sikora, 1992).

Random screening of microorganisms for biocontrol of plant parasitic nematodes without regard to characteristics related to nematode antagonism has a low probability of success because of the large number of organisms that have to be tested (Becker *et al.*, 1988). Potential biological control agents of soil-borne plant pathogens can be screened *in vitro* or in soil (Merriman & Russell, 1990). *In vitro* bioassays are rapid, space-efficient and repeatable and hence were adopted in the current investigation as a preliminary step to identify the potential ones. Through *in vitro* screening organisms that produce toxic metabolites or those parasitize the target organisms are usually selected. Bacteria that suppress nematodes by modification of root exudates or by induced systemic resistance cannot be identified by this method. The bioassays using free-living nematodes like *Caenorhabditis elegans* that are very sensitive to test organisms can be misleading (Anke *et al.*, 1995) and hence eggs and J2 of root-knot nematodes were chosen. Besides, these two stages are most vulnerable to soil-borne antagonists and their toxic metabolites. Culture medium also is reported to influence the percentage of eggs parasitized (Kim *et al.*, 1998) and hence initial laboratory screening was done on simple water-agar plates.

Though 73.8% of the fungi screened colonized egg masses, only very few isolates showed considerable degree of egg parasitism. Isolates of many of these fungi differed significantly in their ability to parasitize the eggs of different nematode species as reported by earlier workers (Stirling & Mankau, 1979; Kerry, 1981; Khan & Goswami, 2000b; Sosnowska *et al.*, 2001). Generally, eggs in early developmental stages have been found to be more susceptible

to fungal infection (Irving & Kerry, 1986; Lopez-Llorca & Duncan, 1991). No adverse effects have been observed in eggs containing juveniles i.e. in advanced stage of embryonic development. Mere presence of fungi in females or egg masses may not necessarily mean that the fungus is parasitic as shown in females and eggs of *M. incognita* on black pepper in which the plant pathogenic fungi *Phytophthora palmivora* and *Nectria haemococca* were found (Freire, 1982).

Fungi like *Trichoderma*, *Fusarium* and *Aspergillus* are not regular candidates for biological control of nematodes. However, many of these saprophytic fungi are reported as occasional parasites of nematodes in literature indicating that they have an important role to play in the natural control of root-knot nematodes affecting spice crops. In literature there are only very few reports on the nematicidal activity of fungi like *Drechslera* sp. (Charles *et al.*, 2000), *Fusarium* spp. (Pocasangre *et al.*, 2000; Zareen *et al.*, 2001), *Aspergillus* spp. (Ayoub *et al.*, 2000; Goswami *et al.*, 2001; Siddiqui *et al.*, 2001a), and *Trichoderma* spp. (Windham *et al.*, 1989; Santos *et al.*, 1992; Saifullah & Thomas, 1996; Spiegel & Chet, 1998; Sharon *et al.*, 2001). Potential isolates of fungi belonging to all the above groups were obtained in this study too. *Humicola* sp., *Scopulariopsis* sp. and *Scolecobasidium* sp. have been reported as parasites of cyst nematodes (Kuczynska, 1997; Sosknowska & Banaszak, 1998). Parasitism by *Aspergillus tamarii*, *A. ustus*, *Drechslera* sp., *Humicola* sp., and *Scopulariopsis* sp. on root-knot nematode eggs or females, reported in the present study, are new reports.

In contrast, majority of the fungal isolates (98.7%) inhibited egg hatch at varying levels indicating the involvement of mechanisms other than parasitism. The presence of fungi like *Aspergillus* spp., *Fusarium* spp., *Trichoderma* spp. and *Verticillium* spp. in the vicinity of nematode eggs inhibited the egg hatching suggesting an exogenous effect. Scanning electron

microscopic studies showed the partial disintegration of the vitelline layer of *M. arenaria* eggs infected by *V. chlamydosporium* (Morgan-Jones *et al.*, 1983). The enzymatic disintegration of vitelline and chitin layers increased the permeability of eggshell and enhanced the mycelial penetration leading to total disintegration of the egg contents. Thus the results indicated that combination of enzymatic and mechanical distortion of eggshell is of great importance in the penetration of the nematode eggshell by the fungi.

Subsequently, the role of toxic metabolites involved in the dissolution of vitelline and chitin layers of nematode eggs was proved in the bioassays using culture filtrates of selected fungi. In the present study, several fungi produced substances toxic to J2. Both egg parasites and non-parasites produced nematotoxic substances. There are several earlier studies that had shown nematicidal effects of culture filtrates of several nematophagous fungi and actinomycetes (Mishra *et al.*, 1987; Dicklow *et al.*, 1993). Marked inhibition of egg hatching and high juvenile mortality were observed with culture filtrates of *A. niger* (Siddiqui *et al.*, 2001a), *Penicillium* spp. (Ali, 1990; Singh *et al.*, 1991; Khan *et al.*, 2000) and *Trichoderma* spp. (Djian *et al.*, 1991; Sharma & Saxena, 1992; Khan & Saxena, 1997). The culture filtrate treated eggs in egg masses exhibited morphological changes as deformed embryo and vacuolated eggs (Khan & Goswami, 2000c). Toxic metabolites reported include oxalic acid from *A. niger* (Mankau, 1969), acetic acid from *P. lilacinus* (Djian *et al.*, 1991) and mycotoxins and trichothecenes from *Fusarium* spp. (Ciancio, 1995; Zareen *et al.*, 2001; Nitao *et al.*, 2001).

The role of these toxins in infection of nematodes is not fully understood. Hydrolytic enzymes such as proteases, collagenases and chitinases produced by microorganisms showed potential for the control of plant-parasitic nematodes by altering the structure of the cuticle of the nematodes (Miller & Sands, 1977). Serine proteases produced by nematophagous

fungi such as *P. lilacinus*, *V. chlamydosporium* and *V. suchlasporium* have been reported to degrade cysts or eggs of plant-parasitic nematodes, facilitating the penetration of eggshells by the fungi (Lopez-Llorca, 1990; Lopez-Llorca & Robertson, 1992; Bonants *et al.*, 1995; Segers *et al.*, 1996). Chitinolytic enzymes have been reported in fungi like *T. harzianum* (Goldman *et al.*, 1994), *Aspergillus* spp., *P. lilacinus* (Morgan-Jones *et al.*, 1984b), and *Scopulariopsis* sp. (Davila *et al.*, 1999). Many microorganisms, such as the collagenolytic fungus, *C. elegans* (Galper *et al.*, 1991) have been reported to be harmful to plant-parasitic nematodes. Enzymes capable of degrading nematodes are produced by soil microorganisms in response to application of soil amendments such as crab-shell chitin and collagen (Galper *et al.*, 1990; Sarathchandra *et al.*, 1996).

The variability in nematicidal action can be explained as differences in fungal strains, culture media and pH (Cayrol *et al.*, 1989; Kim *et al.*, 1998; Meyer *et al.*, 2000). Variations of toxin production have been observed among strains within a species (Hallmann & Sikora, 1996). Root-knot nematode hatch was higher in potato dextrose broth medium than in water (Nitao *et al.*, 1999). In the present study, the fungus was cultured on Czapek-Dox medium and there are no published reports on its influence on either hatching or nature of culture filtrates.

The effect of rhizobacteria on nematode eggs ranged from inhibition to stimulation. As reported earlier (Becker *et al.*, 1988) the results were highly variable in the present study too. Rhizobacteria interfered with the host-finding process by receptor blockage on roots and (or) modification of root exudates of the host plant, thus hindering the attraction, hatching, or penetration behaviour of nematodes (Oostendorp & Sikora, 1990). They produce specific enzymes or metabolites that were nematicidal or that reduced egg hatch (Spiegel *et al.*, 1991;

The *Trichoderma* isolates (C.22 and C.23) that were superior in reducing root-knot nematode population in cardamom were of *T. harzianum*. *In vitro* bioassays too indicated the nematicidal activity of these isolates. Of late, many others also obtained similar results on using *Trichoderma* isolates (Saifullah & Thomas, 1996; Sankaranarayanan *et al.*, 1998; Nagesh *et al.*, 2001b; Sharon *et al.*, 2001). Various mechanisms like antibiosis, competition, mycoparasitism and enzymatic hydrolysis have been suggested for the biocontrol activity of *Trichoderma* spp. against phytopathogenic fungi (Sivan & Chet, 1992). *T. virens* acts against certain pathogenic fungi through the production of antibiotics (Roberts & Lumsden, 1990). Except competition, all the other mechanisms can potentially be involved in the nematode biocontrol process. The enhanced nematode suppression in native soil suggests the additive role of some microflora present in the soil. Alternately, they might have supported the initial establishment and colonization of the soil by the biocontrol agents. *Trichoderma* isolates, C.20, C.21 and C.23 promoted growth of cardamom seedlings in spite of the nematode infestation. This could be due to the diffusible metabolites released by the fungi. It is reported that some *T. harzianum* isolates have the ability to solubilize insoluble or sparingly soluble minerals by chelation and reduction, which leads to growth promotion (Altomare *et al.*, 1999).

In the second experiment all the *Trichoderma* isolates failed to suppress the nematodes and caused significant improvement in the growth of black pepper plants. The major reason for the poor performance of *Trichoderma* isolates can be their poor establishment in soil. It is reported that *Trichoderma* application in soil affected the penetration of nematodes and did not inhibit the development of nematodes within the roots (Sharon *et al.*, 2001). They also found that the fungus produced metabolites with anti-nematode activity, which could immobilize J2 and thus reduce nematode penetration. In this case large numbers of J2

probably escaped infection because of their high density or the lower concentration of the fungus. The mobility of root-knot nematode J2 may be greater than the growth rate of fungal colonies in soil. So a longer pre-planting incubation period was required to achieve significant nematode control. The nature of the host plant, black pepper, also might have contributed to this. Juvenile penetration into roots of black pepper was reduced only at the beginning of the experiment. The galls due to root-knot nematode infection in black pepper are big in size and therefore the egg masses are embedded in the gall tissues and not accessible to the fungus. Furthermore, frequent high moisture content of the soils in the pots can have a negative effect on sporulation of fungi. The study also revealed the steep decline in *Trichoderma* population towards the end of the experiment. As a result, the subsequent generations of nematodes would have escaped the fungal antagonism, which warrants more frequent application of the biocontrol agents. This study clearly proved that application of *Trichoderma* multiplied on decomposed coffee husk (approximately 10^5 cfu g^{-1}) @ 40g $plant^{-1}$ is too insufficient for black pepper cuttings raised in polythene bags. Repeated applications may be necessary for adequate protection against nematode infestation.

In the greenhouse trial where *V.chlamydosporium* was inoculated in different sequences inoculation of *V.chlamydosporium* first had relevance as it enabled the fungus to get established and challenge the nematode infection. In this experiment, plants that received the nematode inoculum first succumbed to their attack as the fungus was inoculated later (60 days). Even simultaneous inoculation of the fungus and nematodes was able to subside the nematode problem to some extent. Presence of nematodes in roots may stimulate root colonization by *V. chlamydosporium* (Leij *et al.*, 1992c). The percentage of egg masses colonized by *V.chlamydosporium* depends on the abundance of egg masses on the root and on the degree of their exposure to the fungus (Bourne *et al.*, 1996). Lack of aggressive rhizosphere

colonization may be one reason for the inconclusive results obtained here. Fungal parasites of nematodes that can grow well on roots and in the rhizosphere will have a greater probability of contacting nematode hosts than those that are poor colonizers. Besides the cardamom seedlings used in this study were grown up plants (one year old) and hence the establishment of *V.chlamydosporium* in these plants might be poor. Therefore, it can be concluded that biocontrol agents should be applied early to young seedlings for ensuring a healthy crop.

V. chlamydosporium isolates showed marked differences in their ability to colonize roots as well as nematode eggs (Leij & Kerry, 1991). The rhizosphere colonization in non-sterilised soil varied with the fungus strain and with the crop plant (Bourne *et al.*, 1994). It is more effective on less susceptible host plants that produce small galls (Bourne *et al.*, 1996; Kerry & de Leij, 1992; Viaene & Abawi, 2000). Cardamom, therefore, is an ideal plant that can be protected against root-knot nematode attack by applying fungal biocontrol agents.

The experiment using *P. lilacinus* too clearly demonstrated the efficacy of this fungus in checking the root-knot nematode multiplication. The result goes hand in hand with the findings of Cabanillas *et al.* (1989a). They got the greatest protection against *Meloidogyne* when *P. lilacinus* was delivered into soil 10 days before planting and again at the time of planting. In other studies too it is reported that introducing the fungus earlier to nematode inoculation increases the control of nematodes (Mousa *et al.*, 1995; Campos & Campos, 1997). For effective biological control, more than one application of fungi at proper time is needed. But according to Jatala (1986) single application of *P. lilacinus* may be sufficient to establish the fungus in the soil. Since the fungus is an egg parasite the stage of egg development is probably important in timing the delivery of the fungus into the soil. *P. lilacinus* probably will

be more effective against *M. incognita* during early stages of egg development especially at the time of appearance of the first stage larvae rather than during advanced stage. This assumption may help us to explain the difference in results obtained when the fungus is applied before and after nematode inoculation in the previous experiment.

Generally, increase in the dose of *P. lilacinus* was accompanied by an increase in egg infection, decrease in the gall index, number of eggs per egg mass and the final soil population of nematodes with a corresponding increase in plant height and root length as was shown in an experiment on tomato (Khan & Goswami, 2000a). The egg infection increased from 30.4% at 2 g *P. lilacinus* on rice kg^{-1} soil to 62.0% at the highest inoculum concentration (10 g fungus kg^{-1} soil). On the contrary, in this study high dosage (10 g $plant^{-1}$) was found detrimental to the growth of the plant and more studies are needed to prove the toxic effect of coffee husk to young cardamom seedlings.

The non-significant differences in growth improvement or reduction in root galling, on treating the black pepper seedlings with *P. fluorescens*, can be due to many reasons. First of all the beneficial effects of rhizobacteria might not have been expressed because of the short duration of the experiments (two months). Though not statistically significant, many isolates improved the growth and reduced the nematode damage. However, these experiments did not demonstrate the mechanism by which *P. fluorescens* reduced the nematode population in such cases. Generally siderophore and antibiotic production are often proposed as the mechanism of action of fluorescent pseudomonads (Kloepper & Schroth, 1981; Thomashow *et al.*, 1990). The phenazine antibiotics alter hatch, attraction or host recognition by nematodes (Sikora, 1992). The bacteria are also reported to bind the root surface carbohydrate moieties with lectin and thereby interfere with the host recognition by

nematodes (Oostendorp & Sikora, 1990). The extent and intensity of bacterial colonization determines whether sufficient metabolites will be produced to inhibit, interrupt or stimulate certain processes in the nematodes life cycle (Becker *et al.*, 1988; Weller, 1988; Defago & Keel, 1993). The seedlings were raised and the bacteria were introduced only one month after germination. The late introduction of bacteria in all these experiments (except the last one) might have affected the colonization level and thereby the synthesis of metabolites.

Similarly, inoculum density, light quality, watering, soil microbial community, plant nutritional status, soil pH, temperature and survival of applied bacteria are also reported to influence the antagonistic activity of these rhizobacteria (Weller, 1988; Oostendorp & Sikora, 1989; Deacon, 1991). Intergeneric and interspecific competition between rhizobacteria may influence bacterial colonization on the root surface (Racke & Sikora, 1992). In addition, bacterial traits essential for nematode antagonism may have been lost due to spontaneous mutations on artificial growth media. Similar results were observed against root-knot nematodes (Zavaleta-Mejia & van Gundy, 1982; Becker *et al.*, 1988; Santhi & Sivakumar, 1995; Santhi *et al.*, 1999); citrus nematode (Santhi *et al.*, 1998); cyst nematodes (Gokte & Swarup, 1988; Oostendorp & Sikora, 1989) and rice root nematode (Ramakrishnan & Sivakumar, 1998). It is also reported that nematodes like *Meloidogyne*, which are with multiple generations and cause damage throughout the season, are more difficult targets for *P. fluorescens* (Sikora, 1992). Under greenhouse conditions, soil drenches with the aqueous cell suspension or cell-free culture of *P. aeruginosa* resulted in a considerable reduction in nematode population densities in soil and subsequent root-knot development due to *M. javanica* (Siddiqui & Haq, 2001).

The increase in bacterial inoculum correspondingly decreased root galling and root-knot nematode population in cardamom. Consequently, there was significant improvement in growth of cardamom seedlings. The role of *P. penetrans* in suppressing plant parasitic nematodes has been tested in many crops (Chen & Dickson, 1998). The reduction in nematode levels was inversely proportional to the inoculum levels of *P. penetrans* in crops like peanut (Chen, Z.X. *et al.*, 1997). However, the high incidence of nematode juveniles in soil with low spore encumbrance was quite surprising. Single application of *P. penetrans* was not sufficient to sustain the bacterial inoculum in soil. Watering is reported to affect the spore distribution in soil (Davies *et al.*, 1991).

The field experiment to evaluate the performance of *V. chlamydosporium* and *P. penetrans* was conducted in a cardamom nursery located at Appangala, Kodagu, Karnataka. The results of this trial clearly proved that *V. chlamydosporium* caused a significant decline in root-knot nematode population indicating the establishment of this natural antagonist in soil. However, corresponding increase in the growth of cardamom plants was not observed. This was mainly because of the inability of the fungus to reduce the initial invasion of roots by the infective second stage juveniles and the damage they cause to plant growth. Besides, low population levels of root-knot nematodes at the initial phase of the experiment might have affected the establishment of the fungus. The fungus is more abundant on roots infected by nematodes compared with those that are healthy (Kerry, 2001). *V. lecanii* also was not effective against *M. incognita* on cantaloupe at the low nematode inoculum levels (Meyer, 1999). The host status of the plant species also plays a key role in the establishment of the fungus (Bourne *et al.*, 1996; Bourne & Kerry, 1999).

On the other hand, *P. penetrans* failed to induce any change in the nematode population or in the growth of cardamom seedlings indicating the non-suitability of this biocontrol agent in situations where the initial nematode level is very high and rapid kills are needed. Amplification of *P. penetrans* to suppressive levels requires longer duration. Oostendorp *et al.* (1991) reported three years as the minimum period for the bacterium to yield adequate control. Mild nematode infestation in beds treated with *P. penetrans* alone was mainly because of the mode of inoculation of the bacteria. As *P. penetrans* was introduced to soil as an aqueous suspension of infected juveniles of the nematode, bacterial endospores were not available initially to check the nematode invasion into roots. Minimum one or two life cycles of the root-knot nematodes should be completed for the endospores to get released. In addition the initial inoculum would have contained some healthy nematodes devoid of any endospore attachments. In a similar experiment Davies *et al.* (1988), on inoculating J2 attached with endospores, observed reduction in J2 invasion and total nematode population only after the emergence of second generation of nematodes. *P. penetrans* is a mesophilic bacterium with an optimum temperature between 28°C and 35°C (Hatz & Dickson, 1992; Serracin *et al.*, 1997). Relatively high temperatures generally favour endospore attachment, germination and pathogenesis (Chen & Dickson, 1998). Therefore, temperature below 25°C may not be ideal for their development and multiplication. Initial nematode density also plays a crucial role in the establishment and multiplication of *P. penetrans* (Gowen *et al.*, 1998). This may be one of the reasons for the poor performance of this obligate nematode parasite at Appangala.

In the other experiment using *Trichoderma* isolates and *P. lilacinus*, both the fungal bioagents controlled the incidence of rhizome rot, caused by soil-borne fungi like *Pythium vexans* and *R. solani* and root-knot nematode infestation in cardamom nurseries. Suppression

of soil-borne pathogens by *Trichoderma* spp. is well established in several crops and the reported mechanisms involved are competition, mycoparasitism and production of antibiotics (Mukhopadhyay, 1987; Sivan & Chet, 1992). Similarly, there are reports that some strains of *P.lilacinus*, a known egg parasite of nematodes, have antagonistic activity against fungi and bacteria (Brian & Hemming, 1947; Cartwright & Benson, 1995). *P. lilacinus* is a good root colonizer and rhizosphere competitor (Cabanillas *et al.*, 1989a). The supremacy of combined application of *P.lilacinus* and *T. harzianum* in controlling nematodes is noteworthy. In a similar study, Khan *et al.* (1997) applied both these beneficial fungi on potted papaya. Though each biocontrol agent applied alone was able to improve plant vigour, reduce nematode numbers, and decrease incidence of root rot, the combined application was even more effective. Several such combinations have been reported in the literature (Meyer & Roberts, 2002). However, not all combinations of strains result in significantly improved and consistent disease suppression, as reported in a study using *T. harzianum* and *V. chlamydosporium* (Kok *et al.*, 2001). For improved biocontrol performance to occur, strains combined in preparations should be compatible.

The field experiment laid out in an established black pepper garden at Wynad, Kerala was very unique in several respects. This long-term experiment was conducted with the objective of evaluating the remedial action of three promising biocontrol agents viz. *V. chlamydosporium*, *T. harzianum* and *P. penetrans* in comparison to application of pesticides on root-knot nematode infestation in black pepper vines (Panniyur 5). *V. chlamydosporium* was highly effective compared to *T. harzianum* in suppressing nematodes and increasing the yield of black pepper. *T. harzianum* is reported to have a limited capacity to grow in the rhizosphere (Chao *et al.*, 1986; Ahmad & Baker, 1987). Moreover, it is suggested that the main anti-nematode activity caused by *T. harzianum* takes place in soil and not within roots

(Sharon *et al.*, 2001). In a crop like black pepper which is highly susceptible to root-knot nematodes and produce very large compound galls, the soil phase of these nematodes is for a very brief spell.

Though the parasitism of eggs by this fungus significantly reduced multiplication of the nematode and provided population control, compared to other treatments, the resultant nematode level in roots was still very high. The biocontrol intervention was attempted in an established and severely infested black pepper garden. When the nematode levels are very high, galls are larger, and fewer egg masses are present external to the roots. Therefore it may take more time for the fungi applied to reduce nematode populations to non-damaging levels. *V. chlamydosporium* did not colonize cells of tomato roots in an experiment and hence *M. arenaria* egg masses that formed inside galls were protected from parasitism by the fungus (Leij & Kerry, 1991). In another study, *V. lecanii* too failed to affect egg numbers inside cantaloupe roots (Meyer, 1999). In spite of this, the yellowing in black pepper plants has come down and there was a steady increase in yield, wherever *V. chlamydosporium* was applied. The yield increase was almost similar to that obtained with nematicides. Many of the nematode antagonistic fungi are reported to be good phosphate solubilizers and their application not only helps in controlling nematodes but also promotes plant growth by increasing the P availability in soil (Somasekhar *et al.*, 1998).

V. chlamydosporium established and survived in the organic soil during the course of the experiment as the reisolation studies proved. This was expected because organic soils have been reported to be a better substrate for the growth of *V. chlamydosporium* than mineral soils (Leij *et al.*, 1993a; Kerry & Leij, 1992; Kerry *et al.*, 1993; Viaene & Abawi, 2000). The tritrophic interaction between root-knot nematodes, *V. chlamydosporium* and the host plant is

very complex (Kerry, 2001). Kerry *et al.* (1984) showed that some isolates of this fungus were good root colonizers. Leij *et al.* (1993b) reported the ability of *V. chlamydosporium* to proliferate extensively on nematode damaged roots and suppress nematode population up to 90%. In addition, the robust chlamydospores ensure the better survival and rhizosphere colonization of this fungus.

P. penetrans, since deployed as a single control measure, failed to give consistent and durable control of nematodes in perennials. It has been evaluated in perennial crops like kiwi (Stirling, 1984; Verdejo, 1992). Under a continuous crop it may require more time to suppress the nematodes (Oostendorp *et al.*, 1991). Besides temperature and pH can also influence the endospore attachment on nematodes (Hatz & Dickson, 1992; Serracin *et al.*, 1997; Orui, 1997; Chen & Dickson, 1998).

Determination of the exact prerequisite conditions for successful infection of the nematode by the applied biocontrol agents is clearly difficult given the number of possible variables; quality of inoculum supply, condition of plant host, colonization potential, rhizosphere competence, condition of the nematodes and the complexity of the soil habitat (Morgan-Jones *et al.*, 1981). Microbial communities are spatially discrete. They tend to occupy the same favoured sites and therefore interactions occur between them. Therefore, these interactions determine the fate of the introduced inoculants that are applied for the biological control of pests and pathogens. Many attempts to establish biocontrol agents in the field soil have failed because of the antagonistic interaction of endemic components of these microflora (Stirling, 1991).

The effectiveness of many biocontrol agents could be improved if soil temperature regimes were favourable to the antagonists. The non performance of some of the promising

biocontrol agents under field conditions also can be explained if information on their ecological requirements is available. The present study has clearly proved the optimum temperature requirements of some of the promising biocontrol agents like *T. harzianum*, *P. lilacinus* and *V. chlamydosporium*. A temperature range of 25°C to 30°C was preferred by isolates of both *T. harzianum* (C.22) and *V. chlamydosporium* while 25°C was found suitable to *P.lilacinus*. There are several reports that the optimum temperature for the growth and multiplication of *T. harzianum* is between 25°C to 30°C (Jalil *et al.*, 1997; Rollan *et al.*, 1999). Similarly on *V. chlamydosporium* too (Zaki & Maqbool, 1993). But most of the isolates reported from temperate regions favoured a low temperature <25°C (Leij *et al.*, 1992b). Isolates of *V. chlamydosporium* collected from different soils differed in their temperature responses (Bursnall & Tribe, 1974; Kerry, 1981; Dackman & Nordbring-Hertz, 1985; Kerry *et al.*, 1986; Kerry, 1995). One strain of *V. chlamydosporium* had grown more rapidly at 25°C where as most other strains had temperature optima of about 18°C. There are many reports that *P. lilacinus* tolerated higher temperatures from 25-35°C (Cabanillas *et al.*, 1989b; Fioretto & Villacorta, 1989; Wang, 1992; Liu *et al.*, 1995; Cannayane & Sivakumar, 2001). The isolate used (Pl.2) in this study was collected from Kodagu, a cooler hill region in Karnataka, and this can be the probable reason for the low temperature preference of this isolate. This confirms that isolates vary in their ecological requirements, which may in turn influence their efficacy. The present study took into consideration only the mycelial growth of these fungi. The optimum temperatures derived at may not be same for sporulation, production of enzymes etc. Sometimes, examination of the influence of temperature on the parasitism of eggs is made difficult by the effects on hatch; egg parasitic fungi do not parasitize active second stage juveniles and conditions which support rapid hatch reduce the chances of infection.

Likewise, pH of soil or carrier media also plays a crucial role in the rhizosphere colonization and survival of the biocontrol organisms. In the present study both the *Trichoderma* isolates preferred an acidic pH, the C.22 isolate distinctly favouring pH range of 4 to 7. In literature too several studies indicate strainal variation in their pH requirements but most of them were active in acidic pH only (Pandey & Upadhyay, 1997; Singh, R.S. *et al.*, 1998). Alkaline pH was generally found suitable for *Fusarium* isolates.

Exploitation of nematode antagonists as biocontrol agents demands that they should be compatible with chemical pesticides that are applied to the crops or soil to control other pests and diseases. Fungi expressing fungicide or pesticide tolerance as well as antagonism to plant parasites are useful for integrated pest management systems involving chemical control. In the current study some selected representative biocontrol agents were evaluated for their compatibility with pesticides like metalaxyl-mancozeb, potassium phosphonate, chlorpyriphos and phorate. Among the four pesticides only metalaxyl-mancozeb showed incompatibility with all the fungi tested while phorate at the recommended dosage was compatible with all the fungi. Generally nematicides are safe chemicals to most of the antagonistic fungi (Olmert & Kenneth, 1974; Crump & Kerry, 1986; Singh & Dhawan, 1998; Kim & Riggs, 1998; Sharma *et al.*, 1999; Hazarika *et al.*, 2000; Jebakumar *et al.*, 2000; Tzortzakakis, 2000; Khan & Goswami 2001). Fungicides like mancozeb and iprodione suppressed radial growth of *P. lilacinus* and *V. chlamydosporium* (Mertens & Stirling, 1993). The adverse effect of chlorpyriphos on fungal antagonists, though reported in another study (Jebakumar *et al.*, 2000), has to be studied critically. Similarly that of potassium phosphonate on *P. lilacinus* too warrants further studies. There are reports that fungicides like mancozeb are compatible with *Trichoderma* spp. (Kay & Stewart, 1994; Sharma *et al.*, 1999) and *V. chlamydosporium* (Sharma *et al.*, 1999). The pesticide tolerance can vary greatly among different

strains of fungal species (Meyer *et al.*, 1990, 1991). Fungicide tolerant strains have been reported in *T. harzianum* against metalaxyl (Sawant & Mukhopadhyay, 1991) and *V. chlamydosporium* against benomyl (Meyer *et al.*, 1991). However, field studies are needed to prove the compatibility of some of the chemicals, especially potassium phosphonate, with antagonistic fungi.

Developing efficacious formulations is high priority in biological control research. *V. chlamydosporium* grows well on many media, including waste materials, over a considerable pH range (Kerry *et al.*, 1986). However, in the present study, the maximum colony-forming units of the fungus *V. chlamydosporium* was produced on sorghum grains, as reported by others (Sankaranaryanan *et al.*, 2001), followed by coffee husk. It grows well on many grains like oat kernels (Godoy *et al.*, 1983a; Rodriguez-Kabana *et al.*, 1984; Kerry *et al.*, 1984), maize (Bourne *et al.*, 1999) etc. The rich energy source in these grains helps the fungus to grow and multiply. However, the rate of multiplication was slow in most of the substrates as the fungus is a slow growing one.

In contrast *Trichoderma* readily multiplied on sorghum, coffee husk and leaf powder. Sorghum and coffee husk are commonly used for mass culture of *Trichoderma* (Indu & Sawant, 1990; Suseela Bhai *et al.*, 1994; Prakash *et al.*, 1999). *P. lilacinus* on the other hand showed maximum multiplication and sporulation on rice grains and ginger leaf powder. Rice grains were found to be the best substrate for *P.lilacinus* in another study too (Trivedi, 1992). The suitability of leaves of certain plant species as substrates for mass multiplication of the nematophagous fungus, *P. lilacinus* was also evaluated (Sosamma & Jayasree, 1999). Ginger leaf powder is reported for the first time as a carrier for any fungus. The essential oils present in the leaves might have boosted the growth and multiplication of the fungus.

On the contrary, turmeric leaf powder was found to be a poor substrate for the multiplication of *P. lilacinus*. This is anticipated because of the antimicrobial activity of curcumin, present in traces in the turmeric leaves. Likewise neem oil cake, saw dust, coir dust and coir pith formulation ('Terracare') were not ideal substrates for the multiplication of this fungus. Saw dust, coir dust and pith substrates are mainly composed of inert materials and hence might not have supported the growth of the fungus. Neem cake, though rich in nutrients, failed to serve as a good substrate for both *P. lilacinus* and *T. harzianum*. Prakash *et al.* (1999) too obtained similar results with *Trichoderma* spp. Saju *et al.* (2002) attributed this to *Penicillium* colonization in the neem oil cake. Coir pith, an agricultural byproduct, once decomposed is nowadays used widely as a soil amendment. Some workers have used this material as a substrate for multiplying biocontrol fungi like *Trichoderma* spp. (Kumar *et al.*, 2000: Saju *et al.* 2002). Vermicompost is another substrate that was proved not to be a good media for the multiplication of *T. harzianum* and *V. chlamydosporium*. The inhibitory role of vermicompost on *T. harzianum* has also been reported previously (Pereira *et al.*, 1998).

Summary

Spices face a variety of production constraints. Among the various pests and diseases of spices, the damage caused by plant parasitic nematodes is quite serious. Root-knot nematodes (*Meloidogyne* spp.) are among the most important group of plant parasitic nematodes, causing damage and yield losses on most of the spices. Significant reductions in growth and yield are noticed in several spice plants infested with root-knot nematodes. Burrowing nematodes (*R. similis*) and lesion nematodes (*Pratylenchus* spp.) are two other important and destructive root parasites of spice crops. Cultural practices, use of resistant cultivars and application of chemical nematicides are primary strategies for nematode management, but yield losses persist with numerous crops. Application of microorganisms, antagonistic to *Meloidogyne* spp., or of compounds produced by these microbes, could provide additional opportunities for managing nematode problems in spices.

Pursuing this objective, a number of fungi and bacteria were collected and screened for their nematode antagonism. Fungi and bacteria from rhizosphere soil and roots were isolated by the standard dilution plate method while they were isolated directly from root-knot nematode egg masses or individual eggs or females. Majority of them (17 isolates) belonged to the genus *Trichoderma*, while 7 isolates were of *Aspergillus* spp., 6 each of *Paecilomyces* spp. and *Penicillium* spp., 5 isolates of *Fusarium* spp., and 2 isolates of *Verticillium* spp. Taxonomic identity of 22 isolates could not be established as there was no sporulation. Besides these some promising isolates were obtained from other sources too. Altogether there were 5 isolates of *Verticillium* spp., 8 isolates of *Paecilomyces* spp., 88 isolates of *Trichoderma* spp. and 48 isolates of various other fungi. Among the 57 bacteria isolated, 17 isolates belonged to different species of *Bacillus* while 12 were *P. fluorescens* isolates. Notable among these were *V. chlamydosporium, T. harzianum, P. lilacinus, P. penetrans* and *P. fluorescens.*

The test fungi were evaluated for their ability to parasitize various life stages of the nematode viz. eggs, juveniles, females and egg masses. Their modes of action have been studied. Egg and female parasitism was the most prominent mechanism of many of these fungal facultative parasites. Sixty-seven out of the 110 isolates screened were not parasitic on females of root-knot nematodes. Significant parasitism on adult females was observed only in 3 isolates viz. *P. lilacinus* (Pl.1), *T. harzianum* (C.22) and *V. lecanii* (Vl). Out of the 149 fungal isolates screened, 115 isolates colonized the gelatinous matrix of root-knot nematode egg masses (77.18%). Fifty-nine isolates showed 50 - 90 % inhibition in egg hatch.

Further, the biocontrol potential of these short-listed microbes was assessed through a series of greenhouse and field trials. Among the 5 isolates of *Trichoderma* evaluated, isolates C.22 and C.23 were found superior in suppressing root-knot nematode populations than the other isolates. Though not significant, most of the isolates induced some degree of growth promotion. However, results were not encouraging with another 6 isolates of *Trichoderma* screened against nematodes affecting black pepper plants. The time of inoculation of biocontrol agents, their dosage and the carrier substance used had significant influence on the results of these greenhouse evaluations. Though a number of fluorescent pseudomonads were evaluated, none of them had any significant influence on any of the plant growth characters or in reducing the plant damage due to root-knot nematode infestation. Many of them were good growth promoters with an increase in growth ranging from 26.7 to 55.6% even when the plants were affected by root-knot nematodes.

The egg parasitic fungus *V. chlamydosporium* and the obligate bacterial parasite *P. penetrans* were able to check the root-knot nematode multiplication in cardamom nurseries. Significant reduction in nematode population was observed in plots where *V. chlamydosporium* was applied.

Although there was no significant improvement in the growth of cardamom seedlings with either of the biocontrol agents, the maximum growth improvement was obtained with *V. chlamydosporium* treatment. Similarly, there was a significant increase in the total biomass of individual cardamom seedlings treated with *P. lilacinus*. But both *Trichoderma* and *P. lilacinus*, either alone or together, significantly improved the number of quality seedlings. The rhizome rot incidence was drastically reduced wherever any of these bioagents was applied. The nematode population was also suppressed in all the treatments compared to that in control plots, the reduction was statistically significant only with the combined application of the above two fungal antagonists. In general, biocontrol agents performed better in solarized soil than in non-solarized beds.

In black pepper, the reduction in yellowing was statistically significant only in the case of vines treated with biocontrol agents. The highest mean yield (5.14 kg vine^{-1}) was obtained in *V. chlamydosporium* treated plots followed by combined application of phorate and potassium phosphonate (4.20 kg vine^{-1}), which were significantly higher than that of control plots. The root-knot nematode population decreased in all treatments compared to the initial population, but the reduction was not statistically significant in any of the treatment. However, the lowest mean population of root-knot nematodes in black pepper roots was observed in phorate + potassium phosphonate treated plants followed by *P. penetrans* treated vines. However, after 4 years of field evaluation, the nematode level was the lowest in *V. chlamydosporium* treated plots.

Results of these tests, though inconsistent due to the varying biotic and abiotic factors, threw significant insights on the potential of such organisms. Biotic factors that influenced the fate of the introduced biocontrol agent included interference of non-target organisms, degree of rhizosphere and soil colonization by the biocontrol agents, initial population levels of the

target nematodes, susceptibility of the host plant to the target pest etc. Notable abiotic factors consisted of climate as well as physical and chemical composition of the rhizosphere.

The studies also threw light on the varied ecological requirements of some of the promising fungi. The growth of fungi like *T. harzianum*, *P. lilacinus* and *V. chlamydosporium* was severely affected at temperature >30°C. While a temperature range of 25-30°C was found suitable for the optimum growth of *T. harzianum* and *V. chlamydosporium*, an optimum temperature of 25°C was preferred by *P. lilacinus*. The studies also indicated that the optimum pH required for their growth varied with species of fungus (biocontrol agent) and also with different isolates of the same fungus.

Among the four pesticides studied for their compatibility with biocontrol agents like *T. harzianum*, *P. lilacinus* and *V. chlamydosporium*, metalaxyl-mancozeb, at all concentrations, was detrimental to all the 3 fungi. Potassium phosphonate was compatible with *T. harzianum* and *V. chlamydosporium* but not with *P. lilacinus*. Chlorpyriphos at high concentrations also had a negative impact on all the three fungi studied.

Studies on mass multiplication of these fungi had shown the suitability of many of the locally available organic substrates. Among the three fungal isolates evaluated, *T. harzianum* and *P. lilacinus* multiplied readily on several solid substrates like sorghum grains, decomposed coffee husk and leaf powders. Ginger leaf powder and neem oil cake also were good substrates while farmyard manure, vermicompost, coir dust and saw dust were poor substrates for any of these fungi.

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