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BIOCONTROL OF ANTHRACNOSE OF BLACK PEPPER (*Piper nigrum* L.) CAUSED BY *Colletotrichum* spp. USING MYCOPARASITES

BY

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2002

DECLARATION

I hereby declare that this thesis entitled "**Biocontrol of anthracnose of black pepper (*Piper nigrum* L.) caused by *Colletotrichum* spp. using mycoparasites**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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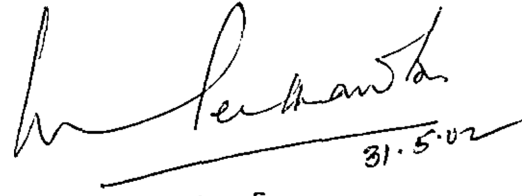

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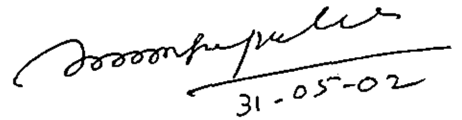

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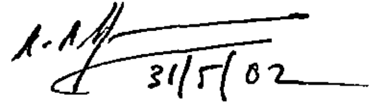

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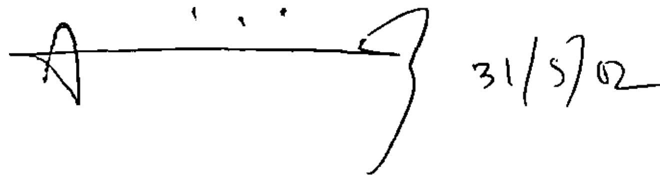

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Dedicated

to

Amma Appa and Ranji

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Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) is a low volume, high value spice crop grown in India and is a major foreign exchange earner for the country. It is popularly known as the 'black gold', which best defines its economic importance. During the year 1999-2000, the share of black pepper in Indian total spice export was Rs. 884.8 crores (43.69 per cent) and Kerala is the leading producer of black pepper in India contributing 96.36 per cent of the total area and 97.33 per cent of the total production (Rajesh *et al.*, 2002).

Productivity of black pepper in India is only 290 kg ha^{-1} as against the 2925 kg ha^{-1} in Malaysia. One of the major reasons attributed to this low productivity is the incidence of diseases such as foot rot, slow decline and anthracnose, especially in the state of Kerala. Among these, the pollu disease or anthracnose caused by *Colletotrichum* spp. is serious and is on the increase in recent years (Sarma *et al.*, 1988). A severe outbreak of this disease was reported in Idukki district of Kerala during 1999 (Sainamole *et al.*, 2000). The fungus *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. (teleomorph, *Glomerella cingulata* (Stonem.) Spauld. and von Schrenk) is consistently isolated from the diseased leaves and berries. The disease is seen both in the nursery and main plantations. In the nursery, the initial stages of infection cause severe defoliation. The disease is seen almost throughout the year. In the plantations the disease affects the leaves, spikes and berries. The disease causes 1.93 – 9.54 per cent spike shedding and the percentage loss due to infection ranges between 0.69 – 3.74 and total loss of weight is 0.67-137.75 g per plant (KAU, 2001). The disease causes considerable economic loss owing to reduction in marketability of the economic produce.

At present, the recommended control measure for combating this disease is the application of Bordeaux mixture (1 per cent) or Captafol (0.1 per cent) once before flowering and then at berry formation stage. Use of fungicides for the control of the disease may result in the persistence of fungicide residues on the berries. Nowadays, much emphasis is being given on organically raised black pepper. Under these circumstances, biological control can be adopted as a suitable alternative strategy for managing this disease. Biological control is an integral factor in sustainable crop production. Being ecofriendly, cost effective and best suited for greenhouse crops, this technique of combating plant diseases is gaining momentum. With this in view, the present investigation has been carried out with the following objectives:

1. Isolating fungal antagonists from rhizosphere and phyllosphere of healthy black pepper plants.
2. Testing the antagonistic activity of the fungal isolates against *Colletotrichum gloeosporioides in vitro*.
3. Testing the efficacy of different carrier materials on multiplication and viability of the selected fungal antagonists.
4. Formulating the selected antagonists in suitable carrier material.
5. Assessing the viability of the antagonists in the prepared formulations.
6. Testing the efficacy of the formulations on black pepper cuttings under greenhouse conditions.
7. Assessing the effect of the antagonist formulations on the growth of black pepper.

Review of

Literature

2. REVIEW OF LITERATURE

The pollu disease or anthracnose of black pepper (*Piper nigrum* L.) caused by *Colletotrichum* spp. is a serious disease, which is on the increase in recent years (Sarma *et al.*, 1988). During June 1999, there was an epidemic of anthracnose in the high ranges (above 1000 meter MSL) of Idukki district (Sainamole *et al.*, 2000). The disease was first identified in North Malabar of Kerala as 'berry spot' and 'berry split' by Ramakrishna Ayyar (1921). Later, the disease was named as 'fungal pollu' and the causal organism was identified as *Colletotrichum gloeosporioides* (Rao, 1926). Maximum damage due to the disease is noticed during August – September period (Unnikrishnan *et al.*, 1987). Fungal pollu caused 1.93 – 9.54 per cent spike shedding and the percentage loss due to infection ranged between 0.69 – 3.74 and total loss of weight was 0.67 – 137.75 g per plant (KAU, 2001).

Anthracnose of the green berries causes significant yield losses of 10 – 20 per cent in India in shaded pepper (Sarma *et al.*, 1988; Radhakrishnan and Naik, 1983). Although chemical control of the disease through the use of fungicides may lessen the severity of this disease, continuous application of chemicals causes long term damage to the environment. Moreover, the Ministry of Commerce is popularizing products developed without chemical application. There is strong evidence that natural biological control provides protection against many foliar diseases in crop plants (Blakeman and Fokkema, 1982). In recent years, direct application of antagonistic

microorganisms to control foliar and root infecting pathogens has gained momentum (Whipps, 1992).

2.1 The pathogen, *Collelotrichum gloeosporioides* (Penz.) Penz. and Sacc.

The fungus has been described by Sutton (1992) as follows :

Teleomorph : *Glomerella cingulata* (Stonem.) Spauld and von Schrenk

Colonies variable, greyish white to dark grey, aerial mycelium even and felted or in tufts associated with conidiomata, reverse unevenly white to grey or darker especially with age. Setae present or absent. Sclerotia absent but immature ascomata may be mistaken for sclerotia. Conidia formed in pale salmon masses, straight, cylindrical, apex obtuse, base truncate, 12-17 x 3.5 – 6 μm.

2.2 Variability in *Colletotrichum* spp.

A review of literature on the morphological characters of *Colletotrichum* spp. revealed great variability among different isolates.

The conidia of *C. gloeosporioides* may be oblong or cylindrical with rounded ends as observed by several workers (Prasad and Singh, 1960; Prasad, 1962; Swarup and Mathur, 1964; Singh and Katiyar, 1969; Vyas and Joshi, 1976). The length of conidia of *C. gloeosporioides* was found varying from 7 μm as reported by Lele and Asha Ram (1969) on loquat to 23-28 μm as observed by Prasad and Verma (1966) on *Sweitenia macrophyllata*.

Chackao *et al.* (1978) observed variation in the radial growth of two isolates of *Colletotrichum truncatum* (Schwein) Andrus and Moore within and between different media. Variation in colony colour has been reported by

Bhardwaj and Singh (1986) in four isolates of *C. truncatum* derived from different hosts. Singh and Shukla (1986) observed variation in the mycelial dry weight of *C. truncatum* corresponding to varied temperature and pH.

Pathogenic variability in isolates of *Colletotrichum graminicola* (Ces.) Wilson from different sorghum species and areas have been reported on the basis of differential reaction on foliage of sorghum cultivars (Ali and Warren, 1987; Cardwell *et al.*, 1989). Smith and Black (1990), compared the morphological, cultural and pathogenic characteristics of twenty-four isolates of *Colletotrichum* from strawberry and found distinct variations among the same. Variations have been reported in cultural characteristics, morphology and virulence of several foliar isolates of *C. graminicola*, *C. sublineolum* Henn. and *C. gloeosporioides* from India (Pande *et al.*, 1991; Rao *et al.*, 1998; Mathur *et al.*, 1999; Thakur *et al.*, 1999).

Mathur *et al.* (2000) compared thirty eight isolates of *C. graminicola* for morphological and pathogenic diversity and suggested that the evolution of variability for pathogenicity traits in the fungus is due to heterokaryosis and subsequent somatic recombination. Maziah and Bailey (2000) analysed the morphological and cultural variation among twelve *Colletotrichum* isolates obtained from tropical forest nurseries and found that most of the isolates showed variation in their conidial size and appressorial shape and size, suggesting the existence of different forms of *Glomerella cingulata*. Marley *et al.* (2001) classified fifty isolates of *C. sublineolum* into nine morphological groups on the basis of growth in culture and morphological characteristics. Mathur and Totla (2001) distinguished six patho types of *C. graminicola*, the causal agent of sorghum anthracnose, on the basis of

virulence and symptoms on grain and foliage. Mathur *et al.* (2001) reported that five single-lesion isolates of *C. graminicola* from sorghum and their derivatives varied significantly for morphological traits like colony colour, growth pattern, spore size and sporulation rate on oat meal agar medium. Sharma and Kaushal (2001) investigated the cultural and morphological characters of populations of eleven monoconidial isolates of *C. truncatum* and observed definable variations among them.

2.3 Survival of *Colletotrichum* spp.

Facultative saprophytes like *Colletotrichum* can survive in crop debris, soil etc. for varying periods depending upon the environmental conditions.

Dasgupta (1989) reported that the survival of *C. capsici* (H. Syd.) E. Butl. and Bisby causing anthracnose of betelvine, during unfavourable seasons, is possible through competitive saprophytic ability. Hegde *et al.* (1989) isolated *C. gloeosporioides*, the causal agent of anthracnose of arecanut, from dried and diseased arecanut leaves which had been on the ground for eight months or more. Palarpawar and Ghurde (1989) studied the perpetuation of *C. curcumae* (Syd.) Butler and Bisby and observed that the viability of the pathogen was retained upto nine months when naturally infested turmeric leaves were buried in the soil.

The survival of conidia of *Colletotrichum acutatum* Simmonds ex Simmonds in plant debris and in soil has been reported to vary depending on soil moisture and temperature (Eastburn and Gubler, 1992). Wilson *et al.* (1992) reported that *C. acutatum* survived in infected strawberry fruits even after exposure to winter conditions for three months. Dillard and Cobb (1993)

demonstrated that the bean anthracnose fungus, *C. lindemuthianum* (Sacc. and Magnus.) Briosi and Cav. overwintered four months in bean debris placed 0 to 20 cm deep in soil in New York state. Misra and Sinha (1996) reported that the sorghum anthracnose pathogen, *C. graminicola* survived for five months when infected leaves were stored in nylon bags buried in the soil.

Dillard and Cobb (1998) showed that *C. coccodes* (Wallr.) S. Hughes. survived in infected roots and fruits of tomato plants, which served as the primary source of inoculum. Yoshida and Shirata (1999) examined the over wintering survival of *C. dematium* (Pers. : Fr.) Grove., the causal agent of mulberry anthracnose and suggested that the pathogen can over winter in infected mulberry leaves which later on served as a primary source of inoculum in the following year. As yet, no studies are available on the survival of *C. gloeosporioides* causing anthracnose of black pepper.

2.4 Biological control of *Colletotrichum* spp.

The possibility and potential for controlling plant pathogenic fungi with antagonistic microorganisms have long been considered and studied.

2.4.1 Fungal antagonists of plant pathogens

The most exhaustively studied microorganism as a biocontrol agent is *Trichoderma* spp. The antagonistic potential of *Trichoderma* sp. was first demonstrated by Weindling (1932) on *Rhizoctonia solani* Kühn, a soil borne plant pathogenic fungus. The antagonistic properties of *Trichoderma* spp. have been revealed by several workers (Haran *et al.*, 1995; Chet, 1998). Most of the studies remain centered on the use of the antagonistic properties of *T. harzianum* Rifai. for biocontrol purposes (Sivan and Chet, 1982). It is known

that *T. harzianum* has antagonistic properties against *Sclerotium rolfsii* Sacc., *Pythium* spp., *Fusarium* spp., *R. solani* etc. (Elad *et al.*, 1983). Later on, other *Trichodema* spp. and antagonistic fungi have been extensively studied and utilised in the biological control of many plant diseases (Chet and Inbar, 1994; Sen, 2000).

Singh *et al.* (1978) recorded the control of *C. dematium* f. sp. *truncata* through direct parasitism under *in vitro* conditions by *Acremonium sordidulum* (Drechsler) Subram. The inhibitory action of *Aspergillus niger* Van Tieghem, *A. flavus* Link. and *Trichoderma viride* Pers. ex. S.F. Gray on *R. solani* Kühn infecting rice was demonstrated by Gokulapalan and Nair (1984). Cristinzio (1987) reported the antagonistic property of *Trichoderma* spp. and *Chaetomium* spp. against *Phytophthora capsici* Leonian. Padmakumari (1989) found *A. niger*, *Chaetomium globosum* Kunze, *Gliocladium virens* Miller, Gidders and Foster and *T. harzianum* to be antagonistic to *R. solani* under *in vitro* conditions. In dual culture tests on PDA, *A. niger* showed antagonism to *Macrophomina phaseolina* (Tassi.) Goid, *Phoma glomerata* (Corda) Wollenweber and Hochapfel, *Curvularia lunata* (Wakker) Boedjin, *Dreschlera oryzae* (Breda de Haan) Subram. and Jain. and *Alternaria alternata* (Fr.: Fr.) Keissl. (Gajbe and Lanjewar, 1991). *In vitro* tests showed that *A. niger* isolates inhibited the growth of *Fusarium oxysporum melonis* (Sacc.) Snyder and Hansen and *Fusarium solani* (Mart.) Sacc. (Sharma and Sen, 1991).

Mukherjee and Sen (1992) reported *Aspergillus terreus* Thom., *A. fumigatus* Fres. and *Penicillium citrinum* Thom. to be antagonistic under *in vitro* conditions to *Macrophomina phaseolina*, a soil borne plant pathogen.

Vaishnav *et al.* (1992) observed that *A. flavus, A. niger* and *Penicillum* sp. were parasitizing sugarcane smut whips from 10 – 15 days after the onset of monsoon till December. The growth of the mycoparasites reduced the viability and secondary spread of smut teliospores. Germination of teliospores of Karnal bunt pathogen, *Neovossia indica* (Mitra) Mundkur was significantly reduced by culture filtrates of *T. viride, Gliocladium virens* and *Aspergillus* spp. in lab experiments (Aujla and Kaur, 1993). Similarly, applications of spore suspensions of *A. terreus* Thom. reduced infection by sheath blight pathogen, *R. solani* in rice, particularly when plants were treated before inoculation with the sclerotia of the pathogen (Gogoi and Roy, 1993). Anandaraj and Sarma (1995) observed that *Trichoderma* spp. and *Gliocladium virens* Miller, Giddeins and Foster were effective against *P. capsici.* An Integrated Disease Management (IDM) with *T. harzianum* as a component for the management of foot rot of pepper reduced the disease incidence from 25 per cent to 15 per cent in the field (Sarma *et al.*, 1996). Combination of fungal antagonists, *Aspergillus* sp. and *Penicillium* sp. was also found to delay foliar infection in black pepper by *P. capsici* (Jubina and Girija, 1997).

Mandal *et al.* (1999) demonstrated the inhibitory effects of *Trichoderma pseudokoningii* Rifai, *T. hamatum* (Bon.) Bain, *Talaromyces flavus* (Klocker) Stock and Samson and *Trichothecium roseum* (Pers.) Link. on the mycelial growth of wheat spot blotch pathogen, *Dreschlera sorokiniana* (Sacc.) Subram. and Jain in dual culture tests. *Trichoderma viride* and *T. harzianum* caused the maximum inhibition of mycelial growth of seed borne *Colletotrichum lindemuthianum* (Sacc. and Magnus) Briosi and Cav. in dual culture studies (Ravi *et al.*, 1999). *Aspergillus niger, A. flavus*

and *T. viride* were found to be potential antagonists of ginger rhizome rot pathogen, *Pythium aphanidermatum* (Edison) Fitzpatrik by dual culture and cell free culture filtrate studies (Shanmugam and Varma, 1999).

In field conditions, application of *Aspergillus terreus* into pressmud amended neutral soil showed highest efficacy in reducing sheath blight of rice caused by *R. solani*. (Das and Roy, 2000). Girija and Jubina (2000) reported that *Bacillus subtilis* and three isolates of *Bacillus* sp. from black pepper rhizosphere were capable of inhibiting mycelial growth and sporulation of *P. capsici* and *C. gloeosporioides*, as well as cause reduction in lesion development by both pathogens on detached foliage of pepper plants. Nallathambi *et al.* (2000) demonstrated the control of *Colletotrichum falcatum* Went., the incitant of red rot of sugarcane by *Trichoderma* spp. and *Pseudomonas fluorescens* (Migula) isolates from sugarcane rhizosphere. The rhizosphere of healthy pigeon pea plants was heavily colonized by *A. niger* and *Penicillium* sp., while diseased roots were colonized by *Fusarium udum* Butler, and *A. niger* showed moderate antagonism by suppressing pathogen colonies on PDA (Pandey and Upadhyay, 2000). Rajan and Sarma (2000) studied the antagonistic properties of *Verticillium tenerum* Nees. and established the disease suppressive activities of *V. tenerum* on *Phytophthora capsici*, the foot rot pathogen of black pepper. Singh and Singh (2000) observed maximum inhibition of growth of *Alternaria solani* (Ell & Mart.) Jones and Grout with the metabolites of *Aspergillus flavus* and *A. terreus* in laboratory tests. Rajathilagam and Kannabiran (2001) reported the antifungal effect of non-volatile antibiotics extracted in chloroform of *T. viride* against *C. capsici*. A perusal of the literature revealed that no studies were available

on the antagonistic reactions of *Aspergillus* spp. or *Trichoderma* spp. on *Colletotrichum gloeosporioides* in black pepper.

2.5 Mechanism of action of antagonists

T. harzianum produces cell well lytic enzymes which cause lysis of mycelia of *R. solani* (Elad *et al.*, 1983). Chu and Wu (1981) observed that the hyphae of ten isolates of *T. pseudokoningii*, three isolates of *T. longibrachiatum* Rifai, one of *T. hamatum* (Bon.) Bain, two of *T. harzianum* and five of *Pencillium* spp. could coil around the hyphae of *R. solani* which consequently lost their contents and collapsed. Sharma and Gupta (1982) reported that inhibition of *Colletotrichum* sp. under *in vitro* conditions is due to the production of lytic enzymes by *Streptomyces rochei*. The penicilli and aspergilli act through antibiosis or as a competitor of the pathogen at the infection court (Cook and Baker, 1983). Michail *et al.* (1986) reported that the culture filtrates of *T. longibrachiatum* decreased the mycelial dry weight, sporulation and spore germination of *Fusarium solani* (Mart.) App. & Wollenw. in culture.

Lenne and Brown (1991) suggested that antibiotics produced by bacterial antagonists were chiefly responsible for the inhibition of *C. gloeosporioides* and subsequent reduction in anthracnose lesions on the surface of *Stylosanthes guianensis* Sw. in Pucallpa, Peru. Reeny (1995), in her studies with *T. viride*, found that it controlled *C. capsici* in vegetables by penetration of hyphal cells. Sen *et al.* (1995) recorded that *A. niger* strain AN 27 inhibited spore germination and caused aberration and lysis of chlamydospores and sclerotia of *Macrophomina phaseolina* (Tassi.) Goid, *Fusarium oxysporum* Schlech. and *Sclerotinia sclerotiorum* (Lib.) de Bary.

Inbar *et al.* (1996) observed coiling and disintegration of hyphae of *Sclerotinia sclerotiorum* by *T. harzianum.*

Mondal (1998) showed that *Aspergillus niger* AN 27 has the attribute of rhizosphere competence which leads to the control of many soil borne plant diseases. Mondal and Sen (1999) described the production of hydroxamate and catecholate groups of siderophores by *Aspergillus niger* AN 27 effective against various soil borne plant pathogens. The unsterilized culture filtrate of *A. niger* changed the colony colour, suppressed the secondary sporidial formation and inhibited the germination of teleutospores and sporidia of the sorghum pathogens, *Sphacelotheca cruenta* (Keuhn.) Potter and *Sporisorium sorghi* (Rajasab and Saraswathi, 1999). A local isolate of *T. harzianum* (ITCC No. 4542) directly attacked and lysed the mycelium and sclerotium of *Sclerotium rolfsii* in brinjal when the two fungi were grown in dual culture in petriplates (Singh and Singh, 2000). Bunker and Mathur (2001) demonstrated that the hyphae of *T. aureoviride* Rifai coiled over the hyphae of *R. solani* in dual culture, thus resulting in emptying of contents of hyphae of the latter. Godwin-Egein and Arinzae (2001) reported that the mechanisms of antagonism employed by *T. harzianum* against *F. oxysporum* were competition, lysis and hyperparasitism.

2.6 Mass multiplication and formulation of fungal antagonists

For the biological control of plant pathogens, it is necessary to mass produce the promising antagonists rapidly as spores, mycelia or mixtures which can be achieved with liquid media in agitator stirred fermentors (Papavizas *et al.*, 1984). To improve the efficacy of microbial antagonists,

several formulations of biocontrol agents have been developed, which are being produced commercially in many countries (Papavizas, 1985).

Backman and Kabana (1975) developed a diatomaceous earth granule impregnated with a 10 per cent molasses solution as a suitable system for growth and delivery of *T. harzianum* to peanut fields against soil-borne diseases. Several isolates of *Trichoderma* spp. can develop large amounts of biomass containing conidia and chlamydospores in both liquid and solid media containing inexpensive ingredients like molasses and brewer's yeast (Lewis and Papavizas, 1983). Fravel *et al.* (1985) reported a method of encapsulation of potential biocontrol agents in an alginate dry matrix. Lewis and Papavizas (1985) prepared a pelletized formulation of wheat bran or kaoline clay in an alginate gel containing conidia, chlamydospores and fermentation biomass of several isolates of *Trichoderma* and *Gliocladium*. Application of wheat bran saw dust preparation of *T. harzianum* or *T. koningii* Oud. brought out an excellent control of damping off of tomato and eggplant and wilt and root rot of lentil under field conditions (Mukhopadhyay, 1987).

Lewis *et al.* (1991) devised a biocontrol formulation system in which vermiculite and powdered wheat bran were mixed with wet or dry fermentor biomass of *Trichoderma* sp. or *Gliocladium virens*, moistened with 0.05 N HCl and dried before storage. Montealegre *et al.* (1993) proposed a liquid fermentation method for the large scale production of *T. harzianum* biomass. Daigle and Cotty (1995) developed a method of alginate pelletting for the delivery of atoxigenic *Aspergillus flavus* strains to furrow-irrigated cotton in desert environments. Alginate prills containing *Talaromyces flavus* (Klocker) Stock and Samson with a clay carrier (pyrax) reduced the impact of

Verticillium dahliae Kleb. on potato and egg plant (Fravel *et al.*, 1995). Similarly, Lewis *et al.* (1996) showed that alginate prills with *Gliocladium virens* biomass significantly reduced damping off of zinnia caused by *R. solani* and *Pythium ultimum.*

Nakkeeran *et al.* (1997) standardised the storage conditions to increase the shelf-life of *Trichoderma* formulations. Prasad *et al.* (1997) highlighted the superiority of PDA as a medium for biomass production of *T. harzianum,* when compared to V-8 juice and molasses and brewer's yeast.

Five isolates of *Cladorrhinum foecundissimum* Sacc. and Marchal added to soilless mix as ten day old fresh bran preparations (1.0 per cent w/w) significantly reduced damping off of egg plant and pepper caused by *Rhizoctonia solani* (Lewis and Larkin, 1998). Prasad and Rangeshwaran (1999) evaluated a modified granular formulation containing powdered wheat bran, kaolin, acacia powder and biomass of *T. harzianum, T. virens* and *Gliocladium deliquescens* Sopp. and observed that all the granules significantly reduced chickpea damping off caused by *R. solani. T. harzianum* is successfully multiplied on a large scale on decomposed coffee husk in Koorg district in Karnataka state and applied to the coffee plantations against soil-borne diseases (Girija Ganeshan *et al.*, 2000). Kumar *et al.* (2000) recorded that, in partly decomposed and sterilized coconut coir pith, the population of *Trichoderma* spp. increased from 10^4 c.f.u. to 10^7 c.f.u. per gram of coir pith in ten days. Prasad and Rangeshwaran (2000) found that kaolin and talc are better carriers of *T. harzianum,* while evaluating different carrier materials for their effect on the shelf life of the fungus.

2.7 Method of application of biocontrol agents

Different methods of application of biopesticide formulations have been tested to control crop diseases caused by various plant pathogens. Strawberry fruits were protected against storage rot by spraying the plants in the field beginning at early flowering with aqueous suspension of conidia of *T. viride* and *T. polysporum* (Link ex Pers.) Rifai (Tronsmo and Dennis, 1977). Tschen and Kuo (1981) reported that coating of mungbean seeds with a culture of the bacterium, *Bacillus megaterium*, could control damping off disease affecting the crop. De Oliveira *et al.* (1984) demonstrated that the application of *T. harzianum* to the soil as a conidial suspension at 10^6 conidia per ml during the transplanting period significantly reduced the severity of white rot of garlic caused by *Sclerotium cepivorum* Berk.

Biles and Hill (1988) obtained significant reduction in leaf spot of wheat caused by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex. Dast. by foliar application of *T. harzianum* at the rate of 10^8 spores per ml. Zhou and Reeleder (1989) found that foliar application of spore suspension of *Epicoccum purpurascens* Link. significantly reduced white mold incidence on snap bean. Similarly, foliar application of *Coniothyrium minitans* Campbell conidial suspension (10^6 conidia per ml) significantly reduced the viability of sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary produced on diseased bean plants (Trutmann *et al.*, 1991).

Gliocladium virens and *T. longibrachiatum* applied to soil as wheat bran dust preparation along with organic amendments survived well in soil and reduced the population of rice sheath blight pathogen (Baby and Manibhushanarao, 1993). Application of spore suspensions of *A. terreus*

Thom. reduced infection by sheath blight pathogen, *R. solani* in rice, particularly when plants were treated before inoculation with sclerotia of the pathogen (Gogoi and Roy, 1993). Sixty four per cent reduction in incidence of bacterial leaf blight of rice was obtained by foliar application of *Aspergillus* sp. at the rate of 10^6 spores per ml (Saikia and Chowdhury, 1993). Tosi and Zazzerini (1994) suggested that there was an increased antagonistic effect when fungal isolates were added to soil as air dried inoculum rather than as seed treatment in rust infested safflower seeds. Jackson *et al.* (1994) reported that spore suspensions of *Penicillium chrysogenum* Thom. isolates significantly reduced the development of chocolate spot lesions of *Botrytis fabae* Delacr. on faba beans. Muskmelon seedlings raised from soils treated with Kalisena SL, a formulation of *A. niger* AN 27, showed fifty six per cent resistance to *Fusarium oxysporum melonis* without the physical presence of *A. niger* in the root zone (Angappan *et al.*, 1996). Krishnamurthy and Gnanamanickam (1998) obtained 60 per cent suppression of rice blast, when *Pseudomonas fluorescens* strain Pf 7-14 was applied as foliar sprays in seed bed and field experiments. Five isolates of *Cladorrhinum foecundissimum* Sacc. and Marchal added to soilless mix as ten days old fresh bran preparations significantly reduced damping off of egg plant and pepper caused by *R. solani* (Lewis and Larkin, 1998).

Harman (2000) demonstrated that foliar spray of *T. harzianum* strain T-22 controlled powdery mildews on Catharanthus and pumpkins and *Botrytis cinerea* Pers. on strawberry and grapes. Huang *et al.* (2000) demonstrated that foliar application of spore suspension of *Coniothyrium minitans* at the rate of 30 x 10^{11} spores per hectare reduced the incidence of white mold of

dry bean caused by *Sclerotinia sclerotiorum* by fifty six per cent. Sen (2000) obtained 80 per cent control of *Fusarium* wilt of musk melon by treating the seeds with Kalisena SD at the rate of eight gram per kilogram and soil with Kalisena SL at the rate of thirty gram per pit. Susheelabhai *et al.* (2000) showed that soil application of *T. harzianum* in carrier media at the rate of one kg per plant at 23 x 10^8 c.f.u. per gram twice a year was effective in reducing the soil population level of *Phytophthora* species as well as reducing the incidence of capsule rot upto eighty three per cent in cardamom.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Survey on the incidence of anthracnose

Survey was conducted in the pepper plantations of Southern and Central parts of Kerala during February – July 2000 to study the occurrence of fungal pollu. Diseased plant samples viz., leaves and spikes were collected at fortnightly intervals from the black pepper plantations of the following districts viz., Thiruvananthapuram (Amboori, Balaramapuram, Vellayani, and Peringamala), Kollam (Thenmala and Kulathupuzha), Alappuzha (Kayamkulam), Ernakulam, Kozhikode, Thrissur and Malappuram (Perinthalmanna). Ten random samples were collected from three plantations from each location. Isolation of the pathogen was done following standard procedures of tissue isolation on potato dextrose agar medium. The isolates were numbered from C1 to C11 and the pathogen was identified based on the cultural and morphological characteristics.

3.2 Isolation of the pathogen

Colletotrichum gloeosporioides, causing anthracnose (fungal pollu) of black pepper was isolated from the naturally infected black pepper leaves and spikes. For isolation of the pathogen, portions of the leaf and spikes showing fresh typical symptoms were used. The materials were cut into small pieces, surface sterilized with 0.1 per cent mercuric chloride solution and repeatedly washed in three changes of distilled water. They were then transferred to potato dextrose agar medium (PDA) in sterile petridishes and incubated at room temperature (28 ± 5^0C) for 48 hrs. On the second day, the visible fungal

growth from the infected tissues were purified by the hyphal tip method and transferred to PDA slants. The isolates were maintained by subculturing at periodical intervals and stored under refrigeration.

3.3 Pathogenicity test

The pathogenicity of the isolates was proved following Koch's postulates. Two months old black pepper cuttings raised in poly bags were used for the study. The leaves of these cuttings were inoculated on both surfaces with seven day old culture of the pathogen containing mycelium and conidia. Humidity was provided by placing a thin layer of moist cotton over it. The fungus was then re-isolated from the leaf portions exhibiting typical disease symptoms and characters studied.

3.4 Cultural and morphological variability in *C. gloeosporioides*

Ten days old cultures of each isolate was used for the study. Cultural and morphological characteristics viz., colony diameter, type of growth, colour, sporulation and conidial morphology were studied.

3.4.1 Colony morphology

Three PDA plates for each isolate were inoculated by placing uniform inoculum disc of five mm diameter in the centre of a plate and incubated at $28 \pm 5\ ^{0}C$. Colony colour and sporulation density were measured 15 days after incubation. Mycelial dry weight of each isolate was observed by culturing the fungus on potato dextrose broth. Fifty ml of broth was poured in 250 ml conical flasks and sterilized by autoclaving at 1.04 kg cm^{-2} for 20 minutes. Five millimeter culture discs of each isolate were inoculated in the broth and the flasks were incubated at room temperature for 10 days. Mycelium was

filtered on Whatman filter paper No.1 and dried in the oven at 60^0C and weighed the next day onwards till constant weights were obtained.

3.4.2 Morphological characteristics of *C. gloeosporioides* isolates

Conidial size was measured using ocular micrometer after calibrating the microscope. The average conidial size of each isolate was measured.

3.5 Pathogenic variability of *C. gloeosporioides*

Young, healthy leaves of black pepper variety Karimunda collected from the Instructional Farm, College of Agriculture, Vellayani were used for the experiment. The eleven isolates of *C. gloeosporioides* were grown on PDA for seven days by incubating at room temperature. The black pepper leaves were inoculated on both surfaces with the isolates of the pathogen using agar discs with mycelia and conidia as inoculum and incubated at room temperature after providing adequate moisture and humidity. Observations were taken on the lesion development in all the leaves at fourth, sixth and eighth day after inoculation. Based on the nature and period of symptom development, the isolates were classified into three groups viz., highly virulent, semivirulent and mildly virulent.

3.6 Survival of *C. gloeosporioides* in infected black pepper leaves

The method described by Yoshida and Shirata (1999) was used for this study with slight modifications.

3.6.1 Survival of *C. gloeosporioides* on infected leaves in soil

Diseased black pepper leaves of variety Karimunda collected from black pepper plantations of the Instructional Farm, College of Agriculture, Vellayani were used for the study. The collected leaves were washed

thoroughly in running water and were cut into small bits. Earthern pots were filled with non-sterilized soil from black pepper plantations. Approximately 2 kg soil was taken in each pot. 250 g of the leaf bits were placed in each of these pots at a depth of 30 cm from the surface and covered with soil. The pots were then placed outdoors and watered on alternate days. The experiment was replicated thrice.

The leaves buried in the soil were collected from each pot at an interval of 30 days for a period of 180 days. The bits were plated on PDA as per the standard technique. Survival of the pathogen was determined by the development of viable fungal colonies on PDA medium after incubation at room temperature.

3.6.2 Survival of *C. gloeosporioides* on black pepper leaves under laboratory conditions

Naturally infected black pepper leaves collected form the Instructional Farm, College of Agriculture, Vellayani were used for this experiment. These were thoroughly washed in running water and air dried for 24 hours. The air dried leaves were placed in brown paper covers of size 32 x 26 cm, sealed tightly and kept under laboratory conditions at room temperature. Samples were taken from these covers at 30 days interval for a period of 180 days. The survival of the pathogen was determined as described under 3.5.1.

3.7 Isolation of mycoflora from the black pepper phyllosphere

Dilution plate technique (Timonin, 1940) was done for the isolation of mycoflora from the black pepper leaf surface. Disease free leaf samples collected from disease free vines and from infected gardens were used for the

isolation procedure. Disease free vines were selected from among the infected vines in the garden. Leaf bits were cut using a sterilized blade. One gram of the leaf bits were transferred to 100 ml of sterile distilled water in a 250 ml conical flask and shaken for 20 minutes in a rotary shaker. From this, the final dilution of 10^4 was prepared. One ml of this solution was plated on Martin's Rose Bengal agar medium for isolating fungi in the phyllosphere. The plants were then incubated at room temperature for 48-72 hrs. After the incubation period, the fungal colonies were examined and transferred to PDA plates and subsequently purified by the hyphal tip culture method. The purified cultures were then stored under refrigerated conditions for identification studies and subsequent antagonism studies.

3.8 Isolation of mycoflora from the black pepper rhizosphere

Soil samples were collected from the rhizosphere of black pepper vines from disease free areas. The rhizosphere mycoflora were obtained by dilution plate technique and the pure cultures of the fungi were maintained for identification studies and antagonistic studies.

3.9 *In vitro* screening of the antagonists against *C. gloeosporioides*

The fungal antagonists were tested for their antagonistic effect against *C. gloeosporioides* by the dual culture technique (Dickinson and Skidmore, 1976). Agar discs of 5 mm diameter cut from the edge of vigorously growing seven day old colonies of *C. gloeosporioides* and the antagonistic fungi were placed 3 cm apart on PDA in a petridish of 90 mm diameter and incubated at room temperature (28 ± 5^0C) for five days. Three replications were maintained for each treatment. The nature of parasitism of the antagonists on

the pathogen was studied at regular intervals. Petridishes inoculated with five millimeter agar discs of *C. gloeosporioides* served as the control.

The percentage inhibition of mycelial growth was calculated using the formula,

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

I = Inhibition of mycelial growth

C = Growth of the pathogen in control plates (cm)

T = Growth of the pathogen in dual culture (cm)

(Vincent, 1927).

3.9.1 Mycoparasitism of the selected fungal antagonists of *Colletotrichum gloeosporioides*

The mechanism of mycoparasitism of the fungal antagonists on *C. gloeosporioides* was studied using the dual culture technique (Dennis and Webster, 1971) was used. Melted PDA was poured in 90 mm sterile petridishes and allowed to solidify. Sterilized cellophane discs of 90 mm diameter were placed over this so as to lie flat on the medium, using a pair of sterile forceps. An agar disc of 5 mm diameter containing the mycelium cut from the margin of an actively growing culture of *C. gloeosporioides* was placed at one end of the petridish and a 5 mm agar disc of the test fungus was placed 3 cm away from it. The plates were incubated at room temperature (28 ± 5^{0}C) for 48 hours. Direct observations were carried out after incubation period under a light microscope. Microscopic observations for hyphal interactions were made by cutting out one sq. cm portions of cellophane

containing intermingling hyphal growth and mounting in cotton blue lactophenol.

3.9.2 Identification and characterization of the antagonists showing maximum inhibition of *C. gloeosporioides*

The selected fungal antagonists were identified by the slide culture technique (Riddel, 1974). Plain agar was melted and poured into sterile petridishes to a thickness of 2 mm and after solidification, blocks of 6 mm were cut out using a sterile needle. One such block was placed at the centre of a sterile microscopic slide and all the four sides of the agar block were inoculated with small bits of the fungal culture. A coverslip was placed on top of the agar block and the slide was kept in a moist chamber and incubated for 48 hours at room temperature (28 ± 5^0C). The coverslip was then gently lifted, a drop of 15 per cent alcohol was placed at the centre, and before drying, the coverslip was mounted on lactophenol cotton blue. The cultures were then examined and identified.

The fungal antagonists were grown on PDA in petridishes and incubated at room temperature for 10 days. Observations were made on colony diameter, colony colour and pigmentation. The fungal cultures were identified at The Agharkar Research Institute, MACS, Pune, Maharashtra.

3.10 Effect of carrier material on multiplication, viability and shelf-life of the fungal antagonists

Five carrier materials were tested. They were :

1) Rice bran

2) Neem cake

3) Talc

4) Vermiculite

5) Biomanure

The biomanure used in the experiment was the compost prepared by Poabs Green Pvt. Ltd., Thiruvananthapuram, from city waste. Twenty five grams each of the above materials were added to 250 ml conical flasks. Ten ml of sterile water was added to each and mixed well. This mixture was sterilized by autoclaving at 1.04 kg cm^{-2} for 20 min. To each of these flasks, 5 mm agar discs containing the mycelia of the fungal antagonists were added aseptically. The flasks were then incubated at room temperature (28 ± 5^0C) for seven days.

To study the effect of carrier material on the multiplication of the fungal antagonists, one gram each of the carrier material containing the fungal growth was taken in a test tube and shaken well with 10 ml sterile water to release all the spores into suspension. The spore count in the suspension was determined using a hemocytometer. To study the effect of carrier material on the viability of the fungal antagonists, the germination of the spores was observed after 24 hours.

3.11 Preparation of talc based formulation of the fungal antagonists

The fungal isolates producing maximum inhibition of the mycelial growth of *C. gloeosporioides* were further mass multiplied and formulated. The fermentation biomass of the selected fungal antagonists were prepared by a slightly modified liquid fermentation process of Papavizas *et al.* (1984).

Stock cultures of the fungal antagonists were maintained on PDA. Discs of 5 mm diameter were inoculated into 500 ml of potato dextrose broth in one litre flasks allowing it to float on the broth. This was incubated at room temperature for 15 days to obtain sufficient propagules in the mycelial mat. The mat was carefully removed and pressed in filter paper to remove the water content and then air dried. This mat was then sun dried for one hour and then powdered in a mixer grinder. The resulting powder was mixed with sterilized talc at the rate of 25 per cent w/w. One per cent carboxy methyl cellulose (CMC) was added to this and stored at room temperature.

3.11.1 Shelf-life of the talc based formulations

Hundred milligram of the talc formulation was mixed thoroughly with 100 ml sterile water by shaking in a rotary shaker for 10 min. One ml of this suspension was pipetted out using a sterile pipette and transferred to a conical flask containing 99 ml sterile water. From this, 1 ml was transferred to 9 ml of sterile water in a test tube and the process was repeated till a dilution of 10^6 was obtained. One ml of this was pipetted aseptically into a sterile petriplate and mixed with 15 ml of Martin's Rose Bengal agar medium. The petridishes were shaken by swirling motion of the hand and incubated at room temperature. The colonies were counted on the fourth day and similar observations were taken at periodic intervals and the number of colony forming units (c.f.u) of the antagonistic fungi per gram of the formulation was calculated.

3.12 Preparation of pathogen inoculum for field application

250 g of rice bran was taken in one litre conical flasks and 10 ml of sterile water was added to this and mixed well. This was sterilized by

autoclaving at 1.04 kg per cm^{-2} for 20 minutes. Agar disc of 5 mm diameter cut from the edge of vigorously growing culture of *C. gloeosporioides* was aseptically transferred into the flasks and incubated at room temperature for seven days. This was used as pathogen inoculum in the field trial.

3.13 Efficacy of the talc-based formulations in the field

Efficacy of talc based formulation of the selected fungal antagonists was tested by using two methods of application viz., soil application and foliar application.

An experiment was laid out at the District Agricultural Farm, Peringamala for this study. Three months old black pepper cuttings showing uniform infection of anthracnose were used for the study. Three cuttings per poly bag containing approximately 2 kg of potting mixture was used. All these cuttings were treated with the pathogen inoculum as soil application and also as foliar spray prior to application of the antagonists. For soil application, bran based culture of *C. gloeosporioides* was applied at the rate of 10 g per poly bag. Foliar spraying was done using conidial suspension of *C. gloeosporioides* containing 10^6 conidia per ml. The black pepper cuttings were sprayed with *C. gloeosporioides* to get maximum infection. The treated cuttings were kept under greenhouse conditions.

3.13.1 Soil application of the antagonists

Talc based formulation of the fungal antagonists was applied to the soil at the base of the black pepper cuttings twice at 15 days interval starting from 10 days after application of pathogen inoculum. The antagonist inoculum was applied at the rate of 10 g per kg soil after raking the soil without disturbing the roots of the

cuttings. This was then mixed properly with soil. The application was done twice at 15 days interval.

3.13.2 Foliar application of the fungal antagonists

Foliar application of the antagonists was done on the same day as that of soil application. For this one per cent aqueous suspension of the formulated product was prepared and sprayed using a hand sprayer. The disease intensity was recorded at periodic intervals. Disease intensity was calculated using a 0 – 7 score chart (Plate 1) where,

0 – No infection

1 – Lesions covering upto 25 per cent leaf area

3 – Lesions covering 26 – 50 per cent leaf area

5 – Lesions covering 51 – 75 per cent leaf area

7 – Lesions covering >75 per cent leaf area

Percentage disease index was calculated using the formula of Mayee and Datar (1986),

$$\text{PDI} = \frac{\text{Sum of grades of each leaf}}{\text{Number of leaves assessed}} \times \frac{100}{\text{Maximum grade used}}$$

3.14 Effect of antagonist formulations on growth parameters of black pepper

After 60 days, the plants were uprooted and observations were made on the following.

i) Number of leaves

ii) Shoot length

Plate 1 Score chart (0 – 7) for anthracnose for black pepper

Plate : 1

The length of the shoot from soil level to tip of each plant was measured in cm.

ii) Root length

After carefully depotting the cuttings from the poly bags, the roots were washed gently in tap water to remove all adhering soil particles and blot dried. The length of the roots were then measured in cm.

iv) Fresh weight

The fresh weight (g) of the plants were taken in a Mettler Single Pan Balance.

v) Dry weight

The plants were dried in the sun to a constant weight for determining the dry weight (g).

Results

4. RESULTS

4.1 Survey on the incidence of black pepper anthracnose

Infected black pepper leaves and spikes were collected from the various black pepper plantations for a period of six months starting from February 2000. The locations surveyed and the pathogens isolated are presented in Table 1 and Fig 1. The isolation studies revealed that all the infected samples were associated with *Colletotrichum gloeosporioides.* Ninety per cent of the leaf and berry samples showed typical anthracnose symptoms. In certain cases association of *C. capsici* was also observed. The other pathogens observed were *Phytophthora capsici, Botryodiplodia* sp. and *Pestalotia* sp. Mixed infection of *C. gloeosporioides* and *C. capsici* were also found in some of the plantations. From the survey, it was confirmed that *C. gloeosporioides* was the most common pathogen associated with black pepper during the investigation.

4.1.1 Symptomatology of the disease

Anthracnose is observed both in the nursery as well as in the main field. Foliar infection caused by *Colletotrichum* spp. on black pepper varied from minute brown or black specks to large blighted areas, resulting in severe defoliation (Plate 2A). Pin head like acervuli of the fungus were also seen on the blighted areas. The blighted areas had papery white or grayish white centres, which break resulting in shot-hole symptoms (Plate 2B). The blighting of foliage is observed both in the nursery as well as in the main

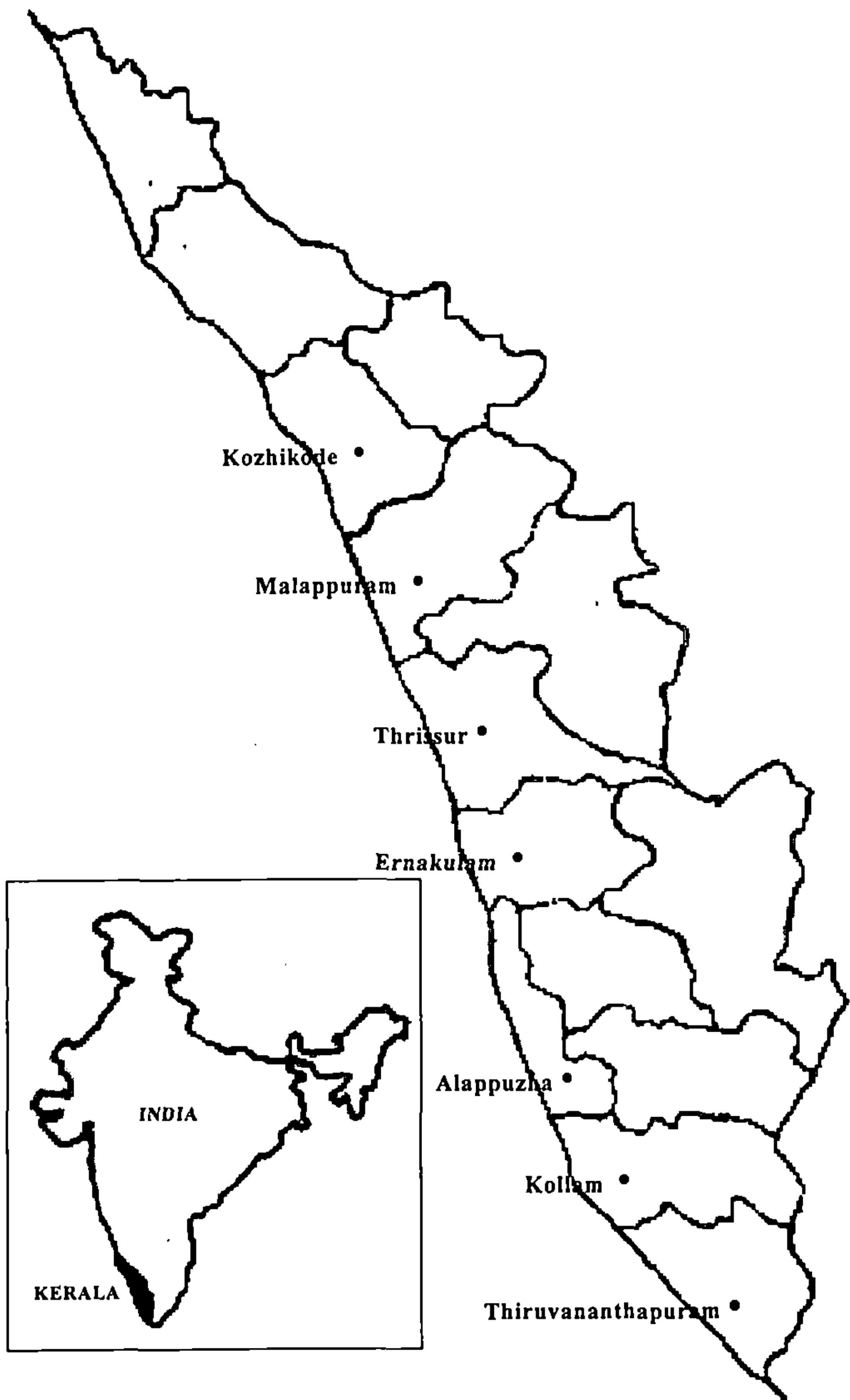


Fig. 1 Map showing the locale of the survey on anthracnose of black pepper

Table 1 Survey on the incidence of anthracnose of black pepper

Location	Pathogens isolated
Thiruvananthapuram	
Amboori	*Colletotrichum gloeosporioides*
Balaramapuram	*Colletotrichum gloeosporioides*, *Phytophthora capsici*
Peringamala	*Colletotrichum gloeosporioides*, *Pestalotia* sp., *Phytophthora capsici*
Vellayani	*Colletotrichum gloeosporioides*, *Pestalotia* sp.
Kollam	
Kulathupuzha	*Colletotrichum gloeosporioides*, *Botryodiplodia* sp., *Colletotrichum capsici*
Thenmala	*Colletotrichum gloeosporioides*, *Phytophthora capsici*
Alappuzha	
Kayamkulam	*Colletotrichum gloeosporioides*, *Botryodiplodia* sp.
Ernakulam	*Colletotrichum gloeosporioides*, *Colletotrichum capsici*
Thrissur	*Colletotrichum gloeosporioides*, *Colletotrichum capsici*
Kozhikode	*Colletotrichum gloeosporioides*, *Colletotrichum capsici*
Malappuram	
Perinthalmanna	*Colletotrichum gloeosporioides*

Plate 2A Defoliation and spike shedding due to anthracnose infection

2B Blighting on leaves as a result of infection by *C. gloeosporioides*

2C Damage on spikes

2D Anthracnose infection on bush pepper

Plate : 2A

Plate : 2B

Plate : 2C

Plate : 2D

field. Many of the affected black pepper cuttings showed severe defoliation leaving only the bare stems.

The pathogen also affects the spikes and berries. On the berries, sunken areas were observed which later resulted in hollow shrivelled berries . Spike shedding and berry shedding were also observed. Damage on spikes resulted in 100 per cent yield loss (Plate 2C). Early infection of the spikes resulted in spike shedding. Early infections resulted in defoliation and blackening of stem which later dries up. Anthracnose is also observed in bush pepper (Plate 2D). The foliar symptoms are typical as those seen in black pepper cuttings as well as in the plantations.

4.2 Isolation of the pathogen

The black pepper anthracnose pathogen was isolated and purified from naturally infected black pepper leaves and berries by following standard procedure. The pathogen associated with anthracnose was identified as *Colletotrichum gloeosporioides* by studying the cultural and conidial morphology.

4.3 Pathogenicity test

Pathogenicity was proved following Koch's postulates on young leaves of the black pepper variety, Karimunda. The symptoms were reproduced which were typical as that of anthracnose of black pepper.

4.4 Cultural and morphological variability of *C. gloeosporioides*

The cultural characteristics of eleven isolates of the pathogen were studied on PDA (Table 2 and Plate 3). Colony growth of C_2, C_{10} and C_{11} was felty, whereas it was cottony in isolate C_1. Isolates C_7 and C_9 exhibited restricted growth, whereas isolates C_4 and C_5 showed appressed growth. In

Table 2 Cultural characteristics of different isolates of *Colletotrichum gloeosporioides*

Isolate	Type of growth	Colony colour	Spore mass colour	Colony *+ diameter (cm)	Mycelium *+ dry weight (mg)
C_1 Amboori	Cottony	Uniformly dark grey	Salmon pink	8.47	295.10
C_2 Balaramapuram	Even felty	Dark grey centre with black margins	Dark pink	8.57	284.06
C_3 Ernakulam	Fluffy centre with appressed margins	Cottony white centre with dark grey margins	Dark pink	7.83	259.47
C_4 Kayamkulam	Appressed	Light grey centre with black margins	Pale salmon pink	8.80	279.45
C_5 Kozhikode	Appressed	Uniformly dark grey	Salmon pink	7.73	239.46
C_6 Perinthalmanna	Fluffy, in tufts	Unevenly grey with black margins	Salmon pink	8.50	306.89
C_7 Kulathupuzha	Irregular, restricted	Dark grey with black margins	Dark pink	7.53	178.75
C_8 Peringamala	Fluffy centre with appressed margins	Greyish white centre with dark grey margins	Dark pink	8.20	227.74
C_9 Thrissur	Restricted with appressed margins	Blackish margins with light grey centre	Pale pink	8.63	268.94
C_{10} Thenmala	Felty	Uniformly dark grey	Salmon pink	8.30	292.95
C_{11} Vellayani	Even felty	Greyish white centre with dark grey margins	Salmon pink	8.90	308.79
CD (0.05)				0.45	9.57

* Mean of three replications
+ Observations on eighth day of incubation

Plate 3 **Cultural characteristics of *C. gloeosporioides* isolates of black pepper**

Plate 4A **Symptoms produced by highly virulent isolates of *C. gloeosporioides* on excised leaves**

4B **Symptoms produced by semi virulent isolates of *C. gloeosporioides* on excised leaves**

4C **Symptoms produced by mildly virulent isolates of *C. gloeosporioides* on excised leaves**

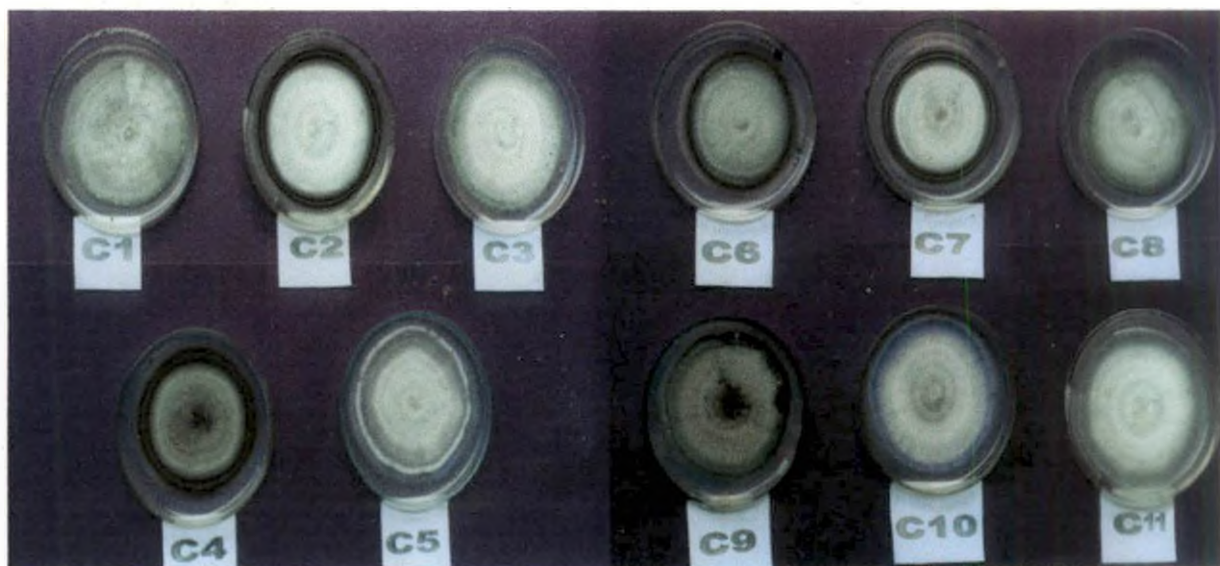


Plate : 3

Plate : 4A

Plate : 4B

Plate : 4C

isolate C_6, growth was fluffy and in tufts, whereas in isolates C_3 and C_8, growth was fluffy in the centre with appressed margins.

The colony colour of the isolates varied from grey to dark grey in the centre, but varied towards the margins, except for isolates C_1, C_5 and C_{10} which were uniformly dark grey. In isolates C_2, C_4, C_6, C_7 and C_9, the margins were black in colour, whereas isolates C_3, C_8 and C_{11} had dark grey margins. C_1, C_5 and C_{10} isolates were uniformly dark grey in colour.

The colour of the spore mass of isolates C_1, C_4, C_5, C_6, C_{10} and C_{11} was salmon pink whereas it was dark pink in the remaining isolates except in C_9 where it was pale orangish pink (Table 2).

Maximum radial growth was of isolate C_{11} which had a colony diameter of 8.9 cm which was statistically on par with isolates C_1, C_2, C_4, C_6 and C_9. Least radial growth of 7.53 cm was of isolate C_7 which was statistically on par with C_3 and C_5 having colony diameters of 7.83 cm and 7.73 cm respectively. The remaining isolates, C_8 and C_{10} had colony diameters of 8.2 and 8.3 cm respectively.

Highest mycelial dry weight was of isolate C_{11} (308.79 mg) which was on par with isolate C_6 (306.89 mg). Least mycelial dry weight was of isolate C_7 (178.75 mg) which was significantly different from all the other isolates (Table 2).

Average length and width of conidia ranged from 13.10 to 16.85 μm and 3.82 to 5.43 μm respectively (Table 3). Isolate C_{10} possessed the largest conidia measuring 16.85 x 5.43 μm. Conidial length of C_{10} was on par with isolate C_3. Shortest conidial length of 13.10 μm was seen in isolate C_6 which

Table 3 Conidial size of different isolates of *Colletotrichum gloeosporioides*

Isolate	Spore size*	
	Mean length (μm)	Mean width (μm)
C_1	14.71	3.86
C_2	14.38	4.76
C_3	16.59	4.34
C_4	16.14	4.76
C_5	13.97	5.17
C_6	13.10	5.20
C_7	15.43	4.49
C_8	15.36	3.82
C_9	15.45	4.20
C_{10}	16.85	5.43
C_{11}	13.83	4.03
CD (0.05)	0.498	0.302

* Mean of three replications with 30 spores per replication

was significantly different from all the other isolates. Broadest conidia was that of isolate C_{10} which was on par with isolate C_5. Smallest conidial width of 3.82 µm was seen in isolate C_8 which was on par with isolates C_1 and C_{11}.

4.5 Pathogenic variability of *C. gloeosporioides*

Based on the time taken for symptom development and nature and spread of the symptoms, the pathogen isolates were grouped into three categories *viz.*, highly virulent, semivirulent and mildly virulent (Table 4 and Plates 4A, 4B & 4C). Isolates C_1, C_4, C_6, C_7 and C_9 were classified as highly virulent types. Isolates C_2, C_5 and C_{10} were the semivirulent types, while the rest *viz.*, isolates C_3, C_8 and C_{11} were classified as mildly virulent types. The most virulent isolates were found to be C_6 and C_1.

4.6 Survival of *C. gloeosporioides*

Studies on the survival of *C. gloeosporioides* causing anthracnose of black pepper showed that the fungus survived for 90 days in infected black pepper leaves in the soil (Table 5 and Fig. 2). Under laboratory conditions, the pathogen survived upto 150 days in the infected black pepper leaves (Table 5 and Fig. 2).

4.7 Isolation of mycoflora from black pepper phyllosphere

The phylloplane mycoflora were isolated by employing the dilution plate technique from all the eleven locations and maintained on PDA slants. The commonly obtained fungi from the phyllosphere was *Aspergillus* spp. This fungus was obtained from nine out of eleven locations. *Penicillium* spp. was obtained from eight out of the eleven locations surveyed. *Fusarium* spp.,

Table 4 Pathogenic variability among isolates of *Colletotrichum gloeosporioides*

Isolate	Nature of symptoms			Lesion * diameter (cm) (8 DAI)	Type
	Days after inoculation				
	4	6	8		
C_1	Dark lesions	Lesions enlarged	Shot holes produced	1.10	Highly virulent
C_2	Dark spots	Slightly spreading	Restricted	0.40	Semivirulent
C_3	Minute spots	Not spreading	Not spreading	0.10	Mildly virulent
C_4	Dark lesions	Enlarged	Large lesions	0.66	Highly virulent
C_5	Small dark spots	Spreading	Spreading	0.40	Semi virulent
C_6	Dark lesions	Lesions spreading	Large spots with grey centre	1.16	Highly virulent
C_7	Dark lesions	Spreading	Large lesions	0.93	Highly virulent
C_8	Minute spots	Not spreading	Not spreading	0.10	Mildly virulent
C_9	Dark lesions	Spreading	Large spots	0.98	Highly virulent
C_{10}	Small dark spots	Enlarged spots	Enlarged spots	0.45	Semi virulent
C_{11}	Minute spots	Not spreading	Not spreading	0.09	Mildly virulent

*Mean of three replications
Lesion size ranging from 0.5 and above – Highly virulent
Lesion size ranging from 0.2 to 0.5 cm – Semivirulent
Lesion size < 0.2 cm – Mildly virulent

DAI – Days after inoculation

Table 5 Survival of *Colletotrichum gloeosporioides* in black pepper leaves in the soil and under laboratory conditions

Days after incubation / burial	Percentage recovery *	
	In the soil	In the lab
0	100.0	100.0
7	75.0	83.3
30	75.0	58.3
60	58.1	50.0
90	33.3	33.3
120	0.0	25.0
150	0.0	8.3
180	0.0	0.0

* Average of three replications

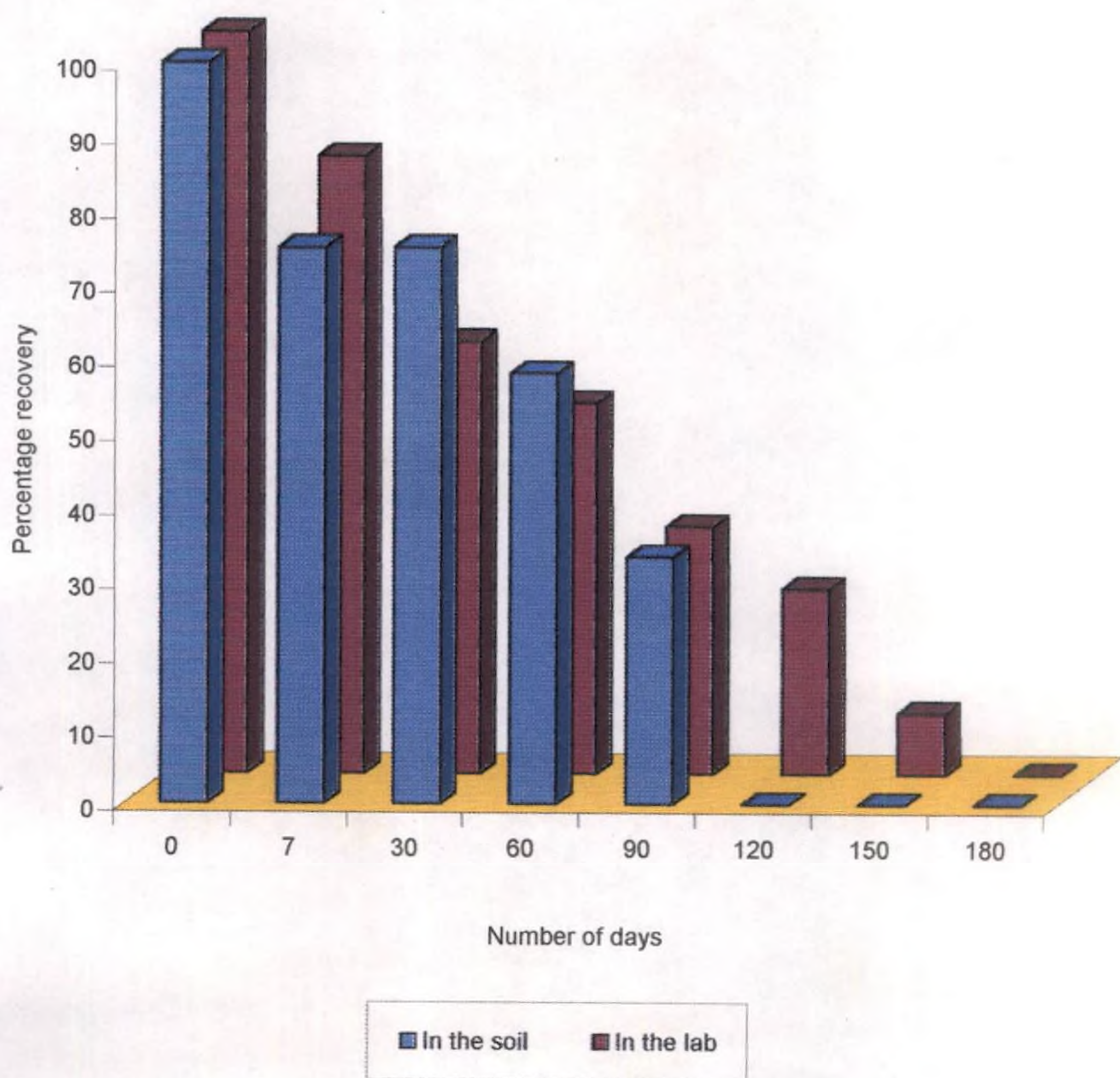


Fig. 2 Survival of C. gloeosporioides in black pepper leaves and berries in the soil and under laboratory conditions

Curvularia spp., *Cylindrocladium* spp., *Botryodiplodia* spp., *Trichoderma* spp., *Rhizopus* sp. and Yeast were also obtained from certain locations (Table 6).

4.8 Isolation of mycoflora from black pepper rhizosphere

The rhizosphere mycoflora were isolated by dilution plate technique from all the eleven locations and maintained on PDA slants (Table 7). The most frequently isolated fungi from the rhizosphere were *Aspergillus* spp., *Penicillium* spp. and *Trichoderma* spp. Other fungi obtained less frequently were *Rhizopus* sp., *Mucor* sp. and *Fusarium* spp.

4.9 *In vitro* screening of fungal antagonists against *C. gloeosporioides*

For dual culture studies, the isolate C_6 was used as the test fungus, since it was highly virulent and fast growing. When the fungi isolated from the rhizosphere and phyllosphere were paired with isolate C_6, some of the fungi showed inhibition of growth at the point of contact with the test organism. Some species of *Aspergillus*, *Penicillium* and *Fusarium* intermingled freely with *C. gloeosporioides* and grew together. The same was noticed in the case of *Botryodiplodia* sp., *Curvularia* sp., *Cylindrocladium* sp., *Mucor* sp. and *Rhizopus* sp. The yeast isolates did not interfere with the growth of the test organism.

The fungi which emerged as potential antagonists of *C. gloeosporioides* caused a clear zone of inhibition between the paired cultures. This included two isolates of *Trichoderma* viz., isolate T_1 from Balaramapuram obtained from the rhizosphere and isolate T_2 from Vellayani obtained from the phyllosphere and two phyllosphere isolates of *Aspergillus* viz., isolate A_1 from Kayamkulam and isolate A_2 from Peringamala. These isolates were

Table 6 Fungi isolated from phylloplane of black pepper

Location	Fungi
Thiruvananthapuram	
Amboori	Yeast, *Penicillium* sp.
Balaramapuram	*Aspergillus* sp., *Fusarium* sp.
Peringamala	*Aspergillus* sp., Yeast, *Botryodiplodia* sp.
Vellayani	*Aspergillus* sp., *Trichoderma* sp., *Rhizopus* sp.
Kollam	
Kulathupuzha	*Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., Yeast
Thenmala	*Aspergillus* sp., *Alternaria* sp., *Penicillium* sp.
Alappuzha	
Kayamkulam	*Aspergillus* sp., *Penicillium* sp.
Ernakulam	*Aspergillus* sp., *Rhizopus* sp., *Curvularia* sp.
Thrissur	*Penicillium* sp., *Aspergillus* sp., *Curvularia* sp.
Kozhikode	*Aspergillus* sp., *Cylindrocladium* sp., *Penicillium* sp.
Malappuram	
Perinthalmanna	*Penicillium* sp., *Botryodiplodia* sp.

Table 7 Fungi isolated from rhizosphere of black pepper

Location	Fungi
Thiruvananthapuram	
Amboori	*Aspergillus* sp., *Penicillium* sp.
Balaramapuram	*Aspergillus* sp., *Trichoderma* sp.
Peringamala	*Aspergillus* sp., *Penicillium* sp., *Mucor* sp.
Vellayani	*Aspergillus* sp., *Fusarium* sp., *Penicillium* sp.
Kollam	
Kulathupuzha	*Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp.
Thenmala	*Penicillium* sp., *Fusarium* sp.
Alappuzha	
Kayamkulam	*Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp.
Ernakulam	*Aspergillus* sp., *Penicillium* sp.
Thrissur	*Aspergillus* sp, *Penicillium* sp.
Kozhikode	*Aspergillus* sp., *Trichoderma* sp., *Rhizopus* sp.
Malappuram	
Perinthalmanna	*Aspergillus* sp., *Penicillium* sp.

selected for further studies. All these isolates completely overgrew and parasitized *C. gloeosporioides* after seven days.

All the four antagonists showed same degree of inhibition of mycelial growth of *C. gloeosporioides* on the third day of incubation (Plates 5A, 5B, 5C & 5D). After seven days, isolates A_1 and A_2 showed maximum inhibition of mycelial growth of *C. gloeosporioides*, whereas isolate T_2 showed the least degree of inhibition and was significantly different from the other three antagonists (Table 8 and Fig. 3).

4.9.1 Identification and characterization of the selected fungal antagonists

On the basis of cultural characters and conidial morphology (Table 9) the four fungal antagonists were tentatively identified as :

T_1 – *T. harzianum* Rifai

T_2 – *T. harzianum* Rifai

A_1 – *A. niger* van Tieghem

A_2 – *A. niger* van Tieghem

The identity of the fungi was confirmed at Agharkar Research Institute, MACS, Pune, India.

4.9.2 Mycoparasitism of the fungal antagonists on *C. gloeosporioides*

All the four fungal antagonists were found to be efficient parasites of *C. gloeosporioides*. The fungi caused excessive granulation, vacuolation and finally disintegration of host hyphae (Plates 6C & 6D). Isolates T_1 and T_2

Table 8 Efficiency of fungal isolates inhibiting *Colletotrichum gloeosporioides in vitro*

Isolate	*Percentage inhibition of mycelial growth *in vitro*		
	3rd day	5th day	7th day
T. harzianum (T_1)	26.72 (5.26)	41.08 (6.48)	49.97 (7.14)
T. harzianum (T_2)	29.42 (5.51)	33.71 (5.89)	43.61 (6.68)
A. niger (A_1)	30.49 (5.61)	51.72 (7.26)	54.08 (7.42)
A. niger (A_2)	27.36 (5.32)	51.25 (7.22)	53.06 (7.35)
CD (0.05)		0.139	0.077

*Average of three replications

Figures in parentheses are $\sqrt{x+1}$ transformed values

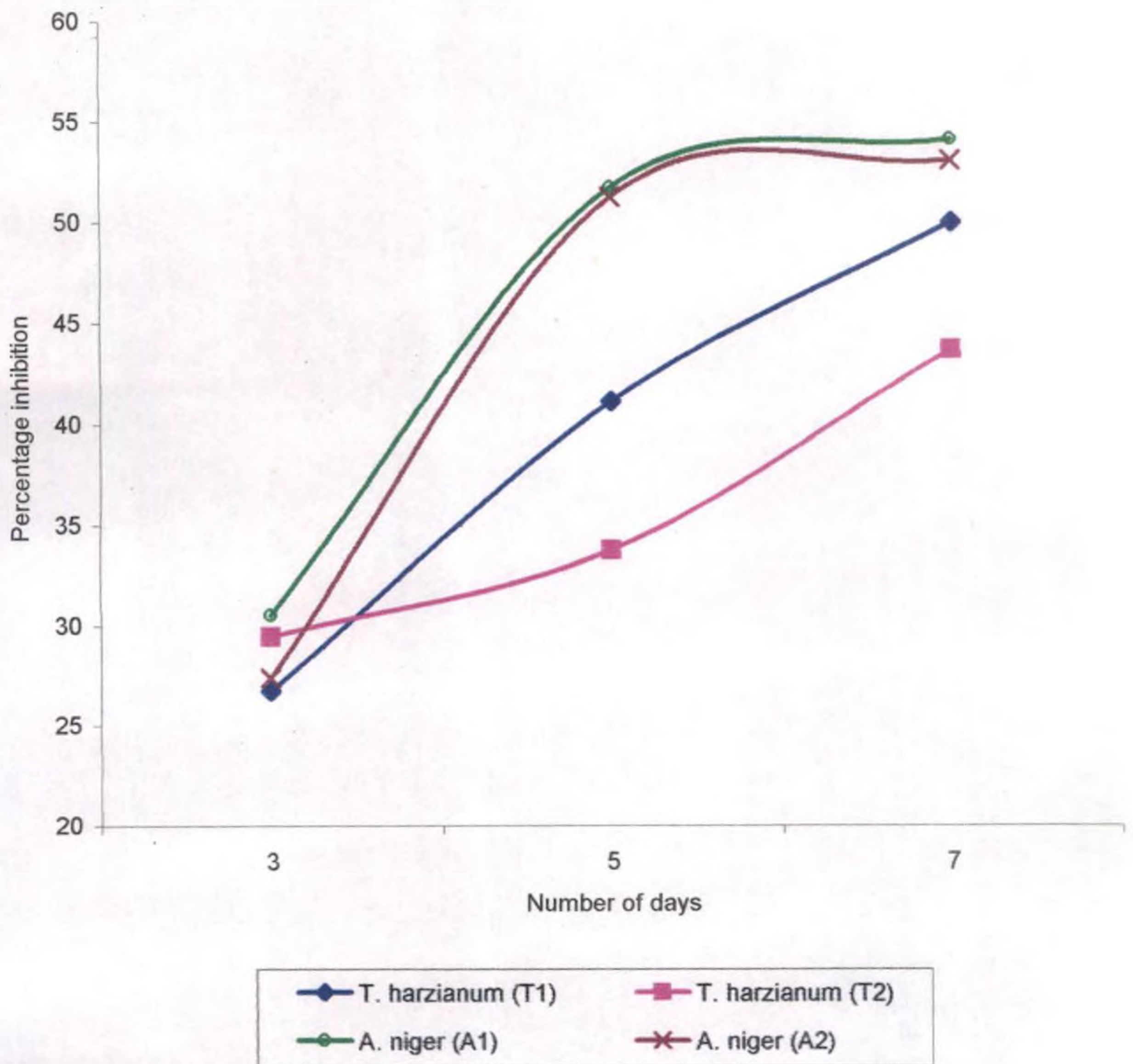


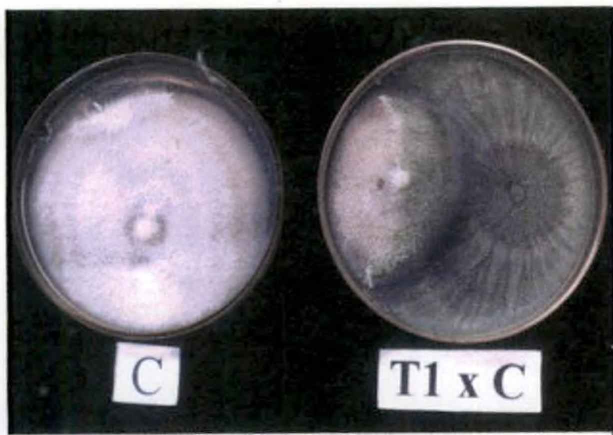
Fig. 3 Efficiency of fungal isolates in inhibiting *C. gloeosporioides in vitro*

Plate 5A Inhibition of *C. gloeosporioides* by *T. harzianum* isolate T_1

5B Inhibition of *C. gloeosporioides* by *T. harzianum* isolate T_2

5C Inhibition of *C. gloeosporioides* by *A. niger* isolate A_1

5D Inhibition of *C. gloeosporioides* by *A. niger* isolate A_2



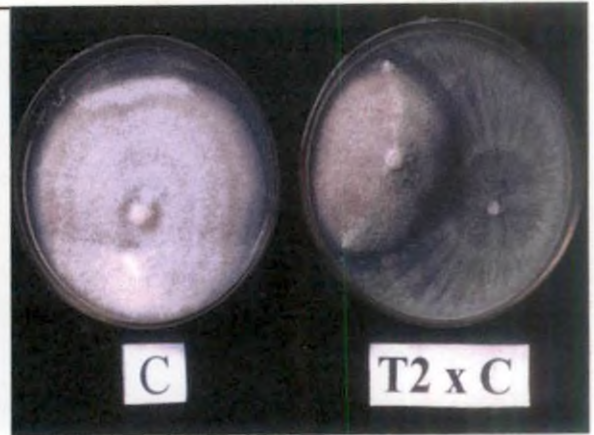
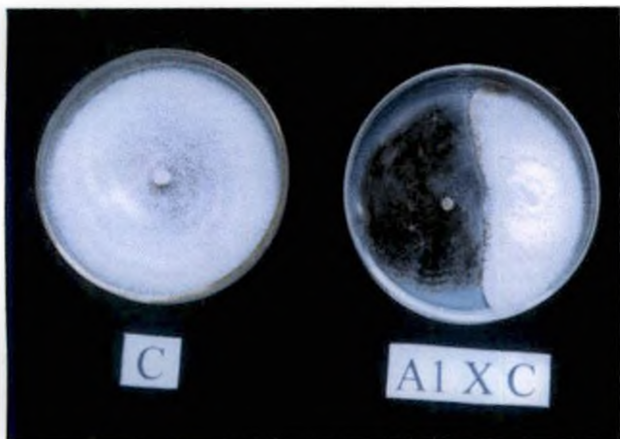


Plate : 5A

Plate : 5B



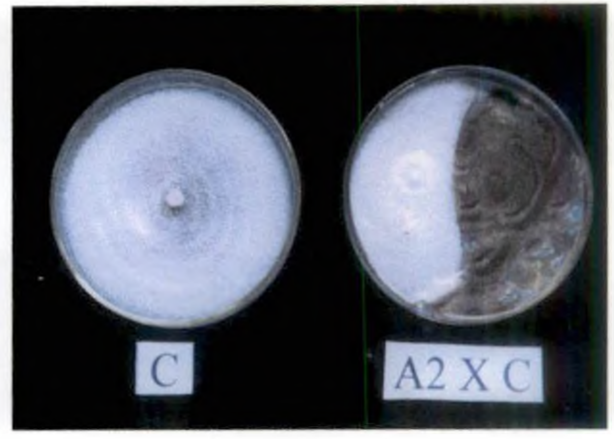


Plate : 5C

Plate : 5D

Plate 6A **Coiling of hyphae of *T. harzianum* isolate T_1 on hyphae of *C. gloeosporioides***

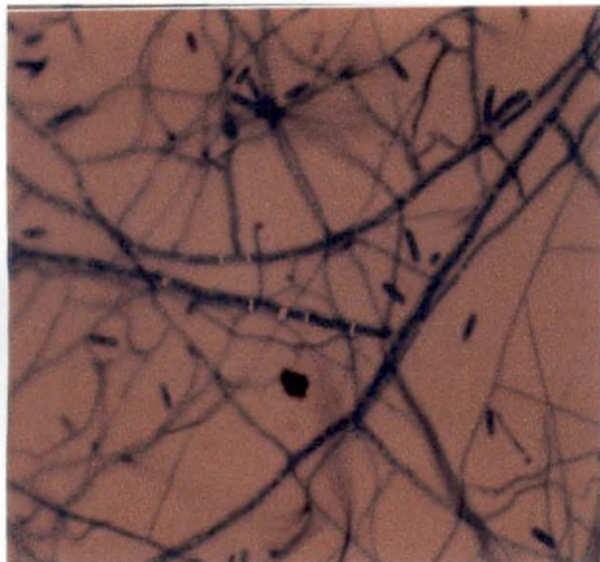
6B **Coiling of hyphae of *T. harzianum* isolate T_2 on hyphae of *C. gloeosporioides***

6C **Vacuolation and granulation of hyphae of *C. gloeosporioides* caused by *A. niger* isolate A_1**

6D **Vacuolation and granulation of hyphae of *C. gloeosporioides* caused by *A. niger* isolate A_2**

Plate : 6A

Plate : 6B



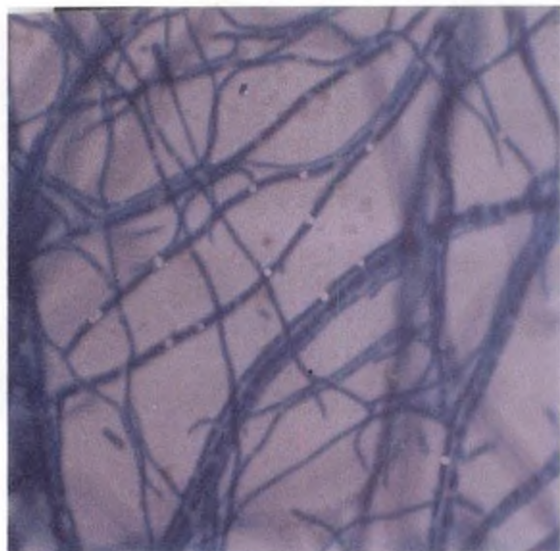


Plate : 6C

Plate : 6D

Table 9 Colony characters of the antagonistic fungi

Fungus	Isolate	Colony colour	Colony diameter (cm)
T. harzianum Rifai	T_1	Dull green	8.63
T. harzianum Rifai	T_2	Dull green	8.96
A. niger van Tieghem	A_1	Black	6.43
A. niger van Tieghem	A_2	Brownish black	7.21

*Average of three replications

Table 10 Effect of carrier materials on multiplication of fungal antagonists

Carrier material	Spore count (10^7 g^{-1}) * +			
	T_1	T_2	A_1	A_2
M_1 (Neem cake)	0.00 (1.00)	0.259 (1.12)	28.14 (5.39)	21.33 (4.73)
M_2 (Biomanure)	0.53 (1.23)	0.00 (1.00)	0.00 (1.00)	5.52 (2.56)
M_3 (Rice bran)	24.09 (5.01)	31.33 (5.69)	47.16 (6.94)	57.46 (7.65)
M_4 (Talc)	12.76 (3.71)	12.53 (3.68)	10.64 (3.41)	13.12 (3.76)
M_5 (Vermiculite)	10.83 (3.44)	0.18 (1.08)	5.09 (2.47)	4.07 (2.25)
CD (0.05)	0.07	0.097	0.197	0.253

* Seven days after inoculation
+ Average of three replications

Figures in parentheses are $\sqrt{x + 1}$ transformed values

were found to coil around and penetrate host hyphae leading to its disintegration and death (Plates 6A & 6B).

4.10 Effects of carrier materials on multiplication and viability of the fungal antagonists

Among the different carrier materials tried, isolate T_1 failed to grow in neem cake and its growth was meagre (0.53×10^7 g^{-1}) in biomanure (Table 10). The best carrier material for T_1 was rice bran (Plate 7A). Biomanure failed to support the growth of isolate T_2 and growth of T_2 was very meagre in neem cake and in vermiculite. As in the case of T_1, the best carrier material for growth of T_2 was rice bran (Plate 7B).

Isolate A_1 failed to grow in biomanure and its best growth was noticed in rice bran (Table 10 and Plate 7C). Unlike A_1, A_2 was found to grow in all the carrier materials, even though its growth was sparse in biomanure and vermiculite. For A_2 also, the best carrier material for growth was rice bran (Plate 7D). Biomanure supported the growth of only isolates T_1 and A_2. Even these isolates grew only very sparsely in this material (Table 10 and Fig. 4).

Neem cake and biomanure were unsuitable for spore germination of all the four fungi tested (Table 11 and Fig. 5). Vermiculite was unsuitable for the spore germination of isolates T_1 and T_2, whereas, spores of A_1 and A_2 germinated in vermiculite. Maximum spore germination for all the four was observed in talc, the highest being 97.28 per cent for A_1. Eventhough growth of the fungi was maximum in rice bran, the germination of the spores was more in talc. Also, the growth rate of all the fungi was more or less the same in talc. Hence, talc was utilised for further studies.

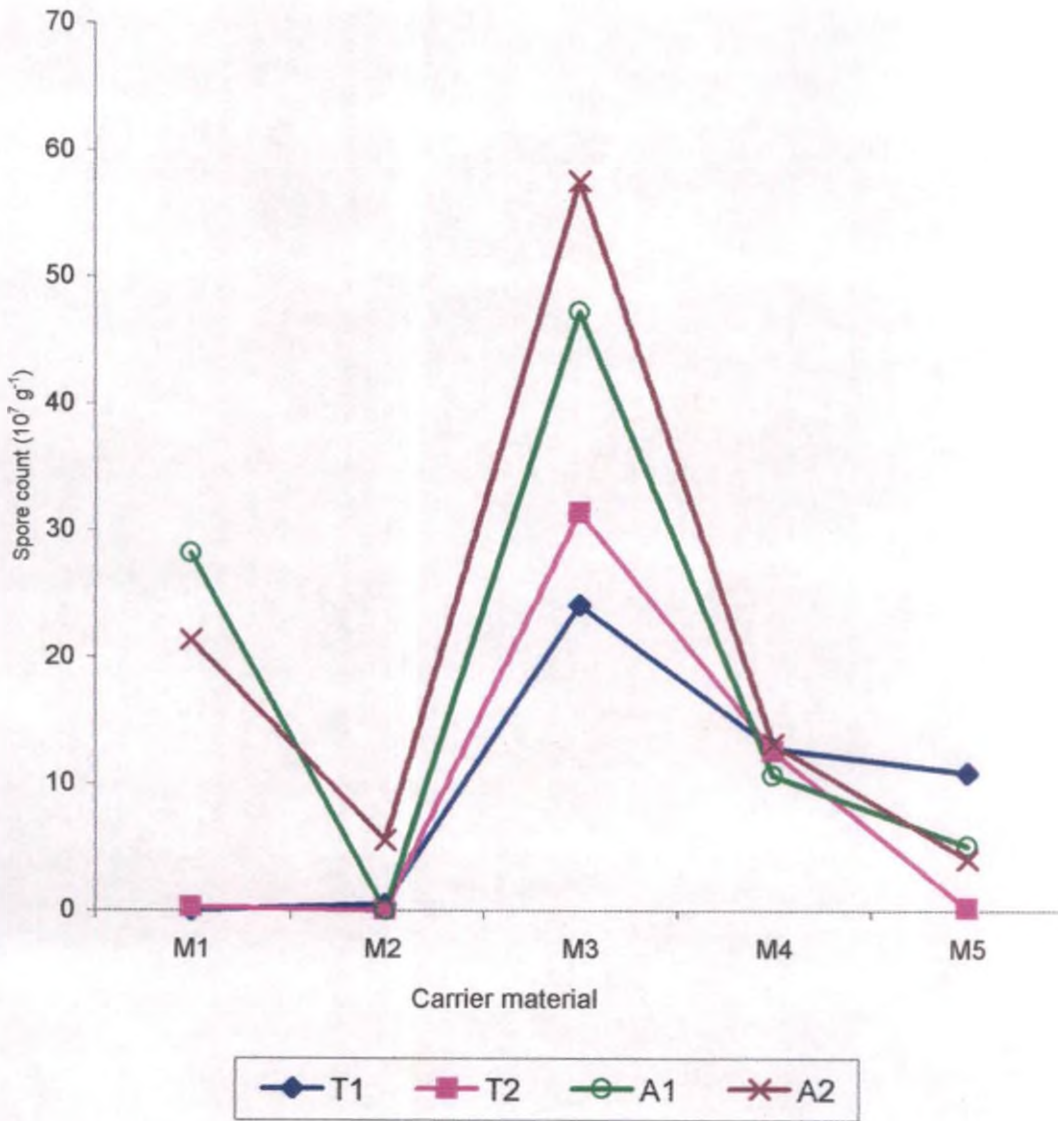


Fig. 4 Effect of carrier materials on multiplication of fungal antagonists

M1 - Neem cake, M2 - Biomanure, M3 - Rice bran, M4 - Talc , M5 - Vermiculite

Table 11 Effect of carrier materials on spore germination of fungal antagonists

Carrier material	Conidial germination (%) * +			
	T_1	T_2	A_1	A_2
M_1 (Neem cake)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
M_2 (Biomanure)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
M_3 (Rice bran)	55.85 (7.54)	43.82 (6.69)	76.50 (8.80)	66.32 (8.21)
M_4 (Talc)	91.76 (9.63)	80.25 (9.01)	97.28 (9.91)	96.34 (9.87)
M_5 (Vermiculite)	0.00 (1.00)	0.00 (1.00)	15.32 (4.05)	11.11 (3.48)
CD (0.05)	0.202	0.552	0.32	0.21

*Observation after 48 hours
+ Mean of three replications (30 spores per replication)

Figures in parentheses are $\sqrt{x+1}$ transformed values

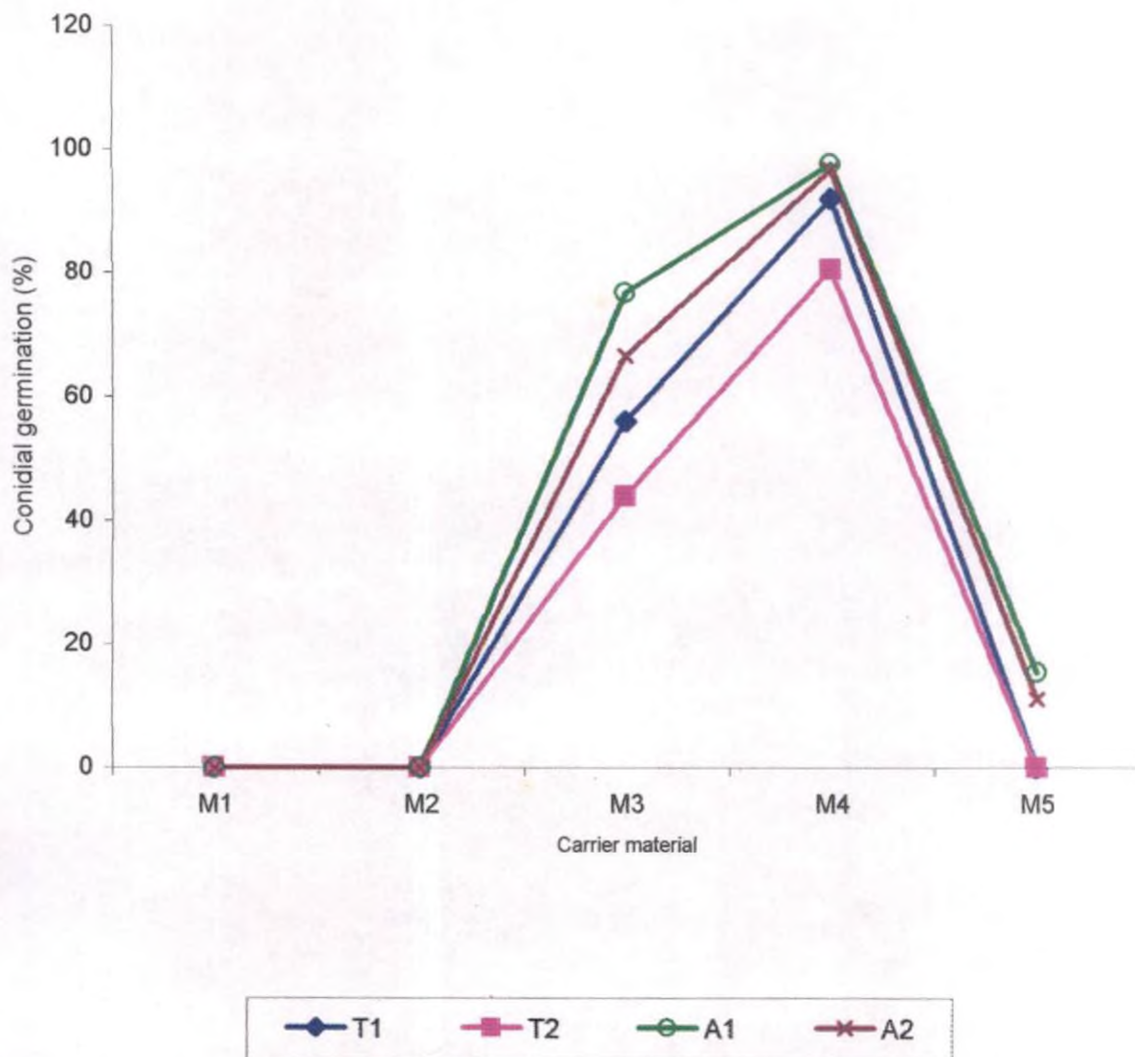


Fig. 5 Effect of carrier materials on viability of fungal antagonists

M1 - Neem cake, M2 - Biomanure, M3 - Rice bran, M4 - Talc , M5 - Vermiculite

Plate 7A Effect of different carrier materials on growth of *T. harzianum* isolate T_1

7B Effect of different carrier materials on growth of *T. harzianum* isolate T_2

7C Effect of different carrier materials on growth of *A. niger* isolate A_1

7D Effect of different carrier materials on growth of *A. niger* isolate A_2

Plate : 7A

Plate : 7B

Plate : 7C

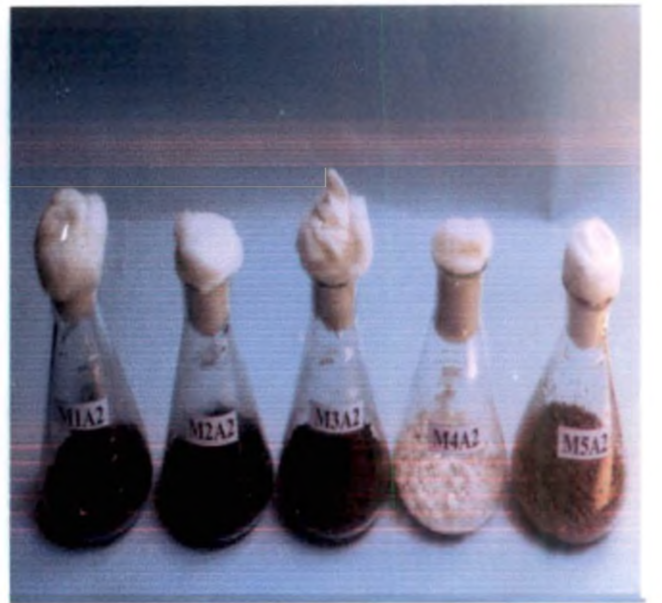


Plate : 7D

4.11 Preparation of talc based formulation of the selected fungal antagonists

Talc based formulations of the four fungal antagonists were prepared by using the fermentor biomass of each of the four fungi.

4.11.1 Shelf-life of the talc based formulations

Viability of the fungal antagonists in talc based formulations stored at room temperature was reduced with length of incubation (Table 12 and Fig. 6). After 30 days of storage, the number of c.f.u. per gram was found to be 44.6 x 10^6, 55.6 x 10^6, 81.26 x 10^6 and 75.65 x 10^6 for isolates T_1, T_2, A_1 and A_2 respectively. More than 30 x 10^6 c.f.u. g^{-1} was noticed for all the antagonists even upto 120 days of incubation. Then, the viability of both the *Trichoderma* isolates was reduced considerably and at the end of 150 days, viability of isolate T_1 was completely lost. As for the *Aspergillus* isolates, they retained their viability even upto 180 days of incubation. Among the two *Aspergillus* isolates, isolate A_2 gave 21.54 c.f.u. g^{-1} compared to 7.51 c.f.u. g^{-1} for isolate A_1 at 180 days of incubation.

4.12 Preparation of pathogen inoculum for field application

For soil application rice bran based inoculum of *C. gloeosporioides* was prepared and for foliar spraying, conidial suspension of *C. gloeosporioides* containing 10^6 conidia per ml was prepared. This was then applied on to the black pepper cuttings prior to the application of antagonist formulation.

4.13 Efficacy of the talc-based formulations in the field

A combination of two methods of application *viz*., soil application and foliar spray was done on black pepper cuttings in poly bags. As the pathogen survived in the soil, the fungal antagonists were applied to the soil for

Table 12 Survival of fungal antagonists in talc based formulation

Period (days)	Viability (10^6 c.f.u. g^{-1}) *				CD (0.05)
	T_1	T_2	A_1	A_2	
30	44.63 (6.76)	55.60 (7.52)	81.26 (9.07)	75.65 (8.76)	0.79
60	37.55 (6.21)	48.65 (7.05)	68.54 (8.34)	68.99 (8.37)	0.59
90	31.99 (5.74)	43.89 (6.70)	40.32 (6.43)	56.95 (7.61)	0.38
120	30.30 (5.60)	34.28 (5.94)	32.29 (5.77)	39.61 (6.37)	0.46
150	0.00 (1.00)	4.90 (2.43)	20.25 (4.62)	27.62 (5.35)	1.45
180	0.00 (1.00)	0.00 (1.00)	7.51 (2.92)	21.54 (4.75)	0.613

*Mean of three replications

Figures in parentheses are $\sqrt{x + 1}$ transformed values

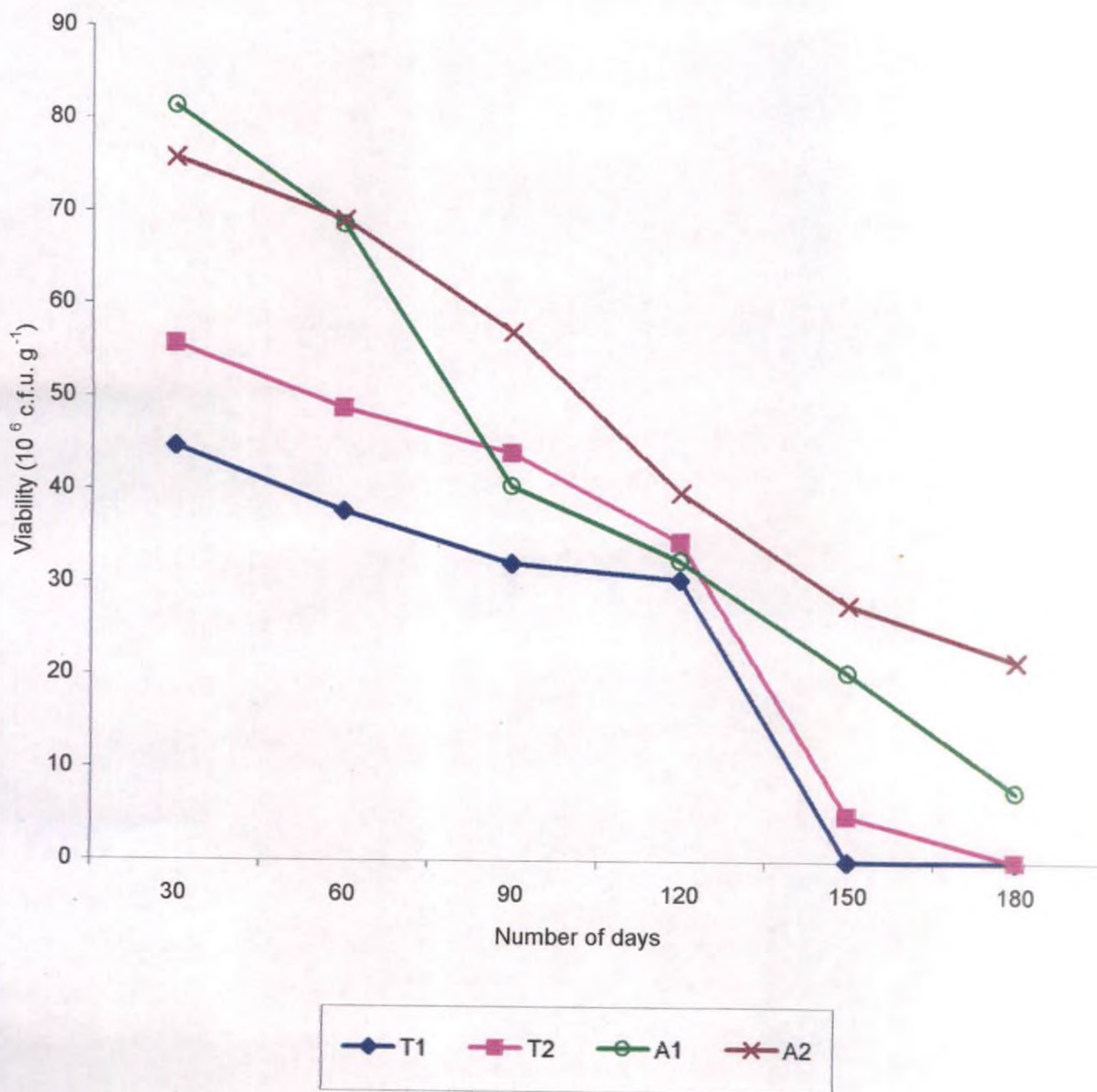


Fig. 6 Survival of fungal antagonists in talc based formulation

efficient control of the disease. As an added protection of the cuttings and to obtain better spread of the antagonists, foliar application was also done.

At 20 days after antagonist application, there was no significant difference between the treatments and control plants (Table 13 and Fig. 7).

At 40 days after application, highest disease intensity (62.36 %) was observed in control plants which was significantly different from the other treatments. However, there was no significant difference among the four antagonists. A similar trend was also noticed at 60 days after application of antagonists (Table 13 and Fig. 7). Compared to a disease intensity of less than 25.11 per cent in antagonist treated plants, the control plants showed a high disease intensity (64.70 %) with marked defoliation during this period.

4.14 Effect of antagonist formulations on growth parameters of black pepper

The number of leaves, shoot length, root length, fresh weight and dry weight of the pepper cuttings were taken 60 days after application of the talc-based formulations and compared with the control plants. In general, the application of fungal antagonists promoted the growth of black pepper cuttings (Plates 8A, 8B, 8C & 8B).

The number of leaves observed in T_1, T_2, A_1 and A_2 (13.0, 16.67, 14.67 and 13.67 respectively) were on par with each other, but were significantly different from the control plants (5.00 per plant) (Table 14).

Application of antagonists had no effect on shoot length of black pepper cuttings. All the treatments including control were on par with each other (Table 14 and Fig. 8).

Table 13 Effect of talc based formulation on disease inhibition

Treatment	Percentage disease index (%)*		
	20 DAA	40 DAA	60 DAA
T_1	43.22 (6.65)	17.75 (4.33)	24.50 (5.05)
T_2	18.71 (4.44)	14.44 (3.93)	16.31 (4.16)
A_1	36.45 (6.12)	16.06 (4.13)	21.75 (4.77)
A_2	37.69 (6.22)	15.24 (4.03)	25.11 (5.11)
C	32.99 (5.83)	62.36 (7.96)	64.77 (8.11)
CD (0.05)		1.29	1.74

*Average of three replications

Figures in parentheses are $\sqrt{x + 1}$ transformed values

T_1 – *T. harzianum* (T_1) soil application + foliar spray
T_2 – *T. harzianum* (T_2) " "
A_1 – *A. niger* (A_1) " "
A_2 – *A. niger* (A_2) " "
C – Control - *Colletotrichum gloeosporioides* (foliar spray + soil application)
DAA – Days after application of the talc based formulations

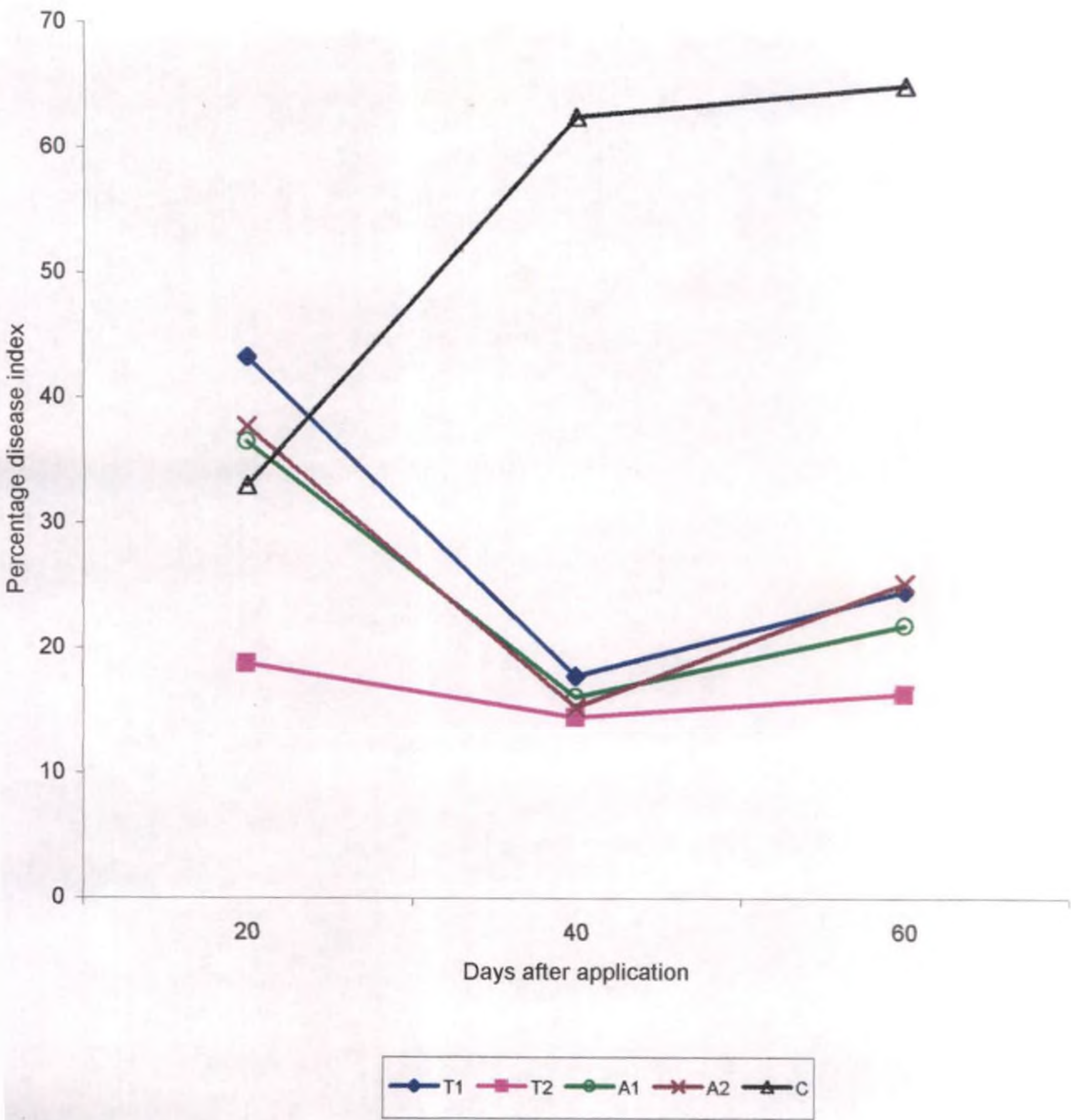


Fig. 7 Effect of talc based formulations on the incidence of anthracnose of black pepper in the field

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Table 14 Effect of talc based formulations on growth parameters of pepper +

Treatment	No. of * leaves	Shoot * length (cm)	Root * length (cm)	Fresh * weight (g)	Dry weight* (g)
T_1	13.00	45.23	24.53	14.45	3.36
T_2	16.67	35.83	29.27	26.75	6.49
A_1	14.67	53.40	39.73	29.40	5.82
A_2	13.67	38.97	27.33	20.53	3.67
C	5.00	21.87	14.07	9.68	1.37
CD (0.05)	5.93		10.64	9.47	2.31

*Average of three replications
+ Observations taken 60 days after treatment

Plate 8A Effect of formulation of *T. harzianum* isolate T_1 on incidence of anthracnose

8B Effect of formulation of *T. harzianum* isolate T_2 on incidence of anthracnose

8C Effect of formulation of *A. niger* isolate A_1 on incidence of anthracnose

8D Effect of formulation of *A. niger* isolate A_2 on incidence of anthracnose

Plate 9A Effect of formulation of *T. harzianum* isolate T_1 on growth parameters of black pepper cuttings

9B Effect of formulation of *T. harzianum* isolate T_2 on growth parameters of black pepper cuttings

9C Effect of formulation of *A. niger* isolate A_1 on growth parameters of black pepper cuttings

9D Effect of formulation of *A. niger* isolate A_2 on growth parameters of black pepper cuttings

Plate :8A

Plate :8B

Plate :8C

Plate :8D

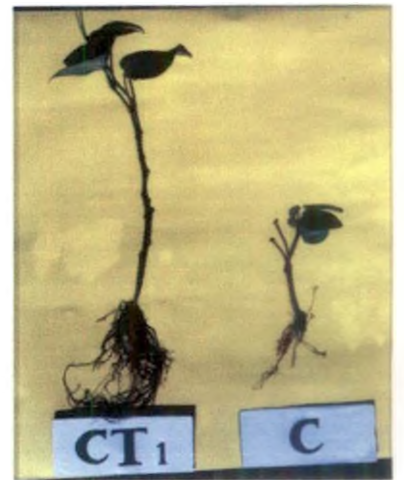
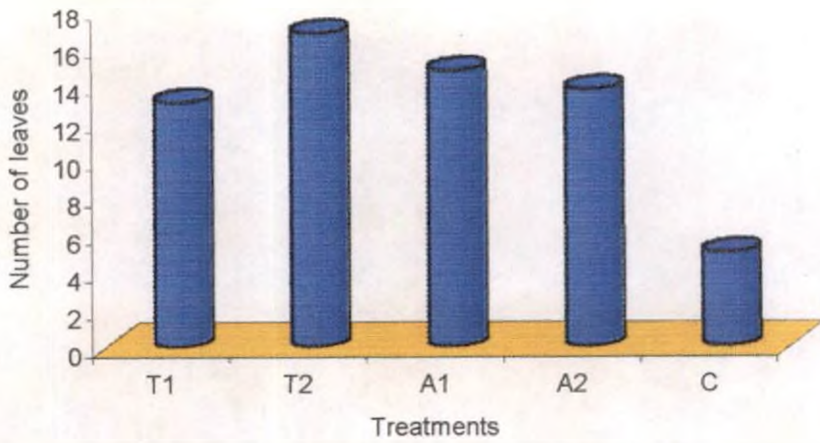


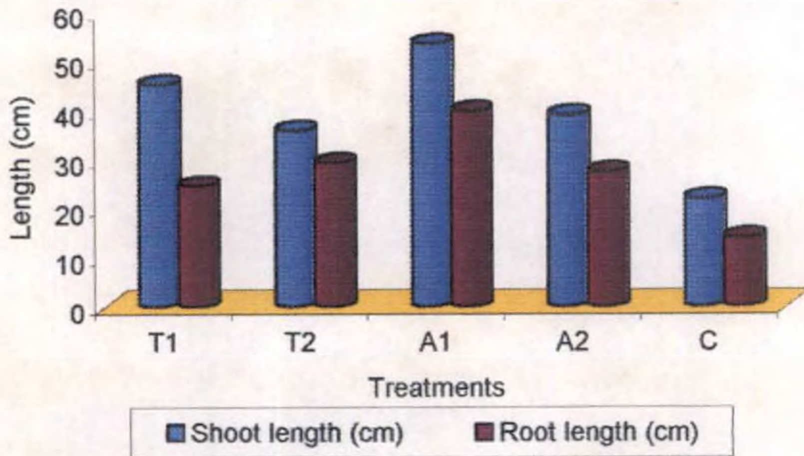
Plate :9A

Plate :9B

Plate :9C

Plate :9D





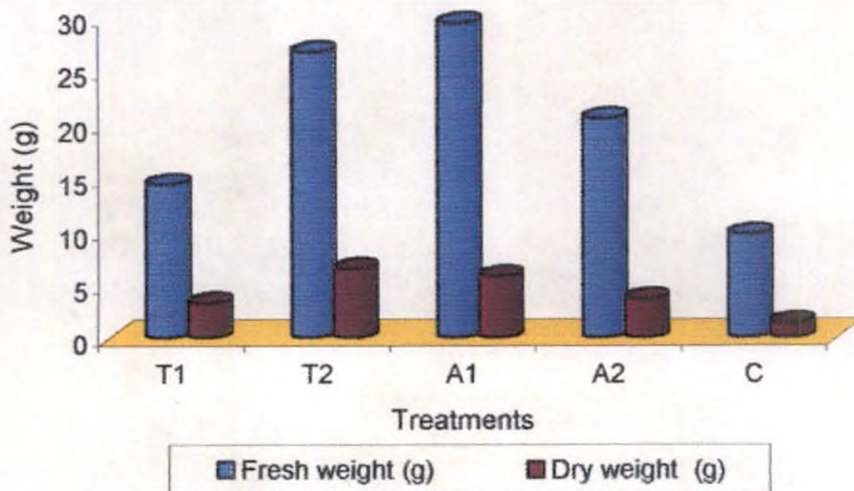


Fig. 8 Effect of talc based formulations on growth parameters of black pepper

Maximum root length was seen in the treatments A_1 (39.73 cm) and T_2 (29.27 cm) (Plates 9C & 9B). Treatment T_1 was not significantly different from control while treatment A_2 was on par with T_1, but was significantly different from control (Table 14, Fig. 8 and Plats 9A & 9D).

Application of fungal antagonists increased the fresh weight of plants. The highest value for mean fresh weight (29.4 g) was recorded in A_1 which was significantly higher than in control (9.68 g) and on par with A_2 (20.53 g) and T_2 (26.75 g) (Table 14 and Fig. 8). The treatment T_1 was statistically on par with control.

A comparison of dry weights of plants showed that treatments T_1, A_2 and control had the least dry matter content while, the treatment T_2 gave maximum dry weight (6.49 g), which was on par with A_1 (5.82 g) (Table 14 and Fig. 8). T_1 and A_2 also increased the dry weights (3.36 and 3.67 g respectively), but were statistically comparable with control (1.37 g).

Discussion

5. DISCUSSION

A search for resident antagonistic fungi having potential for the management of *Colletotrichum* spp., the incitant of anthracnose of black pepper was carried out in the present investigation. *Colletotrichum gloeosporioides* (Penz.) Penz.and Sacc. has a very wide host range causing foliar blights or anthracnose in numerous crops (Sutton, 1992). Black pepper (*Piper nigrum* L.) has also been recorded as a host of this pathogen (Rao, 1926). Recently, an epidemic of anthracnose on pepper was reported by Sainamole *et al.* (2000) in Idukki district of Kerala. The quality and quantity of the economic produce i.e., the berries is affected by shrinking and shrivelling due to berry infection. Large scale defoliation occurring as a result of infection is another characteristic symptom which reduces the vigour of the plant. Defoliation is found to be very severe in the nursery also. Fungicides like Bordeaux mixture and Captafol are currently applied for combating anthracnose of black pepper (Sarma *et al.*, 1988; KAU, 1996). But, continuous application of chemicals causes long term damage of the environment and human health. Attempts are being made to find out ecofriendly techniques to combat the disease, as organically produced pepper fetches a premium price in the international market.

Biological control is gaining importance in plant disease management as it is an ecofriendly and low cost technique. Prospects for the success of biological control in greenhouse crops like black pepper are often good because these crops are subjected to careful management and the crop environment in many instances can be precisely monitored to favour the

biological control activity. Under these circumstances, biological control seems to be a suitable alternative strategy for managing anthracnose of black pepper.

In the present study, survey was conducted for six months starting from February 2000 on the incidence of black pepper anthracnose, which revealed that anthracnose affects black pepper plantations all over the State and almost throughout the year. The pathogen associated was confirmed as *Colletotrichum gloeosporioides*. The genus *Colletotrichum* is a highly evolved genus comprising of numerous species which showed morphological and pathogenic variability among different isolates infecting the same crop (Ali and Warren, 1987; Pande *et al.*, 1991; Mathur *et al.*, 1999). The variability in the colony characters, pigmentation, conidial morphology, pathogenicity etc. in the present investigation also indicates the same conclusion.

Variation in radial growth, colony colour and mycelial dry weight among isolates of *C. trancatum* has been reported in earlier studies by several workers (Chackao *et al.*, 1978; Bhardwaj and Singh, 1986; Singh and Shukla, 1986). Marley *et al.* (2001) classified fifty isolates of *Colletotrichum sublineolum* into nine morphological groups on the basis of growth in culture and morphological characteristics. Morphological and pathogenic diversity was obtained among eleven isolates of *C. gloeosporioides* infecting black pepper from eleven locations. Mathur *et al.* (2000) suggested a genetic basis for the evolution of morphological and pathogenic variability in *Colletotrichum graminicola*.

Many species of *Colletotrichum* survived for long periods in crop debris, soil etc. and thus served as source of inoculum for infection in the subsequent seasons or years (Dasgupta, 1989; Hegde *et al.*, 1989; Dillard and Cobb, 1998; Yoshida and Shirata, 1999). Infected crop debris was found to be a source for the survival of the pathogen during off-season (Dasgupta, 1989; Palarpawar and Ghurde, 1989; Wilson *et al.*, 1992; Misra and Sinha, 1996; Urena-Padilla *et al.*, 2001). *C. gloeosporioides* causing anthracnose of black pepper survived for three months on infected black pepper leaves in soil and upto five months in infected leaves kept under laboratory conditions. This suggests that the pathogen can survive in soil and crop debris in the field in the absence of host plant. The survival of *Colletotrichum* spp. for shorter periods in the soil may attributed to factors such as soil pH, soil moisture, temperature and soil microorganisms. The absence of the above mentioned deleterious factors in laboratory conditions might have enhanced the survival of *C. gloeosporioides* for longer periods in the lab. This study indicates that under undisturbed conditions, the pathogen can survive for more than 150 days, which in turn favours the pathogen for further infection.

Efficient microbial antagonists are popularly used in the biological control of many foliar pathogens (Blakeman and Fokkema, 1982; Cristinzio, 1987; Anuratha and Gnanamanickam, 1990; Anandaraj and Sarma, 1995). In spite of extensive research efforts in the direct application of antagonistic microbes for controlling foliar and root infecting pathogens, very little work has been done under field conditions (De Oliveira *et al.*, 1984; Mew and Rosales, 1986; Biles and Hill, 1988; Zhou and Reeleder, 1989; Angappan *et al.*, 1996; Harman, 2000).

In the present investigation, resident fungi isolated from the phylloplane and rhizosphere of black pepper were tested for their antagonistic effects against the anthracnose pathogen, *C. gloeosporioides*. Among them, two isolates of *Trichoderma harzianum* Rifai (T_1 and T_2) and two isolates of *Aspergillus niger* van Tieghem (A_1 and A_2) caused maximum inhibition of growth of *C. gloeosporioides* in dual culture plates, when the fungal isolates were tested for antagonism on the pathogen. The use of *Trichoderma* spp. as an efficient antagonist of several plant pathogens is well established (Weindling, 1932; Harman and Taylor, 1980; Haran *et al.*, 1995; Chet, 1998). *T. harzianum* is a potential biocontrol agent having antagonistic properties against several plant pathogens (Sivan and Chet, 1982; Elad *et al.*, 1983; Padmakumari, 1989; Ravi *et al.*, 1999; Hazarika *et al.*, 2000). *T. harzianum* is also a potent antagonist of *Phytophthora capsici* causing foot rot of black pepper (Sarma *et al.*, 1996). *A. niger* is a very efficient antagonist of many plant pathogenic fungi and is gaining importance as a biocontrol agent (Padmakumari, 1989; Gajbe and Lanjewar, 1991; Sharma and Sen, 1991; Sen *et al.*, 1995; Shanmugam and Varma, 1999; Pandey and Upadhyay, 2000). Pratibha Sharma *et al.* (2001) reported the antagonistic effect of commercial and lab formulations of *T. harzianum* and *A. niger* on *Sclerotinia sclerotiorum* causing rots in cauliflower.

The *T. harzianum* isolates parasitized the hyphae of *C. gloeosporioides* by coiling around the hyphae of the latter. Granulation, penetration and disintegration of the host hyphae were the other types of parasitic activity observed in the lab experiments. The disintegration of the pathogen hyphae observed may be due to the lytic activity of the mycoparasites.

Elad *et al.* (1983) demonstrated that *T. harzianum* produces cell wall lytic enzymes which caused lysis of mycelia of *R. solani. T. harzianum* employed lysis and hyperparasitism as mechanisms of antagonism on the hyphae of several plant pathogenic fungi (Singh and Singh, 2000; Godwin-Egein and Arinzae, 2001). Inbar *et al.* (1996) observed coiling and disintegration of hyphae of *Sclerotinia sclerotiorum* by *T. harzianum*. In the present study the *A. niger* isolates caused granulation, vacuolation and lysis of host hyphae leading to the disintegration of the latter. *A. niger* strain AN27 inhibited spore germination and caused aberration and lysis of chlamydospores and sclerotia of several plant pathogenic fungi (Sen *et al.*, 1995). Under scanning electron microscope, *A. niger* was seen coiling around and sporulating on hyphae of *Pythium aphanidermatum, Fusarium oxysporum* and *Macrophomina phaseolina* and sometimes the pathogen hyphae were vacuolated and dead without invasion (Sen, 2000). The inhibitory effects of the metabolites of *A. niger* on several plant pathogens has been well documented (Mondal, 1998; Mondal and Sen, 1999; Rajasab and Saraswathi, 1999; Singh and Singh, 2000). The inhibition of growth of the test organism observed in the present study may also be due to the production of metabolites by *A. niger*.

In the present study, five carrier materials were tested for their effect on multiplication and viability of the four fungal antagonists. In lab studies, the highest biomass for all the four fungi was obtained when rice bran was used as the carrier material. The presence of growth promoting constituents in rice bran induced or favoured the growth of the antagonists. The best carrier material for maintaining the viability of the fungi was found to be talc. The role of talc in maintaining the viability of antagonists over a period of

time has been observed in earlier studies (Vidyasekharan and Muthamilan, 1995; Nakkeeran *et al.*, 1997; Smitha, 2000).

Trichoderma sp. can produce large amounts of biomass containing conidia, chlamydospores and mycelia in liquid media (Papavizas *et al.*, 1984; Nakkeeran *et al.*, 1997; Lewis *et al.*, 1998). In the present study, potato dextrose broth was used as the starter fermentation medium. PDA has been reported as a superior media for biomass production of *Trichoderma* sp. by Prasad *et al.* (1997).

To improve the efficacy of microbial antagonists, several formulations of biocontrol agents have been developed which are being produced commercially in many countries (Papavizas, 1985). Several solid substrate formulations have been developed to deliver *Trichoderma* spp. against many plant pathogenic fungi (Backman and Kabana,1975; Lewis and Papavizas, 1985; Mukhopadhyay, 1987; Lewis *et al.*, 1991; Prasad and Rangeshwaran, 1999; Kumar *et al.*, 2000). *Aspergillus* spp. also have been formulated by inexpensive means (Daigle and Cotty, 1995; Sen, 2000). In the present investigation talc based formulations of all the four fungi antagonistic to *C. gloeosporioides* were prepared by using the fermentor biomass.

Viability of the fungal antagonists in talc based formulations stored at room temperature was reduced with length of incubation. A population of 7.5 x 10^6 c.f.u. per gram of the talc based formulation was recovered for *A. niger* isolate A_1 at 180 days after storage at room temperature. For isolate A_2, the viable count at the same time was 21.54 x 10^6 c.f.u. per gram. Survival ability of the *Trichoderma* isolates varied. Isolate T_1 survived for 120 days while isolate T_2 retained viability for 150 days in the talc based formulation.

Reduction in the viability of talc based formulation of *Trichoderma* from 31 x 10^7 c.f.u. per gram to 13 x 10^7 c.f.u. per gram in 75 days period when stored at 20–30°c was reported by Nakkeeran *et al.* (1997). Talc based formulation of fungal antagonists has much practical advantage, in that it can be easily supplied to the farmers for field application. Several formulations of fungal antagonists have been proposed for the control of many aerial and root diseases of plants (Papavizas, 1985; Lewis and Papavizas, 1985; Vidyasekharan and Muthamilan, 1995; Nakkeeran *et al.*, 1997; Lewis *et al.*, 1998; Sen, 2000). In the present study also, talc based formulation of the fungi were used for field application.

Talc based formulations of the fungal antagonists were tested for their efficacy in suppressing black pepper anthracnose by soil application combined with foliar spray. Since the pathogen survived in the crop debris in soil, soil application of the antagonists seemed to be ideal for controlling the disease in the early stages of crop growth. Tosi and Zazzerini (1994) suggested that there was an increased antagonistic effect when fungal isolates were added to the soil as air-dried inoculum rather than as seed treatment in rust infested safflower beds. Soil application of antagonist formulation has been employed on several occasions to control soil borne plant diseases (De Oliveira *et al.*, 1984; Baby and Manibhushanrao, 1993; Angappan *et al.*, 1996; Lewis and Larkin, 1998; Susheelabhai *et al.*, 2000). The soil inhabiting fungus, *Trichoderma* has been frequently used for biological control in aerial environments (Tronsmo, 1986; Dubos, 1987). Comparisons of the leaf and root habitats show that leaf is a much more dynamic environment (Andrews, 1992). Spurr and Knudson (1985) used bacteria from soil and other habitats

for the control of *Alternaria* and *Cercospora* leaf spots of tobacco. Fluorescent pseudomonads from soil suppressed leaf blotch and rust in wheat seedlings in green houses (Levy *et al.*, 1988).

The present study indicates that apart from reducing the soil inoculum of the pathogen, it is essential to check the aerial phase of the disease by foliar application with the antagonists. Therefore, a combination of soil application and foliar spray was used for the delivery of the formulations of *A. niger* and *T. harzianum* for checking the anthracnose disease of black pepper. Introduction of mycoparasites onto the young shoots and foliage early in the season, before the start of anthracnose epidemic, significantly reduced the rate of disease development. Biles and Hill (1988) obtained significant reduction in leaf spot of wheat caused by *Cochliobolus sativus* by foliar application of *T. harzianum* of the rate of 10^8 spores per ml. Zhou and Reeleder (1989) found that foliar application of spore suspension of *Epicoccum purpurascens* significantly reduced white mold incidence on snap bean. Foliar application of fungal antagonists has been extensively studied with respect to foliar pathogens (Tronsmo and Dennis, 1977; Trutmann *et al.*, 1991; Krishnamurthy and Gnanamanickam, 1998; Harman, 2000; Huang *et al.*, 2000). Foliar application of *Aspergillus* sp. has also been documented (Gogoi and Roy, 1993; Saikia and Chowdhury, 1993).

Soil application of talc based product of *T. harzianum* and *A. niger* isolates @ 10 g per kg soil followed by foliar spray with one per cent suspension of talc based formulation at 15 days interval, starting from ten days after application of pathogen inoculum, effectively checked the occurrence and spread of anthracnose of black pepper under field conditions.

Suppression of disease development by the fungal antagonists was maximum at 40 days after application of the formulations. Though there was an increase in disease intensity of black pepper cuttings in the field at 60 days after antagonist application, plants treated with the formulations were much less affected by the disease compared to the control plants, treated with pathogen alone. Lewis *et al.* (1998) demonstrated that chlamydospores of *Trichoderma* spp. or *Gliocladium virens* in the biomass germinated and the young hyphae grew on the bran which suppressed the spread of the pathogen and reduced its inoculum potential. Harman (2000) demonstrated that foliar spray of *T. harzianum* strain T-22 controlled powdery mildews on *Catharanthus* and pumpkins. He suggested that T-22 must be applied at least once every ten days when disease pressure is high, since it cannot grow on and colonize newly formed leaf tissue. Seed treatment and soil application of *A. niger* formulation, Kalisena resulted in 93 per cent control of charcoal rot of potato caused by *Macrophomina phaseolina* and the sustainability of *A. niger* in potato rhizosphere was proved by its massive increase of over 150 per cent towards the end of crop growth (120 days) under field conditions (Mondal, 1998). The efficiency of *T. harzianum* and *A. niger* in inhibiting the mycelial growth and sclerotial production of *Sclerotinia sclerotiorum* and controlling stalk rot of cauliflower under greenhouse conditions has been reported (Pratibha Sharma *et al.*, 2001).

In the present study, treatment of black pepper cuttings with the talc based formulations of the fungal antagonists viz., *T. harzianum* and *A. niger* had marked influence on plant growth and disease incidence. Jubina and Girija (1998) found that *Pseudomonas fluorescens* and *T. harzianum* did not

have any effect on growth promotion of pepper plants. The results of the present investigation were contradictory to this finding, as *T. harzianum* isolates promoted promoted growth of black pepper cuttings when compared with the control plants. So also did *A. niger* isolates. In field experiments, yields were greater in onion plants treated with *T. harzianum* strain T-22 (Harman, 2000).

In the present investigation, it was observed that *A. niger* isolates, especially isolate A_1 promoted better growth of black pepper cuttings with increased shoot length, root length and fresh weight when compared with the other treatments. Seed and soil treatments with *A. niger* formulation Kalisena increased the biomass of cauliflower seedlings by 45 and 141.5 per cent respectively (Sen *et al.*, 1998). They noticed a similar growth promotion in brinjal also. Two growth promoting compounds were isolated from *A. niger* AN27 by NMR, IR and mass spectral analysis and these compounds increased the germination, root and shoot length and biomass of cauliflower (Mondal *et al.*, 2000). The increased plant growth and biomass production observed in the present study may be attributed to the production of such plant growth promoting metabolites by *A. niger*.

From the present investigation, it is evident that isolates T_1, T_2, A_1 and A_2 were effective in suppressing anthracnose incidence and promoting growth of black pepper cuttings in the greenhouse / nursery. Such fungal antagonists have immense scope in the nursery establishment of black pepper by warding off pathogens and boosting up crop growth.

The selected antagonists have to be further tested in the black pepper plantations to derive any lucid conclusions. The interaction effects of the

different antagonists, their spectrum of activity, development of fungicide resistant/tolerant strains and effective delivery systems, safety of the antagonists towards the animal fauna, their persistence in soil etc. is also to be studied in detail before commercializing the product.

Summary

6. SUMMARY

Black pepper (*Piper nigrum* L.), the 'black gold', is one of the most important spice crops of Kerala which is infected by a number of fungal diseases among which anthracnose is a serious disease which affects the leaves, spikes and berries and is gaining importance in recent years. The disease is seen in bush pepper, in the nursery as well as in black pepper plantations. Unlike foot rot, the disease is seen throughout the year. The present investigation was undertaken to evolve a suitable biocontrol strategy for managing this disease.

A survey conducted in eleven different locations of the state revealed that *Colletotrichum gloeosporioides* is the major pathogen associated with the disease. Significant morphological and pathogenic variability was observed among the different isolates, of which C_6 was the most virulent and fast growing isolate. Studies on the survival of the pathogen revealed that it survived for three months on infected black pepper leaves in soil and for five months on infected black pepper leaves under laboratory conditions.

Rhizosphere and phyllosphere antagonists were assessed for their potential in controlling *C. gloeosporioides*. Rhizosphere and phyllosphere mycoflora of healthy black pepper plants were isolated and screened for their efficacy in inhibiting *C. gloeosporioides in vitro*. Among them, two isolates of *Trichoderma* sp. – T_1 and T_2 and two isolates of *Aspergillus* sp. – A_1 and A_2 were observed to be superior in inhibiting the pathogen. The *Trichoderma* isolates were identified as *T. harzianum* Rifai and *Aspergillus* isolates as *A. niger* van Tieghem.

Studies on the mode of mycoparasitism of *T. harzianum* isolates on *C. gloeosporioides* revealed that these isolates coiled around and penetrated the host hyphae causing granulation and lysis. The *A. niger* isolates caused granulation, vacuolation and lysis of the pathogen hyphae leading to their disintegration.

Five carrier materials were studied for their effect on the mass multiplication and viability of the fungal antagonists. Among these, rice bran was observed to be the best carrier for mass multiplication of the four antagonistic fungi, whereas talc was best for maintaining the viability of the four antagonistic fungi.

The four antagonists were mass multiplied and formulated in talc for application in the field. The fungi were mass multiplied by a modified liquid fermentation technology and the fermentation biomass consisting of mycelia and conidia was mixed with talc. Potato dextrose broth was used for the initial mass multiplication in this experiment.

Studies on the shelf life of the talc based formulations revealed that the viability of the antagonists was reduced with time of incubation at room temperature. The *A. niger* isolates were viable for upto 180 days whereas the *T. harzianum* isolates were viable only for 150 days.

A greenhouse trial was conducted to evaluate the efficacy of the talc based formulations in the field at The District Agricultural Farm, Peringamala, Thiruvananthapuram. A combination of two methods of application viz., soil application and foliar spray was done on black pepper cuttings in polybags showing uniform disease intensity.

Soil application @ 10 g per kg of potting mixture followed by foliar spray with one per cent suspension of the talc based formulation of the antagonists twice at 15 days interval starting from 10 days after pathogen inoculation was very effective in reducing the intensity as well as checking the spread of anthracnose. At 60 days after application, all the four antagonists were equally effective in suppressing the disease, while control plants showed high disease intensity with marked defoliation.

All the four fungal antagonists promoted the growth of black pepper cuttings, in general. Thus, the two isolates of *T. harzianum* (T_1 and T_2) and the two isolates of *A. niger* (A_1 and A_2) had the dual function of disease suppression as well as plant growth promotion.

This investigation forms the first report of the application of *A. niger* and *T. harzianum* as biocontrol agents for management of anthracnose of black pepper.

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*Original not seen

BIOCONTROL OF ANTHRACNOSE OF BLACK PEPPER (*Piper nigrum* L.) CAUSED BY *Colletotrichum* spp. USING MYCOPARASITES

BY

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ABSTRACT OF THE THESIS
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ABSTRACT

An investigation was done for exploiting potential biocontrol agents for the management of anthracnose of black pepper. Survey conducted on the incidence of anthracnose disease in eleven different locations of the state during February – July 2000 revealed that *Colletotrichum gloeosporioides* was the most common pathogen associated with this disease. Distinct variability in morphological characters and pathogenicity was exhibited by the different isolates of the pathogen. The most virulent isolate was isolate C_6 from Perinthalmanna. The pathogen survived for three months on infected leaves in the soil and upto five months on infected leaves under laboratory conditions.

A study was conducted to isolate potential mycoparasites of *C. gloeosporioides* from the rhizosphere and phyllosphere of both healthy and diseased black pepper plants from the various locations surveyed. Among the fungi isolated, two isolates of *Trichoderma harzianum* Rifai (T_1 and T_2) and two isolates of *Aspergillus niger* van Tieghem (A_1 and A_2) were found to be most effective in inhibiting *C. gloeosporioides in vitro*. After mass multiplication, the selected fungal antagonists were formulated in talc, which was found to be the best carrier material for maintaining the viability of the antagonists. The shelf life of the talc based formulations were 150 days and 180 days for the *T. harzianum* isolates and *A. niger* isolates respectively. The antagonistic fungi were tested separately under greenhouse conditions by a combination of two methods of application viz., soil application and foliar spray. Greenhouse studies indicated that all the four isolates were equally

effective in suppressing the development of the disease in black pepper cuttings. Application of the talc based formulations of the four isolates in the soil @ 10 g / kg soil followed by foliar spray with one per cent suspension of the talc based formulations twice at 15 days interval starting from 10 days after pathogen inoculation was very effective in controlling the disease under greenhouse conditions. In general, application of the fungal antagonists promoted the growth of black pepper cuttings. Isolate A_1 produced maximum shoot length, root length and fresh weight in the treated black pepper cuttings. The results of the study assigned dual roles as biocontrol agents and plant growth promoters for all the four fungal antagonists.