MANAGEMENT OF FOLIAR BLIGHT OF AMARANTHUS (*Amaranthus tricolor* L.) CAUSED BY *Rhizoctonia solani* Kühn USING MICROBIAL ANTAGONISTS

BY

SMITHA K.P.

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DECLARATION

I hereby declare that this thesis entitled "**Management of foliar blight of amaranthus (*Amaranthus tricolor* L.) caused by *Rhizoctonia solani* Kühn using microbial antagonists**" 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.

Vellayani,

24-7-2000

SMITHA K.P.

CERTIFICATE

Certified that this thesis entitled "**Management of foliar blight of amaranthus (*Amaranthus tricolor* L.) caused by *Rhizoctonia solani* Kühn using microbial antagonists**" is a record of research work done independently by Ms. Smitha K.P. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Vellayani,
24.7.2000

Dr. Kamala Nayar
(Chairman, Advisory Committee)
Associate Professor
Department of Plant Pathology
College of Agriculture, Vellayani
Thiruvananthapuram

APPROVED BY:

CHAIRMAN

Dr. KAMALA NAYAR

MEMBERS

1. Dr. S. BALAKRISHNAN

2. Dr. C. GOKULAPALAN

3. Dr. P. RAJENDRAN

EXTERNAL EXAMINER

(V. P. Potty)

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INTRODUCTION

Amaranthus, the most nutritious leafy vegetable of the tropics, is a rich and cheap source of many of the vitamins and minerals. Apart from its nutritive value, absence of any serious pest or disease has made it a popular leafy vegetable cultivated throughout India.

However a ravaging disease, hitherto unrecorded in India has been recently observed in the crop, in Kerala (Kamala *et al.*, 1996). The fungus *Rhizoctonia solani* Kuhn (teleomorph; *Thanatephorus cucumeris* (Frank) Donk) was consistently isolated from diseased leaf tissue which infects more than 90 per cent of the plants in the field. The disease causes considerable economic loss owing to reduction in marketability of the produce.

At present the recommended control measure for this foliar blight disease is spraying of mancozeb (Indofil M-45, 0.4 per cent) at fortnightly intervals . But chemical control of the disease is quite hazardous on account of the persistence of fungicide deposited in the consumable produce, viz., the leaves. Mancozeb belongs to the ethylene bis dithiocarbamate group of fungicides. Its degradation product is ethylene thiourea,which is a suspected carcinogen . Under these circumstances biological control can be adopted as a suitable alternative strategy for managing this disease of amaranthus.

Therefore, the present investigation has been carried out with the following objectives

1. Isolation and screening of fungal and bacterial isolates from rhizosphere and phylloplane of amaranthus for *in vitro* antagonism against the leaf blight pathogen.
2. Formulating the selected antagonists in an inert carrier material, viz., talc.
3. Assessing the efficacy of the different methods of application of the selected fungal and bacterial antagonists under green house condition.
4. Testing the efficacy of microbial formulation in field trial .
5. Assessing the influence of chemical inducers of systemic resistance, viz., salicylic acid on microbial antagonists

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Foliar blight caused by *Rhizoctonia solani* is a serious disease of the leafy vegetable amaranthus (*Amaranthus tricolor* L.) in Kerala (Kamala *et al.*, 1996). Incidence of collar rot caused by *R. solani* in amaranthus has been reported by Roy (1975). Aerial blights caused by *R. solani* have been previously reported on vegetables like radish, cabbage, spinach and sugarbeet (Baker, 1970; Galindo *et al.*, 1983; Shew and Main, 1985; Jana *et al.*, 1990). Koike and Subbarao (1999) reported leaf blight of endive and escarole caused by *R. solani* in California. Although chemical control of the disease through the use of fungicides may lessen the severity of this aerial blight disease (Jana *et al.*, 1990; KAU, 1996; Gokulapalan *et al.*,1999) continuous application of chemicals causes long term damage to the environment. In 1987, large quantities of leafy vegetables exported from Malaysia to Singapore were rejected due to high levels of ethylene bis dithiocarbomate residues in the produce by application of the fungicide mancozeb (Mah *et al.*, 1988). There is strong evidence that natural biological control provides protection against many foliar diseases in the crop plants (Blakeman and Fokkema, 1982).

2.1 Biological control of *R. solani*

The possibility of controlling pathogenic fungi with antagonistic microorganisms has long been considered and studied. Several fungal and bacterial antagonists like *Trichoderma* spp., *Gliocladium* spp., *Bacillus subtilis* and fluorescent *Pseudomonas* spp. have been found to be effective in checking the disease caused by *R. solani* on crops viz. rice, pea and cotton (Elad *et al.*,

1982). In the recent years direct application of antagonistic microorganisms to control foliar and root infecting pathogens has gained momentum (Whipps, 1992).

2.1.1 Fungal antagonists of *R. solani*

The most exhaustively researched microorganism as a biocontrol agent is *Trichoderma* spp. Weindling (1932) first demonstrated that *Trichoderma viride* was parasitic on and antagonistic to *R. solani*. The fungus was found to readily parasitise and kill the hyphae of *R. solani*. Hadar *et al.*, (1979) observed that an isolate of *T. harzianum* could directly attack *R. solani* and a wheat bran culture of the fungus could control damping off of bean, tomato and egg plant seedlings caused by *R. solani*. *T. hamatum* effectively reduced the seedling disease of radish and pea caused by *R. solani* under field conditions (Harman and Taylor, 1980).

Biological control of *R. solani* affecting carnations was achieved by using the antagonistic fungus *T. harzianum* (Elad *et al.*, 1981). They also used *T. harzianum* for controlling *R. solani* causing black root rot of strawberries under field conditions. Sportelli *et al.*, (1983) employed *T. viride* in their studies on the biological control of fungal diseases of tomato caused by *R. solani* under greenhouse conditions. *T. viride*, *Aspergillus niger*, *A. flavus* and *Rhizopus* sp. exhibited inhibitory action on *R. solani* infecting rice (Gokulapalan and Nair, 1984). Tomato fruit rot caused by *R. solani* was considerably reduced by FB of various isolates of *Trichoderma* and *Gliocladium*, but not when equal or higher numbers of their conidia were added to soil (Lewis and Papavizas, 1984). Venkatasubhaiah *et al.*, (1984) found that *T. harzianum* was an effective biocontrol agent of *R. solani*, the incitant of collar rot of coffee seedlings.

The biology, ecology and potential for biocontrol of *Trichoderma* and *Gliocladium* have been exhaustively reviewed by Papavizas (1985). Manibhushanrao *et al.* (1987) found that *Gliocladium virens* and *T. longibrachiatum* isolated from paddy fields of Kerala and Tamil Nadu were antagonistic to *R. solani.* Padmakumari (1989) in her studies found *T. harzianum* and *T. viride* to be antagonistic to *R. solani* under *in vitro* conditions. Further studies with pot culture experiments showed the effectiveness of *T. viride* and *T. harzianum* as antagonistic organisms in reducing the intensity of sheath blight of rice by restricting the survival of *R. solani.* An isolate *of T. harzianum* was capable of controlling rice sheath blight pathogen *R. solani* Kuhn (Kumaresan and Manibhushanrao, 1991).

The antagonistic effect of *T. harzianum, T. viride* and *Aspergillus terreus* on *R. solani* causing sheath blight of rice was tested by Bhuyan *et al.* (1994). Bailey and Galligan (1997) noticed that *T. viride* inhibited the evolution of infection efficiency of *R. solani. Trichoderma harzianum, T. hamatum, T. viride* and *Gliocladium virens* were effective in reducing root rot of French bean caused by *R. solani* (Abraham Mathew and Gupta, 1998). Isolates T1 301 and T1 304 of *T. longibrachiatum* with fungicide resistance developed by mutagenesis were efficient antagonists against *Venturia inaequalis* (Palani and Lalithakumari, 1999).

Rhizoctonia solani causing wilt of patchouli (*Pogostemon cablin)* was successfully controlled using *Glomus aggregatum* in combination with *Gliocladium virens* (Anonymous, 2000).

2.1.2 Biocontrol of *R. solani* by bacteria

The bacterial biocontrol agents improve plant growth by suppressing either major or minor pathogens of plants. (Cook and Rovira, 1976; Weller, 1988; Defago *et al.*, 1990). Exhaustive studies were conducted for the control of soil borne diseases using *P. fluorescens* (Cook and Rovira, 1976; Howell and Stipanovic, 1979; Sneh *et al.*, 1984 ; Gutterson *et al.*, 1986; Stutz *et al.*, 1986; Ganesan and Gnanamanickam, 1987 ; Liftshitz *et al.*, 1987; Walther and Gindart, 1988 ; Anuratha and Gnanamanickam, 1990 ; Callan *et al.*, 1990; Weststeijn, 1990 ; Van Peer *et al.*, 1991; Lemanceau *et al*, 1992; Gamliel and Katan, 1993).

Fluorescent pseudomonads occur widely as phyllosphere and rhizosphere inhabitants. Recently some strains of fluorescent pseudomonads were found to suppress foliage diseases also. Austin *et al.* (1977) showed that *P. fluorescens* isolated from leaves of *Lolium perenne* were antagonistic to the pathogen *Drechslera dictyoides*. *P. cepaciae* isolated from conidia of *Bipolaris maydis* obtained from infected corn leaves successfully controlled *Cercospora* leaf spot of peanut and *Alternaria* leaf spot of tobacco (Blakeman and Fokkema, 1982). Mew and Rosales (1986) isolated fluorescent bacteria from paddy fields as well as rice plants which inhibited mycelial growth of *R. solani in vitro* and suppressed sheath blight disease of rice in the field, by seed treatment.

Many studies have indicated the potential of fluorescent pseudomonads in inhibiting or displacing sheath blight pathogen (*R. solani)* at the root surfaces thereby protecting the root health of perennial and annual crops such

as cotton, tobacco, potato, flax, radish, cucumber, wheat and rice (Howell and Stipanovic, 1979., Kloepper and Schroth, 1981., Kloepper, 1983., Mew and Rosales, 1986., Chen and Tin, 1992). *Pseudomonas aeruginosa* and *P. fluorescens* inhibitory to *R. solani* increased plant growth of rice (Sakthivel *et al.*, 1986; Podile *et al.*, 1990). Efficient strains of *P. fluorescens* isolated from rice rhizosphere protected IR 20 and TKM 9 seedlings from infection by *R. solani* in green house tests (Devi *et al.*, 1989).

Basim and Katircioglu (1990) studied the *in vitro* antagonistic effect of some *Bacillus subtilis* isolates against plant pathogenic fungi. *B. subtilis* isolates AB-2 and AB-27 were found to be most antagonistic against *R. solani.* Gokulapalan and Nair (1991) reported the antagonism of *Bacillus chromobacterium, Propionibacterium* and *Rothia* sp. to *R. solani in vitro.*

Rosales *et al.* (1993) found that 23 bacterial strains isolated from paddy inhibited mycelial growth of *R. solani in vitro.* The strains of bacteria were identified as *Bacillus subtilis, B. laterosporus, B. pumilus, Pseudomonas aeruginosa* etc. *Bacillus cereus* strain R_2 isolated form rice plants strongly inhibited *R. solani* AG-1 and suppressed sclerotial germination. Ninety nine isolates of fluorescent pseudomonads from roots of beans (Ph*aseolus vulgaris)* showed *in vitro* antagonism against *R. solani* (Wolk and Sarkar, 1993).

Brannen (1997) reported the suppression of *R. solani* infection of cotton by a biocontrol product containing *B. subtilis. Pseudomonas* sp. and *Burkholderia* sp. showed strong antifungal activity to plant pathogens including *R. solani* and this ability was correlated with the production of pyrrolnitrin by the bacteria (Hammer *et al.*, 1997).

Mew *et al.* (1999) compared two bacterial isolates P-91 and *B. subtilis* B-916 with Jingangmycin for the ability to control sheath blight of rice. *B. subtilis* strain B-916 successfully controlled rice sheath blight under field conditions.

2.2 Mechanism of action of fungal antagonists

Trichoderma harzianum produces cell wall lytic enzymes which causes the lysis of mycelia of *R. solani* (Elad *et al.*, 1980). Chu and Wu (1981) observed that the hyphae of ten isolates of *T. pseudokoningii*, three isolates of *T. longibrachiatum*, one of *T. hamatum*, two of *T. harzianum* and five of *Penicillium* spp. could coil around the hyphae of *R. solani* which consequently lost their contents and collapsed. They also reported that some of the antagonists could penetrate and grow within the hyphae of *R. solani*. Hyphal interaction between *T. harzianum* and *R. solani* results in the destruction of N-acetyl glucosamine by chitinase leading to osmotic imbalances and intracellular disorders on plasma membrane and cytoplasm aggregation of *R. solani* (Benhamou and Chet, 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* in culture. *T. harzianum* readily parasitised the hyphae of *R. solani* leading to coiling, penetration followed by disintegration and death of the mycohost (Gokulapalan, 1989). Kumareshan and Manibhusanrao (1991) suggested that the ability of *T. harzianum* to control rice sheath blight pathogen *R. solani* may be attributed to aggressive coiling of host hyphae.

Trichoderma harzianum and *T. viride* controlled the root rot of *Phaseolus vulgaris* caused by *R. solani*. *T. viride* produced more effective non-volatile compounds than *T. harzianum* on cellophane. *T. harzianum* possessed hooks, pincer shaped structures, coils and penetration sites on *R. solani* (Roberti *et al.*, 1993).

Jagpal Singh and Singh (1993) observed that volatile and non-volatile compounds produced by *T. reesei* (*T. longibrachiatum*) inhibited the growth of *R. solani* by 71 per cent *in vitro*. Spore germination and radial growth of *R. solani* were inhibited by cell free culture filtrates of *Gliocladium virens* and an isolate of *Streptoverticillium* (Mishra and Narain, 1994). Horvath *et al.* (1995) studied the production of soluble antifungal metabolites by the biocontrol fungus *T. harzianum* in connection with the formation of condiospores. Jayasuriya *et al.* (1996) observed that *T. longibrachiatum* (isolate DZE 10) completely inhibited *Rigidoporus lignosus*, the cause of white rot disease on rubber, on agar by producing volatile inhibitory metabolites. *T. harzianum* isolate Th 008 secreted Trichodermin (MW-292) and a small peptide (MW-879) in culture which were antagonistic to the mycelial growth of *R. solani* isolate 2B-12 (Bertagnolli *et al.*, 1998).

2.3 Taxonomy of the genus *Trichoderma*

Rifai (1969) identified nine species of *Trichoderma* which is an authentic record of the characterization of the genus. The species description as proposed by Rifai is presented below.

Trichoderma spp.	Colony	Pigmentation	Hyphal diameter in µ	Chlamydospore	Conidiophore	Phialide	Spore
T. piluliferum Webster and Rifai	Slow growing, smooth, pure white	No pigments	10	Formed infrequently, subglobose	In compact tufts	Short and plump, flask shaped 4.6 - 6.5 x 2.8 - 3.5 µ	Globose, smooth walled, colourless 2.5 - 3.5 µ
T. polysporum (Link ex Pers.) Rifai	Slow growing, smooth, pure white	No pigments	1.8 - 10	Formed infrequently, subglobose	In compact tufts	Short and plump 4 - 6.5 x 3 - 3.5 µ, whorl like arrangements	Ellipsoidal, colourless 2.8 - 3.7 x 1.8 - 2 µ
T. hamatum (Bon.) Bain	Slow growing, smooth, Whitish to greyish green	No pigments	2 - 9	Formed infrequently	Highly ramified , form compact tufts	4 - 6.5 x 3 - 4 µ	Smooth walled 3.8 - 6 x 2.2 - 2.8 µ
T. Koningii Oud	Rapid growth, smooth surfaced which turns hairy later, dull-dark green	No pigments	2 - 10	Intercalary chlamydospores	Much branched, in ring like zones	Nine pin sphaed 7.5 - 12 x 2.5 - 3.5 µ, in false verticils	Elliptical, smooth walled 3 - 4.8 x 1.9 - 2.8 µ
T. aureoviride Rifai	Very slow growth, smooth surfaced	No pigments	1.5 - 8	Scarce	In distinct, compact tufts	Long and slender 7 - 14 x 2 - 2.5 µ	Obovoid, smooth walled 3 - 4.3 x 2 - 3 µ
T. harzianum Rifai	Rapid growth, smooth surfaced which turns hairy later, dull green	No pigments	1.5 - 12	Intercalary or terminal	Much branched, dendroid and form loose tufts	In false verticils, short, skittle shaped 5 - 7 x 3 - 3.5 µ	Subglobose, smooth walled 2.8 - 3.2 x 2.5 - 2.8 µ
T. longibrachiatum Rifai	Rapid growth, smooth at first which turn floccose, olive green	Yellowish green, pigments secreted in some isolates	2 - 10	Numerous , terminal and intercallary	Tufted and arise in circular zones, simple branching	Bottle shaped, abruptly attenuate towards the apex 6 - 14 x 2.5 - 3 µ	Elliptical, smooth walled 3.6 - 6.5 x 2.2 - 3 µ
T. pseudokoningii Rifai	Rapid growth, smooth, greenish to bright green	Yellowish pigments secreted	1 - 10	Infrequently	Formed in distinct zones	In false whorls, skittle shaped 5.5 - 8 x 2.7 - 3.5 µ	Subcylindrical or oblong, smooth walled 3.4 - 4.6 x 2 - 2.5
T. viride Pers ex S.F. Gray	Rapid growth, smooth at first which turn hairy later	No pigments	1.5 - 12	Formed often	Compact to loose tufts, form continuous or broken ring	In false whorls 8 - 14 x 2.4 - 3 xµ	Globose or obovoid, rugose wall 3.6 - 4.5 µ

2.4.1 Mass multiplication and formulation of *Trichoderma*

For the biological control of soil borne plant pathogens it is necessary to mass produce the promising antagonists rapidly in the form of 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).

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). High numbers of mature chlamydospores may prolong the shelf life of preparations (Lewis and Papavizas, 1984). 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*. The ability of certain cations (Ca^{2+}) to form gels with aqueous solutions of sodium alginate can improve delivery technology for *Trichoderma* and *Gliocladium* (Papavizas, 1985).

Berberich (1987) reported the widespread use of biocontrol agents like *Gliocladium* and *Trichoderma* in the pellet form to achieve 75-95 per cent reduction of *R. solani*, a fungal pathogen affecting approximately 250 economically important crops. Application of wheat bran sawdust preparation of *T. harzianum* or *T. koningii* brought out an excellent control of damping off

of tomato, egg plant 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 *T.* spp. 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 *Trichoderma harzianum* biomass. Jagpal Singh *et al.* (1996) observed that *T. harzianum* (isolates MTR 35 and local), *T. reesei* (*T. longibrachiatum*), *T. viride* and *Epicoccum purpurascens* grew and sporulated abundantly on wheat bran + malt medium. Nakkeeran *et al.* (1997) standardised the storage conditions to increase the shelf life of *Trichoderma* formulations. Vermiculite bran acid FB of *T. viride* recorded the highest mean population in milky white bags (205 x 10^6 cfu/g). 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 medium. Conidia of *Gliocladium roseum* can be suspended in water + surfactant Triton X 100 or formulated as powder (Sutton *et al.*, 1997). Lewis *et al.* (1998) prepared a formulation in which commercially manufactured cellulose granules (Biodac) were mixed with a sticker and fermentor produced biomass of the isolates of *T.* spp. and *G. virens*. The chlamydospores in the biomass were activated with dilute acid before application.

Viability of isolates Th-87 and TR1-4 remained high over a 24 week period in VBA-FB stored at 5^0C. There were 10^6 and 10^7 cfu per gram of VBA-FB at that temperature. Lewis *et al.* (1991). Papavizas *et al.* (1984)

observed that less than one third of the propagules of *Trichoderma* in the fermentor biomass were viable at 15 and 20 degrees of the eight weeks. In the talc based formulation of *Trichoderma* from an initial population of 31 x 10^7 cfu per gram, there was a decline in the population to 13 x 10^7 cfu per gram in 75 days when stored at 20-30^{0}C (Nakkeeran *et al.*, 1997).

2.4.2 Development of formulation of bacterial antagonist

A talc based formulation of *Pseudomonas fluorescens* was prepared by mixing 48 hour old growth of the bacteria in King's B broth with sterilised talc @ 400 ml / kg talc along with five g of the sticker carboxy methyl cellulose (Vidhyasekaran and Muthamilan, 1995 ; Kamala, 1996). Lazzaretti and Bettiol (1997) prepared a biological product containing *Bacillus subtilis* cells (60g) and metabolites (60g) and transformed it into a WP formulation with clay (480g), surfactant (7.29 g) and water (2400 ml). The best disease control was observed in experiments with *R. solani* on rice.

Schmiedeknecht *et al.* (1997) demonstrated the control of stem canker and black scurf of potato caused by *R. solani* by different suppressive strains of *B. subtilis* in green house and field experiments. Seed potatoes treated with *B. subtilis* formulated as water soluble granules reduced black scurf up to 67 per cent. *Bacillus cereus* and *P.fluorescens* survived in peat or vermiculite/clay formulation for at least 150 days and effectively controlled *Rhizoctonia* damping off in green house trials (Gasoni *et al.*, 1998).

Krishnamurthy and Gnanamanickam (1998) conducted a study focusing on the development of an effective formulation of *Pseudomonas putida* for increased shelf life. Of the various combinations tested methyl cellulose : talc

in the ratio 1 : 4 was found to maintain the bacterium in a viable state for up to ten months.

2.5 Method of application

Different methods of application of biopesticide formulations have been tested to control diseases caused by *R. solani*. 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* (Tronsmo and Dennis, 1977). *Trichoderma hamatum* conferred suppression when mixed with a soil conducive to *R. solani* (Chet and Baker, 1980). In green house tests isolates of *Trichoderma pseudokoningii*, *T. longibrachiatum*, *T. hamatum* and *Penicillium* sp. increased emergence and decreased severity of infection by *R. solani* when pea seeds were treated with a binding substance and antagonist (Chu and Wu, 1981). Soil augmentation with wheat bran consisting of *T. viride* was found to give appreciable control of sheath blight disease while aerial application of *T. viride* helped to check the spread of the disease in rice (Gokulapalan, 1989). Application of wheat bran - saw dust preparation of *Gliocladium virens* (isolates G_1 and G_2) and *Trichoderma longibrachiatum* (isolates T_1 and T_2) to soil infested with *R. solani* brought about considerable decline in ground nut root rot. Isolate T_1 of *T. longibrachiatum* gave 65.6 per cent control of ground nut root rot (Sreenivasaprasad and Manibhushanrao, 1990). *Gliocladium virens* and *Trichoderma longibrachiatum* applied to soil as wheat bran dust preparation along with organic amendments survived when in soil and reduced the population of rice sheath blight pathogen (Baby and Manibhushanrao, 1993).

Tosi and Zazzerini (1993) suggested that there was a more antagonistic effect when fungal isolates were addded to the soil as air dried inoculum rather than as seed treatment in rust infested safflower seeds. Harman (2000) demonstrated that foliar spray of *T. harzianum* strain T-22 controlled powdery mildews on *Catharanthus* and pumpkins, *Botrytis cinerea* on strawberry and grapes.

Tschen and Kuo (1981) reported that coating of mung bean seeds with a culture of the bacterium *Bacillus megaterium* could control damping off disease affecting the crop, caused by *R. solani*. Mew and Rosales (1986) observed reduction in sheath blight disease incidence in rice when a suspension of *P. fluorescens* was sprayed on plants. Gnanamanickam and Mew (1992) reported that strains 4-15 and 7-14 of *P. fluorescens* afford 59 and 47 per cent leaf blast reduction in rice variety UPLRI-5 which received 3 sprays (500 ml/sqm/spray) with bacteria (10^8 cfu/ml) in addition to seed coated with bacterial suspension. Muthamilan (1994) and Kamala (1996) obtained control of sheath blight disease when rice seedlings were dipped in peat based and talc based inoculum respectively of *P. fluorescens*. They also demonstrated that foliar spray alone with *P. fluorescens* suppressed rice sheath blight.

2.6 Combination of biocontrol agents

Hubbard *et al.* (1983) observed that the biocontrol activity of *Trichoderma hamatum* applied to *Pythium* seed rot of peas in a New York soil was significantly reduced by indigenous populations of fluorescent pseudomonads because of competition for iron.

Fluorescent Pseudomonads and non pathogenic isolates of *Fusarium oxysporum* were effective in inducing suppressiveness to *Fusarium* wilt of cucumber when added to soil together but ineffective when added separately (Park *et al.*, 1988).

Large populations of indigenous fluorescent pseudomonads were associated with decreased populations of *Trichoderma* spp. and decreased take-all suppression in Western Australia (Simon and Sivasithamparam, 1988).

Aphanomyces root rot suppression was not significantly different when seeds were treated with a combination of *T. harzianum* and *P. fluorescens* 2-79 RN_{10} compared to treatment with *T. harzianum* alone (Dandurand and Knudsen, 1993).

Application of a mixture of biocontrol agents would more closely mimic the natural situation and might broaden the spectrum of biological activity, enhance the efficacy and reliability of control (Duffy and Weller, 1995). Realizing the advantage of combining more than one antagonist, several products containing two antagonists have been developed eg., Binab T (*T. viride* + *T. harzianum*) Antagon Combi (*T. viride* + *P. fluorescens*) (Jayarajan, 1996). Duffy *et al.* (1996) demonstrated in field trials that the combination of *Trichoderma koningii* and *Psuedomonas fluorescens* Q 29z-80 increased yields compared to Q29z-80 alone but not different from *T. koningii* alone.

2.7 Induced resistance

Induced resistance is heightened resistance in a plant towards pathogens as a result of a previous treatment with non-pathogenic organisms attenuated pathogen or a chemical that is not itself a pesticide (Deverall and Dann, 1995).

significant reduction in anthracnose lesion size in cucumber when the plants were inoculated with *Colletotrichum orbiculare* (Berk and Mont).

Symptoms of halo blight caused by *Pseudomonas syringae* pv. *phaseolicola* were markedly suppressed in bean plants associated with L forms of the pathogen established by seed inhibition (Amijee *et al.*, 1992). Seed treatment with rhizobacterial strains reduced the symptoms on cucumber plants inoculated with cucumber mosaic virus (Liu *et al.*, 1992). Systemic induced resistance against root pathogen (*Pythium aphanidermatum*) in cucumber was obtained by application of *Pseudomonas* spp. to a root system spatially separated from the pathogen inoculated root (Zhou and Paulitz, 1994).

Colonization of tobacco roots by *Pseudomonas fluorescens* strain CHAO resulted in a reduction of TNV leaf necrosis showing its ability to induce systemic resistance (Maurhofer *et al.*, 1994). The dual nature of the PGPF as inducers of resistance and growth promoters was demonstrated by Meera *et al.* (1995).

Krishnamurthy and Gnanamanickam (1997) reported the induction of systemic resistance in rice by strains of *Pseudomonas* leading to suppression of sheath blight caused by *R. solani*.

Jubina and Girija (1998) reported that *P. fluorescens* and *T. harzianum* did not have any effect on growth promotion of pepper plants.

Xylanase from *Trichoderma reesei* (*T. longibrachiatum*) induced shrinkage of cytoplasm, condensation of nucleus and finally cell death which were accompanied by typical defense response in tobacco plants (Yano *et al.*, 1998).

isonicotinic acid (INA) have been shown to induce resistance responses in a variety of plants (Kauss *et al.*, 1993; Delancy *et al.*, 1994; Conrath *et al.*, 1995). Conrath *et al.* (1995) assessed SA and chlorinated analogue (4 CSA) for their ability to control *Sclerotinia sclerotiorum* on Kiwi leaves. Chlorinated derivatives of SA have been shown to stimulate disease resistance in tobacco. Spraying of different concentrations of salicylic acid on young coconut palms were not effective in reducing symptom development by *Pestalotia palmarum* (Praveena, 1999).

2.7.2 Biotic elicitors

Induced resistance has been achieved in many plant pathogenic systems by treatment with non-pathogenic microorganisms, avirulent races of pathogens, inactivated pathogens and metabolites released by the pathogen (Schonbeck and Dehne, 1986; Kloepper *et al.*, 1992). Bacteria are well known to induce protective responses in host plants. Biological control of Dutch elm disease with *Pseudomonas* spp. was highly successful in preventive treatments (Myers and Strobel, 1983 ; Murdoch *et al.*, 1984 ; Shi and Brasier 1986 ; Scheffer *et al.*, 1989).

Systemic resistance was induced against *Pyricularia oryzae* in rice by inoculation of the first leaf with incompatible bacterial pathogen *Pseudomonas syringae* pv. *syringae* (Smith and Metraux, 1991). Induced resistance to fusarial wilt of carnation was found when roots of carnation were treated with *Pseudomonas* sp. strain WCS 417r before the stem of the plants were inoculated with *Fusarium oxysporum* f. sp. *dianthi* (Van Peer *et al.*, 1991). Wie *et al.* (1991) reported that cucumber seeds treated with certain strains of PGPR resulted in a

2.7.1 Abiotic elicitors

Certain chemicals with no direct antibiotic effect can induce resistance in plants. These include natural products such as salicylic acid (SA) (White, 1979) and synthetic immunomodulators such as 2, 6 - dichloroisonicotinic acid (INA) (Metraux *et al.*, 1991). Injection of SA into cucumber leaves or petioles was found to induce chitinase gene expression as well as peroxidase activity (Rasmussen *et al.*, 1991). Salicylic acid, a product of phenyl propanoid pathway has been implicated as a signal in systemic acquired resistance (Raskin, 1992).

An increase in the concentration of SA was reported to be associated with induced resistance in dicotyledonous plants. This compound induces resistance after exogenous application and may be an endogenous signal for induced resistance (Malamy *et al.*, 1990; Metraux *et al.*, 1991). Reduction in anthracnose lesion size, total necrotic lesion area and penetration of *Colletotrichum lagenarium* into SA treated cucumber cotyledons was reported by Rasmussen *et al.* (1991). Exogenous application of salicylic acid was also effective in inducing resistance to the downy mildew pathogen, *Pseudoperonospora cubensis* (Okuno *et al.*, 1991).

Resistance induced by the chemical SA was demonstrated in a variety of plant pathogen combinations (Malamy and Klessig, 1992; Uknes *et al.*, 1992). Exogenous application of SA increases the resistance of several plants, including cucumber and tobacco, to many types of pathogens, strengthening speculation that SA is the primary signal for induced resistance (Enyedi *et al.*, 1992). SA and chemically related derivatives including 2, 6 dichloro

2.8 Molecular and biochemical changes associated with induced systemic resistance

The number and concentration of peroxidase isoenzymes in bean hypocotyls increased in response to fungal infection by *R. solani* (Wasff *et al.*, 1984). Sub-inhibitory quantities of the bacterial metabolites like HCN or 2,4-diacetyl phloroglucinol might induce stress necessary to activate plant defense mechanism against the pathogen (Defago *et al.*, 1990). Van Peer and Schippers (1992) observed the accumulation of phytoalexins only in the stem segments of bacterized plants and concluded that cell surface components present in the lipopolysaccharides of the bacteria are inducing factors. Smith and Metraux (1991) reported that systemically acquired resistance was not associated with an increase in activities of the enzymes phenylalanine ammonialyase, peroxidase or chitinase after challenge inoculation with *Pyricularia oryzae* in plant previously treated with *Pseudomonas syringae.* pv. *syringae.* Tohamy *et al.* (1993) reported that the amount of free phenols increased in plants infected with *Sclerotium cepivorum* and *Fusarium oxysporum* f. sp. *cepae* and in plants treated with *Trichoderma harzianum* and *T. longibrachiatum* in the presence of one of the pathogens but the amount of total phenols decreased in the same treatment. Treatment with *P. fluorescens* caused increase in activities of PO isozyme and PAL in tobacco while chitinase activity was limited (Schneider and Ullrich, 1994). Chitinase and peroxidase activities were increased in both root and leaf tissue of cucumbers grown in the presence of *T. harzianum* strain 203 (Yedidia *et al.*, 1999). Howell *et al.* (2000) suggested that a major mechanism in the biological control by

Trichoderma virens of cotton seedling damping off incited by *R. solani* appears to be the induction of host resistance as indicated by peroxidase activity and terpenoid synthesis, in seedling roots by the biocontrol agent prior to attack by the pathogen.

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Isolation of the pathogen

Rhizoctonia solani Kuhn causing foliar blight of amaranthus was isolated from naturally infected amaranthus plants collected from the Instructional Farm, College of Agriculture, Vellayani. For isolation of the pathogen, portions of the leaf showing fresh typical symptoms were cut into small bits, surface sterilized with 0.1 per cent mercuric chloride and washed in three changes of sterile distilled water. They were then plated on potato dextrose agar medium (PDA) (Appendix I) in sterile petridishes and incubated at room temperature (25 ± 5^0C). On the second day fungal growth from the infected tissue was purified by the hyphal tip method and transferred to PDA slants. The isolate was maintained on PDA slants by subculturing periodically. This pure culture of the fungus was used throughout the study.

3.2 Pathogenicity test

Pathogenicity of the isolate was proved following Koch's postulates. *Rhizoctonia solani* was grown on PDA for seven days. Leaves of the susceptible variety of amaranthus, Arun, raised in earthen pots were inoculated on the lower surface with the mycelia of the pathogen grown in petri dish. Humidity was provided by placing a thin layer of moist cotton over it. The fungus was then reisolated from the leaf portions exhibiting typical disease symptoms in the artificially infected plants.

3.3 Isolation of microflora from phylloplane

Dilution plate technique (Johnson and Curl, 1972) was done for the isolation of microflora from the leaf surface. Leaf samples collected from disease free area were used for the isolation procedure. Fungi in the phylloplane were isolated by plating one ml of washing from 10^4 dilution on Martin's rosebengal agar medium (Martin, 1950) (Appendix I). Bacteria were isolated by plating one ml of washing from 10^6 dilution on soil extract agar (Appendix I) and King's B medium for fluorescent pseudomonads (Appendix I). The plates were incubated at room temperature for 48 h to 72 h. After the incubation period the microbial colonies were examined and the representative phylloplane microorganisms were maintained on their respective agar media slants for further studies. Fungal and bacterial cultures were maintained on potato dextrose agar (PDA) and nutrient agar (NA) (Appendix I) respectively. Isolates of fluorescent *Pseudomonads* spp. were specifically maintained on King's B medium.

3.4 Isolation of microflora from the rhizosphere

Soil samples were collected from the rhizosphere of amaranthus plants from disease free area. The rhizosphere microflora were obtained by dilution plate technique as mentioned under 3.3 (Johnson and Curl, 1972).

3.5 *In vitro* screening of the antagonists against *R. solani*

The fungal isolates were tested for their antagonistic effect against *R. solani* by the dual plate method outlined by Skidmore and Dickinson (1976). Agar blocks of 3 mm diameter containing seven-day-old growth of mycelia of *R. solani* and the fungi were placed 3.5 cm apart on PDA in a petridish and

incubated at room temperature for five days. Four replications were maintained for each treatment. The paired cultures were examined at regular intervals and the nature of the reaction of the antagonist on the pathogen was studied. Control plates without the inoculation of any antagonist was also simultaneously maintained.

The bacterial isolates were tested for their antagonistic effect against *R. solani* by the dual plate method (Utkhede and Rahe, 1983). Nutrient agar (NA) medium was allowed to solidify for one hour in sterilized petridishes. Then each bacterial isolate was streaked at one end of the petridish 2 cm from the edge over NA 24 h prior to pathogen inoculation. Just opposite to the bacterial streak 3.5 cm away, a 3 mm disc of the pathogen was placed. Four replications were maintained. A control was also maintained. The inhibition zone was measured after 48 hr. For testing the antagonism of fluorescent *Pseudomonas* sp. King's medium B (KMB) (King *et al.*, 1954) was used instead of nutrient agar.

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).

3.6 Mycoparasitism of the selected fungal antagonist on *R. solani*

To study the mechanism of mycoparasitism of the fungal antagonist on *R. solani*, a dual culture technique of Dennis and Webster (1971) was used. In 90 mm sterile petridishes, sterile PDA was poured 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 five mm diameter containing the mycelium of *R. solani* taken form an actively growing culture of the fungus was placed on one end of the petridish and five mm agar disc of the test fungus was placed two cm away from it. The plates were incubated at room temperature for seven days. Direct observations were carried out after incubation period under a light microscope at the zone of hyphal contact. Microscopic observation for hyphal interaction was also made by cutting out one sq.cm portions of cellophane containing intermingling hyphal growth and mounting in cotton blue lactophenol. Photomicrographs of different mechanisms of mycoparasitism exhibited by the antagonist of *R. solani* were prepared.

3.7 Characterisation of fungal isolates

The fungal isolates obtained form the phylloplane and rhizosphere of amaranthus plants were grown on PDA in petridishes and incubated at room temperature for 10 - 14 days. Observations were made on colony diameter, colony colour, pigmentation and conidial dimensions.

3.7.1 Characterisation of the selected fungal isolate showing maximum inhibition of *R. solani*.

The fungus was grown on PDA and the colony characters were observed. The characters were studied in detail using slide cultures (Riddel, 1950). Observations were made on hyphal width, conidiophore width, phialide dimension, spore size and colour.

3.8 Characterisation of the bacterial isolates

Preliminary tests conducted for the identification of the bacterial isolates, which were tested for their biocontrol efficacy against *R. solani* included.

3.8.1 Gram's staining

The isolates were stained for Gram's reaction.

3.8.2 Colony characteristics

Colony characteristics were studied by plating bacteria on nutrient agar (NA). The bacterial cultures were streaked on NA poured in petridishes and incubated at room temperature. After 24 h observations were made on shape, elevation and margin of colonies.

3.9.1 Preparation of talc based formulation of fungal antagonist

The fungal isolate producing maximum inhibition of the mycelial growth of *R. solani* was further mass multiplied. The fermentation biomass (FB) of the selected fungal isolate (*Trichoderma longibrachiatum*) was prepared by a slightly modified liquid fermentation process of Papavizas *et al.(* 1984). Stock culture of *Trichoderma longibrachiatum* was maintained on PDA. Discs of 3 mm diameter was inoculated into 500 ml portions of potato dextrose broth in one litre flasks allowing it to remain submerged. The flasks were shaken on a rotary shaker for 15 days at room temperature. At the end of incubation period the FB was separated from the aqueous broth by filtering through a muslin cloth. Fungal mats obtained were pressed to remove water and air dried. It was then dried under sun for half an hour and then powdered in a mixer grinder. The resulting powder was mixed with sterilized talc in polypropylene bags @ 10 per cent w/w. One per cent carboxy methyl cellulose (CMC) was added to this. The population of the fungus in the product was estimated at monthly intervals.

3.9.2 Preparation of talc based formulation of bacterial antagonist

Talc based formulation of the selected bacterial isolate was prepared by following the method of Vidhyasekaran and Muthamilan (1995). The bacterial isolate (P1) was multiplied in King's Broth (King *et al.*, 1954) which was previously autoclaved at 1.4 kg per cm^2 for 20 min. A loopful of the bacterium (P1) was inoculated into the broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature.

Hundred grams of talc powder was taken in each polypropylene bag. One gram of CMC was added to this, sealed and autoclaved at 1.4 kg/cm^2 for two hours. 40 ml of 48 hr grown inoculum was added, mixed under aseptic conditions and the polypropylene bag was sealed and stored at room temperature. The population of the antagonist was periodically estimated by dilution plate method.

3.10 Assessing the efficacy of different methods of application of fungal and bacterial antagonists

Efficacy of talc-based formulation of the selected fungal and bacterial antagonist in suppressing foliar blight disease of amaranthus was tested in a pot culture experiment by different methods of application, viz., soil application, seedling root dip and foliar spray.

3.10.1 Soil application

Talc based formulation of the fungal and bacterial antagonists were applied to the soil at the rate of 30 g per two kg soil taken in pots of diameter

20 cm . The formulation was mixed well with soil before transplanting the seedlings of the susceptible amaranthus variety 'Arun'.

3.10.2 Seedling root dip

Amaranthus seedlings were separately dipped in 0.5 per cent of aqueous suspension of the talc based formulation of fungal and bacterial antagonists at the time of transplanting. The seedlings were dipped for half an hour to ensure that the roots are completely immersed in inoculum and planted in earthen pots.

3.10.3 Foliar spray

One per cent aqueous suspension of the formulated product was prepared and sprayed three times at 15 days interval starting from five days prior to pathogen inoculation (The pathogen was inoculated 10 days after transplanting).

The experiment was laid out in completely randomised design (CRD) maintaining three replication for each treatment. The following treatments were included.

T_1 - Soil application
T_2 - Seedling root dip
T_3 - Foliar spray
T_4 - Soil application + seedling root dip
T_5 - Soil application + foliar spray
T_6 - Seedling root dip + foliar spray
T_7 - Soil application + seedling root dip + foliar spray
T_8 - Mancozeb (Indofil M-45, 0.4 per cent)
T_9 - Untreated check

The pathogen *R. solani* was inoculated 10 days after transplanting the seedlings. Plants were scored for disease severity based on the 0-9 scale (KAU, 1996) (Plate 1).

Grade	Description
0	No infection
1	1 - 10 per cent of leaf area infected
3	11 - 25 per cent of leaf area infected
5	26 - 50 per cent of leaf area infected
7	51 - 75 per cent of leaf area infected
9	>76 per cent of leaf area infected

Percentage disease index was calculated using the formula suggested by Mayee and Datar (1986).

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

3.11 Testing the efficacy of the formulation of fungal and bacterial antagonists in combination with an abiotic elicitor

The efficient method of application of the formulations of fungal and bacterial antagonists were tested separately and in combination with the abiotic elicitor of defense response, viz., salicylic acid. 2 mM solution of salicylic acid was used for spraying. The experiment was laid out in CRD maintaining three replication for each treatment.

The treatments tested were as follows.

T_1 - Fungal antagonist formulation by the best method of application

T_2 - Bacterial antagonist formulation by the best method of application

T_3 - Fungal + bacterial formulation by the best method of application

Plate 1 Symptoms of amaranthus foliar blight based on 0-9 scale (KAU, 1996)


0
1
3
5
7
9

T_4 - Foliar spray with salicylic acid (2 mM) five days prior to pathogen inoculation

T_5 - Salicylic acid + fungal formulation

T_6 - Salicylic acid + bacterial formulation

T_7 - Salicylic acid + fungal + bacterial formulation

T_8 - Mancozeb (Indofil M-45, 0.4 per cent)

T_9 - Untreated check

The pathogen *R. solani* was inoculated 10 days after transplanting. Plants were scored for disease severity based on 0-9 scale (KAU, 1996). Percentage disease index was calculated as mentioned under 3.11.3.

The treatment which recorded the lowest disease incidence was tested further under field conditions.

3.12 Mass multiplication of *Trichoderma longibrachiatum* in bran for field application

500 g of rice bran was mixed with 250 ml of water and autoclaved in polypropylene bag for 2 hr. Five grams of talc based formulation of *T. longibrachiatum* was added to the bran and incubated at room temperature. After three days the entire bran was covered with white mycelial growth of *T. longibrachiatum*. It was mixed well with bran and applied in the field @ 30 g/plant.

3.13 Testing the efficacy of the microbial formulation in field trial

Field trial was conducted during April-June at the Instrucitonal Farm, College of Agriculture, Vellayani. The trial was laid out in Randomised Block Design (RBD) with seven replications maintaining a plot size of 6 x 1 m^2. *Rhizoctonia* blight susceptible variety 'Arun' was used in the experiment (Plate 2). Three treatments tested in the field were as follows.

Plate 2 View of the experimental plot


Management of foliar blight of
amaranthus (Amaranthus tricolor L.)
caused by Rhizoctonia solani Kühn
using microbial antagonists
DESIGN HERE REPLICATION 7 PRETTY SIGN

T_1 - Soil application + foliar spray of *Trichoderma longibrachiatum*.

T_2 - Treated check using mancozeb (Indofil M-45, 0.4 per cent)

T_3 - Untreated check (only pathogen inoculation)

The pathogen was inoculated 30 days after sowing. Observations on disease intensity, yield in each harvest, number of leaves per plant, root and shoot length and weight of leaves per plant were recorded.

3.14 Survival of *Trichoderma longibrachiatum* in the phylloplane

To study the survival of *Trichoderma longibrachiatum* in the phyllosphere of amaranthus plants, 30-day-old amaranthus plants were sprayed with one per cent suspension of the formulated product. Leaf samples were taken at 1, 7, 14 and 21 days after spraying and survival of *Trichoderma longibrachiatum* was estimated. One gram of leaf sample was taken and serial dilutions were prepared. One ml of aliquot was transferred from the dilutions (10^{-3} and 10^{-4}) to sterilized pertridishes over which 15 ml of melted and cooled PDA supplemented with streptomycin sulphate was poured and incubated for 48 hr. Typical colonies of *Trichoderma longibrachiatum* which appeared were counted.

3.15 Induction of defense mechanism after application of *Trichoderma longibrachiatum*

Trichoderma longibrachiatum multiplied in bran was applied to soil (30 g / 2 kg) and foliar spray with one per cent talc based product of *T. longibrachiatum* was given on susceptible amaranthus cultivar Arun as described in previous experiments. Treated check using mancozeb (Indofil M-45, 0.4 per cent) and untreated check were also maintained. Pathogen was inoculated five days after treatment. Leaf samples were collected at 1, 3 and 5 days after inoculation and analysed for the changes in the activity of enzymes viz., phenyl alanine ammonia-lyase, peroxidase and total phenolic content.

3.15.1 Estimation of phenylalanine ammonia lyase (PAL)

PAL activity was analysed based on the procedure described by Dickerson *et al.* (1984). The enzyme extract was prepared by homogenising 1 g leaf sample in 5 ml 0.1 M sodium borate buffer (pH 8.7) (Appendix II) containing a 0.05 g of polyvinyl pyrrolidone using chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min. at 4°C. The supernatent was used for the assay of PAL activity. The reaction mixture contained 3 ml of 0.1 M sodium borate buffer pH 8.7, 0.2 ml enzyme extract and 0.1 ml of 12 mM L-phenyl alanine prepared in the same buffer. The blank contained 3 ml of 0.1 M sodium borate buffer pH 8.7 and 0.2 ml enzyme extract. The reaction mixtures and blank was incubated at 40°C for 30 min. and reaction was stopped by adding 0.2 ml of 3 N HCl. The absorbance was read at 290 nM in a spectrophotometer.

PAL activity was expressed as microgram of cinnamic acid produced/min/g on fresh weight basis.

3.15.2 Estimation of peroxidase (PO)

PO activity was determined according to the procedure described by Srivastava (1987).

Leaf sample of 200 mg was homogenised in one ml of 0.1 M sodium phosphate buffer (pH 6.5) (Appendix II) to which a 0.05 g of polyvinyl pyrrolidone (PVP) was added. The homogenisation was done at 4°C using a pestle and mortar. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 min. at 4°C. The supernatent was used as the

enzyme extract for the assay of PO activity. The reaction mixture consisting of 1 ml of 0.05 M pyrogallol and 50 µl enzyme extract was taken in both reference and sample cuvettes, mixed and kept in a spectrophotometer and the reading was adjusted to zero at 420 nm. The enzyme reaction was started by adding 1 ml of 1 per cent hydrogen peroxide (H_2O_2) into sample cuvettes and the change in absorbance was measured at 30s interval.

3.15.3 Estimation of total phenol

The phenol content was estimated following the procedure described by Bray and Thorpe (1954). One gram of leaf sample was ground in 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min, supernatent was saved and residue was extracted with five times the volume of 80 per cent ethanol and centrifuged. The supernatent was saved and evaporated to dryness. The residue was dissolved in 5 ml distilled water. An aliquat of 0.3 ml was pipetted out and made upto 3 ml with distilled water. Folin-Ciocalteau reagent (0.5 µl) was added and 2 µl of 20 per cent $Na_2 CO_3$ solution was added to each tube after 3 min. This was mixed thoroughly and kept in boiling water for 1 min. This was cooled and absorbance was measured at 650 nm against reagent blank. Standard curve was prepared using different concentrations of catechol and expressed in catechol equivalents as microgram per gram leaf tissue on fresh weight basis.

3.16 Statistical analysis

The data generated from the experiments were subjected to analysis of variance (ANOVA), after appropriate transformations wherever needed.

RESULTS

RESULTS

4.1 Isolation of the pathogen and pathogenicity test

Symptoms of the disease in the green house and field began as small irregular whitish cream spots on the foliage which enlarged especially under high percentage of relative humidity. Gradually the spots became translucent with irregular margins (Plate 3). Severely infected leaves showed shot-hole symptoms finally leading to defoliation. On the undersurface of the leaves powdery masses of the hymenial layer of *Thanatephorus cucumeris* was observed (Plate 4). Infection was restricted to the foliage.

The foliar blight pathogen *Rhizoctonia solani* was isolated and purified from naturally infected amaranthus plants collected from Instructional Farm, College of Agriculture, Vellayani. The pathogen was identified as *R. solani* following microscopic examination of morphological characters. The isolates of the fungus were similar in morphology and had light brown mycelium which produced few or no sclerotia on PDA (Plate 5). Growth range averaged 8 mm of radial growth per day at 28 $\pm$ 2^{0}C. Microscopic examination of the diseased tissue revealed the presence of both the anaphase (*R. solani*) and the teleophase (*T. cucumeris*) (Plates 6 and 7) of the fungus. Hyphal thickness measured 6.2 to 15.5 µm. The basidia measured 9.3 to 15.5 x 6.2 to 9.3 µm and the basidiospores measured 6.2 to 9.3 µ x 6.2 to 7.8 µm in size. The spores were borne on sterigmata measuring 3.1 to 7.08 µ x 2.3 to 3.1 µm. Three to four sterigmata were produced on each basidium.

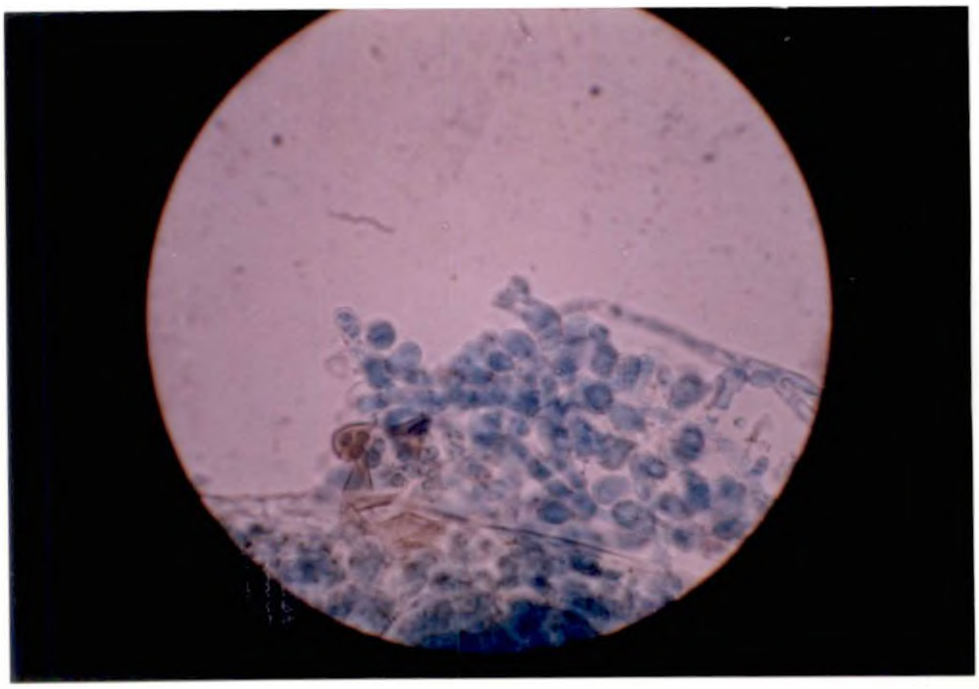
Plate 3 Symptoms of foliar blight disease on amaranthus leaves

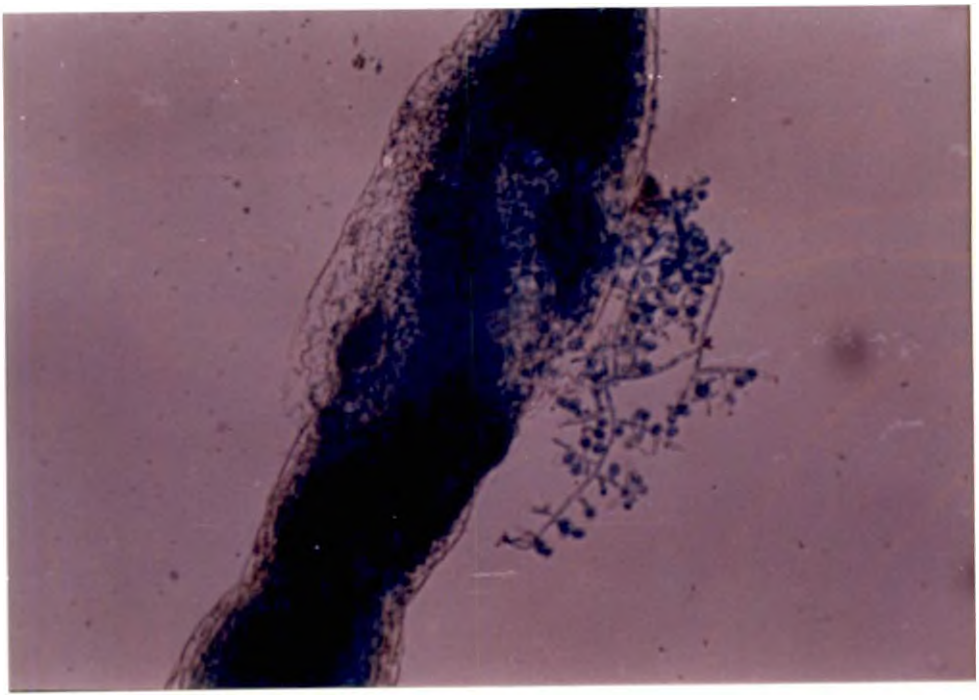
Plate 4 Hymenial layer of *Thanatephorus cucumeris* on the under surface of the leaf

Plate 5 Culture of *Rhizoctonia solani* inciting foliar blight of amaranthus

Plate 6 C.S. of infected leaf showing the perfect stage (*T. cucumeris*) of the pathogen

Plate 7 Basidia and basidiospores of *Thanatephorus cucumeris*





The pathogenicity of the isolate was proved following Koch's postulates. The isolate induced severe symptom when inoculated on the susceptible variety of amaranthus.

4.2 Isolation and identification of microflora from phylloplane and rhizosphere of amaranthus

The phylloplane and rhizosphere microflora were isolated by dilution plate technique and maintained on respective agar media slants. The fungal colony isolated and identified are described in Table 1 a.

Table 1 a Characters of fungal isolates

Fungi	Colony diameter (cm) in 3 days	Colony colour	Conidial character
Trichoderma harzianum Rifai	8	Dull green	Subglobose or ovoid, smooth surfaced, pale green, 3.25 x 2.6 µ
Trichoderma longibrachiatum Rifai	9	Olive green	Ellipsoidal to subcylindrical, smooth surfaced, pale green 4.62 - 5.5 x 2-3.08 µ
Aspergillus niger Van Teighem	2.5	Black	Conidia irregularly roughened
Aspergillus aculeatus Iizuka	2.5	Brownish black	Globose and Echinulate
Aspergillus wenti Wehmer	1.5	Dull-yellowish green	Ellipsoidal 6.6 to 8.25 µ
Fusarium oxysporum Schlecht	3	White with violet tinge	Macroconidia 5 septate, 13-21 x 3.25-4 µ. Microconidia – ellipsoidal 5 x 2.5 µ

The bacteria isolated and characterised are given in Table 1b.

Table 1b Characters of bacterial isolates on nutrient agar

Bacterial isolate	Colony characteristics			Cell morphology	Gram reaction
	Form	Elevation	Margin		
P_4	Rhizoid	Flat	Undulate	Rod	Negative
A	Circular	Flat	Entire	Rod	Negative
S	Irregular	Raised	Curled	Rod	Negative
L	Circular	Flat	Entire	Rod	Negative
C	Circular	Convex	Entire	Rod	Negative
P_1	Rhizoid	Raised	Undulate	Rod	Negative

4.3 *In vitro* screening of the isolates against *R. solani*

4.3.1 Fungal isolates

When the fungal isolates were paired with *R. solani*, some of the fungi were found to have a cessation of growth at the point of contact with the test organism. Eg., *Aspergillus* spp. *Fusarium* sp intermingled freely with *R. solani* and grew together. The fungi which emerged as potential antagonists of *R. solani* caused a clear zone of inhibition between the paired cultures. This included two isolates of *Trichoderma* viz. isolate A and B (Plate 8). These two isolates of *Trichoderma* spp. completely over grew and parasitized *R. solani* after seven days. *Trichoderma* sp. isolate B obtained from rhizosphere of amaranthus showed maximum inhibition of the mycelial growth of *R. solani* (Table 2).

Plate 8 Inhibition of *R. solani* by *Trichoderma* spp.

1. *R. solani*
2. *R. solani* x *Trichoderma* isolate B
3. *R. solani* x *Trichoderma* isolate A

Plate 9 Inhibition of *R. solani* by fluorescent *Pseudomonas* sp. P 1

1. *R. solani*
2. *R. solani* x fluorescent *Pseudomonas* sp. P 1


1
2
3

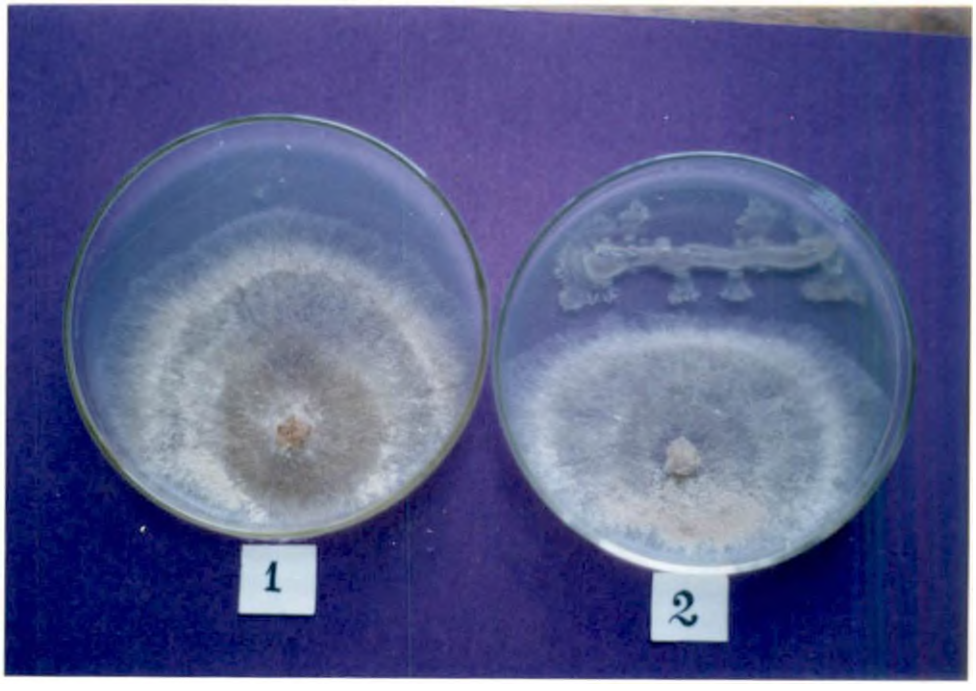

1
2

Table 2 Efficacy of fungal isolates in inhibiting *R. solani in vitro*

Isolate	Percentage inhibition of mycelial growth at 48 h *
Trichoderma harzianum (Isolate A)	40.78 (6.83)
T. longibanchiatum (Isolate B)	50.86 (7.13)
Aspergillus niger (Isolate A1)	12.72 (3.52)
Aspergillus aculeatus (Isolate A2)	7.86 (2.80)
Aspergillus wenti (Isolate A3)	22.42 (4.74)
Fusarium oxysporum (Isolate F)	14.49 (3.81)
CD (5 %)	1.7

* Mean of four replications

Table 3 Efficacy of bacterial isolates in inhibiting *R. solani in vitro*

Isolates	Inhibition zone at 48 h (mm) *
P_4	13.69 (3.70)
A	9.59 (3.09)
S	12.24 (3.49)
L	13.29 (3.73)
C	14.70 (3.83)
P_1	28.18 (5.31)
CD (5%)	0.44

* Mean of four replications

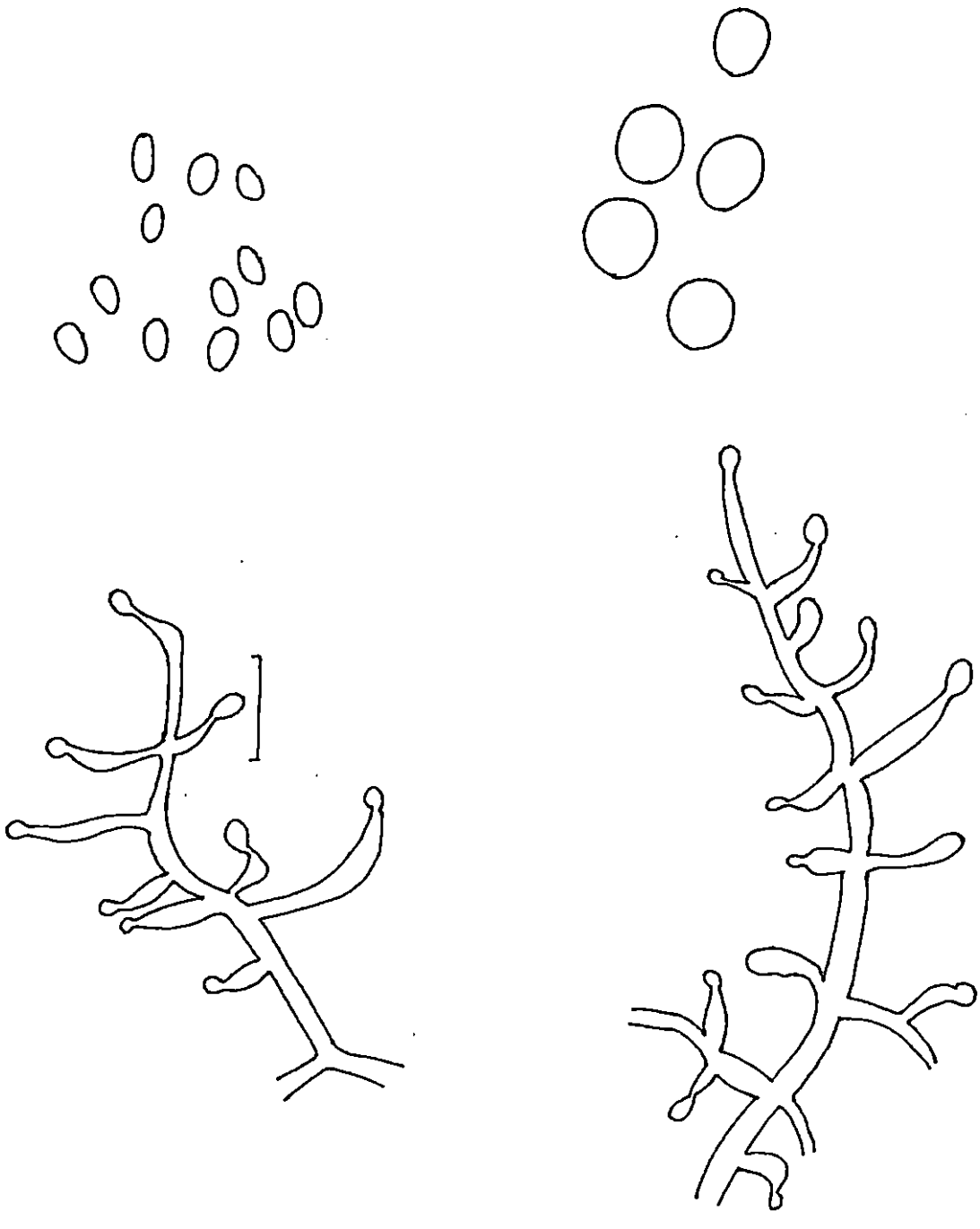
Figures in parantheses are $\sqrt{x}$ transformed values

4.3.2 Bacterial isolates

The bacterial isolate P_1 (fluorescent *Pseudomonas* sp.) obtained from the rhizosphere of amaranthus showed maximum inhibition of *R. solani* (Plate 9). Different isolates showed different degrees of antagonism towards *R. solani in vitro* (Table 3). The isolates P_4, L, S and C showed a clear zone of inhibition demarcating the growth of test fungus and the bacteria. The isolate A was the least effective in inhibiting the pathogen.

4.4 Characterisation of the selected *Trichoderma* sp. (isolate B)

The colonies grew very rapidly reaching a diameter of 3 cm in 24 hr. The colonies appear smooth at first, translucent or watery white, later becoming floccose, especially towards the margin of the petridish (Plate 10). The conidial areas gradually change their colouration from whitish green to light olive green. Pigments are secreted into the medium so that the reverse of the colony have lemon yellow colour (Plate 11). Mycelium is septate, smooth walled, hyaline, 4-9 μ in diameter. Numerous terminal chlamydospores are formed together with intercalary ones which remain submerged in the medium. They are globose or broadly ellipsoidal, smooth-walled, hyaline and measure 4.65 - 6.75 μ. Conidiophore branching is simple (Plate 12). The phialides are mostly formed singly, alternately or irregularity along the main branch. The phialides are 7.7 - 12.3 μ x 1.54 - 2.31μ, bottle shaped abruptly attenuate towards short conical apices. The phialospores are 4.62 - 5.5 x 2 - 3.08 μ, elliptical or ellipsoidal to subcylindrical, smooth walled and pale green (Fig.1). On the basis of the above observed characters the isolate B was identified as *Trichoderma longibrachiatum* Rifai.



Trichoderma longibrachiatum

FIG. 1

Plate 10 Culture of *T. longibrachiatum*

Plate 11 Pigment secretion by *T. longibrachiatum*

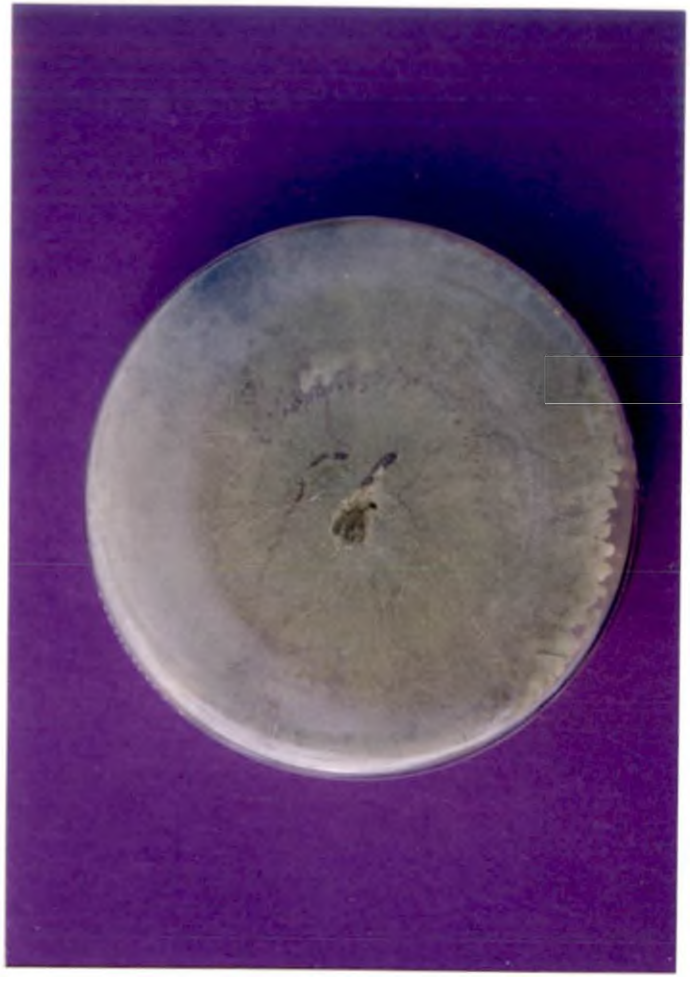


Plate 12 Photomicrograph of *T. longibrachiatum*

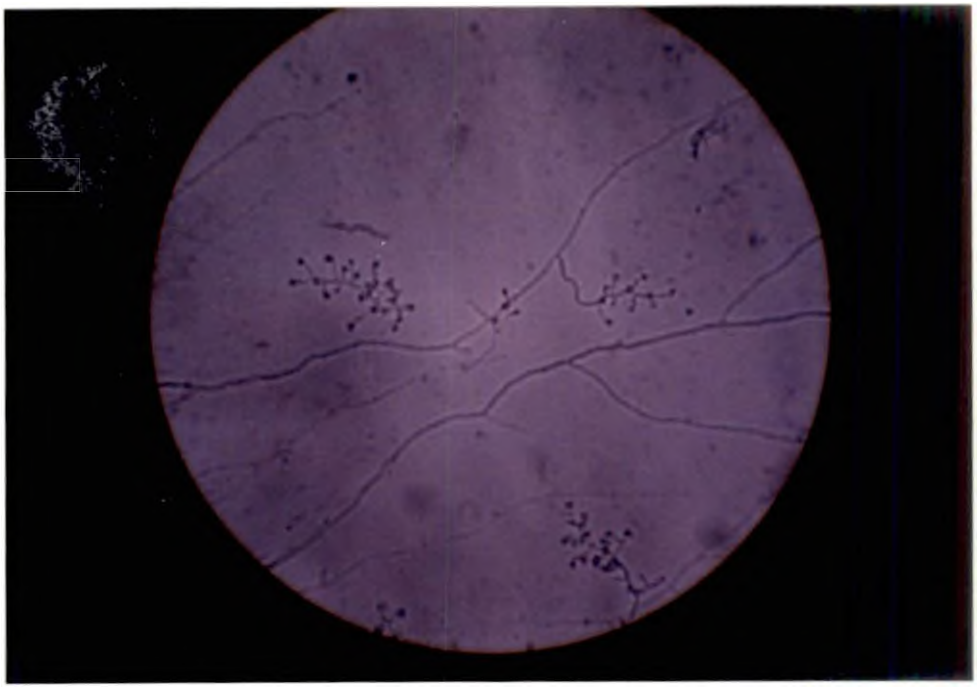
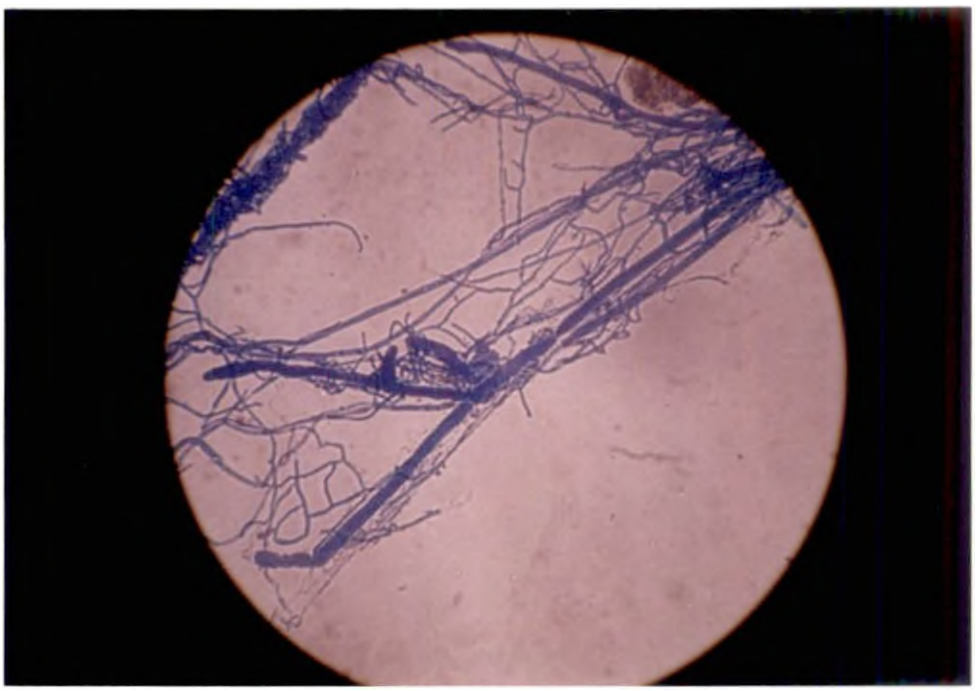


Plate 13 Mycoparasitism of *R. solani* by *T. longibrachiatum*

a) Disintegration

b) Coiling



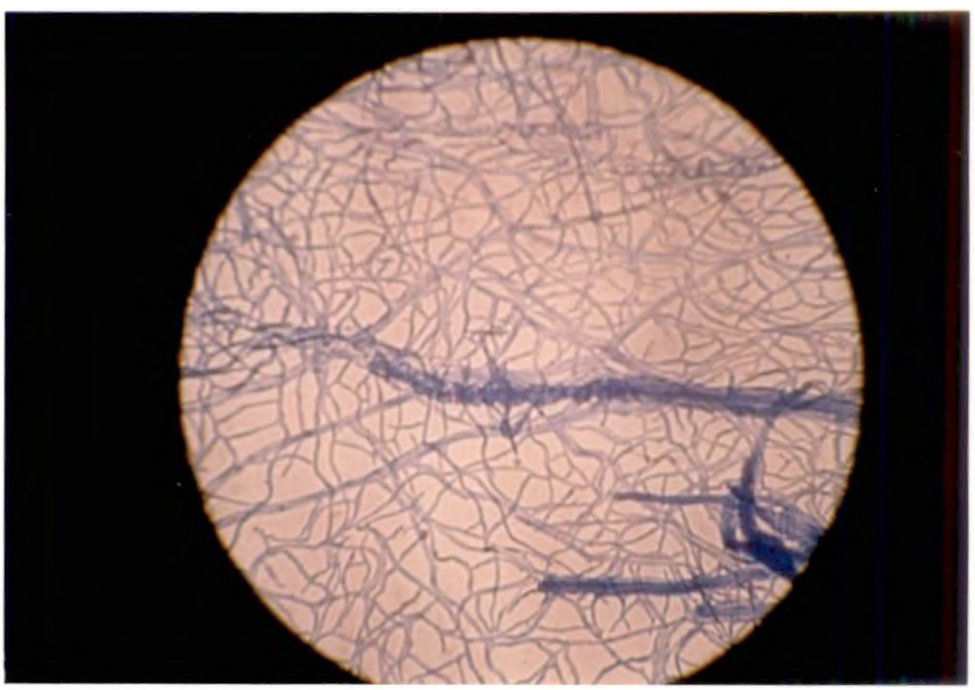
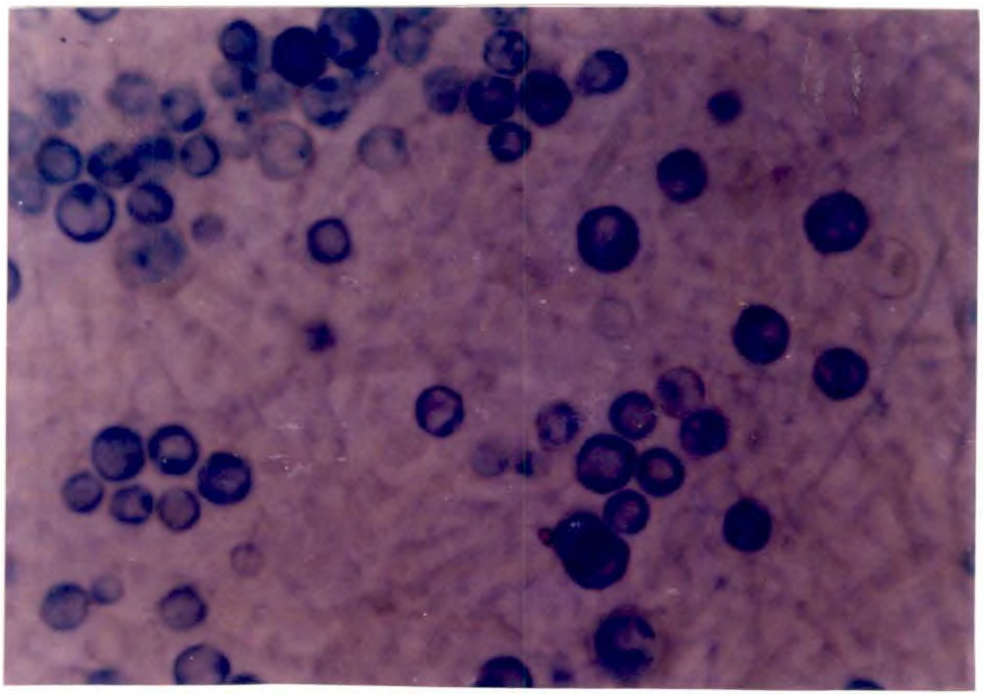


Plate 14 Chlamydospores of *T. longibrachiatum*



4.5 Mycoparasitism of *Trichoderma longibrachiatum* on *R. solani*

T. longibrachiatum proved to be an efficient parasite of *R. solani*. The fungus was found to cause excessive granulation, vacuolation and finally disintegration of host hyphae (Plate 13a). It was aso found to coil around and penetrate host hyphae leading to its disintegration and death (Plate 13b.).

4.6 Preparation of talc based formulation of the selected antagonist

4.6.1 Fungal antagonist

The fungal isolate showing greatest inhibition of *R. solani* viz., *Trichoderma longibrachiatum*, was mass multiplied by modified liquid fermentation technology in one litre conical flasks. 15 days of the fungal growth in 500 ml of potato dextrose broth produced one gm dry mat which when mixed with talc @ 10 per cent w/w contained 10^7 colony forming units per gram. The fermentation biomass consisted of mycelial fragments, immature chlamydospores and mature chlamydospores (Plate 14). After three days of incubation few chlamydospores were formed. At six and 10 days, about half of the spores were mature chlamydospores and at 15 days about 75 per cent of the spores were mature chlamydospores.

Viability of the chlamydospores in the talc based formulation stored at room temperature was reduced with length of incubation. After four weeks of storage 15 per cent of the propagules lost viability and after 15 weeks 32 per cent of the propagules lost viability (Table 4a, Fig. 2). In subsequent studies one to two month old formulations were used.

Table 4a Survival of *Trichoderma longibrachiatum* in talc based formulation

Days	cfu/g (x 10^6)
30	47
60	40
90	33
120	32

Table 4b Survival of fluorescent *Pseudomonas* sp. P 1 in talc based formulation

Days	cfu/g (x 10^{10})
30	9.46
60	1.46
90	1.43
120	1.20
150	0.90

Fig. 2 Survival of *T. longibrachiatum* in talc based formulation

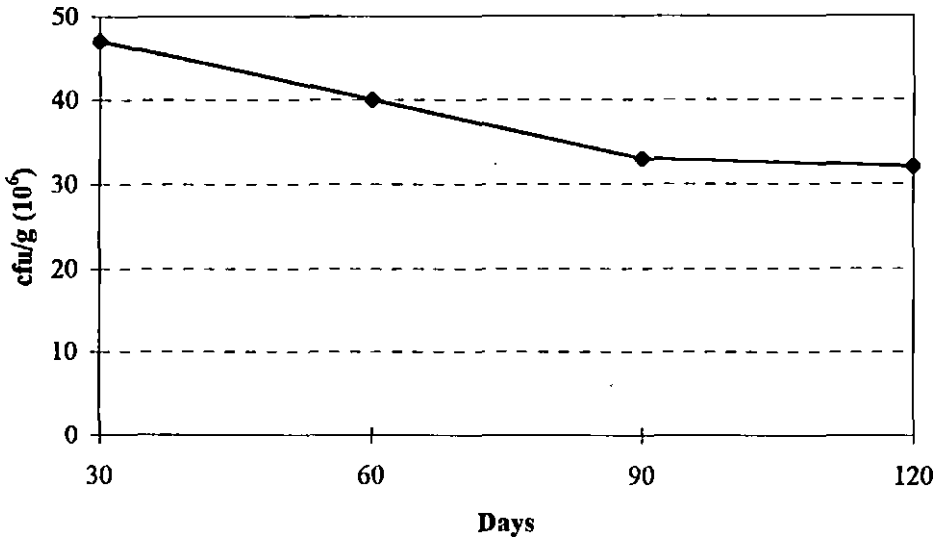
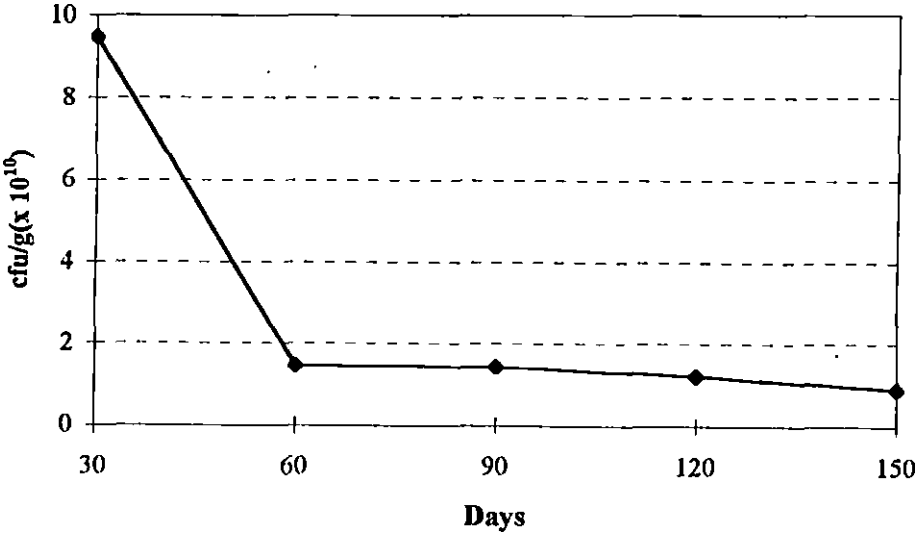


Fig. 3 Survival of fluorescent *Pseudomonas* sp. P1 in talc based formulation



4.6.2 Bacterial antagonist

A talc based formulation of the most efficient bacterial isolate, viz., P_1 (fluorescent *Pseudomonas* sp) was prepared. One gram of the formulation contained 9.46×10^9 cfu. After four weeks the number of cfu/g was reduced to 1.46×10^{10} when stored at room temperature. This remained stable upto a period of 16 weeks (Table 4b, Fig. 3). At 20 weeks the number of cfu/g was reduced to 9×10^9.

In all the studies freshly prepared formulation was used.

4.7 Assessing the efficacy of the method of application of the formulation

4.7.1 Fungal antagonist

Among the different methods of application tested, seedling root dip followed by foliar application was the most effective in reducing the disease intensity. The combinations soil application followed by foliar spray and soil application plus seedling root dip were also almost as effective as seedling root dip followed by foliar spray. A similar reduction in disease intensity was also obtained in the combined application of seedling root dip and soil application followed by foliar spray. Mere soil application or seedling root dip were not effective compared to other methods of application. Foliar spray with mancozeb (0.4 per cent) was as effective as soil application with *T. longibrachiatum* in checking the disease intensity (Table 5, Fig. 4).

4.7.2 Bacterial antagonist

Mere seedling root dip in the talc based formulation of fluorescent *Pseudomonas* sp. (P1) was as effective as seedling root dip followed by foliar

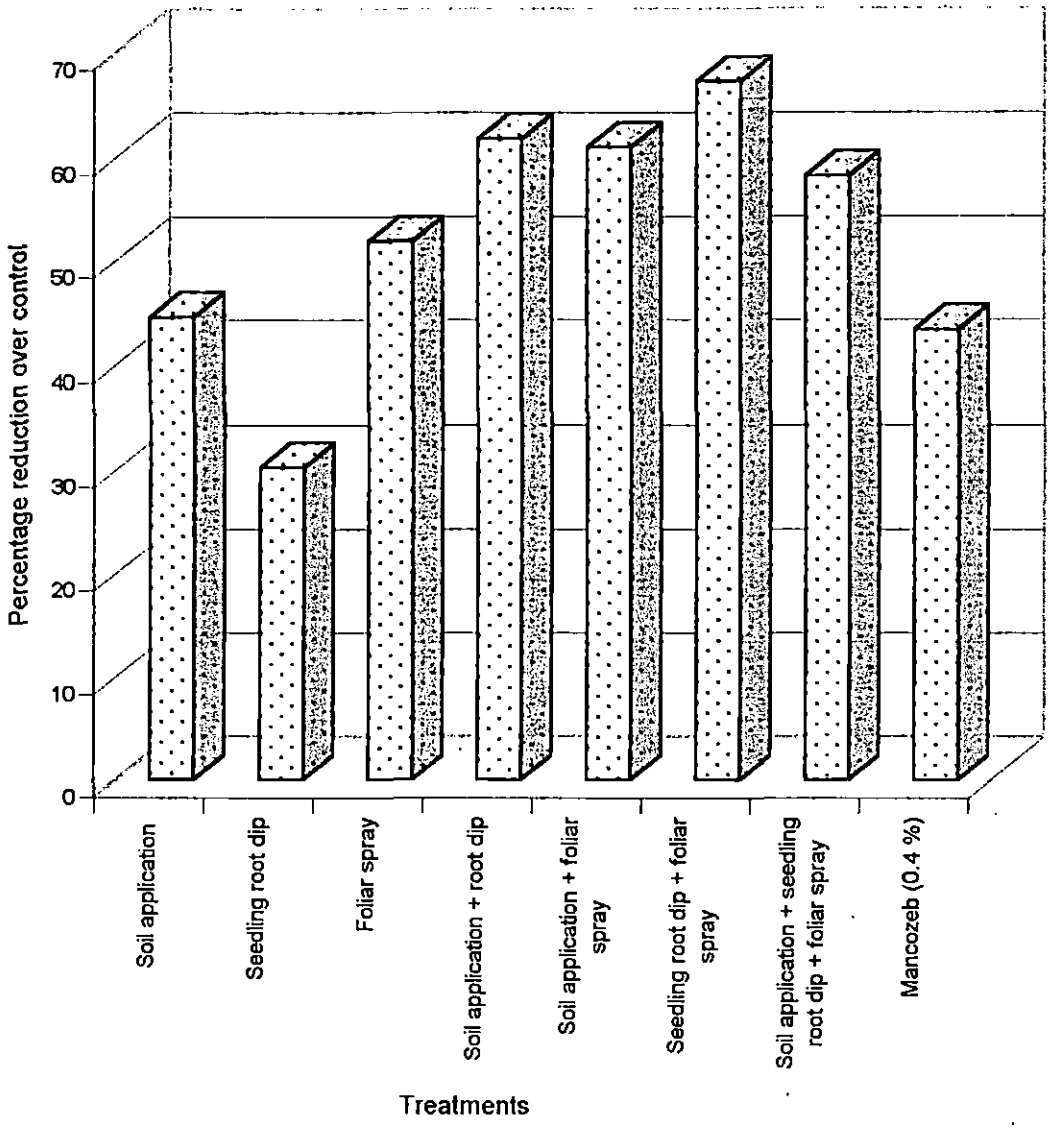
Table 5 Efficacy of different methods of application of talc based formulation of *Trichoderma longibrachiatum* on foliar blight disease intensity in amaranthus

Treatment	Percentage disease index *
Soil application	7.17 (2.68)
Seedling root tip	9.02 (3.00)
Foliar spray	6.22 (2.49)
Soil application + root dip	4.95 (2.23)
Soil application + foliar spray	5.06 (2.25)
Seedling root dip + foliar spray	4.24 (2.06)
Soil application +seedling root dip + foliar spray	5.41 (2.33)
Treated check using Mancozeb (0.4 %)	7.33 (2.71)
Untreated check	12.91 (3.59)
CD (5 %)	0.53

Figures in parantheses are $\sqrt{x}$ transformed values

* Mean of three replications

Fig. 4 Efficacy of methods of application of talc based formulation of *Trichoderma longibrachiaturm* on foliar blight disease intensity



spray with the product in reducing the disease severity. Soil application followed by foliar spray, mere foliar spray and a combination of all the three methods of application were also found to be effective in checking the disease intensity. Mere soil application with P_1 product or a combination of soil application and seedling root dip was not effective in checking the disease. Foliar spray with P_1 product was as effective as foliar spray with mancozeb (Table 6, Fig. 5).

Incidence of disease in plants treated with the formulation by soil application followed by foliar spray was almost as effective as seedling root dip followed by foliar spray. As amaranthus is a seldom transplanted crop the former treatment was choosen for subsequent studies.

4.8 Testing the efficacy of fungal and bacterial antagonist in combination with an abiotic elicitor, viz., salicylic acid

The selected efficient method of application, viz., soil application followed by foliar spray, with the fungal and bacterial antagonist was tested separately and in combination with the abiotic elicitor salicylic acid. Maximum control of the disease was observed when talc based formulation of *T. longibrachiatum* was applied to soil followed by foliar spray. Combined application of the elicitor separately with *T. longibrachiatum* and fluorescent *Pseudomonas* sp. and with a mixture of formulations of *Trichoderma* and fluorescent *Pseudomonas* sp. were also effective. Though mere application of fluorescent *Pseudomonas* sp. was effective in reducing the disease a combination of fluorescent *Pseudomonas* sp. and *T. longibrachiatum* formulation without SA was ineffective. The elicitor alone also was not effective in reducing the disease intensity (Table 7, Fig. 6).

Table 6 Efficacy of different methods of application of talc based formulation of bacterial antagonist on foliar blight disease intensity in amaranthus

Treatment	Percentage disease index *
Soil application	7.68 (2.77)
Seedling root dip	5.41 (2.33)
Foliar spray	5.89 (2.43)
Soil application + seedling root dip	9.01 (3.00)
Soil application + foliar spray	5.77 (2.40)
Seedling root dip + foliar spray	4.18 (2.05)
Soil application +seedling root dip + foliar spray	5.82 (2.41)
Treated check using Mancozeb (0.4 %)	7.33 (2.71)
Untreated check	12.91 (3.59)
CD (5 %)	0.32

Figures in parantheses are $\sqrt{x}$ transformed values

* Mean of three replications

Fig. 5 Efficacy of methods of application of talc based formulation of fluorescent *Pseudomonas* sp. on foliar blight disease intensity

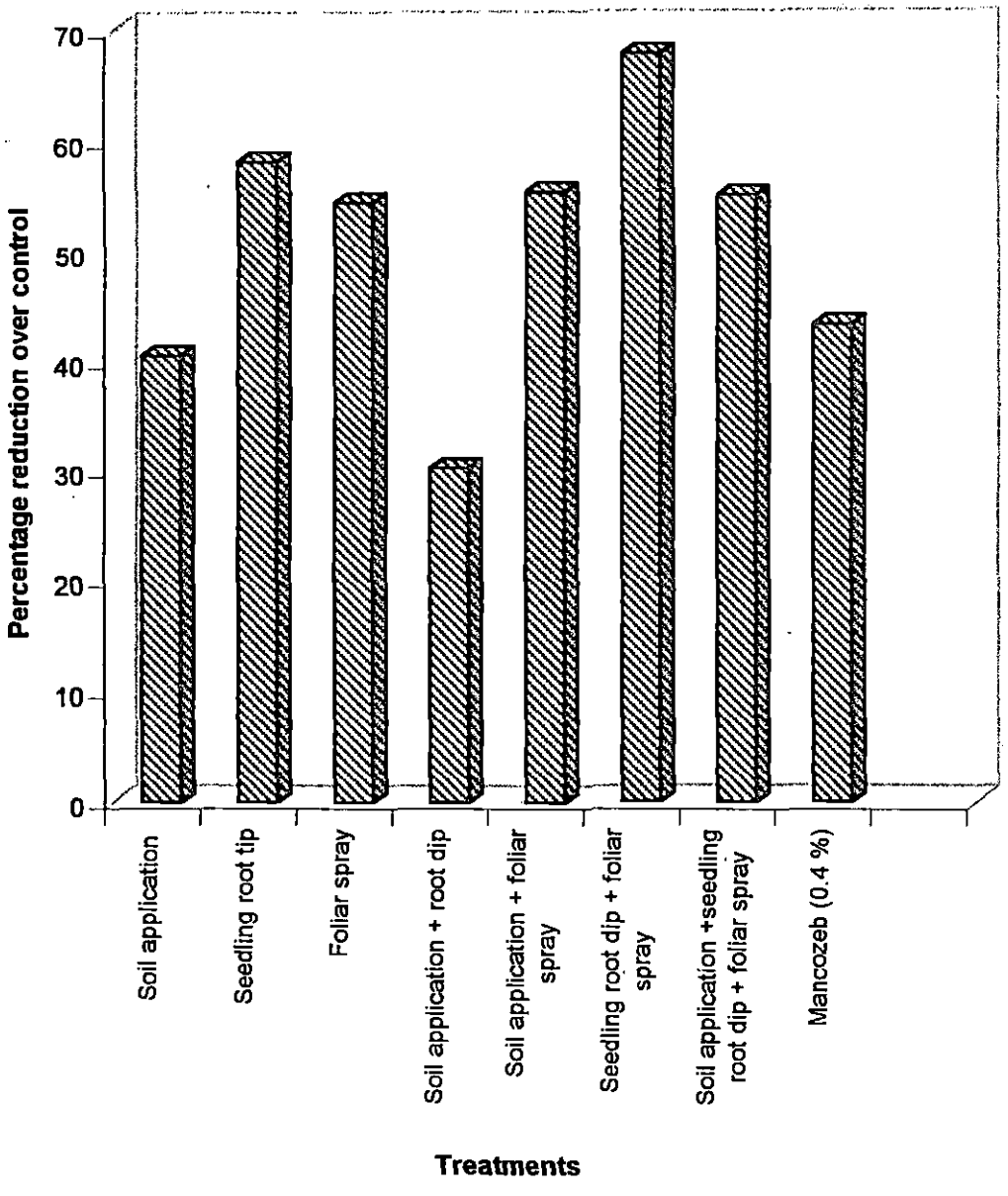


Table 7 Efficacy of formulation of fungal and bacterial antagonist in combination with abiotic elicitor (salicylic acid)

Treatment	Percentage disease index *
Trichoderma longibrachiatum (SA + FS)	0.89 (0.94)
Fluorescent *Pseudomonas* sp. (SA + FS)	2.02 (1.42)
Trichoderma longibrachiatum (SA + FS) + Fluorescent *Pseudomonas* sp. (SA + FS)	3.06 (1.75)
Salicylic acid (foliar spray)	2.41 (1.55)
Trichoderma longibrachiatum (SA + FS) + Salicylic acid (FS)	1.59 (1.26)
Fluorescent *Pseudomonas* sp. (SA + FS) + Salicylic acid (FS)	1.68 (1.29)
Trichoderma longibrachiatum (SA + FS) + Fluorescent *Pseudomonas* sp. (SA + FS) + Salicylic acid (FS)	1.39 (1.18)
Mancozeb (0.4 %) (foliar spray)	1.71 (1.31)
Untreated check	3.62 (1.90)
CD (5 %)	0.43

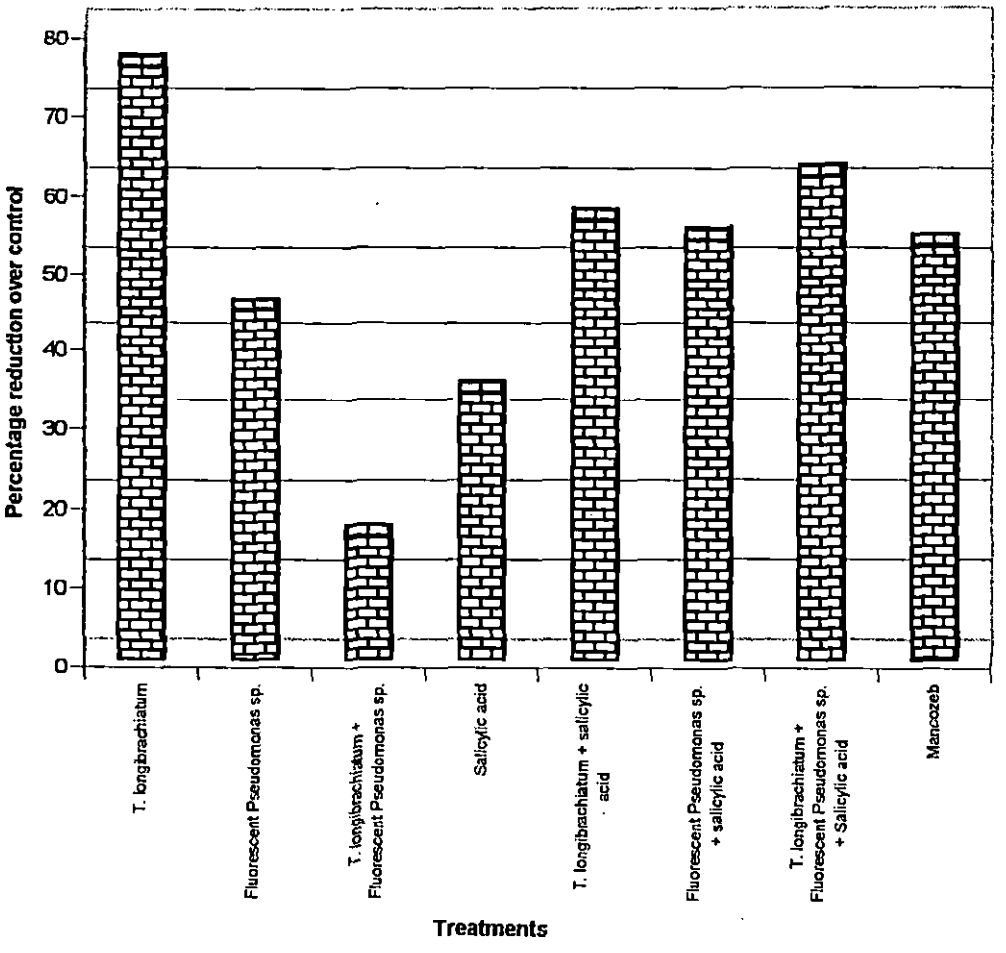
Figures in parantheses are $\sqrt{x}$ transformed values

* Mean of three replications

SA – Soil application

FS – Foliar spray

Fig. 6 Efficacy of formulation of fungal and bacterial antagonists in combination with salicylic acid on foliar blight disease intensity



4.9 Mass multiplicaiton of *T. longibrachiatum* in bran for field application

T. longibrachiatum was multiplied in bran for soil application in the field experiment. On the third day after adding *T. longibrachiatum* to bran whitish mycelial growth of the fungus covered the surface of the bran. It was applied to the soil in field @ 30 g per plant.

4.10 Efficacy of microbial formulation in field trial

The best treatment from the final pot experiment was tested in a field trial to assess the efficacy of the microbial formulation under *in vivo* conditions. At 40 days after sowing, the treatment soil application followed by foliar spray with *T. longibrachiatum* was found to be more effective than treatment with mancozeb in reducing the disease intensity (Plate 16). At 60 days after sowing there was an increase in disease intensity in all the treatments and the plants treated with *T. longibrachiatum* showed the lowest disease incidence. Mancozeb treatment also had similar effects (Table 8, Fig. 7).

There was no effect on growth parameters and yield due to treatment with *T. longibrachiatum* (Table 9).

4.11 Survival of *T. longibrachiatum* in the phylloplane

The population of *Trichoderma longibrachiatum* in the phylloplane was assessed at weekly intervals starting from first day after spraying the talc based formulation. On the first day after spraying the population was 18 x 10^3 cfu/g of leaf. After one week the population decreased slightly and it remained

Plate 15 Field trial on biological control of foliar blight of amaranthus


R_3T_3
R_3T_2
R_3T_1

Table 8 Efficacy of the formulation of *T. longibrachiatum* in field trial

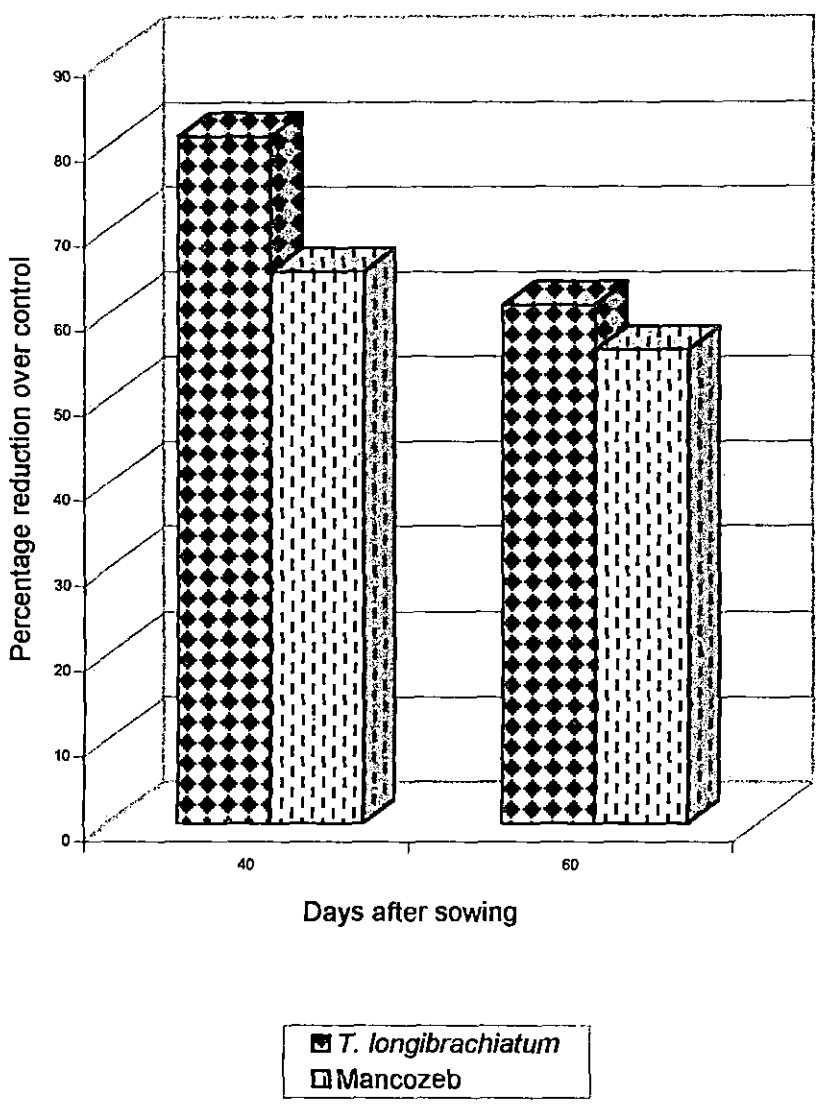
Treatment	Percentage disease index *	
	40 DAS	60 DAS
Trichoderma longibrachiatum (soil application + foliar spray)	2.857	14.443
Mancozeb (0.4 per cent foliar spray)	5.238	16.39
Untreated check	14.919	36.98
CD (5 %)	5.22	11.99

*Mean of seven replications

Table 9 Effect of *T. longibrachiatum* on yield and yield contributing parameters

Treatment	Yield of cuttings (g)	Weight of leaves (g)	Number of leaves	Shoot length	Root length
Trichoderma longibrachiatum	426.29	177.86	255.71	131.14	31.00
Mancozeb (0.4 %)	386.43	177.57	246.14	122.57	25.71
Untreatd check	410.57	172.14	250.43	119.86	28.14
CD (5 %)	66.72	26.57	41.78	12.89	6.16

Fig. 7 Efficacy of *Trichoderma longibrachiatum* on foliar blight disease intensity under field conditions



constant till third week. Twenty one days after spraying only 2 x 10^1 cfu/g of leaf was noticed (Table 10, Fig.8).

4.12 Induction of defense mechanism

4.12.1 Phenylalanine ammonia lyase

There was an increase in PAL activity one day after inoculating leaves of untreated amaranthus plants with *R. solani*. Similar increase was observed due to soil application followed by foliar spray with talc based formulation of *T. longibrachiatum* after inoculating amaranthus leaves with *R. solani*. PAL activity was less in plants sprayed with mancozeb and inoculated with *R. solani*. On the third day after inoculation of *R. solani* the trend in PAL activity was the same as on first day in treated and untreated plants. However there was higher induction of PAL on the third day in each treatment. On the fifth day increase in PAL activity was observed in plants inoculated with *R. solani* but it was higher in plants sprayed with mancozeb (Table 11, Fig. 9).

4.12.2 Peroxidase

Peroxidase activity was high due to soil application followed by foliar spray with talc based formulation of *T. longibrachiatum* at one day after inoculating the plants with *R. solani* compared to other treatments. Induction of peroxidase activity was similar on the third day after inoculation with *R. solani*. Change in peroxidase activity was same in all the treated plants on the fifth day after inoculating with *R. solani* (Table 12, Fig. 10).

Table 10 Survival of *T. longibrachiatum* in the phylloplane

Days after spraying	cfu/g of leaf
1	18×10^3
7	10×10^2
14	5×10^2
21	2×10^1

Fig. 8 Survival of *T. longibrachiatum* in the phylloplane

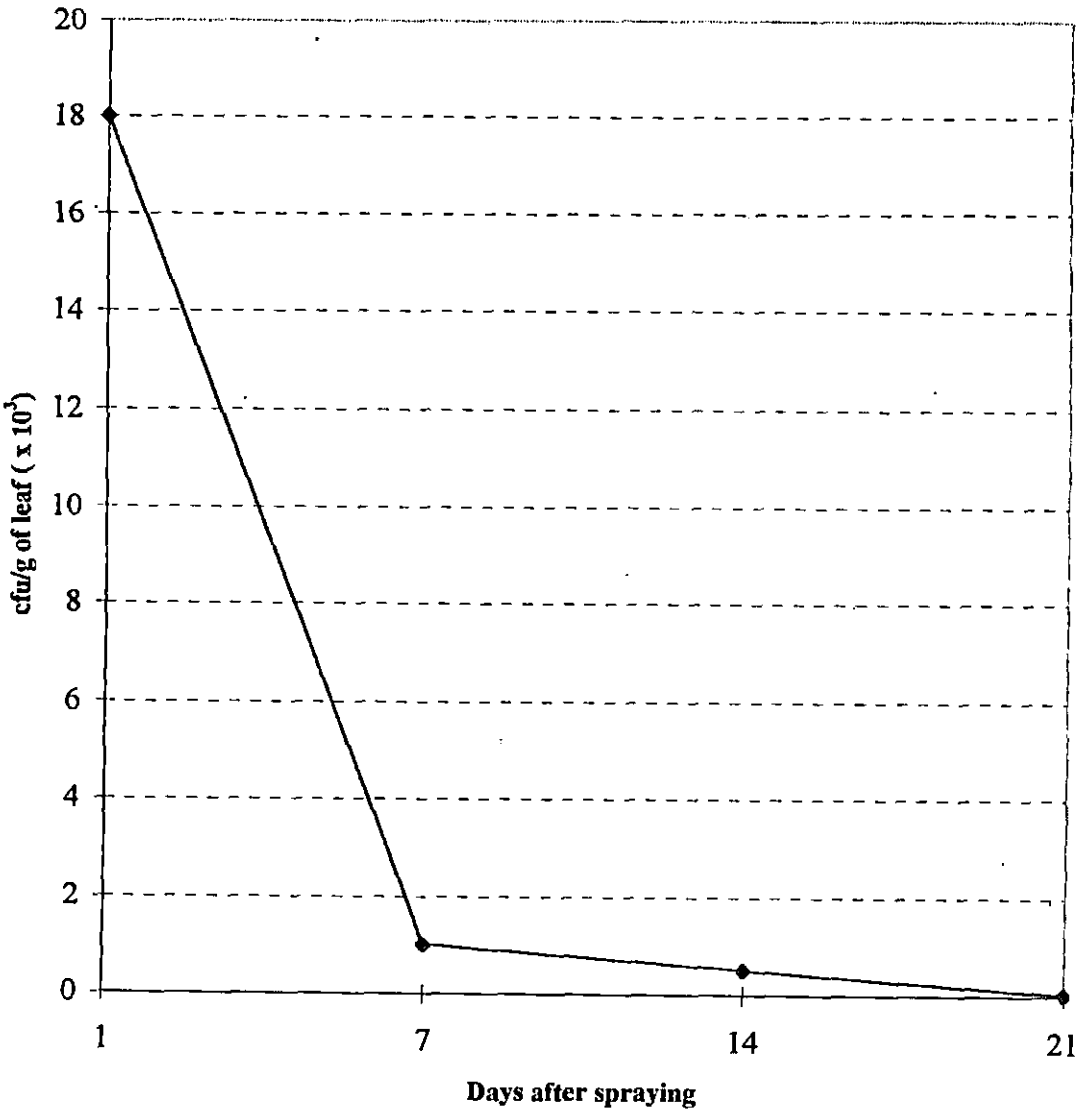


Table 11 Effect of application of *Trichoderma longibrachiatum* on changes in phenylalanine ammonia lyase (PAL) activity in amaranthus leaves inoculated with *Rhizoctonia solani*

Treatment	PAL activity (n mol transcinnamic acid/min/g fresh weight) at different days after inoculation with *R. solani*		
	1	3	5
T. longibrachiatum + *R. solani*	111.86	306.39	339.75
Mancozeb + *R. solani*	85.60	214.81	384.25
Untreated control (*R. solani* alone)	123.69	316.06	342.99
Untreated uninoculated control	101.32	98.25	99.97
CD (5 %)	17.76	39.39	19.29

Table 12 Effect of *Trichoderma longibrachiatum* on changes in peroxidase activity in amaranthus leaves inoculated with *Rhizoctonia solani*

Treatment	Peroxidase activity in units (1 unit = change in absorbance of 0.001 /minute / g fresh weight) at different days after inoculation with *R. solani*		
	1	3	5
T. longibrachiatum + *R. solani*	13.20	12.98	16.475
Mancozeb (0.4 %) + (*R. solani*)	7.40	9.02	17.38
Untreated control (*R. solani* alone)	9.22	7.70	14.43
Untreated uninoculated control	9.35	9.36	8.93
CD (5 %)	4.36	1.97	5.05

Fig. 9 Effect of application of *T. longibrachiatum* on changes in PAL activity

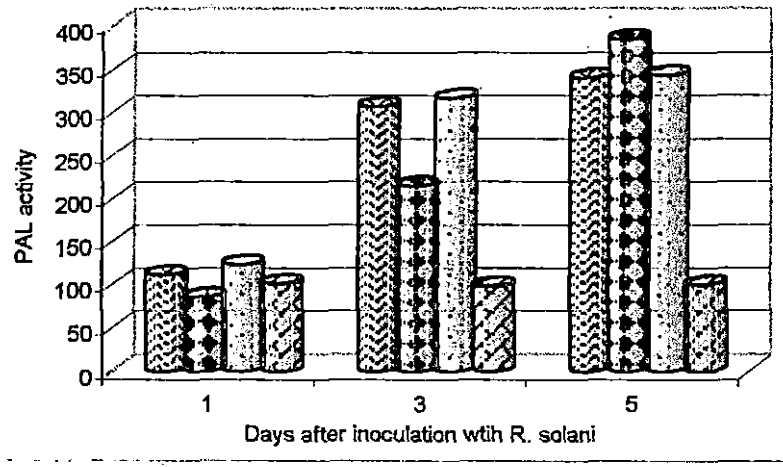
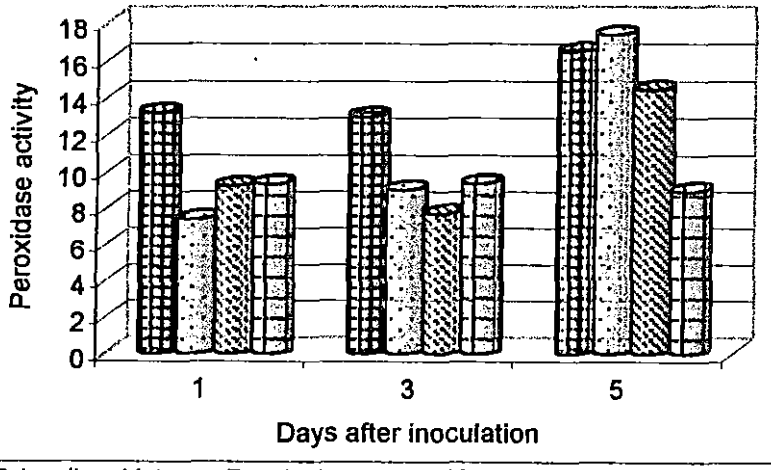


Fig. 10 Effect of application of *T. longibrachiatum* on changes in peroxidase activity



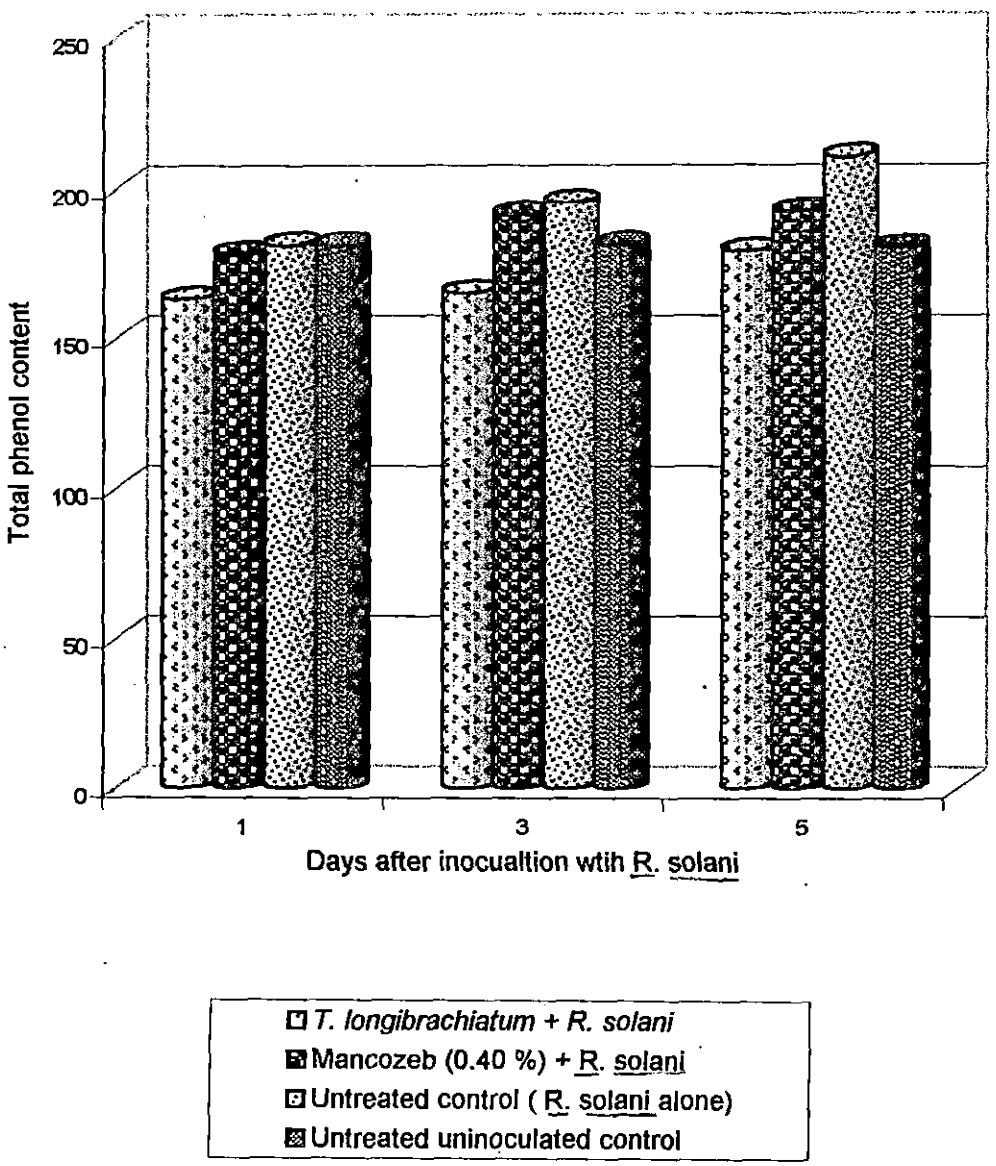
4.12.3 Total phenol

The total phenol content was low in plants treated with *T. longibrachiatum* prior to inoculation with *R. solani* on the first day after inoculating the pathogen. Similar result was observed in plants treated with mancozeb also. On the third day after inoculating *R. solani* the total phenol content increased in plants treated with mancozeb as well as in the plants inoculated with *R. solani* alone. There was no increase in phenol content in the plants treated wtih *T. longibrachiatum*. Similar trend was observed on the fifth day after inoculating *R. solani*. There was a slight increase in the total phenol content in the plants treated with *T. longibrachiatum* on the fifth after inoculation (Table 13, Fig. 11).

Table 13 Effect of *Trichoderma longibrachiatum* on the total phenol content in amaranthus plants inoculated with *R. solani*

Treatment	Total phenol content (μ g catechol equivalent/g fresh weight) at different days after inoculation with *R.solani*		
	1	3	5
T. longibrachiatum + *R. solani*	163	165	179
Mancozeb + *R. sonani*	178	191	192
Untreated check (*R. solani* alone)	181	195	210
Untreated uninoculated check	181	181	180

Fig. 11 Effect of application of *T. longibrachiatum* on total phenol content



DISCUSSION

DISCUSSION

Rhizoctonia solani Kuhn is an ubiquitous soil-borne pathogen with a very wide host range. Teleomorph of this pathogen (*Thanatephorus cucumeris*) causes foliar blights of numerous crops (Galindo *et al.*, 1983 ; Shew and Main, 1985; Jana *et al.*, 1990 ; Koike and Subbarao, 1999). Amaranthus (*Amaranthus tricolor* L.) has also been recorded as a host of this pathogen (Roy, 1975) on which symptoms of foliar blight were recently observed in Kerala (Kamala *et al.*, 1996). The quality of the produce is greatly reduced by the numerous creamy white spots which appear on the leaves as a result of infection by the pathogen *R. solani*. Fungicides like mancozeb are currently applied for combating diseases caused by this pathogen on several leafy vegetables (Jana *et al.*, 1990 ; KAU, 1996 ; Gokulapalan *et al.*, 1999). In 1987, large quantities of leafy vegetables exported from Malaysia to Singapore were rejected and destroyed due to high levels of ethylene bis dithiocarbamate residues in the produce by the application of the fungicide mancozeb (Mah *et al.*, 1988). Under these circumstances biological control seems to be a suitable alternative strategy for managing this disease of amaranthus.

Efficient microbial antagonists are popularly used in the biocontrol of soil borne pathogens. (Cook and Rovira, 1976 ; Stipanovic, 1979 ; Blakeman and Fokkema, 1982 ; Elad *et al.*, 1982 ; Sportelli *et al.*, 1983 ; Papavizas, 1985 ; Michail *et al.*, 1986 ; Gutterson *et al.*, 1986 ; Defago *et al.*, 1990 ; Anuratha and Gnanamanickam, 1990 ; Mew *et al.*, 1999). In spite of the 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 (Mew and Rosales, 1986 ; Elad *et al.*, 1990 ; Whipps, 1992 ; Harman *et al.* 1990 ; Kohl *et al.*, 1998 ; Mew *et al.*, 1999 ; Harman, 2000).

In the present investigation a few isolates of bacteria and fungi were tested for their antagonistic effects against the foliar blight pathogen *R. solani*. Among the bacteria, a fluorescent *Pseudomonas* sp. Isolated on King's B medium consistently showed highest *in vitro* antagonism aganist *R. solani*. The inhibitory effect of fluorescent pseudomonads has been well documented (Howell and Stipanovic, 1979 ; Kloepper and Schroth, 1981 ; Kloepper, 1983 ; Mew and Rosales, 1986 ; Shakthivel *et al.*, 1986 ; Devi *et al.*, 1989 ; Podile *et al.*, 1990 ; Chen and Tin, 1992 ; Hammer *et al.*, 1997). Isolate B of *Trichoderma* sp. most efficiently inhibited growth of *R. solani* in dual culture plates when fungal isolates were tested for antagonism against the pathogen. *Trichoderma* as an efficient antagonist to the pathogen *R. solani* have been recorded in numerous studies (Hadar *et al.*, 1979 ; Harman *et al.*, 1980 ; Elad *et al.*, 1982 ; Venkatasubhaiah *et al.*, 1984 ; Gokulapalan and Nair, 1984 ; Padmakumari, 1989 ; Kumaresan and Manibhushanrao, 1991).

Following the key of Rifai (1969) the isolate B of *Trichoderma* was identified as *T. longibrachiatum*. *T. longibrachiatum* is a potential biocontrol agent having antagonistic properties against several plant pathogens (Michail *et al.*, 1986 ; Manibhushanrao, 1989 ; Jagpal Singh and Singh, 1993 ; Palani and Lalithakumari, 1999). *T. longibrachiatum* efficiently parasitised the hyphae of *R. solani* causing granulation, vacuolation and disintegration of host hyphae, in the experiments conducted in the laboratory. Prior to the lysis of

host hyphae, coiling and penetration of the hyphae of *R. solani* by *T. longibrachiatum* was also observed. Chu and Wu (1981) demonstrated that *T. longibrachiatum* coiled around the hyphae of *R. solani* which led to the collapse and death of host hyphae. The inhibitory effects of the metabolites of *T. longibrachiatum* on several pathogens has been will documented (Michail *et al.*, 1988 ; Jagpal Singh and Singh, 1993 ; Jayasuriya *et al.*, 1996).

For the experimental production of biocontrol fungi a slightly modified liquid fermentation technology of Papavizas *et al.* (1984) was used *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 fermentation medium. Though expensive compared to V-8 juice or brewer's yeast, PDA is superior as a media for biomass production of *Trichoderma* sp. (Prasad *et al.*, 1997).

In the lab studies the highest biomass yield was obtained after 15 days when >75 per cent of the chlamydospores became mature. Papavizas *et al.* (1984) observed that high numbers of mature chalamydospores prolong the shelf life of the preparations. A population of 32 x 10^6 cfu per gm of the prepared talc based formulation of *T. longibrachiatum* was recovered even 120 days after storage at room temperature. In a talc based formulation of *Trichoderma*, the population declined by 31 x 10^7 cfu per gram to 13 x 10^7 cfu per gram in 75 days period when stored at 20 – 30^{0}C (Nakkeeran *et al.*, 1997).

Talc based product of fluorescent *Pseudomonas* P1 was prepared as described by Vidhyasekaran and Muthamilan (1995) and was used for soil application, seedling root dip and foliar spray. The bacteria was observed to

survive well in the product. The population of the bacteria in the product was 9 x 10^9 even after five months of storage at room temperature. Talc based formulation of fungal and bacterial antagonists has much practical advantage as it can be directly and easily supplied to the farmers for field application. Formualtions of fungal and bacterial antagonists have been proposed for the control of many root and aerial diseases (Kloepper, 1980 ; Papavizas *et al.*, 1984 ; Lewis and Papavizas, 1984 ; Lewis *et al.*, 1991 ; Vidhyasekaran and Muthamilan, 1995 ; Nakkeeran *et al.*, 1997 and Lewis *et al.*, 1998).

Formulations of the microbial antagonists were tested for their efficacy in suppressing foliar blight by soil application, seedling root dip and foliar spray. In the present investigation mere root dip or soil application with the talc based formulation of *Trichoderma longibrachiatum* was not very effective in lowering the intensity of foliar blight of amaranthus. However when the above methods of application were followed by foliar spray with the formulation there was appreciable reduction in the disease intensity . Tomato fruit rot caused by *R. solani* was considerably reduced by FB of various isolates of *Trichoderma* and *Gliocladium*, but not when equal or higher numbers of their conidia were added to soil (Lewis and Papavizas, 1984). Baby and Manibhushanrao (1993) demonstrated that soil augmentation with wheat bran consisting of *Trichoderma* sp. reduced sheath blight incidence in rice. Application of wheat bran saw dust preparation of *T. longibrachiatum* to soil infested with *R. solani* brought about considerable decline in groundnut root rot (Sreenivasaprasad and Manibhushanrao, 1990). Tosi and Zazzerini (1993) suggested that there was a more antagonistic effect when fungal isolates were added to the soil as air-dried inoculum rather than as seed

treatment in rust infested safflower seeds. The present study therefore indicates that apart from reducing the soil inoculum of the pathogen *R. solani* inciting the foliar blight disease, it is essential to check the aerial phase of the disease by foliar spray with the antagonist. Therefore, combining soil application and foliar spray is the most effective method in the delivery of the formulation of *T. longibrachiatum* for checking the foliar blight disease. Gokulapalan (1989) observed that soil augmentation with wheat bran consisting of *Trichoderma viride* gave appreciable control of sheath blight while aerial application of *T. viride* helped to check the spread of the disease in rice.

With regard to the bacterial antagonist mere seedling root dip in the talc based formulation was effective in suppressing the foliar blight disease. Control was improved when seedling root dip was followed by foliar spray of the prepared bacterial formulation. Van Peer *et al.* (1991) could protect carnation against fusarium wilt by root bacterization with *Pseudomonas* sp. strain WCS 417r. Muthamilan (1994) and Kamala (1996) had obtained good control of sheath blight disease incited by *R. solani* by dipping the roots of rice seedlings in peat based and talc based inoculum of *Pseudomonas* respectively at the time of transplanting. Foliar application of fluorescent *Pseudomonas* was effective in lowering the intensity of several aerial diseases of crop plants including sheath blight of rice (Muthamilan, 1994 ; Rabindran, 1994 ; Kamala, 1996). In this investigation, the method of soil application followed by foliar sparay, which was equally effective in reducing the disease intensity, was adopted for delivering the microbial antagonist to the plants, as amaranthus is a seldom transplanted crop.

Combination of strains of biocontrol agents was proposed to improve the performance of biocontrol agent. Results of the present study indicated that combination of *T. longibrachiatum* and fluorescent *Pseudomonas* was less effective in suppressing the disease when compared to the application of either antagonists alone. Hubbard *et al.* (1983) observed that indigenous populations of fluorescent pseudomonads significantly reduced the biocontrol activity of *Trichoderma hamatum* applied to Pythium seed rot of peas in a New York soil because of competition for iron. Large populations of indigenous fluorescent pseudomonads were associated with decreased populations of *Trichoderma* spp. and decreased take-all suppression in Western Australia (Simon and Sivasithamparam, 1988). On the contrary Danduard and Knudsen (1993) demonstrated that a combination of *P. fluorescens* 2-79 plus *T. harzianum* Th z ID1 neither inhibited nor enhanced the biocontrol activity of the latter agent against root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi*. In studies conducted by Duffy *et al.* (1996) although *T. koningii* and fluorescent pseudomonads (Q 29 z-80) were compatible, application of *T. koningii* alone was as effective as the combination.

Foliar spray with the abiotic elicitor of induced resistance salicylic acid (SA) was not effective in lowering the intensity of the blight disease. SA did not have any synergistic affect on the biocontrol acitvity of the antagonists also. Rasmussen (1991) suggested that although SA may have a role as an endogenous inducer of resistance, it is not the systemic signal of induced resistance. Young palms sprayed with1,5, 10, 20 and 50 mM concentrations of SA did not show any delay in symptom development by *Pestalotia palmarum* (Praveena, 1999).

Soil application of *T. longibrachiatum* multiplied in bran followed by foliar spary with the talc based product at 15 days interval effectively checked the foliar blight disease of amaranthus under field conditions. Suppression of the disease by the microbial antagonist *T. longibrachiatum* was maximum at 40 days after sowing (DAS). Although there was a rise in disease intensity of amaranthus plants in the field 60 DAS, plants treated with the formulation of *T. longibrachiatum* were less affected by the disease compared to plants sprayed with the fungicide mancozeb (0.4 per cent). Strawberry fruits were protected against storage rot by spraying strawberry plants in the field, begining at early flowering, with aqueous suspension of conidia of *T. viride* and *T. polysporum* (Tronsmo and Dennis, 1977). 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. Activated Biodac formulation containing chlamydospores of *Trichoderma* spp. reduced the spread of *R. solani* (R-23) in soil less mix which significantly lowered the post emergence damping off of cucumber, brinjal and pepper seedlings (Lewis *et al.*, 1998).

Aerial application with the formulation of *T. longibrachiatum* was given three times from 25 days after sowing up to the final harvest of the crop. In studies on the survival of *T. longibrachiatum* in the phylloplane the population of the fungus steadily declined within a period of 14 days after application of the formulation on the foliage. The spread of *G. virens* isolate TRBG on greengrass blades were greatly limited by the low relative humidity (Yuen *et al.*, 1994). Harman (2000) demonstrated that foliar spray of *T. harzianum* strain T-22 controlled powdery mildews on

Catharanthus and pumpkins ; *Botrytis cinerea* on strawberry and grapes. He suggested that T-22 must be applied at least once every 10 days when disease pressure is high, since it cannot grow on and colonize newly formed leaf tissues.

In the present study treatment of amaranthus with the formulation of microbial antagonist *T. longibrachiatum* did not have any influence on the yield of cuttings, number of leaves or the root-shoot length. Jubina and Girija (1998) found that *P. fluorescens* and *T. harzianum* did not have any effect on growth and yield of pepper plants. In the field experiments, yields were greater in onion planted with *T. harzianum* strain T-22 (Harman, 2000). With the integrated T-22 + fungicide programme, yields were increased 10 per cent relative to the same programme without T-22.

Although the activity of PAL was high on first and third day after inoculating amaranthus plants with *R. solani*, there was a slight reduction in the level of the enzyme in plants which were previously treated with *T. longibrachiatum*. However, the activity of the enzyme PO was high in the inoculated plants in the presence of the fungal antagonist. There was a delay in the induction of PAL and PO in plants sprayed with mancozeb prior to inoculation with *R. solani*. The number and concentration of peroxidase isoenzymes in bean hypocotyl increased in response to infection by highly pathogenic isolate of *R. solani* from okra (Wasfy *et al.*, 1984). Chitinase and peroxidase activities were increased in both root and leaf tissue of cucumbers grown in the presence of *T. harzianum* strain 203 (Yedidia *et al.*, 1999).

The level of total phenols did not increase when the plants were inoculated with *R. solani* in the presence of *T. longibrachiatum*. Tohamy *et al.*

(1993) demonstrated that the amount of free phenols increased in plants infected with *Sclerotium cepivorum* or *Fusarium oxysporum* f. sp. *cepae* and in plants treated with *Trichoderma* spp. *T. hamatum* and *T. longibrachiatum* in the presence of one of the pathogens while the same treatment reduced the amount of total phenols. Howell *et al.* (2000) suggested that the major mechanism in the biocontrol by *T. viride* of cotton seedling damping off incited by *R. solani* appears to be the induction of host resistance, as indicated by peroxidase activity and terpenoid synthesis.

SUMMARY

SUMMARY

Foliar blight caused by *Rhizoctonia solani* has recently emerged as a serious disease affecting the leafy vegetable amaranthus (*Amaranthus tricolor* L.) in Kerala. The present investigation was undertaken to evolve a suitable biocontrol strategy for managing this disease. Rhizosphere and phylloplane antagonists were assessed for their potential in controlling *R. solani*, the incitant of foliar blight disease.

Rhizosphere and phylloplane microflora of healthy amaranthus plants were isolated and screened for their efficacy in inhibiting the foliar blight pathogen *R. solani in vitro*. Among the fungal isolates, *Trichoderma* sp. obtained from the rhizosphere was observed to be superior in inhibiting the pathogen. This effective isolate was identified as *Trichoderma longibrachiatum*. An isolate of fluorescent *Pseudomonas* sp., P1 was identified as the best bacterial antagonist against the pathogen *in vitro*.

Studies on the mycoparasitism of *T. longibrachiatum* on *R. solani* indicated that the fungal antagonist coiled around and penetrated the host hyphae causing granulation, vacuolation and lysis of the latter.

The two efficient antagonists were mass multiplied and formulated in the inert carrier material, talc for application under green house and field conditions. *Trichoderma longibrachiatum* was mass multiplied by a modified liquid fermentation technology and the fermentation biomass consisting of mycelia and chlamydospores was mixed with talc. The bacterial antagonist P1 was mass multiplied in Kings B broth and formulated in talc.

Separate pot culture experiments were conducted to assess the efficacy of different methods of application of the formulated products of fungal and bacterial antagonists *in vivo*. Soil application followed by foliar sparay with one per cent suspension of the talc based formulation of the antagonists was very effective in reducing the intensity of foliar blight caused by *R. solani* and was selected as the mode of delivery of the product of the antagonists in the field.

A green house trial was conducted to evaluate the efficacy of combination of the selected antagonists as well as the influence of the abiotic elicitor,*viz.*, salicylic acid on the biocontrol properties of microbial antagonists. Soil application followed by foliar spray with the talc based formulation of *Trichoderma longibrachiatum* was most effective in lowering the disease intensity. The chemical inducer of defense mechanism viz., SA did not have any synergistic influence on the microbial antagonists in suppressing the disease.

A trial was conducted at College of Agriculture, Vellayani to assess the performance of the microbial formulation under field condition in comparison with the farmers practice of application of the fungicide, mancozeb. Soil application of *Trichoderma longibrachiatum* multiplied in bran followed by foliar spray with one per cent suspension of talc based formulation of *T. longibrachiatum* at 15 days interval upto 60 days after sowing was effective in checking foliar blight disease of amaranthus in the field. There was no appreciable difference in the yield of plants.

This investigation forms the first report of the application of *Trichoderma longibrachiatum* as biocontrol agent for crop disease management in Kerala.

Changes in the levels of total phenol and the enzymes PAL and PO in amaranthus plants due to treatments tested under field conditions were analysed by setting up a separate pot culture experiment. Application of *T. longibrachiatum* prior to inoculating the plants with the pathogen resulted in the earlier induction of the enzymes PAL and PO compared to the plants sprayed with mancozeb. Level of activity of PO was also higher in these plants. The total phenol content was low in the plants treated with *T. longibrachiatum* prior to inoculation with the pathogen *R. solani*.

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APPENDICES

APPENDIX – I

COMPOSITION OF DIFFERENT MEDIA

Potato dextrose agar

Potato	- 200 g
Dextrose	- 20 g
Agar	- 20 g
Distilled water	- 1 L

Martin's Rosebengal agar

Dextrose	- 10 g
Peptone	- 5 g
Potassium dihydrogen phosphate	- 1g
Magnesium sulphate	- 0.5 g
Rosebengal	- 33 mg
Streptomycin solution (1 %) – 3 ml	
Agar	- 15 g
Distilled water	- 1 L
pH	- 7.0

Soil extract agar

Glucose	- 1 g
Dihydrogen potassium phosphate	- 0.5 g
Agar	- 15 g
Soil extract	- 100 ml
Tap water	- 900 ml
Adjust pH to 6.8	

King's B medium

Peptone	- 20 g
Dipotassium hydrogen phosphate	- 1.5 g
Magensium sulphate	- 1.5 g
Glycerol	- 10 ml
Distilled water	- 1 L

Adjust pH to 7.2

Nutrient agar

Beef extract	- 1 g
Yeast extract	- 2 g
Peptone	- 5 g
Sodium chloride	- 5 g
Agar	- 15 g
Distilled water	- 1 L

Adjust pH to 7.2 - 7.4

APPENDIX - II

Buffers for enzyme analysis

0.1 M sodium acetate (pH 4.7)

Stock solutions

A : 0.2 M solution of acetic acid (11.55 ml in 1000 ml)

B : 0.2 M solution of sodium acetate (16.4 g of $C_2H_3O_2$ Na Or 27.2 g of $C_2H_3O_2$ Na $3H_2O$ in 1000 ml).

22.7 ml of A is mixed with 27 ml of B, diluted to a total of 100 ml.

0.1 M Borate Buffer (pH 8.8)

A : 0.2 M solution boric acid (12.4 g in 1000 ml)

B : 0.05 M solution of borax (19.05 g in 1000 ml)

50 ml of A is mixed with 30 ml of B, diluted to a total of 200 ml.

MANAGEMENT OF FOLIAR BLIGHT OF AMARANTHUS (*Amaranthus tricolor* L.) CAUSED BY *Rhizoctonia solani* Kühn USING MICROBIAL ANTAGONISTS

BY

SMITHA K.P.

ABSTRACT OF THE THESIS
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DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI
THIRUVANANTHAPURAM

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ABSTRACT

The possibility of biological control of foliar blight pathogen of amaranthus *Rhizoctonia solani* with microbial antagonists was studied. *Trichoderma longibrachiatum* and a fluorescent *Pseudomonas* sp., P1 were found to be most effective in inhibiting *R. solani in vitro*. After mass multiplication the selected antagonists were formulated in an inert carrier material, viz., talc and were tested separately by different methods of application under greenhouse conditions. Soil application followed by foliar spray with one per cent suspension of the formulated product of the microbial antagonists was selected as the best method of delivering the biocontrol agent in checking the disease *in vivo*. Green house studies conducted indicated that soil application followed by foliar spray with *T. longibrachiatum* was more effective in suppressing the disease than by either combining the fungal and bacterial antagonists or by applying the bacterial antagonist P1 alone. Further, it was revealed that the abiotic elicitor salicylic acid had no synergistic influence on biocontrol properties of the antagonists. Application of *T. longibrachiatum* in the soil followed by foliar spray at 15 days interval starting from 25 days after sowing was very effective in controlling the disease in field conditions. There was no appreciable difference in the yield of plants. There was an earlier induction of the enzymes PAL and PO in plants treated with *Trichoderma longibrachiatum* compared to plants sprayed with mancozeb. Level of activity of PO was high in *R. solani* inoculated plants in the presence of *Trichoderma longibrachiatum*. The content of total phenols in plants treated with *T.longibrachiatum* was found to be low.