"Evaluation of systemic acquired resistance and induced systemic resistance on the suppression of foliar blight disease of amaranthus (*Amaranthus tricolor* L.)"

By

ATHIRA BABU B. M.

(2012-09-101)

THESIS

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DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM - 695 522
KERALA, INDIA

2017

DECLARATION

I hereby declare that this thesis entitled "**Evaluation of systemic acquired resistance and induced systemic resistance on the suppression of foliar blight disease of amaranthus (*Amaranthus tricolor* l.)**" 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, associate ship, fellowship or other similar title, of any other university or society.

Athira

Place: Vellayani
Date: 02-11-2017

Athira Babu B. M.
(2012-09-101)

CERTIFICATE

Certified that this thesis entitled "**Evaluation of systemic acquired resistance and induced systemic resistance on the suppression of foliar blight disease of amaranthus (*Amaranthus tricolor* L.)**" is a record of research work done by **Ms. Athira Babu B. M. (2012-09-101)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Vellayani
Date: 02-11-2017

Dr K. N. Anith
(Chairman, Advisory Committe)
Professor (Microbiology)
Department of Agricultural Microbiology
College of Agriculture, Vellayani

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Athira Babu B. M., a candidate for the degree of B.Sc. - M.Sc. (Integrated) Biotechnology, agree that the thesis entitled **"Evaluation of systemic acquired resistance and induced systemic resistance on the suppression of foliar blight disease of amaranthus (*Amaranthus tricolor* L.)"** may be submitted by Ms. Athira Babu B. M., in partial fulfillment of the requirement for the degree.

Dr. K. N. Anith (Chairman)
Professor (Microbiology)
Department of Agricultural Microbiology
College of Agriculture, Vellayani

Dr. Swapna Alex
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani

Dr. K. B. Soni
Professor and Course Director
B.Sc.- M.Sc. (Integrated) Biotechnology
Department of Plant Biotechnology
College of Agriculture, Vellayani

Dr Roy Stephen
Professor
Department of Plant Physiology
College of Agriculture, Vellayani

2/11/17

EXTERNAL EXAMINER
Dr. Sisha M.S
Professor, School of Biosciences
M.G. University, Kottayam

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
µl	Micro litre
µM	Micro Molar
bp	base pair
cm	Centi Metre
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylene diamine tetra acetic acid
et al.	And other co workers
etc	Etcetera
g	Gram
g-1	Per gram
hrs	Hours
kb	Kilo base
M	Molar
mg	Milli gram
min	Minutes
ml	Millilitre

mM	millimolar
nm	Nanometre
OD	Optical Density
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PVP	Polyvinyl pyrrolidone
rpm	Revolutions per minute
sec	Seconds
Tris HCl	Tris (hydroxy methyl) aminomethane hydrochloride
UV	Ultra Violet
v/v	Volume/volume
w/v	Weight/volume

INTRODUCTION

1. INTRODUCTION

Leafy vegetables are considered to be rich sources of vitamins, minerals and proteins and are included frequently in diet. Amaranthus (*Amranthus tricolor*) is considered to be the cheapest leafy vegetable available in the market, which is a rich source of proteins, minerals especially iron, calcium and phosphorus, vitamin A and C (Rastogi and Shukla, 2013). Foliar blight caused by *Rhizoctonia solani* is a serious threat to amaranth growers (Nayar *et al.*, 1996). The pathogen infects more than 90 percentage of plants in the field and causes considerable economic loss. Although disease management using foliar spray of mancozeb (0.4%) in cow dung supernatant at fortnightly intervals is effective (KAU, 2002), repeated use of chemicals poses health hazards.

Due to increased awareness on the environmental pollution and health hazards of using chemical fertilizers and pesticides, biological control of plant pathogens is gaining more importance these days. Increased use of pesticides and other chemicals have resulted in increase of pest species which are resistant to chemicals. Hence biological control should be integrated with other control measures as the efficacy of different methods vary with time, location and environmental factors (Sharma *et al.*, 2013).

Piriformospora indica, belonging to the order Sebacinales, is extremely versatile in its mycorrhizal associations and its ability to promote plant growth. *P. indica* has shown bioprotective potential against several plant pathogens by imparting induced systemic resistance to the plants (Varma *et al.*, 2012). Decoding of genome of *P. indica* has revealed its potential for application as a plant growth-promoting endophtyic fungus for realizing the targeted improvement in the production of crop plants (Zuccaro *et al.*, 2009).

Systemic acquired resistance can be triggered in plants by using some elicitor chemical compounds (Selim *et al.*, 2014). This particular form of plant resistance can

be activated by biotic and abiotic agents and results in a systemic protection of the entire plant against a spectrum of diseases caused by fungi and bacteria. Plant activators, a category of novel chemicals including Acibenzolar-S-Methyl (a structural analog of salicylic acid) induce the defense capabilities of plants through a unique mode of action that mimics the natural systemic acquired resistance (SAR). The efficacy of ASM was also found in open field conditions, where the plant was protected from the disease (Ryals *et. al.,* 1996; Buonaurio *et. al.,* 2002). This chemical has shown efficiency in reducing the plant diseases caused by a variety of bacterial, fungal and viral pathogens.

Defense mechanisms of plants can be induced by using many biological agents and also by certain chemical compounds. Such a combination of the two types of induced resistance in plants called induced systemic resistance (ISR) and systemic acquired resistance (SAR) have been employed in this study. In the present investigation, an attempt has been made to use *Piriformospora indica* and a chemical activator, Acibenzolar-S-methyl (ASM) both individually and in combinations for the suppression of foliar blight in amaranthus.

The main objective of the study is:

- To study the combined effect of chemical activator Acibenzolar-S-Methyl (ASM) and root colonising endophytic fungus *Piriformospora indica* on plant growth promotion and induction of systemic resistance and suppression of foliar blight disease in amaranthus (*Amaranthus tricolor* L.)

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. AMARANTHUS (*Amaranthus tricolor*)

Amaranthus (*Amaranthus tricolor*), one of the major leafy vegetables known as the poor man's spinach originates from tropical Asia and belongs to the family Amaranthaceae. It is characterized by the C4 cycle. Seedling emerges 3-5 days and flowering may start after 4-8 weeks after sowing. The plant stops growing after 4 months and subsequently dies (Baquar and Olusi, 1988).

Amaranthus tricolor is recognized as an easy-to-grow, productive, tasty and nutritious vegetable. It is used externally to treat inflammations, and internally it acts as a diuretic.

2.2. *Rhizoctonia solani* AND FOLIAR BLIGHT OF AMARANTHUS

Rhizoctonia solani is a multinucleate soil borne fungus which belongs to the large genus Rhizoctonia. The genus is divided into 13 anastomosis groups (AG) that vary in pathogenicity, morphology, ecology and sequence variations (Holcomb and Carling, 2002). *R. solani* is distributed worldwide and causes economic loss by inciting diseases in various crops and ornamentals (Erper *et al.*, 2016). The symptoms of the fungal infection depend on the environmental conditions and the host on which the pathogen attacks (Ogoshi., 1987).

Nayar *et al* (1996) reported the first case of foliar blight in Kerala caused by *R. solani* which became a serious threat to amaranth growers in the state. The disease is characterized by small irregular whitish spots on leaves of amaranthus. Gokulapalan *et al* (2000) stated that high humid conditions facilitate the disease severity which leads to development of extensive translucent light green lesions and shot hole symptoms.

Severe blight in *Zingiber officinale* (ginger), collar rot and web blight in cow pea and leaf spot disease of banana has been reported to be caused by this pathogenic

fungus (Gokulapalan and Girija, 2000; Lakshmanan *et al.*, 1979; Lakshmanan and Mohan, 1989).

2.3. BIOCONTROL OF *Rhizoctonia solani*

Bacterial and fungal antagonists are found to be effective against the pathogenic fungus *R. solani*. Plant Growth Promoting Rhizobacteria (PGPR) which are indigineous to the plant rhizosphere are very important and emerging means of biocontrol of *R. solani* (Akintokun and Taiwo, 2016). Bacterial and fungal isolates belonging to *Bacillus subtilis*, Tric*hoderma harzianum* and *Trichoderma hamatum* were tested against *R. solani* in sugar beet plants and it was observed to be operative. Fungal isolates showed more antagonism than the bacterial ones (Mahmoud, 2016). Commercially available talc formulations of *Trichoderma hazianum* and *Pseudomonas fluorescens* were found to be effective when applied either individually or in combination and it resulted in considerable reduction of sheath blight in rice caused by *Rhizoctonia solani* (Reshu *et al.*, 2017).

Malolepsza *et al* (2017) stated that the fungus, *Trichoderma virens* may have the potential to be developed into a new biofungicide for the integrated management of diseases caused by *R. solani.* This fungus didn't show any direct *in vitro* antagonism against *R. solani* but activated systemic defense responses in tomato plants by increasing the production of defense enzymes in the plants. *Bacillus subtilis* ATCC 117774 has shown good antagonistic potential aginst *R. solani* when tested against the diseases caused in potato (Saber *et al.*, 2015).

2.4. *Piriformospora indica* (*P. indica)*

Piriformospora indica is a cultivable root endophytic fungus discovered in the Indian Thar Desert. It belongs to the order Sebacinales (Verma *et al.*, 1998). Inoculation with the fungus and application of fungal culture filtrate promotes plant growth and biomass production (Varma *et al.*, 1999).

The fungus has a broad host range and colonizes the roots of pteridophytes, bryophytes, gymnosperms and angiosperms (Qiang *et al.*, 2011). The fungal mycelia are almost white or hyaline but older cultures show inconspicuous zonation. *P. indica* produces pear shaped chlamydospores which are found single or in clusters. The fungus colonizes the rhizodermis and does not invade the vascular tissues and the aerial parts of the plant (Deshmukh *et al.*, 2006). It induces systemic resistance to plants against pathogens, toxins and also promotes nutrient uptake, allows plant to survive under different abiotic stresses (Varma *et al.*, 2012).

The colonization of *P. indica* starts with a biotrophic growth phase during which fungal cells are encased by the host plasma membrane. *P. indica* is found more often in moribund host cells where it secretes a large variety of hydrolytic enzymes that degrade proteins (Lahrmann and Zuccaro, 2012).

P. indica was shown to be able to repress H_2O_2 accumulation in the roots and actively suppress immune responses triggered by various microbe associated molecular pattern (MAMPs) (Camehl *et al.*, 2010; Jacobs *et al.*, 2011). The fungus induces systemic disease resistance by enhancing the concentration of antioxidants, ascorbate and glutathione, in the plant body to cope up with the oxidative stress caused by pathogens (Waller *et al.*, 2008; Vadassery *et al.*, 2009). Uptake and transport of phosphorous, with diverse regulatory, structural, and energy transfer roles, are also stimulated by the fungus in the colonized roots of maize (Yadav *et al.*, 2010). Recent studies have also shown that the sulphur metabolism is stimulated by the fungus. Genes which code for several plastid-localized enzymes required for sulphate reduction are upregulated by *P. indica* in *Arabidopsis* roots, and gene inactivation studies confirmed that they are required for the benefits to the plants.

2.5. INDUCED SYSTEMIC RESISTANCE (ISR)

Induced systemic resistance (ISR) of plants against pathogens is a widespread phenomenon that has been investigated with respect to its potential use in plant protection. Induced systemic resistance is the resistance induced by symbionts and orchestrates pathway depending on other hormones such as jasmonate and ethylene (Ross, 1961). Elicited by a local infection, plants respond with a salicylic dependent signaling cascade that leads to systemic expression of a disease resistance that is efficient against fungi, bacteria and viruses (Heil and Bostock, 2002).

It was reported that on the interaction of *P. indica* with *Arabidopsis thaliana* the fungus induces a mode of resistance to microbial pathogens which is similar to that of induced systemic resistance in plants (Molitor and Kogel, 2009).

2.6. SYSTEMIC ACQUIRED RESISTANCE (SAR)

The unspecific systemic immunity acquired by the plants following a preliminary localized infection is known as systemic acquired resistance (SAR). SAR is characterized by activation of a broad spectrum of host defense mechanisms at the local site of initial pathogen attack and also systemically in tissues remote from the site of infection. Systemic induction of resistance through SAR involves the production of a systemic signal at the site of primary infection which is then transferred to other parts of the plant where the defense mechanism gets induced (Schneider *et al.*, 1996). An increase in accumulation of salicylic acid and increased expression of pathogenesis related protein genes (PR genes) is intrinsic with the induction of SAR (Kessman *et al.*, 1994; Sticher *et al.*, 1997).

SAR activators such as salicylic acid and its synthetic functional analogs when applied at suitable concentrations can significantly increase the plant's resistance against pathogens. It may be included in the Integrated Pest Management (IPM) programmes for the management of plant pathogens (Molinari, 2016).

2.7. CHEMICAL ACTIVATOR ACIBENZOLAR S- METHYL (ASM)

Acibenzolar S-methyl (ASM), a structural analog of salicylic acid has been reported to be effective against plant pathogens including fungus, bacteria and virus (Mètrauxs, 2001).

Cools and Ishii (2002) reported that when the first leaves of cucumber plants were treated with the synthetic activator ASM, it protected whole plants from infection with the virulent fungal pathogen *Colletotrichum orbiculare.* The ability of ASM to induce resistance was studied in apple affected by fire blight caused by *Erwinia amylovora* and was reported that ASM promotes induced systemic resistance by increasing defense-related compounds (Brisset *et al.*, 2000). It was reported that the plant activator ASM was found to enhance resistance in tomato cultivars against bacterial wilt in tomato caused by *Ralstonia solanacearum* (Pradhanang *et al.*, 2005).

A study on the mode of action of ASM against sheath blight of rice showed that it exhibited limited fungal toxicity against *R. solani* by reducing the frequency of penetration, colonization of host tissue and spread of hyphae from primary lesions to secondary lesions (Rohilla *et al.,* 2001). Abo-Elyousr and El-Hendawy (2008) found that combined use of *Pseudomonas fluorescens* and ASM not only reduced the pathogen population in bacterial spot disease in tomato but also increased the seedling biomass and tomato yield. Combined use of plant growth promoting rhizobacteria and ASM was found to be beneficial as the growth retardation effect of the plant defense activator was reduced by the growth promoting ability of the rhizobacteria (Nair *et al.*, 2006). Maxson-Stein *et al.*, (2002) reported that the application of ASM as foliar spray has elevated the expression of pathogenesis related protein genes (PR genes) and thereby the production PR proteins in plants. Combined use of plant growth promoting rhizobacteria and ASM was found to be beneficial as the growth retardation effect of the plant defense activator was reduced by the growth promoting ability of the rhizobacteria (Nair *et al.*, 2007).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "evaluation of systemic acquired resistance and induced systemic resistance on the suppression of foliar blight disease on amaranthus (*Amaranthus tricolor* L.)" was carried out at the Department of Agricultural Microbiology and Department of Plant Biotechnology, College of Agriculture, Vellayani.

3.1. ISOLATION OF THE PATHOGEN, *Rhizoctonia solani*

Amaranthus leaves showing typical symptoms of foliar blight caused by the fungus *Rhizoctonia solani* were collected from the fields of College of Agriculture, Vellayani. The infected portion of the leaves were cut into small bits along the growing edge of the infection. The bits were then washed with sterile water several times and were then placed on Potato Dextrose Agar (PDA) plates (Appendix I). Fungal growth was observed after 24 hours of inoculation. The fungus was purified by hyphal tip method wherein the fungal hyphae were cut and transferred to fresh PDA plates under sterile conditions and incubated at 28°C for 5 days and then transferred to PDA slants. The isolate was maintained by periodic subculturing on PDA and stored at 4°C.

3.2. PATHOGENICITY TEST OF THE ISOLATED PATHOGEN

Koch's postulates were proved for confirming the pathogenicity of the isolates of *R. solani.* Five leaves were detached from healthy amaranthus plants. Mycelial discs of *R. solani* were made from five days old culture of the fungus grown on PDA plates. Each of the amaranthus leaves was inoculated with one mycelial disc of *R. solani* on the ventral side. A moistened layer of cotton was placed over the inoculated portion of the leaves to provide humidity. Each leaf was then transferred to sterile petri plates and were closed with the lid. They were then incubated at room temperature (28°C). A control leaf was also maintained under the same conditions but without the fungal inoculation.

The pathogenicity test was done on standing plants also. For this, the upper fully opened two leaves were inoculated on the lower surface with the mycelial discs of *R. solani* made from a five days old culture of the fungus grown on PDA. A thin layer of moistened cotton was placed over the inoculated portion and a polythene cover was used to cover the whole plant to maintain humidity.

Re-isolation of the pathogen was done from the leaves showing typical foliar blight symptoms and the identity of the pathogen was established.

3.3. CULTURING OF *P. indica*

The fungal culture was obtained from Dept of Agricultural Microbiology, College of Agriculture, Vellayani. It was subcultured by placing a mycelial plug cut from the *P. indica* culture on PDA plates and was incubated at 28°C for 7 days. The fungus was also grown in Potato Dextrose Broth by inoculating the broth with mycelial plugs taken from the growing cultures of *P. indica* on PDA plates and was incubated in a shaker at 28°C with a shaking speed of 100 rpm.

3.4. *IN VITRO* EXPERIMENTS

3.4.1. Effect of ASM on *Rhizoctonia solani*

Poisoned food technique was employed to study the effect of ASM on the growth of fungal pathogen *Rhizoctonia solani*. Potato dextrose agar plates incorporated with different concentrations (10ppm, 25ppm, 50ppm, 75ppm, 100ppm, 150ppm and 200ppm) of ASM was prepared. Mycelial plugs of the fungal pathogen taken from an actively growing culture of *R. solani* were placed on the centre of the media and allowed to grow by incubating at 28°C for five days. The growth pattern was studied by comparing with the control plate without ASM ammendment. Observation was taken from the second day of inoculation till the fungus was fully grown on the media by measuring the radii of the fungal mycelia. Percentage mycelial inhibition was calculated using the formula:

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

I = Inhibition of mycelial growth of pathogen

C = Radial growth of pathogen in control plates (cm)

T = Radial growth of pathogen in treated plates (cm)

3.4.2. Screening for *in vitro* antagonism

Dual culture plate assay was used to analyse the ability of *Piriformospora indica* to inhibit the mycelial growth of *R. solani*. *P. indica* was subcultured on PDA plates and incubated at 28°C for seven days. *R.solani* culture isolated from the diseased plants and stored in PDA slants were subcultured on PDA plates and incubated at 28°C for seven days. Mycelial plugs of *P. indica* and *R. solani* from the actively growing cultures were taken and placed 2cm apart on PDA plates and incubated for 1 week at 28^0C and were assessed for inhibition.

3.4.3. Co-culture of *P. indica* and *Amaranthus tricolor*

Potato dextrose agar (PDA), Murashige- Skoog (MS) medium and PDA- MS (1:1) were prepared in bottles. Myclelial plugs of *P. indica* were made from the growing edge of the actively growing culture and placed on the center of the sterile MS, MS-PDA and PDA media in tissue culture bottles. They were incubated at 28°C for 7 days. Amaranthus seeds were sterilized with 4% (w/v) sodium hypochlorite followed by several washes with sterile water. Three seeds were placed on to each of the bottle containing the above medium containing *P. indica* growth and incubated at 28°C. After fifteen days, the root tissues were collected and observed under light microscope after staining with trypan blue for the cololnization with *P. indica*.

3.4.4. Trypan blue staining of root

Roots of amaranthus plants grown *in vitro* were stained following the protocol by Philip and Hayman (1970). Intact plants with roots were carefully picked from the medium and washed with distilled water. Roots were then cut into 1cm bits and boiled in 10% KOH for a minute. After washing in sterile water, the root bits were neutralized using 2% hydrochloric acid. The roots were then stained with 0.5% trypan blue in lactophenol (Appendix) for 10 minutes. The stained root tissues were transferred to destaining solution (Appendix) to remove excess stain. Slides containing the stained root tissues were prepared and mounted on a glass slide. Slides were viewed under a light microscope and colonization of *P. indica* was analysed.

3.4.5. Wheat germ agglutinin- Alexaflour 488 (WGA- AF 488) staining and imaging by confocal microscopy

WGA-AF 488 staining and confocal microscopy was carried out at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram with slight modifications of the procedure of Wright (1984). Both control and *P. indica* inoculated plant roots were collected and were fixed in trichloroacetic acid (TCA) fixation solution containing 0.15% (w/v) TCA in 4:1 (v/v) ethanol/ chloroform for 10 mins (Appendix). The fixation was followed by a wash in 1X phosphate buffer saline (PBS, pH 7.4) (Appendix). The roots were then boiled in 10% KOH for 1 min and then neutralized in 1X PBS for 5 minutes. After neutralization, the root tissues were transferrred to the staining solution containing 100µg ml^{-1} WGA-AF 488 dissolved in 1X PBS (pH 7.4) (Appendix). After overnight incubation in the staining solution, the root tissues were destained by overnight incubation in 1X PBS (pH 7.4). The samples were then viewed by confocal laser imaging on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany). The conjugated WGA-AF 488 was excited at 488nm wavelength and detected at 500- 600 nm and the images were captured at different depths.

3.4.6. DNA isolation and PCR

Genomic DNA from both *P. indica* and plants treated with *P. indica* were isolated and PCR was performed to confirm the colonization of *P. indica* in the roots of amaranthus.

3.4.6.1. Genomic DNA isolation from P. indica

DNA from the fungus was isolated by making some modifications in CTAB method (Satheesan *et al.*, 2012).

Two weeks old culture of the fungus grown in PDB was used for the DNA isolation. The mycelia were filtered and washed with sterile water. 200 mg fungal biomass was ground into fine powder using liquid nitrogen in a mortar and pestle and added to one ml of 2% w/v CTAB buffer (Appendix) preheated at 65°C. It was cooled to room temperature (28°C) and then centrifuged at 10,000 rpm for 5 minutes. Equal volume of chloroform/ isoamyl alcohol (24:1) was added to the supernatant, mixed and centrifuged at 10,000 rpm for 10 minutes. 5 µl of RNase (stock concentration 10 mg ml^{-1}) was added to the upper phase and incubated at 65°C for 15 minutes. Chloroform/ isoamyl alcohol extraction was repeated twice followed by the addition of equal volume of isopropanol to the upper phase to precipitate the DNA. DNA was pelleted by centrifugation at 12,000 rpm for 5 min at room temperature. The pellet was air dried and dissolved in nuclease free water for PCR analysis.

3.4.6.2. Genomic DNA isolation from amaranthus

200mg of plant tissue was ground with liquid nitrogen in approximately 500 µl of CTAB buffer to get a fine paste which was then transferred to a microfuge tube. The CTAB/ plant extract mixture was incubated in a circulating water bath at 55°C for about 15 min. After incubation, the mixture was centrifuged at 12, 000 rpm for 5 min to spin down the cell debris. 250µl of chloroform: isoamyl alcohol (24:1) was added to the supernatant. The contents were mixed and centrifuged at 13000 rpm for 1 minute.

The upper aqueous phase containing the DNA was transferred to a clean centrifuge tube and 50 µl of 7.5 M ammonium acetate was added to each tube followed by the addition of ice cold absolute ethanol. The DNA was precipitated by slowly inverting the tubes several times. The DNA was pelleted by spinning at 12000 rpm for 5 min at room temperature. The pellet was was air dried and resuspended in nuclease free water for PCR analysis.

3.4.6.3. PCR

PCR was performed using PiTEF (*P. indica* transcription elongation factor) primers

PiTEF Forward: 5' TCGTCGCTGTCAACAAGATG 3'

Reverse: 5' GAGGGCTCGAGCATGTTGT 3'

The reaction mixture contained 10 pM of both forward and reverse primers, 0.5 µl of dNTP mix (dATPs, dGTPs, dCTPs and dTTPs), 2.5 µl of 10 X PCR buffer with $MgCl_2$, 1 U Taq polymerase enzyme. The total volume was made up to 25 µl with nuclease free water. The reaction consisted of an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 1 minute, extension at 72°C for 2 min and a final extension at 72°C for 5 min.

Reactants	Volume
Sterile water	19 µL
Buffer	2.5 µL
dNTPs	0.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Taq polymerase	0.5 µL

Template DNA	1.5 µL
Total volume	25 µL

The amplified PCR products were loaded on 1% agarose gel and electrophoresis was done and the bands were viewed with the help of gel documentation system.

3.5. *IN VIVO* EXPERIMENTS

In vivo experiments were conducted under greenhouse conditions using the amaranthus KAU variety Arun.

3.5.1. Treatments

T1: *P.indica*
T2: ASM foliar spray
T3: ASM drenching
T4: *P. indica* + ASM foliar spray
T5: *P. indica* + ASM drenching
T6: Pathogen inoculated control
T7: Absolute control
T8: Chemical control (Mancozeb 0.2 % foliar spray)

Experimental design: CRD
Replications: 3

3.5.1.1. Preparation of P. indica inoculum

P. indica was grown in PDB by inoculating it with the mycelial plugs taken from the growing edge of culture grown on PDA plates and incubated at 28^0C for 15

days. The broth containing the fungal mycelium was strained off to get the mycelia and the same was mixed with the planting medium at the rate of 1% (w/v) for the respective treatments.

3.5.1.2. Preparation of the chemical activator

The chemical activator, ASM was kindly provided by Mathews L. Paret, University of Florida, USA. It was prepared in 75ppm and 100 ppm concentrations for drenching and foliar spray respectively.

ASM solutions were prepared by dissolving 150mg and 200mg Actigard 50WG in 1litre distilled water to get 75ppm and 100ppm concentration of ASM respectively.

3.5.1.3. Production of seedlings

Seeds of Amaranthus KAU var. Arun were surface sterilized using 4% sodium hypochlorite solution for 2 minutes followed by several washes with sterile water and sown in sterile vermiculite perlite mixture (3:1) in portrays. For the treatment with *P. indica*, the planting medium in the nursery was incorporated with fungal mycelium at the rate of 1 % (w/v).

Fifteen days old seedlings were transferred to pots containing garden soil. A layer of sterile soil containing the *P. indica* inoculum was given in between the layers of garden soil for the treatments and the planting was done such that the roots were in contact with the inoculum.

The chemical activator ASM was given both by drenching and foliar spray. A 75ppm ASM solution was applied by drenching the base of the plant at the rate of 5ml/plant. A 100ppm ASM solution was used for the foliar spray application. Both the applications were done twice, on one day after transplanting and one week after transplanting.

3.5.1.4. Challenge inoculation with the pathogen

The challenge inoculation was done after 7 days of ASM application with 5 days old *R. solani* culture grown on PDA plates. Three fully opened leaves of the plants except those of the control plants were selected for the inoculation with the pathogen. Mycelial plugs of uniform radii (2mm) were cut from the plates and placed on the lower surface of the leaves. A thin layer of moist cotton was placed over the inoculated part of the leaf and a polythene bag was used to cover the whole to provide humidity.

3.5.1.5. Observations

Following observations of amaranthus were taken after 5 days of challenge inoculation.

3.2.1.5.1. Disease index

Disease intensity was scored using a 0-9 scale (Plate1).

Grade	Description
0	No infection
1	1- 10 percent of leaf area infected
3	11- 15 percent of leaf area infected
5	26- 50 percent of leaf area infected
7	51- 75 percent of leaf area infected
9	76- 100 percent of leaf area infected

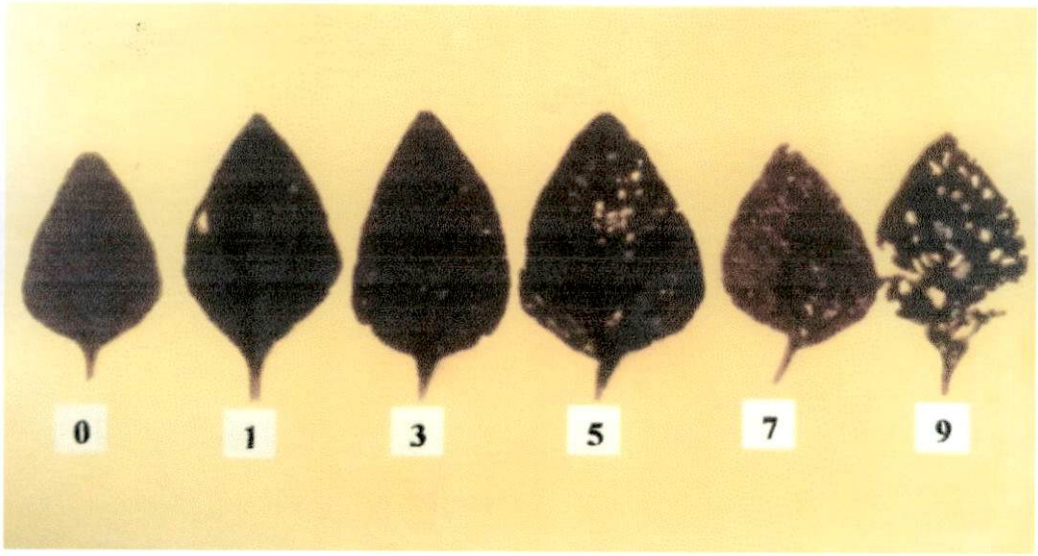


Plate 1. Scale for the scoring of foliar blight of amaranthus

Percent disease index was calculated using the formula

$$\text{Percentage Disease Index (PDI)} = \frac{\text{Sum of individual ratings}}{\text{No. of leaves assessed}} \times \frac{100}{\text{Maximum grade used}}$$

3.5.1.5.2. Percentage root colonization by P. indica

Roots of the plants were taken and cut into 1 cm bits and hundred such bits were viewed under light microscope after staining it with trypan blue following the procedure given by Philip and Hayman (1970) as described in section 3.2.4. Percentage colonization of *P. indica* was analysed.

Percentage root colonization by *P. indica* is given by the formula:

$$\text{Percentage root colonization} = \frac{\text{No. of root bits with chlamydospores}}{\text{Total no. of root bits observed}} \times 100$$

3.5.1.5.3. Shoot length (cm)

After depotting the plants, the length of the shoot of each plant from the ground level to the growing tip was measured.

3.5.1.5.4. Number of leaves

The number of fully opened leaves of each plant was measured.

3.5.1.5.5. Fresh weight of shoot and root (g)

Fresh weight of both shoot and root of each plant was measured separately after depotting.

3.5.1.5.6. Dry weight of shoot and root (g)

Dry weight of the samples was taken after drying them in drying oven at 60°C.

3.5.1.6. Biochemical studies

Leaf samples were taken on 0^{th}, 24^{th} and 48^{th} hour after the challenge inoculation from the plants that received different treatments for estimating changes in activity of defense related enzymes such as phenyl alanine ammonia lyase (PAL), peroxidase and polyphenol oxidase (PPO).

3.5.1.6.1. Phenylalanine Ammonia Lyase (PAL)

PAL activity was estimated using the procedure given by Dickerson *et al.* (1984).

One gram of leaf sample was homogenized in 5 ml 0.1 M sodium borate buffer (pH 8.8) (Appendix) containing 0.05g of polyvinyl pyrrolidone using a chilled mortar and pestle. The homogenate was centrifuged at 10,000rpm for 20 minutes at 4°C. The supernatant was used for the assay. The reaction mixture was prepared by using 3 ml of 0.1 M sodium borate buffer pH 8.8, 0.2 ml of enzyme extract and 12mM L-phenylalanine prepared in the same buffer. The blank contained 3 ml of buffer and 0.2 ml enzyme extract. Both the reaction mixture and blank were incubated at 40°C for 30 minutes. The reaction was stopped by adding 0.2 ml of 3N hydrochloric acid. The absorbance was measured at 290 nm in a spectrophotometer.

PAL activity was expressed as micromoles of cinnamic acid produced per minute per gram of plant tissue.

3.5.1.6.2. Peroxidase (PO)

The procedure described by Srivastva (1987) was used to analyze peroxidase activity.

200 mg of leaf tissue was homogenized at 4°C using a chilled mortar and pestle in one ml of 0.1 M sodium phosphate buffer (pH 6.5) (Appendix) containing 0.05 g of polyvinyl pyrrolidone. The homogenate was centrifuged at 5000 rpm for 15 minutes at 4°C. Both the reaction mixture and blank contained one ml of 0.05 M pyrogallol and 50µl enzyme extract. The sample and blank cuvettes were placed in spectrophotometer with the reading adjusted to zero at 420 nm. The reaction was started by adding one ml of 1% hydrogen peroxide into sample cuvette and change in absorbance was measured at an interval of 30 sec. Peroxidase activity was expressed as change in absorbance of the reaction mixture per minute per gram on fresh weight basis.

3.5.1.6.3. Polyphenol Oxidase (PPO)

PPO activity was estimated using the procedure described by Mayer *et al.* (1965).

200 mg of leaf tissue was homogenized at 4°C using a chilled mortar and pestle in one ml of 0.1 M sodium phosphate buffer (pH 6.5) to which a pinch of polyvinyl pyrrolidone was added. The homogenate was centrifuged at 5,000 rpm for 15 minutes at 4°C and the supernatant was used as enzyme extract. The reaction mixture contained one ml of 0.1 M sodium phosphate buffer and one ml of 0.1 M catechol. Cuvette filled with reaction mixture was placed in spectrophotometer and absorbance was set zero. 50 µl pf enzyme extract was added to start the reaction and change in absorbance was recorded at 495 nm. PPO activity was expressed as change in absorbance of reaction mixture per minute per gram on fresh weight basis.

RESULTS

4. RESULTS

4.1. ISOLATION OF THE PATHOGEN

Rhizoctonia solani, the foliar blight pathogen of amaranthus was isolated from the fields of College of Agriculture, Vellayani. On PDA plates, the mycelial growth was found to be creamish in colour initially which later turned to brownish colour. The fungal culture had zonations and small sclerotia were produced when the fungus completed its growth on the PDA plate (Plate 2). The culture was maintained in PDA slants at 4°C with periodic subculturing.

4.2. PATHOGENICITY TEST OF THE ISOLATED PATHOGEN

Koch's postulates were proved for confirming the pathogenicity of the isolated pathogen. Pale water soaked lesions appeared on the leaves both on the detached leaves and in the standing plants inoculated with the isolated pathogen within 48 hours (Plate 3). On humid conditions, these lesions enlarged and gradually turned translucent with irregular brown margins.

4.3. CULTURING OF *P. indica*

P. indica was grown on PDA plate (Plate 4). The culture was whitish in colour which on ageing turned creamish yellow. It took 10 days for the fungus to grow and spread completely on 90 mm Petri plate.

4.4. *IN VITRO* EXPERIMENTS

4.4.1. Effect of ASM on *R. solani*

The chemical activator ASM did not show any direct inhibitory action on the growth of *R. solani* (Table 1).

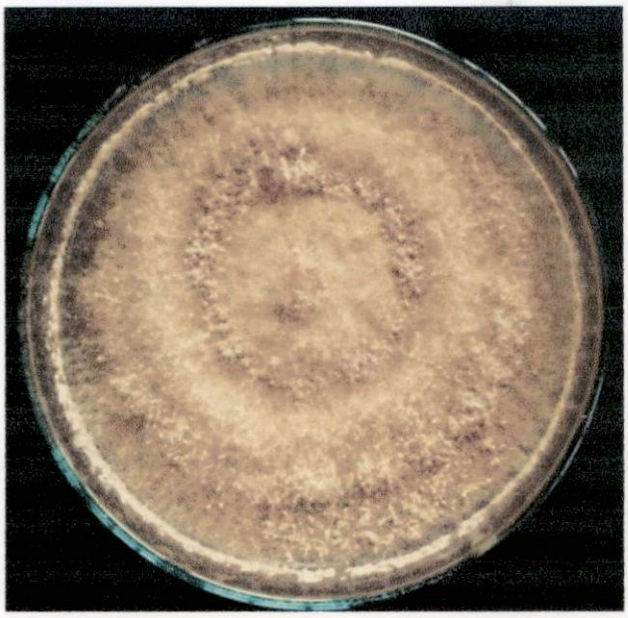


Plate 2. *R. solani* isolated from diseased amaranthus leaves grown on PDA

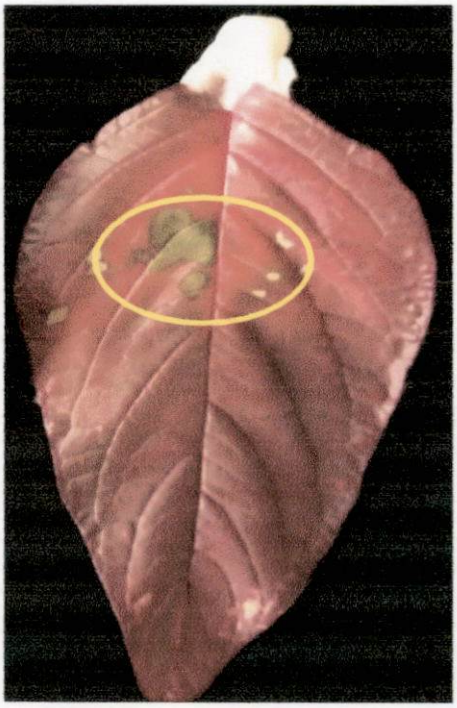


Plate 3. Pathogenicity test done with the isolated pathogen

Table 1. Effect of ASM on the growth of *R. solani*

Concentration of ASM in the medium (ppm)	Diameter of mycelial growth (cm)*	Mycelial inhibition (%)*
10	8.91	0.88
25	8.95	0.44
50	8.99	0
75	8.89	1.11
100	8.85	1.55
150	8.97	0.22
200	8.98	0.11
No ASM	8.99	-

*Mean of three replications having three plates each

4.4.2. Screening for *in vitro* antagonism

P. indica was dual cultured against the pathogen *R. solani* on PDA medium. It was found there was no direct *in vitro* antagonism by *P. indica* against *R. solani* (Plate 5).

4.4.3. Co-culture of *P. indica* and Amaranthus

The endophytic fungus *P. indica* was grown successfully on MS, MS-PDA and PDA media. Amaranthus seedlings were grown along with the fungus in the three media mentioned above. Two methods, trypan blue staining and WGA-AF 488 staining were used for studying the colonization of *P. indica* in the roots. PCR was done using the primers specific for PiTEF gene to confirm the colonization of *P. indica* inside the amaranthus roots.

4.4.3.1. Trypan blue staining of root

After 15 days of seed germination in the medium, colonization was studied by trypan blue staining. Spores of *P. indica* were observed in the cortical cells of the plant root. Dark blue chlamydospores were seen inside the root cells when observed under the light microscope. Mycelia of the fungus were also visible in blue colour (Plate 6).

4.4.3.2. Wheat germ agglutinin- Alexaflour 488 (WGA- AF 488) staining and imaging by confocal microscopy

Chitin specific Wheat Germ Agglutinin- Alexflour 488 (WGA-AF 488) staining and imaging by confocal microscopy showed the fungal colonization in the roots of amaranthus in cortical and epidermal cells (Plate 7).

4.4.3.3. DNA isolation and PCR

Genomic DNA from *P. indica* and *P. indica* colonized amaranthus were isolated. PCR was done using *P. indica* specific TEF gene primers. PCR using the DNA

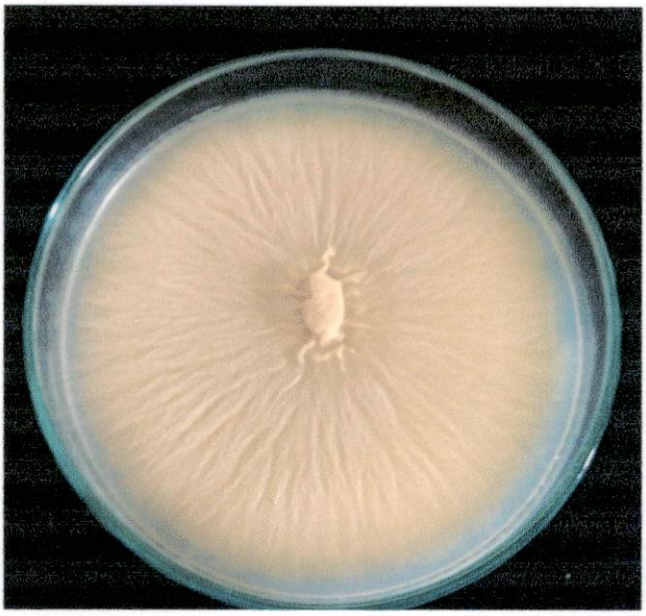
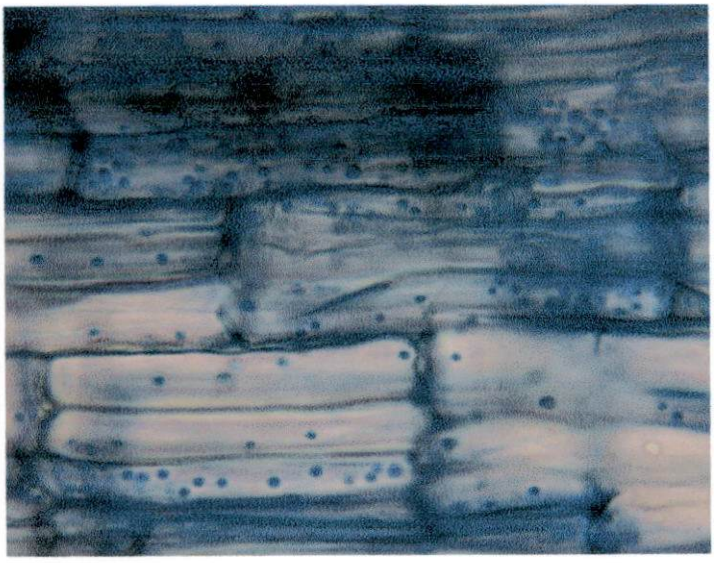
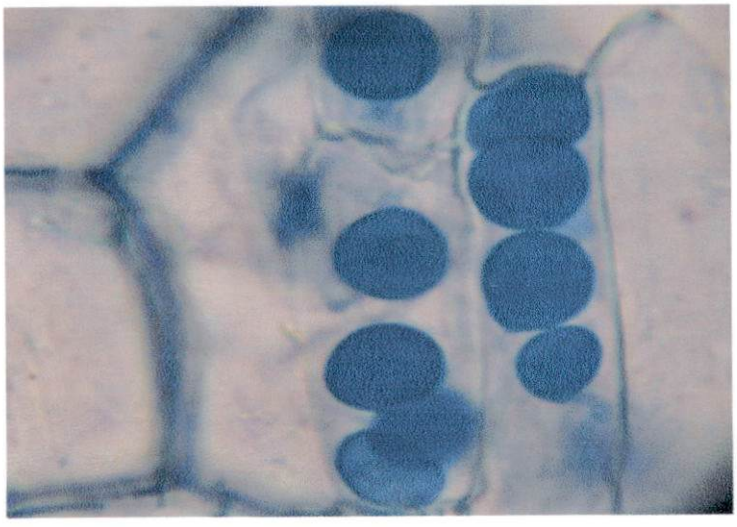


Plate 4. *P. indica* cultured on PDA plate

Plate 5. Dual culture plate assay for screening of *in vitro* antagonism of *P. indica* on *R.solani*



(A)



(B)

Plate 6. Colonization of *P. indica* in amaranthus roots as viewed under light microscope after trypan blue staining (A) Low magnification 10X; (B) 40X magnification

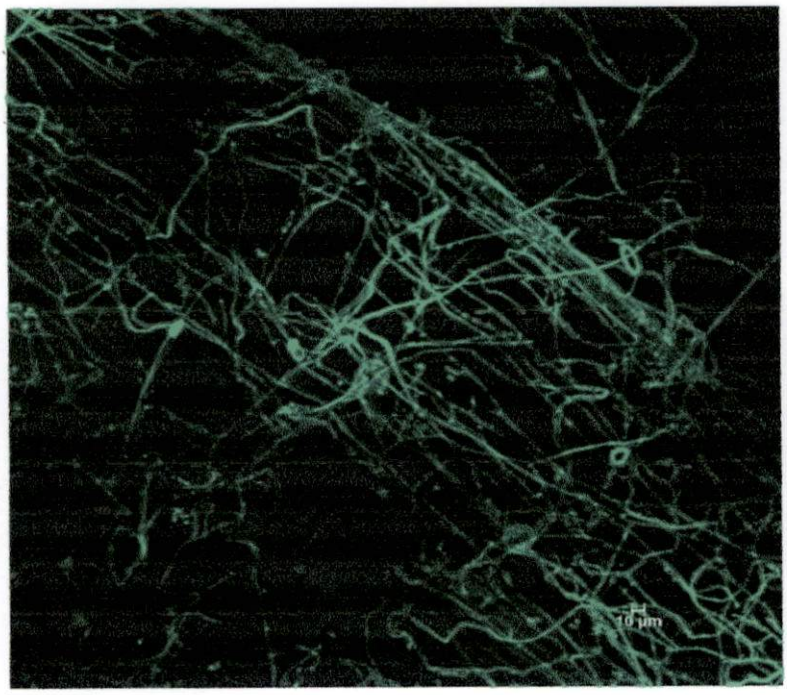


Plate 7. Confocal laser image of WGA-AF 488 stained *P. indica* in amaranthus roots

Table 2: Root colonization of *P. indica* in different treatments

Treatment	Root colonization (%)
P. indica (T1)	48
ASM foliar spray (T2)	ND*
ASM drenching (T3)	ND
ASM foliar spray + *P. indica* (T4)	18.6
ASM drenching + *P. indica* (T5)	4
Pathogen inoculated control (T6)	ND
Absolute control (T7)	ND
Chemical control (T8)	ND

*ND: Not Detected

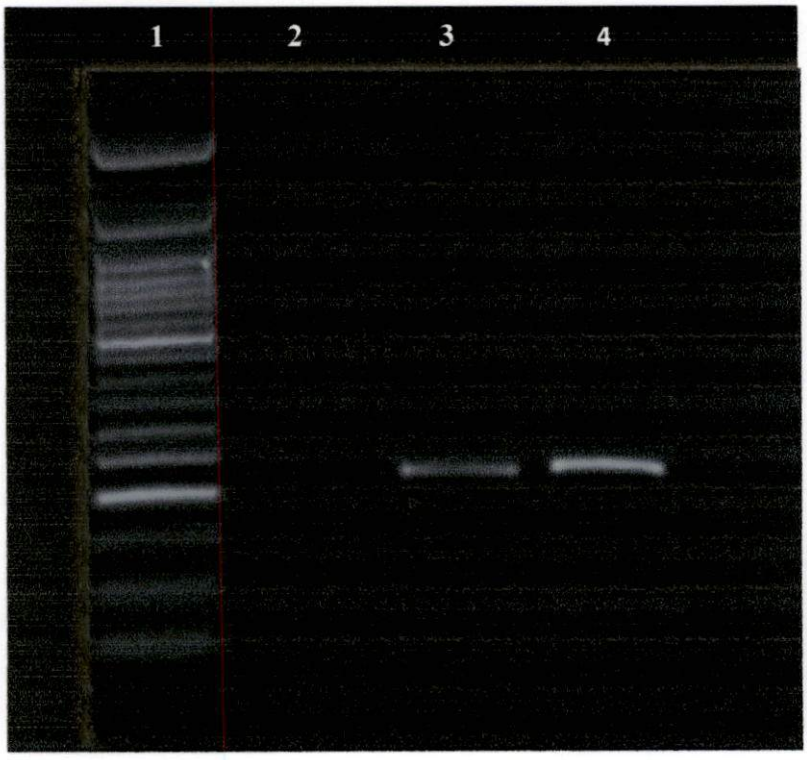


Plate 8. Confirmation of *P. indica* colonization by PCR using PiTEF1 primers Lane 1: 50 bp DNA ladder, Lane 2: DNA from root of uninoculated plants as template, Lane 3: DNA from *P. indica* as template, Lane 3: DNA from roots of *P. indica* inoculated plants as template

isolated from the colonized roots of amaranthus as template produced an amplicon of about 250 bp. These results confirmed the colonization of *Piriformospora indica* in the roots of amaranthus (Plate 8).

4.5. *IN VIVO* EXPERIMENTS

4.5.1. Percentage root colonization by P. indica

Treatment T1 (*P. indica* alone) showed maximum root colonization of 48%. T4 (*P. indica* +ASM foliar spray) showed 18.60% colonization by the fungus and T5 (*P. indica* + ASM drenching) showed colonization of 4% (Table 2).

4.5.2. Disease index

Percentage Disease Index (PDI) was calculated for each of the treatments challenged with the pathogen, *R. solani.* Treatment T7 was the absolute control. T1(treatment with *P. indica* alone) showed delayed disease onset and reduction of 50% disease index was shown on the third day after inoculation. Treatments T3 (ASM drenching) and T4 (ASM foliar spray + *P. indica*) showed no disease progression as the number of days passed (Table 3).

4.5.3. Biometric observations

The following results were obtained for the biometric characters of the plants after different treatments.

4.5.3.1 Shoot length (cm)

Treatment with *P. indica* alone resulted in maximum shoot length of 41.11cm/plant. The mnimum shoot length of 28.23cm/plant was observed for the treatment with ASM foliar spray+ *P. indica* (Table 4).

Table 3: Disease index on 3rd, 4th and 5th day after inoculation with *R. solani*

Treatment	Disease intensity (%)*		
	Day3	Day4	Day5
P. indica (T1)	10.18[d]	15.27[b]	36.11[e]
ASM foliar spray (T2)	28.69[b]	43.05[a]	49.07[c]
ASM drenching (T3)	33.79[b]	44.44[a]	57.40[a]
ASM foliar spray + *P. indica* (T4)	31.79[b]	44.44[a]	45.36[d]
ASM drenching + *P. indica* (T5)	43.96[a]	46.29[a]	55.55[a]
Pathogen inoculated control (T6)	20.36[c]	46.29[a]	51.84[b]
Chemical control (T8)	2.30[e]	5.08[c]	16.20[f]
CD (0.05)	6.20	5.738	2.548

*Mean of three replications having eight plants each

Table 4: Shoot length of amaranthus plants

Treatment	Shoot length (cm/plant) *
P. indica (T1)	41.11^{a}
ASM foliar spray (T2)	31.63^{bc}
ASM drenching (T3)	33.33^{abc}
P. indica +ASM foliar spray (T4)	28.19^{c}
P. indica+ ASM drenching (T5)	28.23^{c}
Pathogen inoculated control (T6)	37.79^{abc}
Absolute control (T7)	34.81^{abc}
Chemical control (T8)	39.59^{ab}
CD (0.05)	7.970

*Mean of three replications having three plants each

4.5.3.2. Number of leaves

The maximum number of leaves were recorded for the treatment involving *P. indica* alone and the minimum was observed for the ASM foliar spray treatment. The two treatments involving the combination of ASM and *P. indica* did not differ significantly (Table 5).

4.5.3.3. Fresh weight of shoot and root (g)

Table 6 gives the shoot and root fresh weight of the treated and control plants. The maximum fresh weight of shoot was obtained for the treatment with *P. indica* alone and the value was found to be 19.38g/plant. However, this did not show any significant difference between the pathogen inoculated control.

The maximum root fresh weight was observed for the *P. indica* treatment and the value was 4.04g/plant. None of the treatments involving ASM application showed any significant difference from each other.

4.5.3.4. Dry weight of shoot and root (g)

Dry weight of shoot for *P. indica* treated plants was found to be maximum with a value of 1.94 g/plant which was not significantly different from the control plants. The four ASM treatments did not differ from each other significantly (Table 7).

4.5.4. Biochemical studies

4.5.4.1. Phenylalanine Ammonia Lyase (PAL)

The treatment which included the combination of *P. indica* and ASM drenching showed maximum phenylalanine ammonialyase activity on the initial hour of pathogen inoculation ie., immediately after the inoculation with pathogen. After 24 hrs, the treatments involving *P. indica* alone and the combination of ASM foliar spray and *P.*

Table 5: Number of leaves of amaranthus plants

Treatments	No. of leaves/ plant*
P. indica (T1)	11.89^{a}
ASM foliar spray (T2)	5.33^{d}
ASM drenching (T3)	7.78^{c}
P. indica +ASM foliar spray (T4)	5.78^{d}
P. indica+ ASM drenching (T5)	5.78^{d}
Pathogen inoculated control (T6)	8.11^{c}
Absolute control (T7)	8.78^{bc}
Chemical control (T8)	10.22^{ab}
CD (0.05)	1.928

*Mean of three replications having three plates each

Table 6. Fresh weight of shoot and root of amaranthus plants

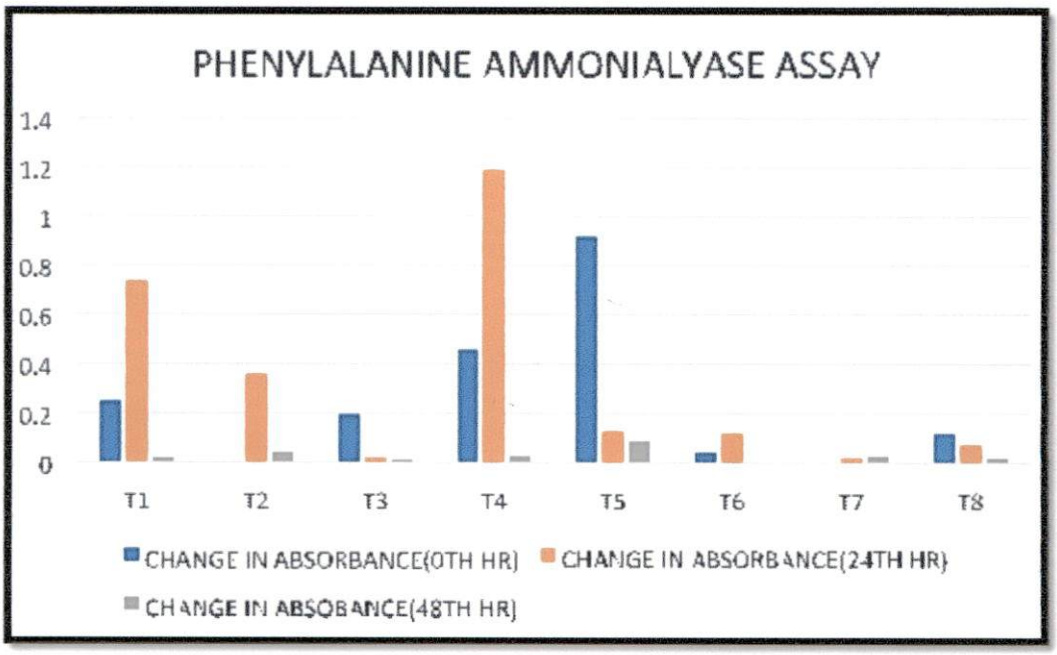
Treatment	Shoot fresh weight (g/plant)*	Root fresh weight (g/plant)*
P. indica (T1)	19.38[a]	4.04[a]
ASM foliar spray (T2)	8.05[b]	0.83[c]
ASM drenching (T3)	10.18[b]	0.81[c]
P. indica +ASM foliar spray (T4)	7.67[b]	1.06[c]
P. indica+ ASM drenching (T5)	9.66[b]	1.35[c]
Pathogen inoculated control (T6)	19.23[a]	3.50[ab]
Absolute control (T7)	10.78[b]	2.80[b]
Chemical control (T8)	17.61[a]	2.92[b]
CD (0.05)	4.986	1.018

*Mean of three replications having three plants each

Table 7. Dry weight of shoot and root of amaranthus plants

Treatment	Shoot dry weight (g/plant)*	Root dry weight (g/plant)*
P. indica (T1)	2.02[a]	0.40[a]
ASM foliar spray (T2)	0.78[b]	0.10[b]
ASM drenching (T3)	1.02[b]	0.13[b]
P. indica +ASM foliar spray (T4)	0.77[b]	0.12[b]
P. indica+ ASM drenching (T5)	1.04[b]	0.16[b]
Pathogen inoculated control (T6)	1.40[a]	0.37[a]
Absolute control (T7)	1.08[b]	0.31[a]
Chemical control (T8)	1.74[a]	0.32[a]
CD (0.05)	0.492	0.119

*Mean of three replications having three plants each



T1: *P. indica*

T2: ASM foliar spray

T3: ASM drenching

T4: *P. indica* + ASM foliar spray

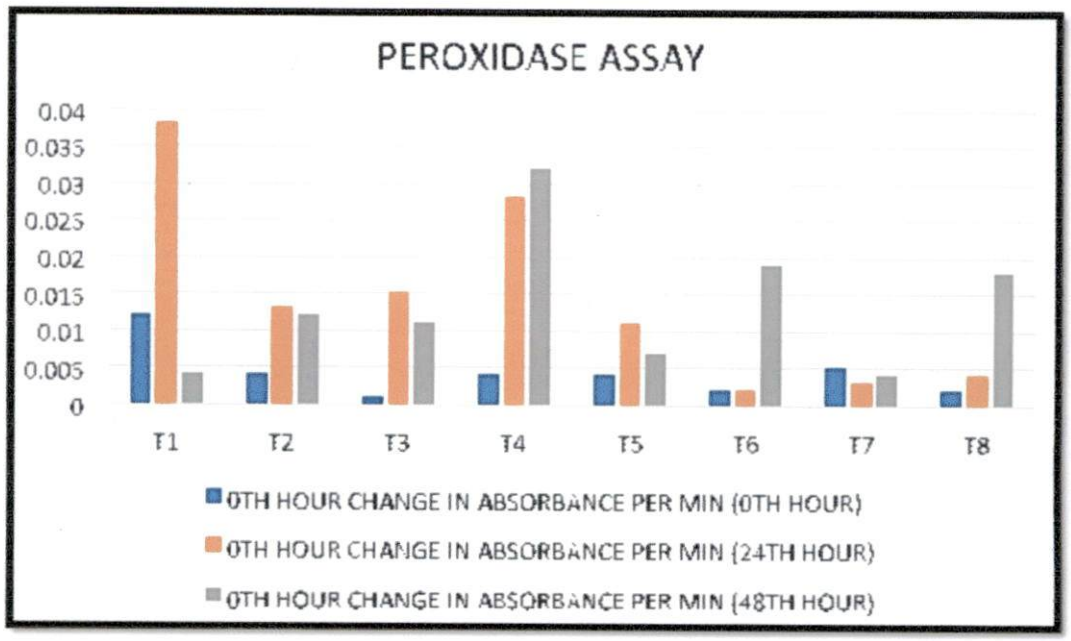
T5: *P. indica* + ASM foliar spray

T6: Pathogen inoculated control

T7: Absolute control

T8: Chemical control

Figure 1. Phenyl Ammonia lyase enzyme activity analysis of amaranthus plants



T1: *P. indica*

T2: ASM foliar spray

T3: ASM drenching

T4: *P. indica* + ASM foliar spray

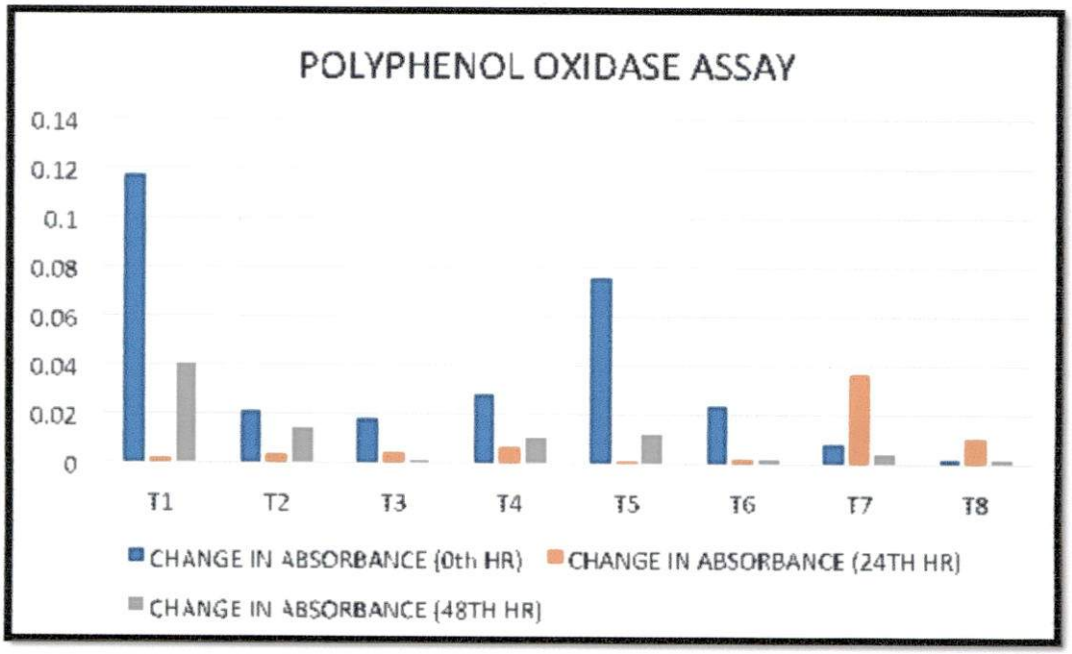
T5: *P. indica* + ASM foliar spray

T6: Pathogen inoculated control

T7: Absolute control

T8: Chemical control

Figure 2. Peroxidase enzyme activity analysis of amaranthus plants



T1: *P. indica*

T2: ASM foliar spray

T3: ASM drenching

T4: *P. indica* + ASM foliar spray

T5: *P. indica* + ASM foliar spray

T6: Pathogen inoculated control

T7: Absolute control

T8: Chemical control

Figure 3. Polyphenol oxidase enzyme activity analysis of amaranthus plants

indica presented increased enzyme activity. However, there was no significant rise in the enzyme activity after 48 hrs (Figure 1)

4.5.4.2. Peroxidase (PO)

On estimation of peroxidase enzyme immediately after the pathogen inoculation, the change in absorbance was maximum for the *P. indica* treated plants. After 24 hrs and 48 hrs of challenge inoculation, the values were found to be maximum for the *P. indica* treated plants and the plants treated with the combination of ASM and *P. indica* (Figure 2)

4.5.4.3. Polyphenol oxidase (PPO)

The treatments with *P. indica* and its combination with ASM drenching showed an increased value of PPO activity on the zeroth hour of challenge inoculation. In the subsequent 24^{th} and 48^{th} hours, there was no significant increase in the PPO activity (Figure 3).

DISCUSSION

5. DISCUSSION

Amaranthus is an agriculturally important crop in many parts of the world. It is of high nutritional value due to the presence of lysine and calcium (Coimbra and Salema, 1994). Foliar blight caused by the fungal pathogen *Rhizoctonia solani* is a serious threat to the amaranth growers and cause about 90% economic loss. As chemical control causes serious environmental pollution and health hazards, biocontrol of phytopathogens is of great importance (Anand *et al.*, 2010)

The present investigation involved the evaluation of a beneficial root endophytic fungus *Piriformospora indica* and a chemical plant activator Acibenzolar-S-methyl (ASM) on suppression of foliar blight of amaranthus caused by the fungus, *Rhizoctonia solani*. Previous works were done at the Department of Plant Pathology, College of Agriculture, Vellayani on the suppression of foliar blight of amaranthus. Smitha (2000) used microbial antagonists like *Trichoderma longibrachiatum* and *Pseudomonas fluorescens* for managing foliar blight. An attempt was made by Priyadarshini (2003) to manage foliar blight by using *Trichoderma harzianum*, *Pseudomonas fluorescens*, *P. indica* and indigineous materials like turmeric powder-baking soda and rice husk ash and colonization of *P. indica* in amaranthus has also been reported. A work was performed to control foliar blight by using plant growth promoting rhizobacteria and ASM (Nair, 2005).

In the current study, the role of the chemical activator ASM and the endophytic fungus *Piriformospora indica* in inducing resistance has been focused. Significance was given to two types of induced resistance in plants which comprises of induced systemic resistance (ISR) and systemic acquired resistance (SAR). The root endophytic mutualistic fungus *Piriformospora indica* is known to trigger resistance in plants. ASM is a chemical activator which elicit resistance in plants against various plant pathogens. ASM is a synthetic structural analog of salicylic acid which is an important component in plants defence mechanism (Tripathi and Pappu, 2015). ASM is found to induce

multiple resistance to fungal and bacterial diseases (Ishii, 2003). Attempts were made to induce resistance against *R. solani* in amaranthus plants by using the beneficial root endophyte *P. indica* and the chemical activator ASM. These were given as individual treatments which encompassed ASM drenching, ASM foliar spray and *P. indica* incorporation in the planting medium and in combinations to evaluate their effect on the suppression of foliar blight caused by *R. solani*.

The effect of different concentrations of ASM on the growth of the pathogen was tested *in vitro* using the poisoned food technique. 10ppm, 25ppm, 50ppm, 75ppm, 100ppm, 150ppm and 200ppm concentration of ASM were prepared and incorporated with PDA media. It was observed that ASM had no inhibitory activity on the growth of *R. solani in vitro*. It was earlier reported that ASM had no significant inhibitory effect on fungal pathogens *in vitro* (Gullino *et al.*, 2000; Akinwunmi *et al.*, 2001; Geetha and Shetty, 2002).

Two staining procedures, trypan blue staining and WGA-AF 488 staining were used to estimate the colonization of *P. indica* in amaranthus. After WGA-AF 488 staining and confocal microscopy, it was observed that *P. indica* colonization was more effective in plants grown *in vitro* in the culturing media than under the greenhouse conditions in vermiculite perlite (3:1) mixture. Hence it can be said that coculturing of *P.indica* and amaranthus *in vitro* forms a better system for its effective colonization in the host plants. Colonization of *P. indica* was further confirmed by using PCR using the primers specific for TEF genes in *P. indica*. Successful colonization of *P. indica* was confirmed in *Centella asiatica* by amplifying the species specific PiTEF1 gene of *P. indica* from the whole genomic DNA isolated from the roots of *Centella* (Satheesan *et al.*, 2012).

Treatment T1 (*P. indica* alone) showed maximum root colonization of 48%. T4 (*P. indica* +ASM foliar spray) showed 18.60% colonization by the fungus and T5 (*P.indica* + ASM drenching) showed the least colonization of 4%. In combination with

ASM *P. indica* colonization was found to be lesser. This could be due to possible suppressive effect of ASM on the growth of the root endophyte.

Percentage Disease Index (PDI) was calculated by scoring the disease symptoms. Among the treatments, the plants treated with foliar spray of 0.4% mancozeb showed the least value of PDI. The plants treated with *P. indica* alone expressed a PDI greater than the chemical control but the value was lesser than all other treatments. The treatments in which ASM was applied PDI was found to be higher than the other treatments. Among the four treatments, T5 (*P. indica* +ASM drenching) showed the highest disease index on the third day after inoculation. On the fourth day after inoculation, it was found that disease index was increased by 3% in T5 but in control plants it was increased to almost twice as that on the third day. It was earlier found that ASM had growth retardation effects resulting in the stunted growth of amaranthus (Nair and Anith, 2009). As ASM causes growth retardation effects including decrease in leaf area, even a small lesion in the leaf can attribute to a higher score and result in a higher PDI value when compared to other treatments. However, the progression of the disease was found to be much lesser in the treatments involving ASM. It can be suggested that it might be due to the resistance induced by ASM that the spread of the disease was lowered. *Piriformospora indica* was found effective in reducing the foliar blight disease index in amaranthus plants by 50%, 67% and 30.34% respectively on 3rd ,4th and 5th day of challenge inoculation when compared to control plants. In the treatments involving ASM on the 3rd day after pathogen inoculation PDI was found to more than the control but on the 4th and 5th day there was decrease of 6.99% and 5.34% in PDI of T2 (ASM foliar spray). In both T3 and T4 the decrease in PDI was found to be 3.99 on the 4th day after pathogen inoculation. In T4 (*P. indica* +ASM foliar spray), PDI was decreased by 12.5%. It was noted that the pathogen inoculated control showed an increase in symptom and the entire leaf was covered in lesions due to high severity of the disease.

Most of the previous reports suggest that *P. indica* has attributed to growth promotion and increase in biomass of several plants. It has increased plant height, shoot biomass and number of tillers in *Oryza sativa* when compared with the plants not inoculated with *P. indica* (JinDan *et al.*, 2015). It has enhanced plant growth in tomato plants (Fakhro *et al.*, 2010). Growth parameters including shoot length, fresh weight and dry weight of root and shoot was found to be enhanced in plants treated with *P. indica* which was in conformation with the previous reports. Seed germination rate was also increased in the seeds sown when the planting material was incorporated with the fungus. For plants treated with ASM alone and its combination with *P. indica*, the biometric parameters were observed to be lesser than the control. When the four treatments involving ASM were compared, it was found that the values of the growth parameters were more in the treatment in which ASM was applied as soil drenching both individually and in its combination with *P. indica*. T3 (ASM drenching) recorded a shoot length 33.33 cm/plant, number of leaves 7.78/plant whereas T2 (ASM foliar spray) showed a lesser shoot length of 31.63 cm/plant and 5.3 number of leaves/plant which was lower than the treatment of ASM drenching. It was reported by Prats *et al* (2002) that the increased application of ASM caused a reduction in shoot weight of sunflower plants and current study also gave similar results on plant growth and biometric characters after ASM application.

Effect of the treatments on three defense related enzymes including phenyl ammonialyase (PAL), Polyphenol oxidase (PPO) and peroxidase (PO) was estimated. Earlier works suggested that the activity of defense enzymes varied significantly between inoculated and uninoculated plants when the plants were sprayed with ASM (Cruz *et al.*, 2013). It was reported by Boava *et al.*, (2009) that there was elevation in the defense enzyme production in *Eucalyptus grandis* treated with ASM and *Saccharomyces cerevisiae* extract against the pathogenic fungus *Puccinia psidii* which cause rust in the plants. *P. indica* was shown to induce systemic resitance against rice sheath blight by interfering with H_2O_2 accumulation and activation of antioxidants

(Nassimi and Taheri, 2017). Zine *et al* (2016) suggested that ASM primed seedlings of tomato for enhanced activity of peroxidase and polyphenol oxidase and protects the plants indirectly from the pathogen *Verticillium dahlia* by activating plant defense responses. The disease symptoms of *R. solani* were seen after 24- 48 hours after inoculation in the amaranthus plants. PAL activity was maximum for the T5 and T4 treatments which was a combination of *P.indica* and ASM drenching and foliar spray respectively during the first hour after inoculation. After 24 hours of pathogen inoculation, it was observed that there was a two-fold increase of PAL activity in the treatment where ASM foliar spray along with *P. indica* and it decreased after 48 hours of challenge inoculation. In *P. indica* treated plants, there was a three fold increase after 24 hours and it gradually decreased after 48 hours. Peroxidase enzyme estimation on zeroth hour showed an increased value for *P. indica* treated plants, a three fold increase in PO activity was noted after 24 hours and later it decreased. Almost a same trend was found in the treatments concerning ASM and its combination with *P. indica*. However, individual application of ASM represented a lesser enzyme activity than its combination with the fungus. PPO estimation results showed the same pattern of its activity as in PAL and PO estimation. The current study clearly shows an increment in the defense enzymes in the initial hours after inoculation with the pathogen and later it decreased. This can be because of interaction between the pathogen and the different treatments in its establishment in the host and its progression in the host. The chemical activator and *P. indica* might hinder the pathogen enactment and its spread in the plants.

The present study was the first attempt to exploit the combination of the chemical activator ASM and the root endophytic fungus *P. indica* in growth promotion and impart disease resistance against *Rhizoctonia solani* which causes foliar blight in amaranthus. Further standardization of the dose of ASM and the fungal inoculum is necessary to understand the combined effect of chemical activator and *P. indica* in amaranthus plants against the foliar blight disease.

SUMMARY

6. SUMMARY

Amaranthus is an important agricultural crop of Kerala. The foliar blight disease caused by *Rhizoctonia solani* is a serious threat to the farmers in Kerala as it results in high economic loss. The objective of the current study is to evaluate the combined effect of the root endophytic fungus *Piriformospora indica* and the chemical activator Actigard™ 50 WG containing the active ingredient Acibenzolar-S-methyl (ASM) on the suppression of the foliar blight disease.

The pathogen *R. solani* was isolated from the fields of College of Agriculture, Vellayani and its pathogenicity was confirmed by inoculating the healthy leaves of amaranthus with the isolate and then reisolating the pathogen from the infected plants. Foliar blight disease suppression and plant growth promotion studies were carried out on amaranthus plants using *P. indica* and ASM by applying them individually and in combination of both.

In vitro experiments included screening for direct antagonism of both ASM and *P. indica* on the growth of the pathogen *R. solani.* Poisoned food technique was employed for studying the effect of ASM on *R. solani.* It was found that ASM did not show any direct inhibitory effect on the growth of the pathogen when grown on PDA incorporated with ASM at different concentrations. Dual culture plate assay was used to check whether *P. indica* had any suppressive effect on *R. solani* and it was observed that the pathogen grew over *P. indica* indicating that there is no direct inhibition by *P. indica* on *R. solani.*

In vivo experiments were carried out under greenhouse conditions where plants were treated with the root endophytic fungus *P. indica* and ASM both individually and in combinations of both. ASM was applied as drenching as well as foliar spray. Foliar blight disease intensity was studied and the percentage disease index was minimum in the absolute control followed by chemical control. Among the rest of the treatments, *P. indica* treated plants showed a low disease index of 50%, 67% and 30.34% on the

third, fourth and fifth day respectively of inoculation with pathogen. ASM treated plants showed an increased disease intensity after three days of challenge inoculation. Later T2 and T3showed a decrease of 3.99% PDI on the fourth day after inoculation. In T4 (*P. indica* +ASM foliar spray), PDI was decreased by 12.5% on the fifth day of challenge inoculation. It was recorded that the disease progression was much reduced in the treatments involving ASM when compared to other treatments.

Effect of ASM and *P. indica* on the growth promotion of amaranthus plants was analysed by observing the biometric characters encompassing fresh and dry weight of shoot and root, length of shoot and the number of leaves in each plant of each treatment. Plants inoculated with *P. indica* exhibited maximum values of all the growth parameters and plants treated with ASM and its combination with *P. indica* displayed lower values than the control plants which may be due to growth retardation effects of ASM. Activities of three defense enzymes (peroxidase, polyphenol oxidase and phenylalanine ammonialyase) were estimated by measuring the aborbance at various wavelengths as per the protocols.

Modifications in the dose, concentration and the time of application of *P. indica* and the chemical activator can be useful in better understanding of its combine effect in the suppression of foliar blight. Molecular characterization of different genes involved in the defence mechanism against *R. solani* by the resistance imparted by both the agents can be investigated and the results can be used to exploit its further use in disease management of amaranthus on a large scale.

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7. REFERENCES

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APPENDICES

APPENDIX I

Composition of media used

1. **Potato Dextrose Agar (PDA) (1 litre)**

   Potato - 200 g

   Dextrose - 20 g

   Agar - 20 g

   Distilled water - 1 litre

   pH - 7

2. **Murashige- Skoog (MS) medium (3%)**

   MS media w/o agar – 30g

   Agar - 10 g

   Distilled water - 1000 ml

3. **MS- PDA medium (1:1)**

   3% MS w/o agar and Potato Dextrose Broth (PDB) was prepared separately and mixed in the ratio 1:1. 20 g of agar was added to the 1 litre (1:1 mixture) of MS-PDB and sterilized for use.

APPENDIX II

Solutions for trypan blue staining

1. **Trypan blue staining solution (100 ml)**

   Lactic acid – 20 ml

   Glycerol – 40 ml

   Phenol – 20 ml

   Water – 20 ml

   Trypan blue – 50 mg

2. **Destaining solution (100 ml)**

   Lactic acid – 20 ml

   Glycerol – 40 ml

   Phenol – 20 ml

   Water – 20 ml

APPENDIX III

Solutions for WGA-AF 488 staining

1. **Trichloro acetic acid fixation solution**

   Ethanol - 80 ml

   Chloroform - 20 ml

   Mix the above contents to get a 4: 1 (v/v) ethanol/ chloroform solution.

   Trichloro acetic acid (TCA) – 0.15 g

   Dissolve TCA in 80 ml ethanol/ chloroform solution and make up the volume to 100 ml.

2. **Phosphate Buffer Saline (PBS) pH – 7.4 (10 X) (1000 ml)**

   Sodium chloride- 80 g

   Dibasic sodium phosphate – 11.6 g

   Monobasic potassium phosphate- 2.4 g

   Potassium chloride – 2g

   Sodium azide – 2 g

   Dissolve in 900 ml water, adjust the pH with HCl and make upto 1L.

3. **WGA- AF 488 in PBS (10 ml)**

   WGA- AF 488- 1 mg

   Dissolve in 8 ml 1X PBS and make upto 10 ml.

APPENDIX IV

Buffers for enzyme analysis

1. **0.1 M sodium 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)

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

2. **0.1 M sodium phosphate buffer (pH 6.5)**

   A: 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml)

   B: 0.2 M solution of dibasic sodium phosphate (53.65 g of Na2HPO4. 7H2O in 1000 ml)

   68.5 ml of A is mixed with 31.5 ml of B, diluted to a total of 200 ml.

APPENDIX V

1. CTAB extraction buffer

CTAB - 2%

PVP - 2%

Tris HCl (pH 8) – 100 mM

EDTA – 25 mM

NaCl – 2M

β- Mercaptoethanol- 2%

EDTA was dissolved using sodium hydroxide pellets and pH was adjusted to 8. It was then heated for complete dissolution of EDTA. pH of Tris was adjusted using Conc. HCl. The reagents were autoclaved and stored.

"Evaluation of systemic acquired resistance and induced systemic resistance on the suppression of foliar blight disease of amaranthus (*Amaranthus tricolor* L.)"

By

ATHIRA BABU B. M.

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Abstract of Thesis

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Faculty of Agriculture
Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM - 695 522
KERALA, INDIA

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ABSTRACT

The study enititled 'evaluation of systemic acquired resistance and induced systemic resistance on the suppression of foliar blight disease of amaranthus (*Amaranthus tricolor* L.)' was conducted at the Department of Agricultural Microbiology and Department of Plant Biotechnology, College of Agriculture, Vellayani.

The study was focused on the evaluation of the combined effect of mutualistic fungus *Piriformospora indica* and Actigard™ 50WG whose active ingredient is Acibenzolar-S-methyl, the plant activator which is a synthetic structural analog of salicylic acid. *In vitro* studies were done for testing the antagonism of *P. indica* and ASM on the growth of *R. solani* by making use of dual culture plate assay and poisoned food technique respectively. The results present no direct *in vitro* antagonism on the pathogen by both *P. indica* and ASM. Colonization of *P. indica* in the roots of amaranthus were confirmed by two staining procedures, trypan blue staining and WGA-AF 488 staining. It was further validated by PCR amplification using species specific primers for TEF1 gene of *P. indica*. The results of confocal microscopy suggest that coculture of *P. indica* and amaranthus *in vitro* can be a better strategy for better colonization of the fungus in the roots.

Disease suppression and growth promotion studies were done using different treatments comprising of the fungus and ASM applied individually and in combinations of both. ASM was applied both as foliar spray and root drenching. Colonization study after ASM treatment revealed that ASM application in the root zone of the plants can result in reduction of percentage root colonization of the fungus, *P. indica.*

The minimum disease intensity was found in chemical control followed by plants treated with *P. indica*. *P. indica* was found effective in reducing the foliar blight disease index in amaranthus plants by 50%, 67% and 30.34% respectively on 3rd ,4th

and 5th day of challenge inoculation when compared to control plants. In the treatments involving ASM on the 3rd day after pathogen inoculation PDI was found to more than the control. However, a decrease of 6.99% and 5.34% in PDI of T2 was observed on the 4th and 5th day after inoculation with *R.solani*. Although ASM did not show significant decrease in percentage disease index, the progression of the disease as days passed after inoculation with *R. solani* was very low. This might be due to the resistance imparted by ASM to the amaranthus plants. However, the combinations of *P. indica* and ASM did not display any convincing results in the enhancement of growth parameters when compared to the control as ASM was found to cause a decrease in the plant growth while it imparts resistance to the amaranthus plants against foliar blight caused by *R. solani*.

The current investigation acknowledges the growth promoting effects of *P. indica* and role of *P. indica* and ASM in imparting resistance against the foliar blight disease caused by *Rhizoctonia solani*.