MATERIALS & METHODS

3.1 Collection of pathogenic strains of *Rhizoctonia solani* causing rice sheath blight from various geographic regions

Samples from four rice-growing states viz. Andhra Pradesh, Tamilnadu, Karnataka, and Punjab in India were collected using transect sampling by walking through the field diagonally. Ten samples were collected per field all along the path of the diagonal. A sample usually consisted of a single rice tiller, which either had sheath blight lesions on the sheath, on the leaf blades, or on both.

Isolation of the fungus:

Rice sheaths and leaf blades with sheath blight symptoms were surface-disinfected with 0.5% sodium hypochlorite for 45 sec and rinsed three times with sterile distilled water. Pieces of sheath or leaf blade dried on sterilized filter paper were placed on a Petri dish containing acidified water agar (PH 4.5) with 10% lactic acid (AWA), and incubated at 28°C in the dark. After 2 to 3 days, cultures developed from infected materials were examined microscopically for hyphal characteristics typical of *Rhizoctonia* spp. (Shew, 1985; Sneh *et al.* 1991). All plated samples yielded *Rhizoctonia* spp. and a hyphal tip of each isolate was subcultured onto AWA for further purification. Isolates were transferred to potato dextrose agar (PDA) slants and maintained at 28°C. Following sufficient growth and production of sclerotia, culture tubes were kept at 4°C for short-term storage. Fungal isolates consisting either mycelium or sclerotia were lyophilized and stored at 4°C for long-term storage.

Morphological and cultural diversity of *Rhizoctonia solani*:

Isolates were subcultured onto PDA Petri dishes in triplicate,

incubated at 28°C for 3 weeks, and studied for the cultural characteristics *viz.*, colony characters (colour, appearance, margin, diameter) and sclerotial characters (shape, growth pattern, number, diameter and texture).

3.2 Aggressiveness of the isolates.

Inoculation of *R. solani* isolates was done on *Oryza sativa* cv. MTU 1010, which is susceptible to sheath blight, to investigate the variation in the virulence of different *R. solani* isolates of rice identified in this study and also to identify the most aggressive isolate. Prior to testing, all isolates were passed through rice and re-isolated. Rice seeds were germinated in humid plates at 28°C for 4 days. Germinated seeds were sown in trays in potting compost (grown under greenhouse conditions at 30 ± 4°C with a 16/8 h light/dark photoperiod). Plants were provided with fertilizers at 8, 15, 22, 29, and 36 days with 5 g of $(NH4)_2$ SO_4. Forty-two day-old plants were inoculated with single, small (approx.0.2 mg) immature sclerotium (approx. 4 days old) placed inside the sheath of the second (from top) fully expanded leaf at 5 cm above the soil surface and a few drops of sterile water was added. Inoculation was done in the evening and the inoculated plants were sprayed with water the next morning. (Amita singh *et al.* 2002). For each isolate, eight replicate rice plants were inoculated in a completely randomized experimental design and the experiment was repeated twice. A non inoculated plant with just sterile water sprayed was used as a control for the pathogenicity tests. Immediately after inoculation, all plants were transferred to a plastic moist chamber with 92 to 100% humidity, inside a greenhouse. Disease incidence was recorded 7 days

after inoculation by determining relative lesion height using the following formula (Sharma, 1990)

([highest point a lesion is seen/plant height] × 100)

Isolate identities were confirmed through re-isolation.

3.3 RAPD analysis of Molecular variability among *Rhizoctonia solani* isolates from rice.

Isolates of *R.solani* used in this study were distinguished using the PCR protocol described by Williams *et al.* (1990) using random primers for PCR amplification. Molecular variability in twenty-eight isolates of *R.solani on* rice was studied by RAPD technique. Total genomic DNA from the fungal isolates was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method as follows.

Total genomic DNA extraction

Isolates were grown in a glucose yeast medium (1.0g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 10.0 g glucose, 5.0 g yeast extract, 1.0 ml of 0.5% copper solution, and 1.0 ml of 1% zinc solution per liter of distilled water) for 4 days at 27°C in an orbital incubator at 100 rpm. Cultures harvested by vacuum filtration were washed with sterile distilled water and freeze-dried. Lyophilized mycelium was ground and approximately 50 mg was homogenized with 500 µl extraction buffer (0.15M NaCl, 50mM Tris-HCl, 10mM EDTA and 2% sodium dodecyl sulphate, pH 8). The slurry was incubated at 65°C for 30 min prior to addition of 500 µl phenol: chloroform: isoamyl-alcohol (125:24:1) and centrifuged at 13000 g at 4°C for 1h. The upper aqueous layer was collected and incubated with 25 µl of ribonuclease solution (20 mg ribonuclease [70U mg^{-1}] ml^{-1} TE buffer) for 1 min. An equal volume of chloroform : isoamyl-alcohol (24:1, v/v) was

added, followed by centrifugation at 13000 g at 4°C for 10 min. The upper aqueous layer was collected and DNA was precipitated with 0.54 volume of cold isopropanol. Pellets of DNA were washed in 70% ethanol, dried under vacuum and finally redissolved in 10-50 µl TE buffer, depending on the size of the pellet. Dissolved DNA was stored at -20°C.

Assessment of DNA quality and quantity

The extracted DNA was quantified by running 2 µl of each DNA sample on 0.7% agarose gel with Lambda uncut DNA (30 and 60 ng) to get a final concentration of 15-20 ng/ µl. To assess the quality, the DNA was diluted with TE buffer and loaded on 1% agarose gels in 0.5 X TE buffer (pH 8.0) in a gel electrophoresis apparatus (Biorad, USA) and electrophoresed for 30 min under constant voltage (70 V). The ethidium bromide (final concentration of 0.3 µg/ ml) was added to the molten agarose at the time of gel casting. The gel was visualized on a UV transilluminator, and photographs were taken using a Gel Documentation System (Alpha Innotech Corporation, USA).

Primers and PCR amplification

Thirty Operon oligonucleotide primers were screened for amplification of genomic DNA of *R. solani*. Among them four primers OPC 5, OPC 2, OPA 8 and OPA 11 were selected and used for amplification. The PCR amplifications were carried out in a total reaction volume of 25 µl containing 15-25 ng of genomic DNA. The reaction buffer consisted of 2.5 µl of 10 X PCR buffer [(500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.0)], 0.2 µM each Operon primer (0.2 mM of dNTP mix (d ATP, dTTP, dCTP and dGTP, Fermentas Inc., MD, USA) and 0.2 µl of *Taq* DNA

polymerase (Bangalore Genei, Bangalore, India). The PCR amplifications were performed by using thermal cycler (Biometra) programmed for initial DNA denaturation at 94°C for 5 min followed by 45 cycles at 94°C for 1 min, 45°C for 1 min 72°C for 2 min and a final cycle was performed at 72°C for 7 min. All the amplified DNA products were resolved by electrophoresis on agarose gel (1.5%) in TAE (1 X) buffer, stained with ethidium bromide and photographs were taken by using Gel Documentation System.

Data analysis

DNA fingerprints were scored for the presence '1' or absence '0' of bands of various molecular weight sizes in the form of binary matrix. Data were analyzed to obtain Jaccard's coefficients among the isolates by using NTSYS-pc (version 2.0; Exeter Biological Software, Setauket, NY). Jaccard's coefficients were clustered to generate dendrograms by using the SAHN clustering programme, selecting the UPGMA algorithm in NTSYS-pc (Rohlf, 1993). The similarity values were subjected to Principal Coordinate Analysis also.

3.4 Biological control of *Rhizoctonia solani*

Biological control, which is a part of integrated disease management and eco-friendly alternative to chemicals to control the disease, an attempt was made to isolate the antagonistic candidates from *Pseudomonas fluorescens* and *Trichoderma* spp. and examined their biocontrol potential under *in vitro*, *in vivo* and field against *R. solani*

Collection of soil samples for the isolation of biocontrol agents

Rhizosphere soil samples were collected from the rice fields with sheath blight in different rice growing areas in four states of India. One kilogram of rhizosphere soil samples were collected into clean plastic bags. Five soil samples taken (four at the corner and one at the center) from each study site were mixed thoroughly into one composite sample. Ten-gram sub samples of soil from each composite sample were processed for isolation of fluorescent *Pseudomonas/Trichoderma* isolates. In cases where processing of the samples could not be done on time, the samples were refrigerated (4°C) for not more than two weeks.

3.4.1. Isolation and identification of *Pseudomonas fluorescens* from rice rhizosphere.

To isolate the rhizosphere colonizing *Pseudomonas fluorescens*, one g soil sample was shaken in 100 ml of sterile distilled water for 25 min. Fluorescent pseudomonads were isolated on King's Medium B (KMB). According to the methodology of Schaad (2001), antagonistic isolates of bacteria were identified by biochemical, physiological and biochemical tests.

In vitro* evaluation of the isolates of *Pseudomonas fluorescens* against *Rhizoctonia solani

All isolates of *Pseudomonas fluorescens* were assessed for their biocontrol efficiency under *in vitro* conditions using dual culture technique, and screening for volatile antifungal compounds and extracellular metabolites. The effect of bioagents on the germination and lysis of sclerotia under *in vitro* conditions and in soil was also studied.

Efficacy of *Pseudomonas fluorescens* isolates against *Rhizoctonia solani* in dual culture

R. solani isolated from sheath blight of the rice cultivar MTU 1010 was shown to be highly virulent in a subsequent pathogenicity test. Efficacy of the *P. fluorescens* in inhibiting growth of *R. solani* was tested by streaking each bacterial isolate on one side of a Petri dish containing PDA and nutrient agar medium (Expert, 1995). A 5 mm mycelial disc from 4 day old culture of *R. solani* on PDA and NA was placed at the opposite side of the Petridish and experiments were independently repeated four times. Growth of fungus was inhibited when it grew toward the bacterial colony and the inhibition zone was measured from the edge of mycelium to the bacterial colony edge.

Screening of the isolates of *Pseudomonas fluorescens* for the production of volatile antifungal compounds.

Two hundred fifty micro liters of an antagonistic bacterial suspension (10 $CFUml^{-1}$) was placed in the Petri dish containing KMB and a 5 mm disk of a four day old pure culture of *R. solani* was placed at the center of another Petri dish containing PDA. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at 26° C for 6 days and the growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonist. Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times (Sivan *et al.* 1987). Results were expressed as means of % inhibition of the growth of *R.solani* in the presence and

absence of any bacterial isolate. Percent inhibition was calculated using the following formula (Sivan *et al.* 1987).

% inhibition = 1 - (fungal growth/ control growth)) x 100

Screening of the isolates of *Pseudomonas fluorescens* for the secretion of Extracellular metabolites

This test was performed in 250 ml Erlenmeyer flasks containing 100 ml of sterile nutrient broth (NB). One milliliter bacterial suspension (10^8 CFU ml^{-1}) was added to the flasks containing NB. The flasks were then incubated for 6 days on a rotary shaker at 175 rpm at room temperature (26+2°C). Bacterial cells were pelleted by centrifugation at 5000 g for 12 min. The supernatants were sterilized with 0.22 µm filtrate. 5, 15 and 25% (v/v) of culture filtrates were mixed individually with PDA and a 5 mm disk of a four day old pure culture of *R. solani* was placed in the Petri dish. The experiment was independently repeated four times.

Effect of *Pseudomonas fluorescens* isolates on lysis of sclerotia of *Rhizoctonia solani in vitro*

Two hundred µl of a bioantagonistic bacterial suspension (10 CFU ml^{-1}) were placed in the Petri dish containing KMB. After incubation for 72 h at 26°C, sclerotia of *R.solani* surface sterilized with 2.5% sodium hypochlorite were placed on the bacterial lawn in the Petridish and the lysis of the sclerotia of the pathogen was measured. Each experiment considering a single bacterial isolate was run in triplicates and was repeated at least three times. Control was run by using sterile distilled water instead of bacterial suspension.

Effect of *Pseudomonas* isolates on germination of sclerotia of *Rhizoctonia solani in vitro*

Sclerotia of *R. solani* were surface sterilized with 2.5% sodium hypochlorite. One ml suspension of antagonistic isolates (10 CFU ml^{-1}) were inoculated into the Erlenmeyer flasks containing 50 ml of KMB. The bacterial suspensions in KMB medium were placed for 24 h on a rotary shaker at 175 rpm at room temperature (26+2°C) and 5 sclerotia were inoculated in each bacterial isolate and placed for 24 h on a rotary shaker at 175 rpm at room temperature. After incubation for 3 days at 26°C, the germination of the sclerotia of the pathogen was recorded. Control was run replacing antagonistic suspension by sterile distilled water. Each experiment considering single bacterial isolate was run in triplicates and was repeated at least three times.

Effect of *Pseudomonas* fluorescence on germination of sclerotia of *Rhizoctonia solani* in soil

The effect of *P. fluorescence* on the germination of sclerotia was tested according to the method of Knudsen and Eschen (1991). 5 kg of soil were sterilized and placed in the pot (15 cm in diameter). Sclerotia of *R. solani* were surface sterilized with 2.5% sodium hypochlorite and soil was drenched with 50 ml of a bioantagonistic bacterial suspension (10 CFU ml^{-1}), the pots were placed for 6 weeks at 26°C. After incubation for 3 days at 26°C, the germination of the sclerotia was measured. Each experiment considering single bacterial isolate was run in triplicates and was repeated three times. Control was run with sterile distilled water by replacing suspensions of antagonistic isolates.

3.4.2 Isolation and identification of *Trichoderma* isolates

Trichoderma strains were isolated following the soil dilution-plating technique of Johnson *et al.* (1989). A soil sample of 10 g was added into 250 ml Erlenmeyer flask containing 100 ml of sterilized distilled water and shaken thoroughly for 30 min. One milliliter of this suspension was pipetted aseptically and dispensed in dilution test tubes with 9 ml of sterilized distilled water. Series of soil dilutions of 1:10, 1:100, 1:1000 and 1:10000 were prepared. One milliliter of the desired dilution (10^{-3} and 10^{-4}) was transferred aseptically into a sterile Petri dish and 10-12 ml of melted *Trichoderma* specific medium. The dish was rotated by hand in a broad and slow swirling motion to disperse the soil suspension. Three Petri dishes were provided for each dilution. Plates were incubated at room temperature for 5 days. After incubation, a small portion of mycelium from each fungal colony was transferred into PDA plates and subsequently to PDA slants. Identification of antagonistic *Trichoderma* spp. was done according to the key proposed by Rifai (1969).

In vitro evaluation of *Trichoderma* isolates as biocontrol agents.

All the isolates of *Trichoderma* spp. were individually tested for their antagonistic efficiency against *R. solani* causing sheath blight of rice under *in vitro* conditions. Isolates of *Trichoderma* spp. were also studied for their potentiality of lytic enzyme activity and also for the solubilization of inorganic phosphate.

Screening of *Trichoderma* spp. isolates against *Rhizoctonia solani* in dual culture

All the isolates of *Trichoderma* were individually tested for their antagonistic property against *R. solani* causing sheath blight using the

dual culture technique. Agar blocks of *R. solani* (4 days old) were placed on plates of PDA. The distance between *R. solani* and the *Trichoderma* spp. was about 5 cm. Plates were incubated at room temperature for 5 days. Three plates were provided per isolate. Plates inoculated with *R.solani* alone served as control. Growth of both *R.solani* and *Trichoderma* spp. was measured 5 days after inoculation. Percent inhibition of radial growth (PIRG) was measured using the formula:

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100$$

Where, R_1 is the diameter (cm) of colony of pathogen in control

R_2 is the diameter (cm) of the colony of pathogen in antagonist-tested plate.

Descriptive assessment of the antagonistic activity was scaled as follows:

++++	=	very high antagonistic activity (>75 PIRG)
+++	=	high antagonistic activity (61-75 PIRG)
++	=	moderate antagonistic activity (51-60 PIRG)
+	=	Low antagonistic activity
-	=	no activity.

Scanning Electron M icroscope (SEM) observation of the mycelial interactions of the *Trichoderma* spp. and *Rhizoctonia solani* in dual culture plate

The mycelia of *R.solani* and *Trichoderma* spp. with in overlapping areas in dual culture plates were observed after 4 days of incubation using scanning electron microscope (SEM) at the Microscopy Service Laboratory called RUSKA LAB, Rajendranagar, Hyderabad, India.

Screening of *Trichoderma* spp. for the production of volatile antifungal compounds

The lower lids of two Petri plates containing PDA were inoculated separately with mycelial discs of *Trichoderma* isolates and *R. solani.* The two lids were then inverted one over the other and sealed air-tight with parafilm. Plates inoculated with *R. solani* and inverted over uninoculated plates were treated as control (Denis and Webster, 1971). The assembly was incubated at 28°C with 12 h photoperiod. After 96 h, colony diameter of *R. solani* was measured in all the treatments.

Evaluation of *Trichoderma* spp. for the production of Lytic enzymes

Quantification of lytic enzymes namely Chitinase; β-1,3-glucanases and β-1,4-glucanases in selected *Trichoderma* spp. was made.

Assessment of chitinase activity among the isolates of *Trichoderma* spp.

Mycelial discs from actively growing culture of *Trichoderma* spp. were inoculated on minimal medium containing 1.5% colloidal chitin and incubated for 72 h at 28°C with a12 h photoperiod. The plates were then observed for zone of chitin lysis around the mycelium. The plates without organism were used as control.

Selected bioagents [1ml of the spore suspension of *Trichoderma* (10^6 spores/ml)] were inoculated in minimal medium containing colloidal chitin and incubated for 1 week. The cultures were filtered to remove the mycelial growth, chlamydospores and conidia and subsequently centrifuged at 10000 rpm for 10 min. The supernatant served as crude enzyme source. The activity of chitinase was determined by quantitative

estimation of the reducing sugars produced with the colloidal chitin as appropriate substrate of enzyme assay. The reaction mixture consisted of 0.5 ml of the enzyme extract, 1 ml 10 mM sodium acetate buffer, pH 5.2 and 0.5 ml of 1% of colloidal chitin. The reaction mixture was incubated for 1 h at 37°C using a shaking water bath and the reaction was stopped after 10 min. The agitation mixture was centrifuged at 5000 rpm for 5 min to recover the supernatant containing N-acetylglucosamine as the principal component. The amount of N-Acetyl Glucosamine liberated was measured by a method that uses dinitrosalicylic (DNS) acid reagent, and the absorbance was measured at 540 nm. One unit (U) of enzyme (chitinase) is defined as the amount of enzyme able to liberate 1µ mol of the reducing sugar per minute at the same condition using N-Acetyl Glucosamine as the standard.

Assessment of β 1, 3-glucanase activity among the isolates of *Trichoderma* spp.

Fungal cultures were inoculated with minimal medium supplemented with cellulose (pH-5.0) and incubated for 7 days under shaking condition. Liquid culture was harvested and centrifuged at 5000 rpm for 5 min to recover the supernatant. For β-1,3-glucanase, equal volumes (62.5 µl) of enzyme extract and laminarin (4%w/v) were mixed and incubated for 10 min at 40°C. The amount of reducing sugars released from laminarin was determined by the addition of 370 µl of DNS reagent to the reaction mixture. The solution was heated in a boiling water bath for 5 min, cooled to room temperature. To this solution 4.5 ml of double distilled water was added, vortexed and the absorbance was measured at 500 nm.

Assessment of β-1,4- glucanase activity among *Trichoderma* spp.

For β-1, 4 glucanases, the crude enzyme (50 µl) was mixed with 450 µl carboxy methyl cellulose and incubated at 55°C for 30 min. Reducing sugars were assayed by using DNS method and the absorbance was measured at 500 nm One unit (U) of enzyme is defined as the amount of enzyme able to liberate 1µmol of the reducing sugar per minute at the same condition using glucose as the standard. Substrate blank was also included.

Evaluation of *Trichoderma* isolates for the solubilization of inorganic phosphate

Phoshate solubilization in solid medium.

Pikovskayas agar medium was poured onto sterilized Petriplates. Mycelial discs from actively growing culture of *Trichoderma spp.* were placed at the centre of the plates and incubated at 30 °C for five days. For the comparison standard phosphate solubilizing fungi *Aspergillus niger* was also inoculated at the centre of media. Then the plates were observed for solubilization zones around the growth.

Phoshate solubilization in broth culture

Quantification of the efficiency of inorganic phosphate solubilization in selected *Trichoderma* isolates was made. Bioagents were inoculated [1ml of the spore suspension of *Trichoderma* (10^6 spores/ml)] to 100 ml of Pikovskaya's broth in 250 ml flasks with equal number of uninoculated controls. The flasks were incubated on a mechanical shaker at 28 $\pm$ 2°C for 15 days. The amount of P_i released in the broth was estimated. The fungal broth cultures were filtered through Whatman No. 1 filter paper to remove the mycelial growth, conidia, chlamydospores and insoluble

phosphate. The filtrate was centrifuged at 10000 rpm for 10 minutes and the available P_i content in the supernatant was estimated by phosphomolybdic blue colour method.

3.4.3. *In vivo* evaluation of biocontrol agents against *Rhizoctonia solani*

Biocontrol agents were tested for their efficacy to control sheath blight *in vivo* and screened using the cultivar MTU 1010 very susceptible to sheath blight. The isolates of *Pseudomonas fluorescens* and *Trichoderma* were selected based upon their ability to inhibit the mycelial growth and sclerotial germination of *R. solani in vitro.*

Preparation of antagonistic bacterial inocula

Non-formulated fresh cells of antagonistic bacteria were prepared to evaluate their efficacy against the build up of sheath blight pathogen in green house experiments. Bacterial isolates were grown in KMB to late exponential phase at 27°C with shaking at 175 rpm. Cells harvested by centrifugation (5000 rpm) at 10°C for 15 min were washed twice and resuspended in 0.5% sterile NaCl solution. The bacterial suspension was adjusted turbidimetrically to about 10^8 colony-forming units (CFU ml^{-1}) and 1% carboxy methyl cellulose (CMC) solution was added. This non-formulated fresh bacterial cell suspension in KMB was sprayed on the rice plants with hand held sprayer.

Preparation of antagonistic fungal inocula

Non-formulated spore suspension was prepared for the foliar spray of *Trichoderma* isolates to study their potentiality against the development of sheath blight in green house conditions. *Trichoderma* culture suspensions were prepared from 7 day old well-sporulated culture

plates. Non-formulated spore suspension was prepared by taking 400 ml sterilized 1% sucrose solution and to that 10 ml of concentrated spore suspension was added. The spore concentration was adjusted using haemocytometer to10^8 spores ml^{-1} and 1% carboxy methyl cellulose (CMC) solution was added. This spore suspension was sprayed on the rice plants with a hand held sprayer.

Experimental design and treatment in the greenhouse test

The efficacy of the isolates of *Pseudomonas fluorescens* and *Trichoderma* was investigated under greenhouse conditions. There were ten treatments, which consisted of rice plants (cv. MTU 1010) inoculated with four isolates of *P.fluorescens*, four isolates of *Trichoderma*, one fungicide and one control. The details are as follows:

Treatment with

T1= *Pseudomonas fluorescens* 2 (Pf-2)

T2= *P. fluorescens* 1 (Pf-1)

T3= *P. fluorescens* 9 (Pf-9)

T4= *P. fluorescens* 11 (Pf-11)

T5= *Trichoderma harzianum* S12

T6= *T. viride* 5

T7= *T. viride* 2

T8= *T. viride* 16

T9= Benlate (0.2%)

T10= Pathogen check (only *R.solani*)

Each treatment consisted of three replications (four rice seedlings for one replication). The experiment was arranged in a Complete Randomized Design and the experiment was repeated twice with similar results.

Pot preparation and Sowing

Paddy rice field soil [clay texture (32.4% sand, 18.9% silt, 48.7% clay), pH 7.1, 3.0% organic matter, 0.2% total N, 23.7% $mgKg^{-1}$ available P and 0.6 $mgKg^{-1}$ available K] was used in the pot test. These soil samples were loaded in plastic pots (21 cm in diameter and 18cm in height) and the pot was filled with tap water until the soil was soaked. The water level was maintained above the soil level. After 72 h the soils were agitated manually to break up aggregates and excess water was drained. Soil level in the plastic pots was adjusted to a height of 13 cm so that 5 cm depth of water was retained in each plastic pot. Seeds were sown in pots ≈1cm deep in soil amended with 30 mg N, 9.7 mg P and 18.5 mg K per kilogram of soil in the form of urea, single superphosphate and muriate of potash, respectively. Seedlings that emerged were thinned to four per pot. All plant material was raised in a greenhouse at 25 °± 3°C.

Pathogen inoculation

Pathogen was inoculated in the sheath region of the rice plants. Inoculum of the isolate, *R. solani* 23 was selected based upon its aggressiveness in causing sheath blight in pathogenicity test. Twenty-five day-old plants were inoculated with single, small (approx. 0.2 mg) sclerotium (approx. 4 day old) of isolate *R. solani* 23. The inoculum was placed inside the sheath of the second (from top) fully expanded leaf at 5 cm above the soil surface and all rice plants were inoculated in the same

manner (Amita Singh *et al.* 2002). Inoculation was done in the evening and one day prior to formulation application. A few drops of sterile water were added to the inoculated sheath. All inoculated plants were transferred to a plastic moist chamber with 92 to 100% humidity, inside a greenhouse. The water level in the plastic pot was maintained at the same level through out the experiment.

Formulation application

Non-formulated fresh cell suspension of *P. fluorescens* and spore suspension of *Trichoderma* isolates both maintained at 10^9 cfu/ml and 10^8 cfu/ml respectively were sprayed on the rice seedlings in the plastic pots using a hand sprayer one day after pathogen inoculation. Rice seedlings, sprayed with Benlate at 150ml (@0.2%) were used as a benchmark to compare the efficiency of the formulations. Rice seedlings in each plastic pot inoculated with only tooth picks infested with *R. solani* were used as a control treatment (Pathogen check).

Assessment of disease intensity

Assessment of sheath blight disease in the greenhouse tests was carried out after 7 days of formulation application. Disease intensity was assessed using the formula described by Sharma *et al.* (1990).

Highest Relative Lesion Height (HRLH) =(highest lesion height/the highest plant height) X100.

This index is based on the highest point reached by a sheath blight lesion relative to the highest point of the plant and is adapted from the relative lesion height method described by Ahn *et al.* (1986). The disease intensity was scored for each tiller in 4 plants. The percentage of reduction

of disease incidence over control (Pathogen check) was also calculated using the formula

$$\text{Reduction over control} = \frac{[\text{Disease incidence(\%) in control}] - [\text{Disease incidence (\%) in treatment}]}{[\text{Disease incidence (\%) in control}]} \times 100$$

3.4.4 Field evaluation of the biocontrol agents for the management of rice sheath blight

The isolates of *Pseudomonas fluorescens* and *Trichoderma* that were found effective *in vivo* in suppressing the sheath blight pathogen were selected for further evaluation under field conditions.

Preparation of powder formulation of antagonistic bacterial isolates

Powder formulation of bacterial isolates was prepared by culturing bacteria in KMB to late exponential phase at 27°C with shaking at 175 rpm. Cells were harvested by centrifugation (5000 rpm) at 10°C for 15 min were washed twice and resuspended in 0.5% sterile NaCl solution. The bacterial suspension was adjusted turbidimetrically to about 10^9 CFU ml^{-1}. This bacterial suspension in KMB was mixed with talc powder at 1: 2 ratio. The mixture was left over night for drying under shade and the dried lumps were ground to fine powder. The ground powder was blended with 1% CMC and 0.5% calcium carbonate. The mixture was packed and sealed in a HDPE covers. Colony forming units in formulation was determined by serial dilution plating method. This water-soluble powder formulation containing bacteria at 10^9 spores g^{-1} was dissolved in tap water (10 g l^{-1}) and sprayed on the rice plants with knapsack sprayer.

Preparation of powder formulation of antagonistic fungal isolates

Powder formulation of *Trichoderma* isolates was prepared by growing the fungi in molasses broth. Inoculum from well-sporulated culture plates was added to 250 ml molasses broth and the culture was incubated with shaking condition at 25°C for 3 days. After 3 days of incubation the culture broth with mycelial mass was transferred aseptically to plastic trays of 15x30 cm that were disinfected with 70% alcohol and sterilized under UV light in laminar air flow.

Trays were covered with transparent polythene sheet and incubated for 4 days at 25°C with a photoperiod of 12 h. The well-sporulated mat along with culture broth and chlamydospores in tray was mixed with sterilized talc powder at 1:2 ratio. Another conidial mat that was devoid of culture broth (after draining the culture broth) was taken and mixed with 1:2 ratio mixtures to adjust the spore concentration to 10^8 spores /g. The mixture was left overnight for drying under shade and the dried lumps were ground to fine powder. The ground powder was blended with 1% CMC and 0.5% $CaCO_3$. The mixture was packed and sealed in a HDPE covers. Colony forming units in formulation was determined by serial dilution plating method. This water-soluble powder formulation containing *Trichoderma* spp. at 10^8 spores g^{-1} was dissolved in tap water (10 g l^{-1}) and sprayed on the rice plants with knapsack sprayer.

Experimental Design and Treatments in the field test

The efficacy of the isolates of bioagents against sheath blight was investigated under field conditions at Regional Agricultural Research Station, ANGR Agricultural University, Maruteru, West Godavari District,

Andhra Pradesh, India. There were six treatments in the field trial. Treatment consisted of rice plant (cv. MTU 1010) inoculated with two isolates of *P.fluorescens*, two isolates of *Trichoderma* , one chemical fungicide (Benlate @ 0.2%), one day after pathogen inoculation. Rice plants inoculated only with *R.solani* were used as a control treatment. Each treatment consisted of three replications, with twelve rice hills for one replication. The experiment was arranged in a Randomized Complete Block Design (RBD). The treatments are as follows:

T1= Treatment with *Pseudomonas fluorescens* 2

T2= *P. fluorescens* 1

T3= *Trichoderma harzianum* S12

T4= *T. viride* 2

T5= Benlate (0.2%)

T6= Pathogen check (only *R. solani*)

Rice Field Preparation

The rice plot had clay texture (32.4% sand, 18.9% silt, 48.7% clay), pH 7.1, 3.0% organic matter, 0.2% total N, 23.7% mg Kg^{-1} available P and 0.5% mg Kg^{-1} available K. It was flooded with water and ploughed until the soil aggregates were broken up. Excess water was drained 2 days later and the field partitioned into 3 blocks. Each block was further partitioned into 6 sub plots (2X2cm) by 30 cm width earth embankment to prevent water movement among the subplots. The rice field was flooded again and the water level was maintained by opening and closing a small gate on each subplot embankment. The experimental plot was surrounded by a buffer zone of approximately 10 m of fallow soil.

Rice Seedlings and Rice Plant Preparation

Rice seedlings (cv.MTU 1010) were raised in the seedling bed. Rice cv. MTU 1010 was selected for this field trial because it is very susceptible to sheath blight disease. All the subplots were applied with recommended dose of N:P:K @ 120:60:60 Kg/ha in the form of urea, single superphosphate and muriate of potash. 50% of the nitrogen and 100% of the recommended P & K were supplied at the time of transplantation and remaining 50% of the nitrogen was applied as top dressing, 45 days after transplantation. After 14 days, these rice seedlings were transplanted into the rice field with 20 cm spacing between and within rows in each 2X2 cm subplot. Eight rows and eight columns of rice seedlings were planted in each subplot, with two rice seedlings planted at each hill site. The two rows and two columns of rice plants planted adjacent to the each embankments in each subplot were used only as guard rows. The four rice plants in the inner most rows and columns were used for disease assessment, while the eight other adjacent rice plants (in the cross configuration surrounding these four inner most rice plants) were used for yield assessment.

Pathogen Inoculation in the field

Only twelve rice hills in the centre of each subplot were inoculated with the pathogen in the efficacy test in the field trial. Rice plants were inoculated according to the methodology described by Amita singh *et al.* (2002) after 45 days of transplanting rice seedlings to the field. The water level was maintained at the same level throughout the experiment.

Formulation Application

Subplots were identified with its respective treatments which were arranged randomly and applied with water soluble powder formulations, one day after inoculating the pathogen. The water soluble powder formulation of bioagents was sprayed to the rice hills using a knapsack sprayer. Rice plants sprayed with fungicide (Benlate @ 0.2%) were used as a benchmark to compare the efficacy of the formulations. Rice plants inoculated with only 20 g of sterile rice seed infested with *R. solani* were used as a control treatment (Pathogen check).

Number of viable cells in bacterial formulations

Viable cells in the bacterial formulation were counted with the drop plate method (Zuberer, 1994). This was carried out to determine the number of viable bacteria in 1 g of the formulation after storage at 30°C. The number of viable bacteria was assessed 20 days and 90 days after producing the formulation. The plates were incubated at room temperature (28 $\pm$ 2°C) for 2 days after which colony-forming units were counted. Bacterial numbers were the average of six replications per dilution.

Number of viable spores of in fungal formulations

Viable spores in the fungal formulation were counted with the serial dilution plating method. This was carried out to determine the number of viable spores in 1 g of the formulation after storage at 30°C. The number of spores was assessed 20 days and 90 days after producing the formulation. The plates were incubated at room temperature (28 $\pm$ 2°C) for 2 days after which colony-forming units were counted. Spore numbers were the average of six replications per dilution.

Disease Assessment in the field

For sheath blight assessment, only the 4 innermost rice hills were considered for scoring the disease intensity and sheath blight symptoms were assessed 7 days after formulation application. The disease intensity was scored using the formula described by Sharma *et al.* (1990).

Yield assessment

For rice yield assessment, rice plants were harvested from the remaining eight hills at the end of the experiment, 110 days after transplanting. Panicles were cut at the base of the uppermost inner-node and the weight of these panicles was assessed. The data were extrapolated to calculated mean yield per plot and mean yield per hectare.

Statistical Analysis

The data obtained were subjected to analysis of variance and the means separated by using Duncun's Multiple Range Test (Peterson, 1985) and ANOVA. Tests were used to establish significant differences. Values in percentages were analyzed statistically after carrying out angular transformation.

The experiment carried out on various aspects of sheath blight of rice caused by *Rhizoctonia solani* with reference to collection of pathogenic isolates and study of cultural, morphological, pathogenic and molecular variability and evaluation of biocontrol agents against the pathogen under *in vitro*, *in vivo* and field conditions.