EXPLOITATION OF HYPOVIRULENCE IN *Rhizoctonia solani* Kühn FOR MANAGEMENT OF SHEATH BLIGHT IN RICE (*Oryza sativa* L.)

BY

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "**Exploitation of hypovirulence in *Rhizoctonia solani* Kühn for management of sheath blight in rice (*Oryza sativa* 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, associateship, fellowship or other similar title, of any other university or society.

Vellayani,

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CERTIFICATE

Certified that this thesis entitled "**Exploitation of hypovirulence in *Rhizoctonia solani* Kühn for management of sheath blight in rice (*Oryza sativa* L.).**" is a record of research work done independently by Mr. Ranjit A. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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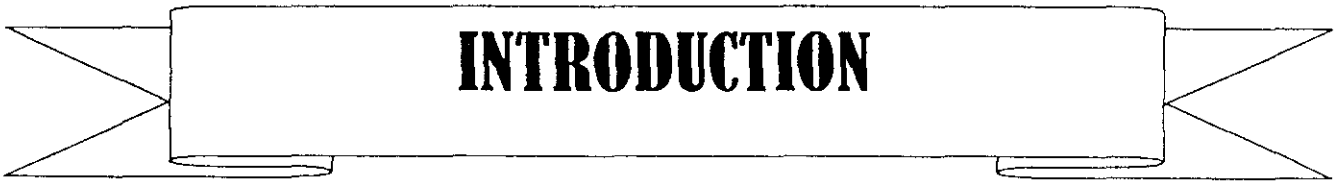

INTRODUCTION

1.INTRODUCTION

Sheath blight of rice, incited by *Rhizoctonia solani* Kühn, is one of the major constraints of rice production coinciding with the adoption of high yielding varieties and intensive agronomic practices. Under favourable environmental conditions for disease development, besides sheath, the entire rice plant involving the foliage gets blighted. The loss in grain yield is estimated to reach up to 42 per cent and an additional economic loss may be incurred by up to 20 per cent due to reduction in milling quality.

Much knowledge is available on the pathogen as well as its control. The chemical methods though effective, have been found to be costly. Further, they also pose environmental hazards. As a part of the integrated disease management programmes and low input systems, biological control is increasingly gaining importance. The present work envisages a novel strategy by utilising weaker strains of the pathogen *R. solani* to suppress the sheath blight disease.

Isolates of *R. solani* with reduced virulence have been reported (Castanho and Butler, 1978 a). Hypovirulence in *R. solani* is cytoplasmically inherited. Such isolates were found to have decreased growth rate and sclerotial production (Girija, 1995). Castanho and Butler (1978 b) expatiated the potential utilisation of such isolates for biological control of diseases caused by *R. solani*. Promising results have been obtained on biological control of *R. solani* using hypovirulent strains in several crops. Co-inoculation of hypovirulent isolates of *R. solani* with virulent ones could reduce disease when tested on crops like sugar beet and cabbage, (Castanho and Butler, 1978 b), bean plants (Poromarto,1998) and potato (Bandy and Tavantzis, 1990).

Induction of resistance in rice to sheath blight using avirulent *R. solani* has been found effective against virulent isolates (Kalaiselvi *et al.*, 1986). The hypovirulent isolates of *R. solani* have been found to produce atypical symptoms and lesser quantity of virulence related enzymes (Girija, 1995). The isolates of *R. solani* obtained from the phyllosphere of rice were found to inhibit sheath blight of rice (Shahjahan *et al.*, 1997). The capacity of the fungus to anastomose will offer immense potentiality to transfer the hypovirulence factor to the virulent strains. If found successful, the hypovirulent strain would offer life long protection to the crop for a practical biocontrol to the much dreaded peril of rice scenario.

The present work was undertaken to explore the variability in *R.solani* with a view to locate weakly virulent/hypovirulent isolates and to test their suitability for biocontrol of sheath blight of rice. Work was undertaken in the following lines :

1. Screening of *R.solani* isolates for hypovirulence.
2. Screening of hypovirulent isolates for vegetative compatibility with virulent isolate of *R. solani* .
3. Preliminary screening for hypovirulent isolates for *in vitro* antagonism.
4. Effect of hypovirulent isolates on seed germination and seedling vigour of rice.
5. Effect of hypovirulent isolates on growth of rice plants.
6. Effect of hypovirulent isolates on suppression of sheath blight disease in rice.
7. Preliminary studies on the elucidation of mechanisms of biocontrol.
8. Formulation of a suitable carrier material for hypovirulent isolates.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Sheath Blight

Sheath blight of rice is a devastating disease of common occurrence in all rice growing tracts, which becomes more pronounced when rice is grown under intensive production system. This disease is found to incur reduction in grain yield up to 50 per cent (Lee and Rush, 1983). Miyake (1910) reported sheath blight disease for the first time from Japan. The first detailed report on this disease in India was made by Paracer and Chahal (1963) from Gurdaspur district of Punjab. The disease has now spread far and wide, and has become prevalent in all rice growing tracts of India. Nair and Sathyarajan (1975) reported the disease from Pattambi in Kerala.

2.2 Etiology

The causal agent of sheath blight of rice has been identified as *Rhizoctonia solani* Kühn by Park and Bertus (1932). The perfect stage of the fungus was reported to be *Thanatephorus cucumeris* (Frank) Donk (Talbot, 1970). The sclerotial state is common and is principally carried through soil. The teleomorph stage has been first reported from India by Saksena and Chaubey (1972). However, the occurrence of basidial stage is rare in standing rice crop (Singh and Pavgi, 1969).

2.3 Morphology

Duggar (1915) observed that young hyphal branches were inclined to the direction of growth and constricted at the point of union of the main

hyphae. Branching of the young hyphae at right angles, later bending towards the direction of growth was observed by Palo (1926).

R. solani exists primarily as sterile mycelium that is colourless when young, but turns yellowish or light brown with age. *R. solani* is characterised by (i) multinucleate cells in young vegetative hyphae. (ii) branching near the distal septum in young vegetative hyphae (iii) a prominent septal pore apparatus (iv) constriction of the branch base (v) formation of a septum in the branch near the point of origin and (vi) shade of brown pigmentation (Parmeter and Whitney, 1969).

2.4 Variability in *R. solani*

R. solani is a collective taxonomic species, consisting of more or less unrelated strains, which can be distinguished from each other based on different characteristics.

2.4.1 Based on morphological and cultural characters

Morphological and cultural characters are frequently used to differentiate isolates of *R.solani*. Sethofer and Jermoljev (1950) identified eight biological forms of *R. solani* from potato tubers which differed morphologically in culture. Kernkamp *et al.* (1952) concluded that the species comprised of an indefinite number of races differing culturally and pathogenically. Certain races appear to be more crop specific. Sadowski (1970) distinguished many strains of *R.solani* differing in cultural characters, ecological requirements and pathogenicity during field and laboratory experiments. Gokulapalan and Nair (1983) have described the morphological characters, anastomosis reaction and pathogenicity of four isolates of

R.solani. Vijayan and Nair (1985) categorised *R.solani* isolates into four groups based on anastomosis, mycelial colour, growth and sclerotial size. Bansal *et al.* (1990) reported that the seven isolates of *R.solani* collected from different host plants, exhibited variability in cultural characters on groundnut seed agar medium at different temperatures. Variations were found in the virulent *R.solani* isolates in mycelial characteristics like texture, colour, growth and hyphal diameter, and production , distribution and size of sclerotia (Basu and Gupta, 1992). Monga and Sheo (1994) categorised thirteen isolates of *R.solani* causing root rot of cotton into four distinct groups on the basis of cultural characteristics.

2.4.2 Based on pathogenicity and growth characters

Wide variation was seen in the growth rate and colony morphology of *R.solani* isolates among various grasses and legumes with little specificity in pathogenicity (Luttrel, 1962). Based on the growth characteristics and pathogenicity Griesbach (1975) assigned isolates of *R. solani* from ten different weeds and potato to a single group, whereas, isolates from wheat and cowpea were grouped separately. Two strains varying in pathogenicity from rice and *Brassica campestris* var *cunifolia* were identified in Assam (Roy, 1993). Das and Balakrishnan (1983) reported that an isolate from cowpea anastomosed with isolates from soybean , green gram, periwinkle and rice, indicating that the two isolates were genetically related. Eight isolates of *R.solani* obtained from different rice growing tracts of India showed considerable variability in mycelial growth, sclerotial production and pathogenicity (Girija, 1993). Mazzola *et al.* (1996) reported that four out of eight isolates of AG-8 obtained from wheat, caused severe root rot and others

caused minimal or no damage. Mazzola (1997) reported variability in pathogenicity of *Rhizoctonia* spp. isolated from apple roots and orchard soils.

2.4.3 Based on cellular nuclear number (CNN)

Cellular nuclear number of *Rhizoctonia* spp. is found to show variation. The cells of *R.solani* are generally multinucleate. Parmeter *et al.* (1967) differentiated *Rhizoctonia*-like organisms, which were binucleate with *Ceratobasidium* teleomorph from multinucleated *R. solani* which produced the teleomorph, *Thanatephorus cucumeris.* The number of nuclei may vary from three to 28 in young cells but decrease with age (Butler and Bracker, 1970). Hyphal cells of *Rhizoctonia* spp useful in biocontrol of root disease of cotton contained two nuclei (Sneh *et al.*, 1991).

2.4.4 Based on hyphal compatibility

Parmeter *et al.* (1969) stated that the *R.solani* was a collective taxonomic species consisting of at least four, more or less unrelated strains, which can be distinguished from each other. In Japan, Ogoshi (1972) grouped 214 isolates of *R.solani* into six groups. Most isolates from groups AG1 were from rice, sugar beet and soil. The members from AG3 were isolated from plants of family Solanaceae. Most of the isolates in AG4 were obtained from leguminous hosts and sugar beet. AG5 and AG6 had members isolated from soil and sugar beet plants, respectively.

2.5 Hypovirulence

Hypovirulence is defined as the reduced ability to cause disease by selected isolates within a population of fungal pathogens (Zhou and Boland,

1997). This phenomenon was noticed by Biraghi in 1953,but was christened "hypovirulence" by Grente (1965).

Hypovirulent strains were defined by Finkler *et al.* (1988) as those strains that infected a wide variety of hosts, but incurred no discernable damage to the host.

This has been reported for a number of plant pathogens (Castanho and Butler,1978 b, Hammar *et al.*,1989, Nuss and Koltin, 1990, Boland,1992, Zhang *et al.*, 1994, Melzer and Boland,1995)

Chestnut trees with abnormally healing cankers were noticed in Como, Italy (Biraghi, 1953). Certain chestnut trees among infected groups stood healthy, despite 85 per cent infection in the shoots. Biraghi (1953) ascribed this to increased resistance of trees on account of repeated pruning. In 1965, Grente isolated atypical strains of *Cryphonectria parasitica*, the causal agent of chestnut blight. Pigmentation and sporulation were lower in these strains than in normal isolates of the pathogen. Grente and Berthelay–Sauret (1978) examined the pathogenic capabilities of the *C. parasitica* isolates and found that the atypical strains were reduced in virulence. Co-inoculation of atypical strains with virulent ones resulted in the healing of the cankers.

A similar phenomenon was reported by Fulbright *et al.* (1985) in Ontario U.S.A. The isolates from the recovering cankers were designated as hypovirulent (HV) types, which showed differences in morphological and cultural characteristics as compared to the virulent (V) isolates. The hypovirulent types had slower growth, showed sectoring and produced

abundant conidia. On frequent subculturing, such cultures produced little growth.

Lindberg (1959) described a disease of *Helminthosporium victoriae* which was characterised by hyphal lysis, slow growth, reduced sporulation and subdued virulence. Lemke (1977) reported a similar kind of degenerative disease in *H.maydis*.

Ophiostoma ulmi, causing Dutch elm disease have been found to have two major subgroups: a highly pathogenic "aggressive strain" and a less pathogenic "nonaggressive strain" (Brasier, 1983 a). These were readily distinguished by their morphologies and temperature optima for growth and difference in physiological properties. Brasier (1983 b) reported a cytoplasmicaly-transmitted disease in an aggressive subgroup of the fungus. The infected isolate was characterised by slow growth, impaired reproductive fitness and the ability to transmit the disease to healthy recipient isolates by hyphal anastomosis.

Several isolates of *Sclerotinia homoeocarpa*, the causal agent of dollar spot of creeping bent grass have been reported to be hypovirulent (Zhou and Boland, 1997). Out of the one hundred and thirty two isolates of the pathogen evaluated for virulence on swords of creeping bent grass, twenty four isolates (18. 2 %) did not show symptom development even after two weeks of inoculation, and thirteen isolates (9.8 %) did not initiate lesions on further incubation for two more weeks. These isolates were considered to be hypovirulent. Melzer and Boland (1995) reported transmissible hypovirulence in *S. minor*

2.5.1 Hypovirulence in *R. solani*

A cytoplasmically controlled degenerative disease in *R. solani* called as "Rhizoctonia decline" was reported by Castanho and Butler (1978 a). They obtained isolates which were debilitated and weakly pathogenic. The isolates, when applied to sites infected with highly infectious strains, were found to reduce the severity of disease.

The cultural characteristics of hypovirulent strains of *R. solani in* sugar beet seedlings have been characterised by Castanho and Butler (1978 a). These strains were white or tan coloured, had floccose mycelia with irregular appearance and produced few or no sclerotia. The virulent cultures on the other hand, had brown mycelia which were appressed to the agar surface, uniform in appearance, producing numerous dark sclerotia and growing rapidly. The hypovirulent isolates were weakly pathogenic. They reduced the severity of damping off of sugar beet when applied to sites infected with highly pathogenic strains.

The hypovirulent strains were later found to be not restricted to few anastomosis groups and were isolated from majority of anastomosis groups in *R. solani* (Ichielevich–Auster *et al.*, 1985 a). Hypovirulent or Non-pathogenic *Rhizoctonia* (Np-R), possessing capacity to protect plants against diseases caused by virulent isolates of different anastomosis groups, were found among different anastomosis groups of binucleate as well as multinucleate *Rhizoctonia* spp. (Finkler *et al.*, 1988, Nuss and Koltin, 1990, Herr, 1995, Sneh, 1999). The binucleate *Rhizoctonia* spp. has been reported to be generally weakly virulent (Hurd and Guisham, 1983) .

2.6 Basis of hypovirulence

Hypovirulence in fungi has been attributed to several causes, but it is often associated with the presence of unencapsidated double- stranded RNA (dsRNA).(Day *et al.*, 1977, Castanho *et al.*,1978, Dodds, 1980, Nuss and Koltin, 1990, Zhou and Boland, 1997).

The hypovirulent phenotype of the fungus is cytoplasmically controlled (Van Alfen *et al.*, 1975) and is associated with high molecular weight dsRNAs (Day *et al.*, 1977). Hypovirulent strains can convert virulent strains to hypovirulent ones by the transfer of dsRNA through anastomosis (Anagnostakis, 1981). He suggested a relationship between the diseased state and dsRNA, following the isolation of three segments of dsRNA from a diseased isolate of *C. parasitica* and the inability to recover any dsRNA from related healthy isolated fragments. This was reinforced by the fact that the dsRNAs could not be isolated from healthy culture, derived by transferring hyphal tips of diseased culture to fresh media.

However Van Alfen *et al.* (1978) opined that hypovirulence can be lost by rapid growth rate or by other factors. Two out of eight hypovirulent isolates did not yield dsRNA on routine purification attempts. Dodds (1980) attributed the strongly debilitating characters of hypovirulent strains to the presence of dsRNA. However, he suggested that the extraction of the dsRNA could be affected by the loopholes in the extraction procedures.

Elliston (1982) recognised two categories of hypovirulence based on the causes: i) cytoplasmic hypovirulence and ii) nuclear hypovirulence. Cytoplasmic hypovirulence (CH) may be due to viruses, plasmids and

organelles that carry genetic elements such as mitochondria. The nuclear hypovirulence (NH) may be caused by mutant nuclear genes, hybrid nuclei, heterokaryons or some extra chromosomal genetic determinants found in the nuclei. Fulbright (1985) reported a transmissible cytoplasmic hypovirus that is not associated with dsRNA. However, presence of dsRNA has been indicated in virulent cultures also. Double *et al.* (1985) reported that one third of the tested isolates of *C. parasitica* from Virginia yielded dsRNA in small amounts. They suggested two types of dsRNA: hypovirulent associated dsRNA and cryptic dsRNA.

Molecular analysis indicated that the dsRNA reduced the accumulation of specific mRNAs and polypeptides.(Powell and Van Alfen, 1987). Accumulation of metabolic oxalate and of several proteins was reduced in dsRNA containing strains of *Cryphonectria parasitica*. These proteins included an extracellular and intracellular laccase , a cell surface protein , a cutinase and a putative mating type pheromone.

The dsRNA (L-dsRNA) obtained from the French hypovirulent strain of *C.parasitica* revealed one large dsRNA with two continuous coding domains (ORFA and ORFB) and multiple defective segments (Shapira *et al.*, 1991). Genetic organisation, expression and replication strategy of the L-dsRNA gave evidence to its viral origin (Choi *et al.*, 1991 a, 1991 b, Shapira *et al.*, 1991).

Choi and Nuss (1992) obtained a full-length cDNA copy of hypovirus associated dsRNA. Chen *et al.* (1993) demonstrated the stable maintenance of integrated viral cDNA through repeated rounds of asexual sporulation and passages through host plant tissue.

Zhou and Boland (1997) found association of dsRNA with hypovirulence in *Sclerotinia homeocarpa*. Out of the 132 isolates of pathogen evaluated for virulence, the hypovirulence phenotype was associated with the presence of dsRNA in at least four isolates. The hypovirulent principle could be transmitted to the virulent isolate and the converted isolate was hypovirulent and contained dsRNA.

dsRNAs were detected in several rust fungi viz., *Puccinia striiformis, P. recondita, P.graminis, P.malvacearum, P.suaveolens and P.helianthi.* However, in rusts, dsRNA was not associated with degeneration of fungal mycelium and did not alter virulence of the pathogen (Zhang *et al.*, 1994).

In *Phytophthora infestans*, dsRNA was found to be associated with increase in virulence (Tooley *et al.*, 1989). In a study conducted on 14 Mexican isolates of *P.infestans* containing dsRNA and eight randomly selected isolates without any dsRNA , with few exceptions, strains which had dsRNA exhibited higher level of virulence.

Various theories have been put forth to explain hypovirulence in *Rhizoctonia* spp. There are relatively few reports on the mode of action of hypovirulent or Non-pathogenic *Rhizoctonia* (Np-R). The suggested theories of the explanation of hypovirulence fall under two broad categories: 1) the involvement of a foreign genetic material, double-stranded RNA, most possibly from a virus and 2) the natural character of the fungal strain itself, considering the vast genetic diversity of *Rhizoctonia* spp.

The earlier works on hypovirulence in *R. solani* was done mostly on the same line as in hypovirulence in *Cryphonectria parasitica* .

Castanho *et al.* (1978) detected dsRNA in the cytoplasm of the *R.solani* strains which showed "Rhizoctonia decline". The degeneration of *Rhizoctonia* was hypothesised to be transmitted through the transfer of dsRNA virus when hyphal fusion occurred between "diseased" and "cured" strains. The dsRNA was found to have a negative effect on the virulence of *R. solani* .

Hashiba *et al.* (1984) associated hypovirulence in *R. solani* to the presence of a small DNA plasmid whereas Martin and Lucas (1984) claimed that the determinants of virulence were located on a large DNA plasmid.

The works on hypovirulent strains of *R. solani* by Finkler *et al.* (1985) yielded contradicting results. Finkler and co-workers could not detect any dsRNA in the hypovirulent strains, but dsRNA of hypoviral nature was detected in the virulent strains. They could also obtain hypovirulent strains from the virulent strains by the hyphal tip culture of virulent strains, but not *vice versa*. This was attributed to the loss of dsRNA segment in the hyphal tip. Further evidence supporting the relationship between dsRNA virus and virulence, was provided by cytoplasmic transmission of virulence from virulent to the hypovirulent strain. The results indicated that the virulent isolates contained dsRNA, whereas, the hypovirulent strains were devoid of dsRNA, and that hypovirulence was not directly related to dsRNA viruses. They indicated that protective effect might be due to the inhibition of virus replication in virulent cultures by the hypovirulent strains.

Bharathan and Tavantziz (1990, 1991) working on the genetic diversity and relationship among dsRNA of *R.solani* found that not all the dsRNA have been associated with hypovirulence. However, they obtained the correlation between specific segment of dsRNA and hypovirulence.

Lakshman and Tavantziz (1994) reported the occurrence of dsRNA in both virulent and hypovirulent isolates which were genetically similar though not identical. Introduction of the character of hypovirulence in virulent isolates coincided with the appearance of three novel dsRNAs. At the same time, reduction in virulence coincided with the loss of two original dsRNAs.

2.7 Use of hypovirulence in biological control and growth promotion

The classical example of exploiting hypovirulence as a biocontrol tool can be seen in the control of chestnut blight caused by *C.parasitica*, which prevented the European chestnut from succumbing to the disease (Anagnostakes, 1981).

Castanho and Butler (1978 b) studied the potential of hypovirulence in *R. solani* as a biological control tool for the management of plant diseases. Sugar beet plants grown in soil infested simultaneously with hypovirulent and virulent strains of *R. solani* showed reduction in damping-off. The addition of viable hypovirulent mycelium to the seed furrows of soil previously treated with virulent strain resulted in five fold decrease in the intensity of damping-off.

Burpee and Goulty (1984) reported the suppression of brown patch disease of creeping bent grass by the isolates of non-pathogenic *Rhizoctonia* spp.

Ichielevich-Auster *et al.* (1985 b) reported the suppression of *Rhizoctonia* incited damping-off disease in cotton by a non-pathogenic isolate of the same fungus. Similarly Sneh *et al.* (1986) reported that a non-pathogenic isolate of *R. solani* increased the growth rate of cotton and protected it from damping off caused by *R. solani*.

Rhizoctonia root rot of snap bean was found to be controlled significantly in field by isolates of binucleate *Rhizoctonia* (Cardosso and Echandi, 1987 a). The effect was found to be superior to that of *Trichoderma hamatum* and *T.harzianum.* Herr (1988) reported the potential use of binucleate *Rhizoctonia* spp. for the control of Rhizoctonia crown and root rot of sugar beet.

Sneh *et al.* (1989) reported that a hypovirulent isolate of *R. solani* afforded high protection rate, ranging from 76 to 94 per cent against virulent isolates of *Rhizoctonia* spp and also induced a higher plant growth response in cotton.

Hypovirulent *R. solani* reduced the severity of disease of *Rhizoctonia* canker in potato (Bandy and Tavantziz, 1990, Escande and Echandi, 1991).

Cubeta and Echandi (1991) reported that a non-pathogenic isolate of *R. solani* was effective in reducing the diseases caused by other pathogens also. They found that the application of avirulent binucleate *R.solani* caused reduction in the severity of *Rhizoctonia* and *Pythium* damping-off of cucumber. Two isolates of *Rhizoctonia* (BNR) spp. evaluated for biocontrol of pre-emergence damping-off caused by *Pythium ultimum* in *Catharanthus roseus* showed significant disease suppression (Burns and Benson,1998). Three to seven day's colonisation period with the BNR gave the highest level (63 %) of biocontrol.

Khan *et al.* (1992) reported that soybean seeds treated with BNR when planted in *R. solani* infested soil, showed decreased disease severity, on all selected cultivars, compared to untreated controls. They also reported that

treatment of BNR caused increased seed germination, better survival of plants and improvement of plant height.

Hare *et al.* in 1994 reported that bean seedlings were protected from infection of virulent *R. solani* by pre-treatment with binucleate isolate of *R. solani.* Suppression of damping off of bean plants caused by *R. solani* by binucleate *Rhizoctonia* spp. was reported (Hare *et al.* 1994 , Haris *et al.*, 1994). Yuen *et al.* (1994) reported biological control of *R. solani* on tall fescue plants by using a weakly virulent isolate of the same fungus. Poromarto. (1998) reported that binucleate *Rhizoctonia* isolates did not cause lesions in soybeans and reduced disease severity caused by the virulent isolate of *R. solani.*

Villajuan-Abagona *et al.* in 1996 found that the damping off disease of cucumber caused by *R. solani* could be controlled using non-pathogenic binucleate isolate of the same fungus.

The isolates of BNR were effective in controlling *Rhizoctonia* stem rot of poinsettia up to eight weeks of transplanting. The severity of disease was 67 per cent lower than that in the infected control (Hwang and Benson, 1998).

Induction of resistance in rice to sheath blight using avirulent *R. solani* were found effective against virulent isolates (Kalaiselvi *et al.*, 1986). The hypovirulent isolates of *R. solani* were found to produce atypical symptoms and lesser quantity of virulence related enzymes in susceptible variety (Girija, 1995). The avirulent isolates of *R. solani* obtained from the phyllosphere of rice were found to inhibit sheath blight of rice (Shahjahan *et al.*, 1997).

2.8 Mechanism of biocontrol

Cook and Baker (1983) have stated "Three fundamental approaches of biological control of plant pathogens involve biological destruction of pathogen, biological protection of plant surface and cross protection or induced resistance". One or more of these mechanisms in the disease suppression by hypovirulent *R.solani* has been investigated by several workers. The information generated on the mechanisms of biocontrol is however, not adequate to give conclusive evidences.

Sneh *et al.* (1989) working on the use of non-pathogenic isolates of *R.solani* (Np-R) for biocontrol have suggested that the isolates might differ in the capability as well as mechanism of protection.

2.8.1 Antibiosis

Absence of antibiosis, lysis, or hyperparasitism was reported by several scientists in the case of *R.solani* (Ichlievich-Auster *et al*, 1985 b., Sneh *et al.*, 1986, Cardosso and Echandi, 1987 a, Poromarto, 1998). No inhibition zone could be observed when hypovirulent culture was dual cultured with virulent cultures. However, Cardosso and Echandi (1987 b) reported that the root exudates of 10- day old binucleate *Rhizoctonia* treated seedlings inhibited hyphal growth and sclerotial germination of *R. solani in vitro.*

2.8.2 Competition

Virulence of *R. solani* as affected by the nutrition of the pathogen has been explained by Weinhold *et al.*(1969). Ichielevich-Auster *et al.* (1985 a) reported that native non-pathogenic *R.solani* had almost the same pattern of growth as that of virulent isolate, and therefore, they might be fit to compete with

the virulent isolates under natural conditions. However, they reported that biological control in the case of hypovirulent *R. solani* was not induced by competition for nutrients since addition of nutrients to plants did not reduce protective effect.

Cardosso and Echandi (1987 a) carried out work on nature of protection of bean seedlings by BNR and showed that the non-pathogenic BNR did not penetrate beyond the epidermal cells. However, they extensively colonized the rhizosphere and rhizoplane of bean seedlings. Ichlievich-Auster *et al.* (1985 b) showed microscopically that the hypovirulent strain formed a thick layer on the surface of the invaded tissue, but did not penetrate deeply, whereas, the virulent strain penetrated the host tissue and quickly reached the vascular system, lysing the host in the process .

Sneh *et al.* (1989) reported that hypovirulent isolates densely colonised the surface roots and hypocotyls of cotton seedlings and the physical removal of the hyphae nullified the protection provided by the isolate. Surface sterilisation of the root hairs with sodium hypochlorite killed the hypovirulent isolate but did not reverse the protective effect. They concluded that hypovirulent strain intensively colonised the root and crown surface of the cotton seedlings and competed with pathogen for infection sites.

2.8.3 Induced systemic resistance (ISR)

Induced systemic resistance is considered to be a general resistance response following inoculation with a non-pathogen or weak pathogen (Kuc and Stobel, 1992).

Ichlievich-Auster *et al.* (1985 a) suggested that protection by hypovirulent strains was acting in or on the plant rather than directly on the pathogen, suggesting operation of induced resistance. The observation that cytoplasmic compatibility was not required for protection added support to this argument. They stated " The stability of avirulence in some strains of *Rhizoctonia* spp. could involve among other mechanisms, an elicitation of natural plant resistance responses – phytoalexins, lignification or suberization"

Cardosso and Echandi (1987 b) reported that disinfection of host surface with sodium hypochlorite killed the hypovirulent culture, but did not reduce the protective effect on the host. They suggested induced resistance to be the major mechanism behind biocontrol by hypovirulent *R.solani.*

Poromarto (1998) reported that the inhibition of hyphal growth of *R.solani* on the surface of soybean tissue inoculated with BNR was due to the induction of novel inhibitory fungal compounds. Formation of papillae during the attempted penetration of cortical cells by BNR was also reported as further evidence for induced resistance.

The role of enzymes in expression of virulence of *R.solani* has been well estabilished (Weinhold and Motta, 1973, Geypen ,1978).

Smith *et al.* (1975) reported the accumulation of phytoalexins in *Phaseolus vulgaris* hypocotyls following infection by *R. solani* . Brookhauser and Weinhold. (1979) had reported that pectinolytic enzymes were related to virulence and that endopectinolyase was very important in initial expression of the symptoms in the case of cotton seedlings. Marcus *et al.* (1986) purified and characterised pectinolytic enzymes produced by virulent and hypovirulent

isolates of *R. solani*. He reported that the enzyme endopectinolyase II was not expressed in hypovirulent isolate.

Sneh *et al.* (1989) reported that Ca^{2+} was significantly higher in radish and cotton seedlings colonized by hypovirulent *R.solani*. The hypovirulent strain did not produce cell wall lytic enzymes such as chitinase and β (1,3) glucanase. Hare *et al.* (1994) obtained higher peroxidase activity in bean plants treated with BNR than that in untreated control. Virulence of *R. solani* was found to be negatively correlated with increased peroxidase activity.

Xue *et al.* (1994) reported enhanced pathogenesis related protein activity in bean seedlings protected with non-pathogenic binucleate *Rhizoctonia* spp.

Girija (1996) reported variation in protein banding patterns of a susceptible rice cultivar due to the inoculation of hypovirulent isolates of *R solani* .The hypovirulent isolate produced unique banding pattern indicating the basic genomic difference between virulent and hypovirulent isolate during the infection of host plants.

Xue *et al.* (1998) reported that treatment with binucleate *Rhizoctonia* elicited a systemic increase in all cellular fractions of peroxidases, 1,3 glucanases and chitinases when compared to control plants. Total peroxidases and glucanases were reported to have a two fold and eight fold increase respectively, in all protected bean seedlings.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Isolation of weakly virulent isolates

3.1.1 Collection of samples for isolation

Soil and plant samples were collected from healthy and diseased rice plants of the different rice growing areas of Kerala i.e. Pattambi, Pilicode, Ambalavayal, Trichur, Moncompu, Changanassery, Kayamkulam, Kottarakkara, Chirayinkil, Vembayam and Vellayani.

3.1.2 Isolation of *Rhizoctonia solani.*

3.1.2.1 Isolation from plant samples

Sheath and leaf of rice plants showing typical or atypical symptoms of sheath blight were used for isolation of *R.solani.* Bits of tissue were surface sterilised with 0.1 per cent mercuric chloride solution for one minute and repeatedly washed in three changes of sterile water. These bits were placed in potato dextrose agar (PDA) medium (Appendix I) in sterile Petri dishes and incubated at room temperature (28 ± 2^{0}C) for 48 hours. When fungal growth was visible, mycelial bits were transferred to PDA slants.

3.1.2.2 Isolation from soil

Isolation of *R.solani* from soil was done by serial dilution plate technique (Waksman, 1922).

One g of thoroughly mixed soil sample was transferred aseptically into a 250 ml conical flask containing 100 ml of sterile water and shaken for 20 min in a mechanical shaker. One ml of the suspension was pipetted out to 99 ml of sterile water in a 250 ml conical flask, under aseptic conditions. The flasks were again shaken for 15 min in a mechanical shaker. One ml of suspension was pipetted out to sterile Petri dishes, melted and cooled.

Rhizoctonia specific selective medium with gallic acid (Appendix I) was poured at the rate of 20 ml per dish and gently rotated for thorough mixing. The Petri plates were incubated at 28 ± 2°C. Typical colonies were transferred to PDA slants and stored at 4°C.

3.1.3 Purification of isolates

Typical colonies of *R.solani* were transferred to PDA slants. Hyphal tip technique (Parmeter *et al.*, 1969) was employed to get pure culture of the isolates. Cultures were maintained on sterile PDA slants in test tubes at 4°C. Twenty-eight cultures were thus obtained.

3.1.4 Pathogenicity tests

Laboratory testing of the isolates of *R.solani* for pathogenicity was done by *in vitro* cut stalk assay (Prasanna Kumari Pillai, 1990) on the susceptible rice variety, Jyothi.

Pieces of seven cm long stalks were excised from the upper portion of tillers at the maximum tillering stage and placed in nine cm Petri plates containing three ml of 20 ppm kinetin solution. Single sclerotium/mycelial bit of each isolate was placed in the leaf axil with a pair of sterile forceps. Optimum humidity was maintained for symptom development. Observation on symptom development was noted three days after inoculation.

3.1.5 Screening of isolates for hypovirulence

Isolates of *R.solani* were subjected to a preliminary screening based on mycelial and cultural characters, and virulence.

3.1.5.1 Preparation of inoculum

The *R.solani* isolates were inoculated at the centre of PDA medium taken in sterilised dishes and incubated at 28 ± 2°C for 48 hours.

3.1.5.2 Studies on mycelial and cultural characters

One cm diameter discs of mycelium were cut from the actively growing region of the isolates grown as described under 3.1.5.1. and inoculated at the centre of PDA medium taken in 9 cm sterile Petri dishes with three replications, and were incubated at 28 ± 2^0C.

Radial growth was measured at 24 h interval till the isolates covered the plates. The colour and appearance of the mycelium, the day of first appearance of sclerotial initials and the number of sclerotia formed were recorded. The observations were carried out for a period of one month for each isolate.

3.1.5.3 *In vitro* cut stalk assay for lesion production

Virulence of the cultures was tested by *in vitro* cut stalk assay as described in 3.1.4. The onset of lesion formation and lesion length were recorded.

3.2.1 Selection of hypovirulent isolates

Based on the cultural characters and virulence, hypovirulent isolates were selected. Isolates without apparent sclerotial production and lesion formation, and normal growth rate were selected as hypovirulent isolates. Thus six isolates were chosen as hypovirulent (HV).

3.2.2 Selection of the most virulent isolate for comparative studies

The isolate with the highest sclerotial production and lesion length was selected for comparative studies, for the rest of the experiments.

3.3 Vegetative compatibility studies

Six isolates were tested for their ability for anastomosis (hyphal fusion) with the virulent isolate by the method of Parmeter *et al.* (1969). Opposing isolates were placed on cellophane film kept on two per cent water agar (Appendix I) in nine cm Petri dishes. One pair of isolates – a hypovirulent isolate and the virulent isolate were tested per dish. One cm mycelial discs from the margin of actively growing young cultures were placed four cm apart in each dish. The dishes were incubated at room temperature (28±2°C) until the advancing hyphae made contact. Such portions of the cellophane with the connecting hyphae were removed, mounted on a microscopic slide, stained with 0.1 per cent cotton blue lactophenol (Appendix I) and examined under 10X of a light microscope for hyphal fusion. The point of contact/fusion of the opposing hyphae was located by tracing hyphae along the direction of growth.

3.4 Dual culture studies with the virulent culture

Dual culture studies were conducted to analyse the interaction patterns of hypovirulent and the virulent strain, according to the method of Skidmore and Dickinson (1976).

Mycelial discs of five mm diameter were cut from periphery of five day old cultures grown on PDA, one from a hypovirulent isolate and the other from the virulent isolate. The discs were placed four cm apart, diametrically opposite on a PDA plate. The mycelia of the two cultures were allowed to meet. Inhibition of mycelial growth was recorded.

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

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

I = Inhibition of mycelial growth

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

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

3.5 Nuclear staining

The cellular nuclear number (CNN) of the isolates was determined by nuclear staining. A modified method of Burpee *et al.*,(1978) was followed for nuclear staining. The stain used was 0.5 per cent trypan blue in lactophenol (Appendix I).

Cellophane discs of eight cm diameter were placed on Petri plates plated with two per cent water agar. The hypovirulent and the virulent cultures of *R.solani* were individually inoculated at the centre of the cellophane discs. The plates were incubated at 28±2°C for five days. Rectangular pieces of cellophane of dimension 3cm x 2cm were carefully cut from different parts of the plate. These pieces were carefully mounted on a drop of trypan blue stain placed at the centre of clean slides and a cover slip was placed over it to hold the cellophane in position. The slide was warmed gently over a spirit lamp till the stain just started to boil. The slide was then allowed to cool and was observed under light microscope at 45X and under oil immersion to examine the cellular nuclear number of the culture. Observations were taken from 25 randomly selected fields.

3.6 Measurement of hyphal width

Hyphal width (μm) of the selected isolates were measured to determine the difference between virulent and hypovirulent isolate. The main runner hypha was located and observations were made from fifteen randomly selected fields. The average of the observations was worked out for each isolate.

3.7 Transmissibility studies

Dual culture experiment and hyphal tip culture studies were set up to determine whether hypovirulence was transmissible. Two separate methods were adopted for dual culture experiment.

3.7.1 Subculturing mycelia from the interface region

Colonized agar discs of the hypovirulent and virulent isolates were placed side by side on a PDA plate, four cm apart and incubated at room temperature until the hyphae of two isolates made contact. Agar plugs were then removed from the zone of contact of two isolates and subcultured on PDA slants.

3.7.2 Modified method of Zhou and Boland (1997)

Agar disc of the hypovirulent isolate was plated in a nine cm Petri dish, two cm from the periphery and allowed to grow for 48 hours. An agar disc of five mm size was then cut from the virulent isolate and placed on the periphery of the growing hypovirulent culture so that the disc just touched the growing mycelium of the hypovirulent culture. The dish was then incubated for another 24 hours. The mycelium growing towards the periphery was then cut as a disc and was subcultured on PDA slants.

The isolated cultures were then plated on PDA plates for comparisons of morphology. The rate of growth, day of sclerotial formation and the intensity of sclerotial formation were recorded. Cut stalk assay was done for such isolates to compare their virulence with the parent isolates.

3.7.3 Hyphal tip culture studies

Hyphal tip cultures were made to test any possible conversion to virulent phenotype as suggested by Castanho *et al.*, (1978).

Hypovirulent cultures were inoculated on two per cent water agar and incubated for 48 hours at 28±2 °C. The growing parts of the mycelium were observed through light microscope at 10X and hyphal tips were located and marked. The marked portions were cut and transferred aseptically to sterile Petri dishes and incubated at 28±2°C. The isolates were observed for conversion to virulent phenotype based on mycelial characters, sclerotial production and lesion production on cut stalks of rice.

3.8 Effect of hypovirulent isolates on seed germination and seedling vigour

3.8.1 Seed germination studies

The effect of hypovirulent isolates on germination of rice seeds was done by the blotter method of Neergaard (1986). The virulent isolate and the hypovirulent isolates were grown on PDA in sterile Petri dishes. Rice seeds of variety, Jyothi were surface sterilised with 0.05% sodium hypochlorite and 30 seeds were placed in the Petri dish containing the respective isolate and kept as such for 48 hours.

The seeds were then mixed with mycelium and sown in Petri dishes on moistened filter paper arranged in three layers. The seeds were incubated for 48 hours at 28± 2°C. Three replicates were maintained per isolate. The total number of seeds germinated was recorded. The germination percentage of the seeds was worked out using the formula,

$$\text{Germination per cent} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown.}} \times 100$$

3.8.2 Studies on effect of hypovirulent isolates on seedling growth

The hypovirulent isolates were inoculated on seedlings of susceptible variety Jyothi, to assess the effect of hypovirulent isolates on seedling growth according to the modified method of Premalatha Dath (1990). An untreated control was maintained for comparative studies. The experiment was set up in a Completely Randomised Design with three replications.

Hypovirulent isolates were grown on PDA for seven days. Seedlings of susceptible variety, Jyothi were raised on plastic trays 25x15x6 cm, filled with sterilised sand up to a height of five cm. Agar discs of one-week-old culture of the isolates were spread over the soil surface at the base of the seedlings at the eighth day after sowing. The height of the plants was recorded 18 days after sowing. The data were analysed statistically for comparison with untreated control.

3.9 Studies on growth promotion by hypovirulent isolates

The hypovirulent cultures were tested in a pot culture experiment conducted at the College of Agriculture, Vellayani, in a Completely Randomized Design, to find out their effect on promotion of crop growth

and to see whether these isolates incited any symptoms of sheath blight under *in vivo* conditions. Six hypovirulent cultures and the virulent culture GR13 were used for the study. The design adopted was C.R.D. with eight treatments and three replications.

The treatments were,

T1	A3	T5	GR18
T2	GR8	T6	GR23
T3	RS9	T7	GR13
T4	A13	T8	Untreated control

Plant height was recorded at 20, 40, 60 and 90 days after transplanting (DAT). The fresh weight and dry weight of the plants were recorded after harvest.

3.9.1 Preparation of inoculum for hypovirulent and virulent *R.solani* cultures

The virulent isolate and the different hypovirulent isolates were grown on rice bran. Rice bran was mixed with water in the ratio 2:1 and 250 g of this mixture was filled in polypropylene bags, sealed and sterilised at 121.1° C at 1.02 kg cm^{-2} for two hours.

Mycelial discs were cut from seven-day old PDA cultures of different isolates. Each bag was inoculated with three five mm mycelial discs and was incubated at 28±2°C for two weeks.

3.9.2 Application of inoculum

Plastic pots of height 25cm and diameter 20cm were filled with clay from rice field, leaving 10 cm gap from the top. Two hundred and fifty g of

the inoculum was mixed with soil in the ratio 1:2 and the rice bran-soil mixture was applied on the top of the filled pots. Eighteen-day-old rice seedlings were transplanted from nursery to the pots. Two hills of three seedlings were planted per pot.

3.10 Assessment of hypovirulent isolates for sheath blight suppression

A pot culture experiment was conducted at the College of Agriculture, Vellayani, with a view to study the effect of hypovirulent isolates on sheath blight suppression and to compare this with the effects of a systemic chemical fungicide and proven biocontrol agents. The control treatments were: plants inoculated with *Trichoderma harzianum,* those inoculated with *Pseudomonas fluorescens* , plants sprayed with carbendazim (0.1%) and an untreated control. The details of the experiment are as follows:

Design : Completely Randomized Design.

Replication: Three.

Treatments: Eleven.

The treatments were

T1	A3 X GR13	T7	GR13
T2	GR8 X GR13	T8	Untreated control
T3	RS9 X GR13	T9	*T.harzianum* X GR13
T4	A13 X GR13	T10	*P.fluorescens* X GR13
T5	GR18 X GR13		
T6	GR23 X GR13	T11	Carbendazim (0.1 %) X GR13

*Where A3,GR8,RS9,A13,GR18 and GR23 are hypovirulent *R.solani* isolates and GR13,the virulent *R.solani* isolate.

3.10.1 Preparation of inoculum.

3.10.1.1 Preparation of inoculum of hypovirulent and virulent isolates

The inoculum of hypovirulent and virulent isolates was prepared as described under 3.9.1.

3.10.1.2 Preparation of inoculum of *T. harzianum*

The original culture of *T. harzianum* was procured from Department of Plant Pathology, T.N.A.U.,Coimbatore. *T. harzianum* was mass-multiplied in rice bran (Elad *et al.*,1983). Rice bran was mixed with water in the ratio 1: 2 and 250 g of this mixture was taken in a polypropylene bag , sealed and autoclaved for two hours at 121.1 ° C and 1.02 $kgcm^{-2}$. Mycelial discs of 10-day-old PDA culture of *T.harzianum* were used to inoculate the rice bran-water mixture. The bags were then incubated for 10 days at 28±2°C.

3.10.1.3 Preparation of inoculum of *Pseudomonas fluorescens*

The stock culture of *P.fluoroscens* was procured from Department of Plant Pathology,T.N.A.U.,Coimbatore. Talc-based formulation of *P. fluorescens* was prepared by following the method of Vidhyasekaran and Muthamilan (1995). The bacterial culture was multiplied in King's broth (King *et al.*, 1954, (Appendix I)) which was previously autoclaved at 1.02 $kgcm^{-2}$ for 20 min. A loopful of the bacterium was inoculated into the broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature.

One hundred g of talc powder was taken in each polypropylene bag. One gram of carboxy methyl cellulose was thoroughly mixed with this, sealed and autoclaved at 1.02 kg cm^{-2} for two hours. Forty ml of 48 hr grown inoculum was added, mixed under aseptic conditions and the polypropylene bag was sealed and stored at room temperature.

3.10.2 Application of inoculum

3.10.2.1 Application of hypovirulent isolates, chemical and biological control treatments.

Plants were raised and the hypovirulent isolates and *T.harzianum* were applied as described in the method under 3.9.2. *P.fluorescens* and carbendazim were applied at 0.2 and 0.1 per cent as foliar spray respectively at 10 and 40 DAT.

3.10.2.2 Application of inoculum of virulent isolate

Seven days after transplanting, the pots were topdressed (IRRI,1986) with 250g of virulent isolate in rice bran (rice bran inoculum : soil in the proportion 1:2 w/w) . A second challenge inoculation of the virulent isolate was given 35 DAT by inserting a sclerotium of the virulent culture was inserted in between the outermost leaf sheath of all treatments except untreated control.

The observations were recorded 20, 40, 60 and 90 DAT. The observations recorded were plant height (cm), lesion size (cm^2) vertical spread i.e., the height of uppermost lesion from the point of inoculation and horizontal spread i.e., number of tillers affected per hill. The following parameters were analysed statistically.

a) Lesion size

b) Vertical spread

c) Horizontal spread

d) Relative Lesion Height (%) = $\frac{\text{Height of the uppermost lesion}}{\text{Height of the plant}} \times 100$

(Ahn *et al.*, 1986)

The relative disease intensity on rice plants was worked out for all treatments from the scale proposed by Ahn *et al.*, (1986)

Relative disease intensity scale

Grade	RLH (%)	Relative disease intensity
0	0	0
1	<20	1
3	20-30	5
5	31-45	25
7	46-65	50
9	>65	100

3.11 Effect of hypovirulent isolate on non-target flora in the rice ecosystem

Effect of hypovirulent isolate of *R. solani* was tested on the other flora in the rice ecosystem as well as on the common crop plants in the rice fallows to detect any averse effects on these non-target plants. The isolate that offered the maximum protection was used for this experiment.

The plants tested are given below

	Botanical names	**Common name/Vernacular names**
Crop plants		
1.	*Amaranthus tricolor* L.	Amaranthus
2.	*Amorphophallus companulatus* L.	Amorphophallus
3.	*Arachis hypogaea* L.	Ground nut
4.	*Colocasia esculenta* L.	Colocasia

5.	*Ipomoea batatus* (L.) Lam	Sweet potato
6.	*Sesamum indicum* L.	Sesamum
7.	*Vigna radiata L.*	Green gram
8.	*Vigna unguiculata* L.(Walp)	Cowpea

Weeds

9.	*Ageratum conyzoides* L.	Appa
10.	*Amaranthus viridis* (L) Notrysag	Kuppacheera
11.	*Axonopus* spp.	Axonopus
12.	*Bulbostylis barbata* SW.	Sooryan
13.	*Cleome viscosa* L.	Kattukaduku
14.	*Commelina benghalensis* L.	Vazhappadathy
15.	*Cynodon dactylon (L.)*	Karuka
16.	*Cyperus rotundus* L.	Muthanga
17.	*Echinochloa colona* (L.)Link	Kavada
18.	*Hydrocotyl asiatica* Urban	Kudangal
19.	*Leucas aspera* Spreng	Thumba
20.	*Monochoria vaginalis* (Burm.f.) Presl	Neerthamara
21.	*Panicum distachyum*. Stapf	Paalpullu
22.	*Panicum repens* L.	Torpedo grass

3.12 Effect of hypovirulent isolates on defense related enzymes

The rice plants inoculated with hypovirulent isolates of *R. solani* were compared with those inoculated with virulent isolate for assessing their effect on the activity of various defense related enzymes. Two hypovirulent isolates: the best performing isolate and the isolate with intermediary performance, were selected for the study.

3.12.1 Effect on peroxidase

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

3.12.1.1 Preparation of enzyme extract

Leaf sample of 200 mg was homogenised in one ml of 0.1 M sodium phosphate buffer (pH 6.5) (Appendix II) to which 0.05 g of polyvinyl pyrrolidone (PVP) was added. The homogenisation was done at 4°C using a pestle and mortar. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 min at 4^0C. The supernatant was used as the enzyme extract for the assay of peroxidase activity.

3.12.1.2 Assay of peroxidase activity

The reaction mixture consisting of one ml of 0.05 M pyrogallol and 50 µl enzyme extract was taken in both reference and sample cuvettes, mixed and kept in a spectrophotometer and the reading was adjusted to zero at 420 nm. The enzyme reaction was started by adding one ml of one per cent hydrogen peroxide (H_2O_2) into sample cuvettes and the change in absorbence was measured at 30 seconds interval. The enzyme activity was expressed as change in optical density per gram fresh weight of plant material per minute.

3.12.2 Effect on phenol oxidase

Activity of phenol oxidase was assessed according to the method of Mukherjee and Ghosh (1975)

3.12.2.1 Preparation of enzyme extract

One gram of leaf material was separately macerated in a prechilled mortar and pestle using three ml of 0.1 M sodium phosphate buffer (P^H 6.5). The homogenate was centrifuged in a refrigerated centrifuge at 12,000 rpm for 15 minutes. The supernatant was used to measure the enzyme activity of phenol oxidase.

3.12.2.2 Assay of phenol oxidase activity

The reaction mixture contained 2.3 ml of phosphate buffer 0.1 M (pH 6.5) (Appendix II). 0.5 ml of catechol 0.01M and 0.2ml of enzyme extract to a total volume of 3.0 ml. The enzyme extract was added to the reaction tubes after adjusting the Spectronic 20 set at 495 nm, to zero absorbence with all contents except the enzyme. The changes in absorbence were recorded at 30 second intervals up to 150 seconds . Controls were maintained with heated enzyme. The enzyme activity was expressed as change in optical density per gram fresh weight of plant material per minute

3.13 Screening of carrier materials

Survival of hypovirulent isolate was tested on different carrier materials for the purpose of mass multiplication of biocontrol agent. The hypovirulent isolate chosen for this study was the one, which offered maximum protection to the treated plants.

The different carrier materials tested were rice bran, oats meal sand medium, paddy straw, saw dust, coir pith and talc. The composition of the carrier materials are given in Appendix III.

All the carrier materials were taken in 250 ml conical flasks and filled up to 100 ml. Mycelial discs of one week old culture of the hypovirulent isolate were used to inoculate all materials except for talc based formulation.

3.13.1 Preparation of talc-based formulation

Potato Dextrose broth (Appendix I) was prepared in conical flasks and sterilised a 1.02kg cm^{-2} for 20 minutes. Hypovirulent *R.solani* cultures were inoculated into potato dextrose broth with a fungal disc cut from a ten-day old culture grown on PDA plated in Petri dishes.. At the end of incubation period, the mycelium was separated from the aqueous broth by filtering through a muslin cloth. Fungal mats obtained were pressed to remove water and air dried. It was then dried under sun for half an hour and then powdered in a mixer grinder. The resulting powder was mixed with sterilized talc in polypropylene bags @ 10 per cent w/w. One per cent carboxy methyl cellulose (CMC) was added to this.

3.13.2 Estimation of population of hypovirulent *R.solani* isolates in the carrier materials

The number of colony forming units from each career material was determined by serial dilution and plate counting method at an interval of ten days for the first three months of inoculation and then at an interval of one month for a total five months. The medium used was Martin's Rose Bengal Agar medium (Appendix I). The population of the fungus was expressed as number of colony forming units (cfu) per gram of carrier materials.

RESULTS

4. RESULTS

Experiments were conducted under *in vitro* and *in vivo* conditions to isolate, screen and test isolates of *Rhizoctonia solani* for hypovirulence, to explore the possibilities of their use in biocontrol as well as to outline their mode of action for disease suppression.

4.1 Detection of weakly virulent isolates of *R.solani*

The results of the experiments conducted to locate weakly virulent isolates of *R.solani* are described below.

4.1.1 Collection of *R.solani* isolates

Twenty-eight cultures were obtained through tissue isolation from plant samples and serial dilutions and plating of soil samples collected from different rice growing tracts of Kerala. The list of isolates with their location of collection and source of material used for isolation are presented in Table 1.

4.1.2 Isolation of *R.solani*

Isolation from the soil and plant samples yielded *R.solani* Kühn, which conformed to the characters, as described by Parmeter and Whitney (1969). All the isolates examined showed young hyphal branches inclined to the direction of growth and constricted at the point of origin from the main hyphae. Branching was from the distal septum.

Pure cultures were obtained by the hyphal tip technique and were maintained at 4°C.

Table 1 : List of R.solani isolates with their locations of collection and sources

Sl no	Isolates	Locations	Sources of material
1	A1	Pattambi, Palakkad	leaf
2	A2	Pattambi, Palakkad	sheath
3	A3	Pattambi Palakkad	soil
4	A5	Pattambi, Palakkad	soil
5	A6	Ambalavayal,Wayanad	leaf
6	A7	Ambalavayal,Wayanad	sheath
7	A13	Ambalavayal,Wayanad	soil
8	GR2	Ambalavayal,Wayanad	soil
9	GR3	Kottarakkara,Kollam	soil
10	GR6	Kottarakkara,Kollam	soil
11	GR8	Moncompu, Alappuzha	leaf
12	GR10	Moncompu, Alappuzha	sheath
13	GR12	Moncompu, Alappuzha	soil
14	GR13	Changanassery, Kottayam	soil
15	GR18	Kayamkulam,Kollam	sheath
16	GR21	Pilicode,Kasaragode	soil
17	GR23	Pilicode,Kasaragode	leaf
18	IS1	Pilicode,Kasaragode	leaf
19	IS2	Pilicode,Kasaragode	soil
20	IS4	Vellayani,Thiruvananthapuram	soil
21	IS5	Vellayani,Thiruvananthapuram	sheath
22	IS6	Vellayani,Thiruvananthapuram	soil
23	RS9	Karamana,Thiruvananthapuram	leaf
24	VA1	Karamana,Thiruvananthapuram	leaf
25	VA2	Karamana,Thiruvanathapuram	leaf
26	VA3	Ulloor,Thiruvanathapuram	leaf
27	VA4	Vembayam,Thiruvananthapuram	leaf
28	VA5	Vellayani, Thiruvananthapuram	leaf

4.1.3. Pathogenicity tests

The isolates of *R.solani* showed differences in symptom production. The results are presented in Table 2. Excepting eight isolates, all the other *R.solani* isolates produced lesions. Symptoms appeared as elliptical lesions, initially dull green and water soaked, later turning brown or straw coloured with dark brown margin characteristic of sheath blight (Plate 1) The isolates *viz* A3, GR6, GR8, RS9, A13, GR18, GR21 and GR23 did not produce typical symptom of sheath blight, but a slight yellowing was noticed around the point of inoculation. (Plate 2)

4.1.4 Screening of *R.solani* isolates for hypovirulence

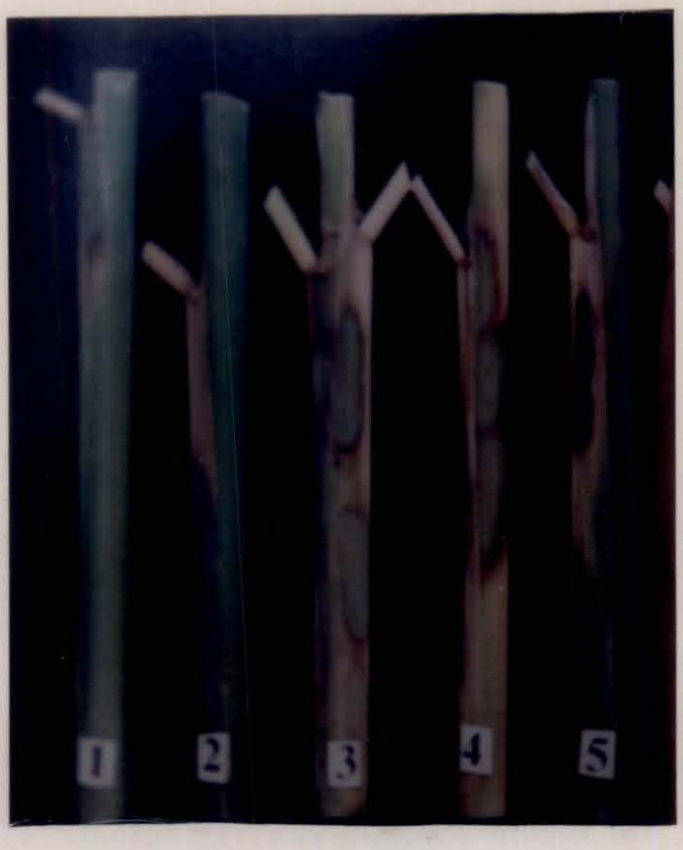
4.1.4.1 Studies on mycelial growth and sclerotial formation

Mycelial growth and sclerotial formation of the different *R.solani* isolates were conducted *in vitro* and the results are presented in Table.3. All isolates showed almost similar growth rate. Majority of the cultures took three days to attain full growth in nine cm diameter Petri dishes. However, four cultures GR6,GR8,GR18 and GR21 required four days to attain full growth.

Sclerotial formation was recorded in culture GR13 after three days of growth. In most of the other cultures, sclerotia were formed between one to two weeks of inoculation. The isolate A2 took 15 days to form sclerotial initials. Eight cultures *viz*, A3,GR6,GR8,RS9,A13,GR18,GR21 and GR23 showed no sclerotial formation.

Plate 1. Effect of inoculation of virulent isolates of *R. solani* on cut stalks of rice variety , Jyothi

Plate 2. Effect of inoculation of hypovirulent isolates of *R.solani* on cut stalks of rice variety ,Jyothi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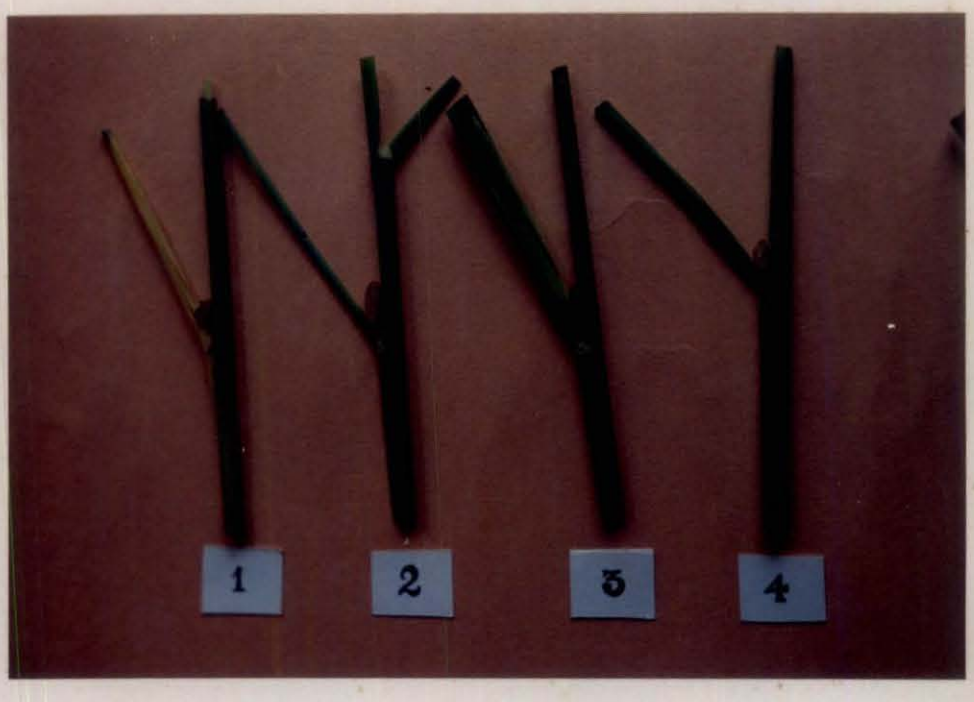
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Table 2 : Pathogenicity of *R.solani* isolates on susceptible rice variety, Jyothi

Serial No.	Isolates	Pathogenicity(+/-)
1	A1	+
2	A2	+
3	A3	-
4	A5	+
5	A6	+
6	A7	+
7	A13	-
8	GR2	+
9	GR3	+
10	GR6	-
11	GR8	-
12	GR10	+
13	GR12	+
14	GR13	+
15	GR18	-
16	GR21	-
17	GR23	-
18	IS1	+
19	IS2	+
20	IS4	+
21	IS5	+
22	IS6	+
23	RS9	-
24	VA1	+
25	VA2	+
26	VA3	+
27	VA4	+
28	VA5	+

+ = Infection on cust stalks - = No infection

4.1.4.1.1 Colour of mycelium

The colour of mycelium varied among isolates (Plate 3). The isolates that did not produce sclerotia were tan coloured with fluffy mycelium, whereas in the sclerotial isolates mycelium was light brown in colour and regularly appressed to the surface. (Table 3)

4.1.4.2 *In vitro* cut stalk assay for lesion production

All the sclerotia forming isolates produced typical lesions of sheath blight (Table 4). The length of the lesion varied from 1 cm to 4.3 cm. The isolate GR13 recorded the highest lesion length i.e.4.3 cm. The non-sclerotial isolates did not produce lesion but showed slight yellowing in the area of inoculation (Plate 2).

4.2 Selection of isolates

The data obtained from the above experiments were used for the selection of hypovirulent isolates and the most virulent isolate of *R.solani*. (Table 5).(Plate 3)

4.2.1 Selection of hypovirulent isolates.

Six *R.solani* isolates producing tan coloured mycelium, without formation of sclerotia and without production of symptoms typical of sheath blight were identified as hypovirulent isolates. Thus, the hypovirulent isolates A3, A13, GR8, GR18, GR23 and RS9 were selected for further studies (Table 5, Plates 3b-e).Two hypovirulent isolates GR6 and GR21 which showed

Table 3 : Mycelial growth and sclerotial formation of *R. solani* isolates

		Mycelial characters			Sclerotial characters	
Sl no	Isolate	Mycelial growth on 3rd day (cm)	Mycelial colour	Mycelial Texture	Sclerotial production	Day of formation of sclerotia
1	A1	9	Light Brown	Appressed	+	7
2	A2	9	Light Brown	Appressed	+	15
3	A3	9	Tan	Fluffy	-	-
4	A5	9	Light Brown	Appressed	+	14
5	A6	9	Light Brown	Appressed	+	-
6	A7	9	Light Brown	Appressed	+	14
7	A13	9	Tan	Fluffy	-	-
8	GR2	9	Light Brown	Appressed	+	14
9	GR3	9	Light Brown	Appressed	+	13
10	GR6	8.1	Tan	Fluffy	-	-
11	GR8	8.5	Tan	Fluffy	-	-
12	GR10	9	Light Brown	Appressed	+	7
13	GR12	9	Light Brown	Appressed	+	13
14	GR13	9	Light Brown	Appressed	+	3
15	GR18	8	Tan	Fluffy	-	-
16	GR21	7	Tan	Fluffy	-	-
17	GR23	9	Tan	Fluffy	-	-
18	IS1	9	Light Brown	Appressed	+	13
19	IS2	9	Chocolate Brown	Appressed	+	12
20	IS4	9	Light Brown	Appressed	+	13
21	IS5	9	Light Brown	Appressed	+	13
22	IS6	9	Light Brown	Appressed	+	13
23	RS9	9	Tan	Fluffy	-	-
24	VA1	9	Light Brown	Appressed	+	14
25	VA2	9	Chocolate Brown	Appressed	+	13
26	VA3	9	Light Brown	Appressed	+	13
27	VA4	9	Light Brown	Appressed	+	13
28	VA5	9	Light Brown	Appressed	+	13

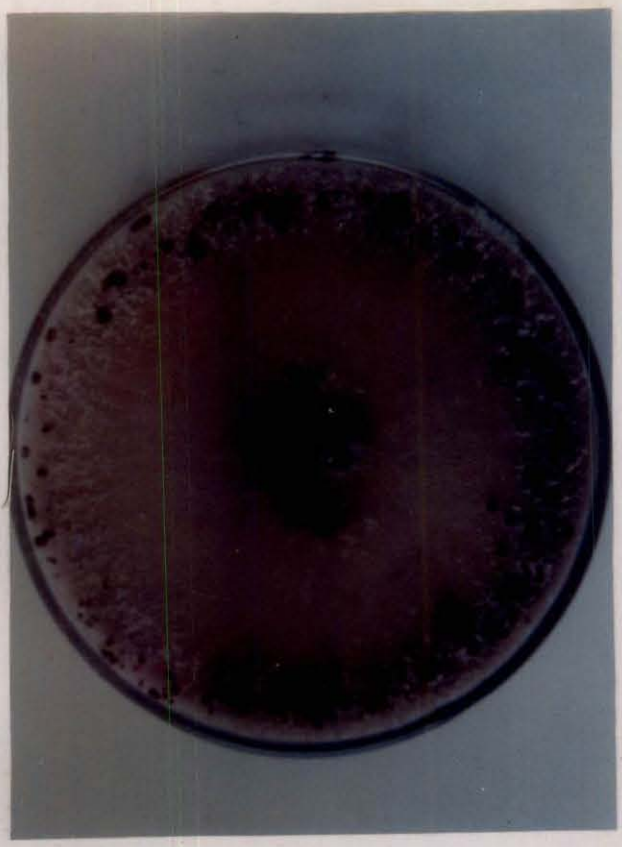
Plate 3. Selected isolates of *R. solani*

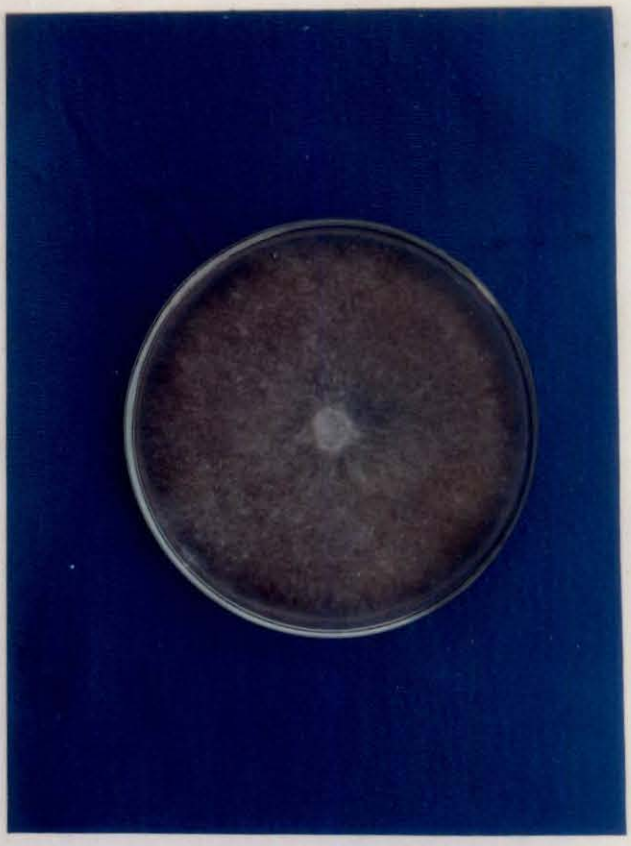
3a . Most viulent isolate
3b –3e Hypovirulent isolates

3a. GR13

3b. GR8

3c GR23



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3d A13 3e RS9

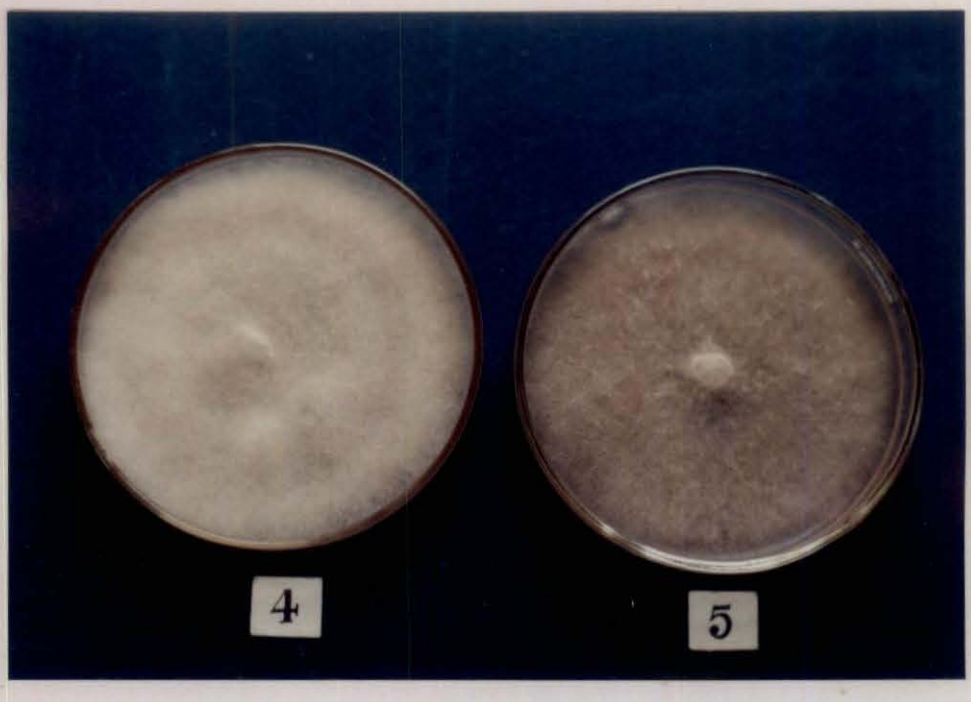
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Table 4 : Effect on lesion development by *R. solani* isolates on *in vitro* cut stalk assay in rice variety, Jyothi

SLNO	ISOLATE	LESION	LESION LENGTH (cm)
1	A1	+	1.2
2	A2	+	1.9
3	A3	-	-
4	A5	+	1.0
5	A6	+	1.0
6	A7	+	1.1
7	A13	-	-
8	GR2	+	1.2
9	GR3	+	1.2
10	GR6	-	-
11	GR8	-	-
12	GR10	+	4.1
13	GR12	+	2.0
14	GR13	+	4.3
15	GR18	-	-
16	GR21	-	-
17	GR23	-	-
18	IS1	+	3.0
19	IS2	+	3.2
20	IS4	+	3.0
21	IS5	+	1.6
22	IS6	+	1.4
23	VA1	+	2.9
24	VA2	+	2.6
25	VA3	+	2.2
26	VA4	+	2.7
27	VA5	+	3.3
28	RS9	-	-

+ = Lesions formed on cut stalks
- = No lesion formation on cut stalks

Table 5 : List of selected isolates

Sl no	Isolates	Hypovirulent/Virulent
1	A3	Hypovirulent
2	GR8	Hypovirulent
3	RS9	Hypovirulent
4	A13	Hypovirulent
5	GR18	Hypovirulent
6	GR23	Hypovirulent
7	GR13	Virulent

lesser growth rate than the virulent isolate and which produced very little growth on subculturing were discarded.

4.2.2 Selection of the most virulent isolate for comparative studies

Isolate GR13 (Plate 3a) was found to possess the maximum values for the virulence related attributes studied. It recorded nine cm of mycelial growth in three days (Table 3). Sclerotial initials were produced in three days in this isolate (Table 3). The maximum lesion length was produced by the isolate GR13, i.e. 4.3 cm (Table 4) Based on all these characters, this isolate (GR13) was chosen as the most virulent isolate for further comparative studies.

4.3 Compatibility studies of hypovirulent isolates with virulent isolates of *R.solani*

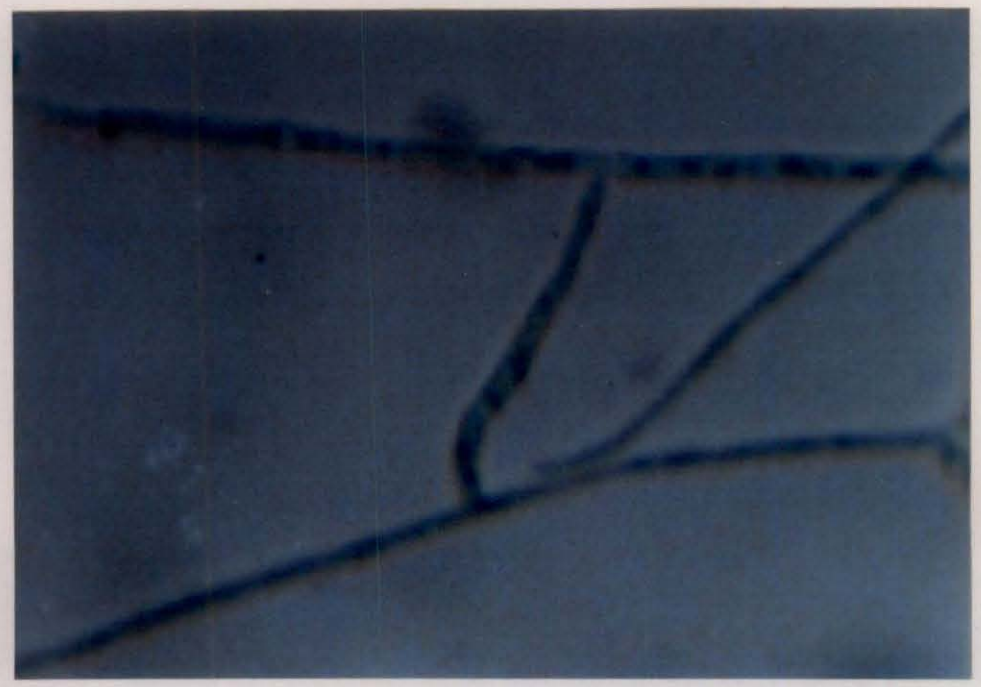
Hyphal fusion was noticed between hypovirulent isolates and the virulent isolate (Table 6) (Plate 4). Two types of hyphal fusion were mainly noticed: i) Tip to sidewall of the hyphae and ii) sidewall to sidewall of the hyphae (Plates 4a-4c). All the isolates except A13 and GR23 showed these kinds of fusions. These isolates when dual cultured with GR13 showed contact fusion. The contact fusion showed only hyphal contact without further hyphal growth, cell wall fusion or cytoplasmic fusion (Plate 4d).

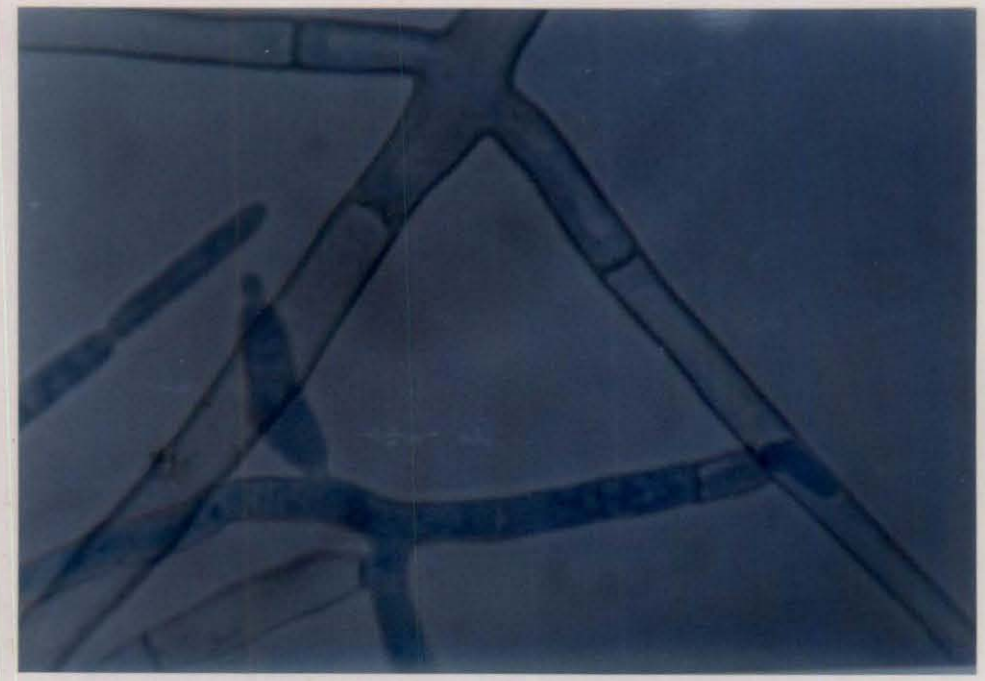
4.4 Dual culturing of hypovirulent and virulent *R.solani*

The effect of hypovirulent *R.solani* isolates on the mycelial growth and sclerotial production of the virulent isolate was tested by the dual culture technique (Plate 5) and the results are presented in the Table 7.

Plate 4. Hyphal fusion between the virulent isolate and hypovirulent isolates of *R.solani*

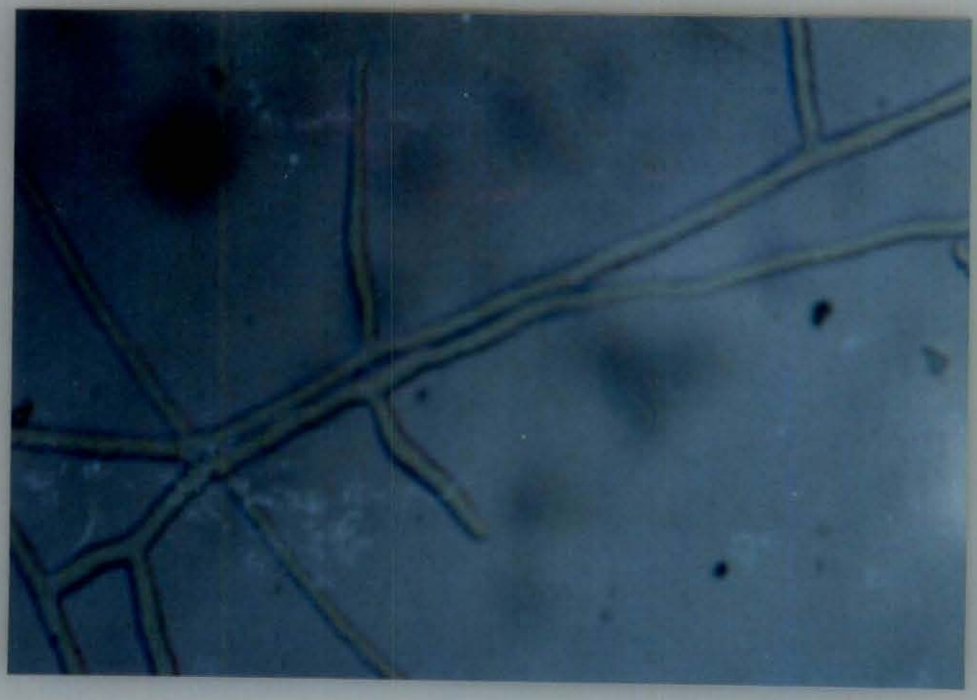
4a-4b Tip to side wall fusion





4c Side wall to side wall fusion

4d Contact fusion



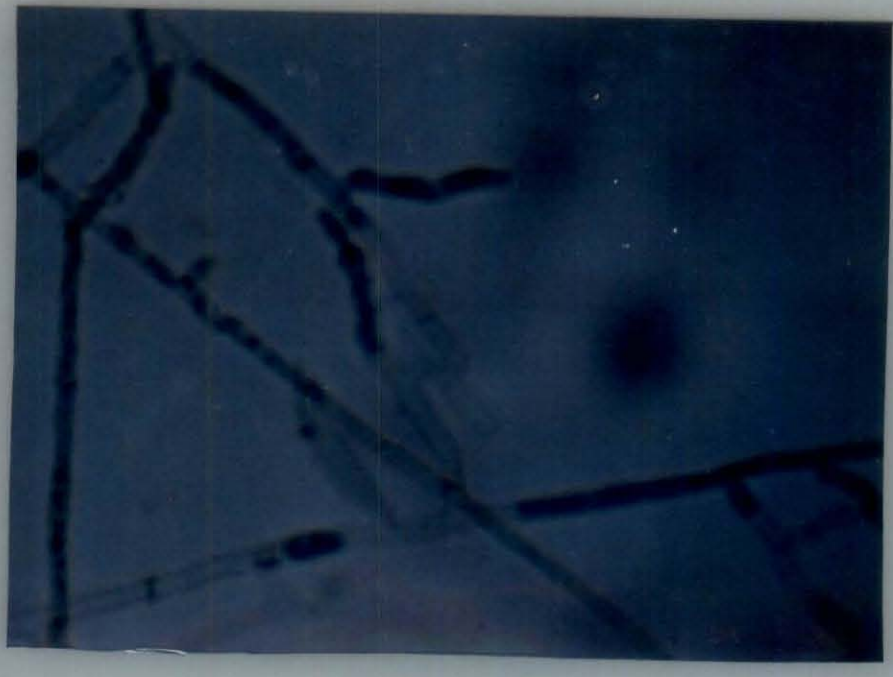


Table 6 : Anastomosis between hypovirulent isolates and virulent isolate (GR13) of *R.solani*

Hypovirulent isolate	Anastomosing reaction with virulent isolate GR 13	Type of hyphal fusion
A3	+	Sidewall to side wall Tip to side wall
A13	-	Contact
GR8	+	Sidewall to side wall Tip to side wall
GR18	+	Sidewall to side wall Tip to side wall
GR21	+	Sidewall to side wall Tip to side wall
GR23	-	Contact
RS9	+	Sidewall to side wall Tip to side wall

+ = Compatible reaction - = Incompatible reaction

Table 7 : Mycelial growth and sclerotial formation of the virulent *R.solani* isolate on interaction with hypovirulent isolates

Hypovirulent isolate	Mycelial growth (cm)	Inhibition(%)	Day of sclerotial formation
A13	2.66	29.2	4
A3	3.10	17	3
GR8	3.26	13.52	4
GR18	2.56	32.09	3
GR23	2.7	27.3	3
RS9	3.06	18.83	3
Control	3.77	-	3

Co–inoculation with hypovirulent isolates caused a reduction in mycelial growth of the virulent isolate GR13 from 13 to 32 per cent in three days of incubation .The virulent isolate was inhibited from further growth by all the hypovirulent isolates. However, there was no lytic zone at the point of contact of the two cultures. Overgrowth of the hypovirulent cultures was also not noticed. The isolates A13 and GR23 had a faster growth rate and were able to suppress the growth of the virulent isolate. No inhibition zone could be identified. The virulent culture continued to produce sclerotia even after contact with the hypovirulent strain. The isolates A13, GR6, GR 8 and GR 21 caused a 24 h delay in the sclerotial initiation by the virulent isolate (Table 7).

4.5 Nuclear staining

Nuclear staining of the *R.solani* isolates revealed the presence of fungal nuclei as minute dark blue round bodies. On observation under high dry magnification objective i.e. 45 X, the cellular nuclear number (CNN) could be detected. The cells of the hyphae of hypovirulent cultures A13 and GR23 were binucleate (Plate 6). The virulent isolate, GR13 along with the rest of the hypovirulent cultures were multinucleate (Table 8, Plate 7).

4.6 Measurement of hyphal width

Dimensions of the hyphal width was found to vary between hypovirulent and virulent isolates (Table 8) . The virulent isolate recorded an average width of 11.6 μm . The multinucleate hypovirulent isolates varied from 9.2 to 10.56 μm. The binucleate hypovirulent isolates showed thinner hyphae the width of which ranged from 6.2 to 6.6μm

Plate 5. Interaction between virulent isolate and hypovirulent isolate of *R.solani*

GR 13 – Most virulent isolate
GR8, A13, GR23 -Hypovirulent isolate

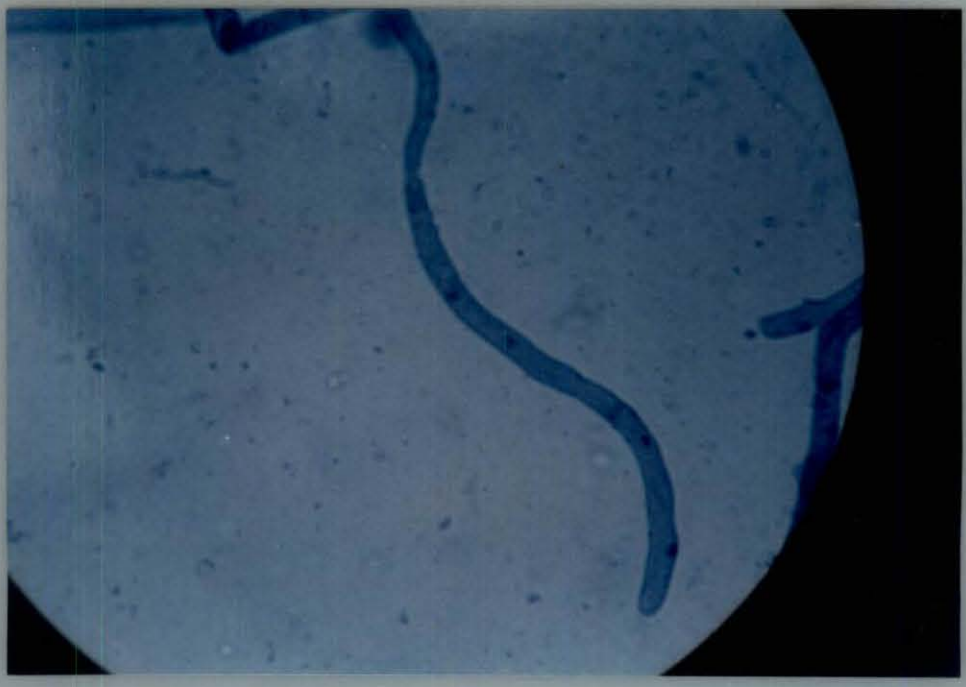
GR 13 x GR 8

GR13 x GR23

GR 13 x A13

Plate 6 Multinucleate condition of hypha of *R.solani*

Plate 7 Binucleate condition of hypha of *R.solani*



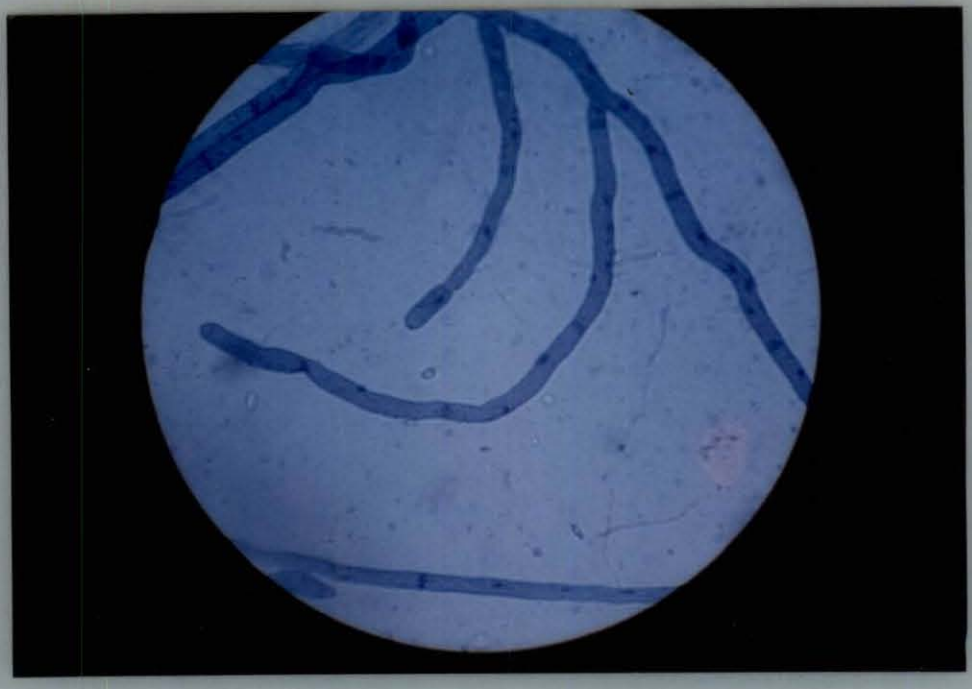


Table 8 : Cellular nuclear number and hyphal width of selected isolates

Isolate	Multinucleate/Binucleate	Hyphal width (μm)
A3	Multinucleate	9.2
GR8	Multinucleate	9.2
RS9	Multinucleate	9.6
A13	Binucleate	6.6
GR18	Multinucleate	10.5
GR23	Binucleate	6.2
GR13	Multinucleate	11.6

Table 9 : Number of hypovirulent / virulent cultures obtained from subcultures of mycelia from interface region

Isolate X GR13	No of hypovirulent cultures obtained	No of virulent cultures obtained
A3	12	13
GR8	13	12
RS9	10	15
A13	14	11
GR18	13	12
GR23	13	12

4.7 Transmissibility studies

Two methods were adopted to study transmissibility of hypovirulence.

4.7.1 Subculturing mycelia from the interface region

The cultures obtained from this method were a mixture of virulent and hypovirulent ones. The frequency of appearance of hypovirulence and virulent stains was almost the same. (Table 9)

4.7.2 Modified method of Zhou and Boland (1997)

The cultures obtained from the second method continued to produce sclerotia (Plate 8). There was no significant reduction in the growth rate or sclerotial production of these cultures than those of the original virulent culture. The characters were stable for all four subcultures. None of the cultures thus obtained produced any symptom on the cut stalks of susceptible rice variety.

4.7.3 Hyphal tip culture studies

The hyphal tip cultures obtained from the hypovirulent isolate did not produce any sclerotia. All the growth characters expressed were in congruence to the original hypovirulent culture. No virulent cultures could be obtained from the hypovirulent isolate.

4.8.1 Effect of hypovirulent isolates on seed germination

Inoculation of hypovirulent isolates did not cause any suppression of germination. The germination of seeds of susceptible variety, Jyothi was recorded

Plate 8. Modified method of Zhou and Boland for hyphal fusion of *R.solani* isolates

on treatment with these isolates. In the untreated control, the germination was 95 per cent. The hypovirulent isolates showed no deleterious effect on the germination of the seeds. The seed germination ranged from 93.5 to 95 per cent (Table 10)

4.8.2 Effect of hypovirulent isolates on seedling vigour

The results of the effect of inoculation of hypovirulent isolates on seedling vigour observed as the seedling height after two weeks of seed germination are presented in the Table 10.

In general, the hypovirulent isolates did not produce any lethal effect on the seedling or delayed its growth. On statistical analysis the effects of the all hypovirulent isolates was found to be on par with untreated control. The maximum seedling height (12.55 cm) was observed on inoculation with GR23. Visual symptoms of disease development were not produced by any of the isolates.

The hypovirulent isolates did not show any correlation with seedling vigour.

4.9 Studies on growth promotion by hypovirulent isolates

Rice variety, Jyothi, was inoculated with the hypovirulent isolates in a pot culture experiment conducted in a completely randomised design for studying their effect on plant height at periodic intervals and on the fresh and dry weight of the plants after harvest. A comparison of the growth parameters with the virulent isolate treated plants and untreated control plants and the results are presented in Table 11.

Table 10: Effect of inoculation of hypovirulent isolates of *R.solani* on germination of rice seeds and seedling vigour

Isolate	Germination (%)	Seedling height (cm)
A3	94.3	11.27
GR8	94.2	12.20
RS9	95	11.63
A13	94.2	12.36
GR18	94.3	10.75
GR23	93.4	12.55
CONTROL	95.1	11.15
CD(0.05)	-	1.57

Table 11 : Effect of inoculation of hypovirulent isolates of *R.solani* on plant height, fresh weight and dry weight of rice variety, Jyothi

	Height of plants(cm)				FRESH WEIGHT (g)	DRY WEIGHT (g)
Treatments	20DAT	40DAT	60DAT	90DAT		
T1	19.67	33.33	54.67	60.00	680	171.87
T2	19.67	40.00	61.00	65.33	705	183.65
T3	19.67	32.33	55.33	61.00	695	173.65
T4	19.67	42.67	63.67	68.33	738	188.66
T5	19.67	34.67	57.33	62.33	720	182.91
T6	21.00	41.00	62.67	68.67	750	193.34
T7	17.33	29.33	44.33	48.67	645	163.41
T8	20.33	39.67	60.67	65.33	740	188.4
CD (0.05)	1.12	1.73	1.96	3.72	22.4	4.89

* Where ,T1 =A3, T2=GR8, T3=RS9, T4=A13 ,T5=GR18, T6=GR23, T7=GR13, T8=CONTROL

None of the hypovirulent isolates induced any symptoms on rice plants. The statistical analysis showed that the hypovirulent isolates differed significantly from the virulent isolate regarding the plant height and fresh and dry weight of the plants. The effects of the treatments GR23 and A13 were found to be either on par or superior to those of untreated control plants at different periods of observation. The height of the plants treated with the hypovirulent isolates A3 and RS9 was significantly less than the untreated control at all intervals indicating a negative effect on the growth of plants. On the contrary, the inoculation of hypovirulent isolates GR23 and A13 caused the highest values for all the growth related parameters (Plate 9, Fig 1-2) .The height, fresh and dry weight of plants were 62.6 cm, 750 g and 193.34g respectively in plants treated with the hypovirulent isolate GR23 whereas in the plants treated with GR13, the virulent isolate, the values for the above characters were 44.33 cm, 645 g and 163.41 g respectively. However, the effects were not statistically significant in comparison to untreated control, in the case of treatment with hypovirulent isolate in all of the observations.

4.10 Assessment of hypovirulent isolates for sheath blight suppression

The effect of hypovirulent isolates on *R.solani* on suppression of sheath blight disease incidence was studied in a pot culture experiment conducted in a completely randomised design. The plants were challenge inoculated with the virulent culture of *R.solani* at the time of transplanting as well as further challenged inoculated at 35 DAT . The effects were also compared with that of untreated control, chemical control

1. GR23 (Hypovirulent)

2. Control

3. GR13 (Virulent)

Effect of hypovirulent isolates of*R.solani* on plant height of rice variety,Jyothi

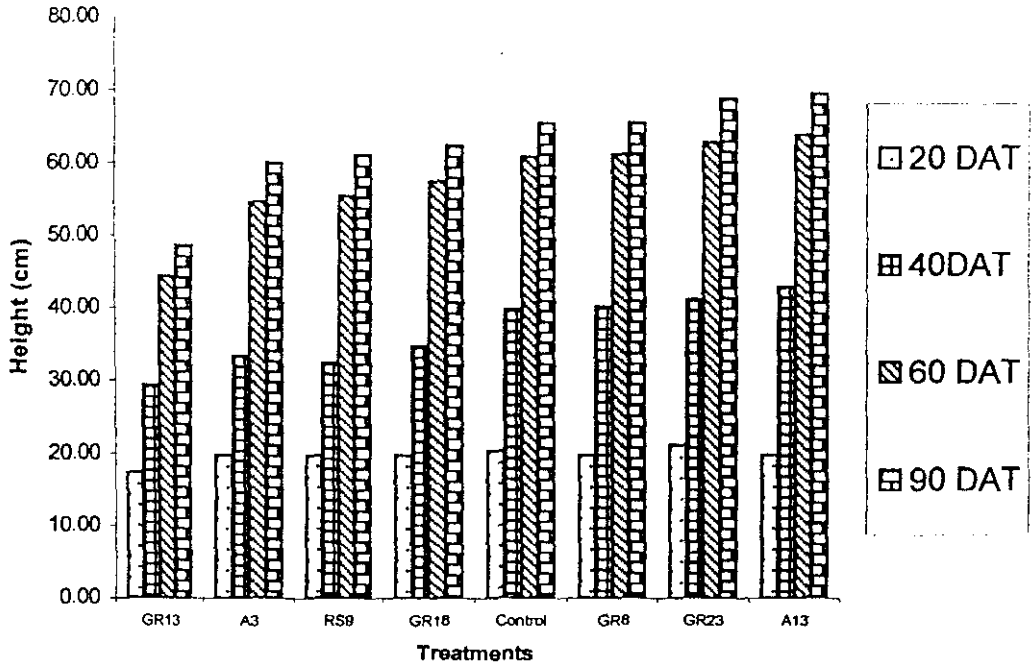
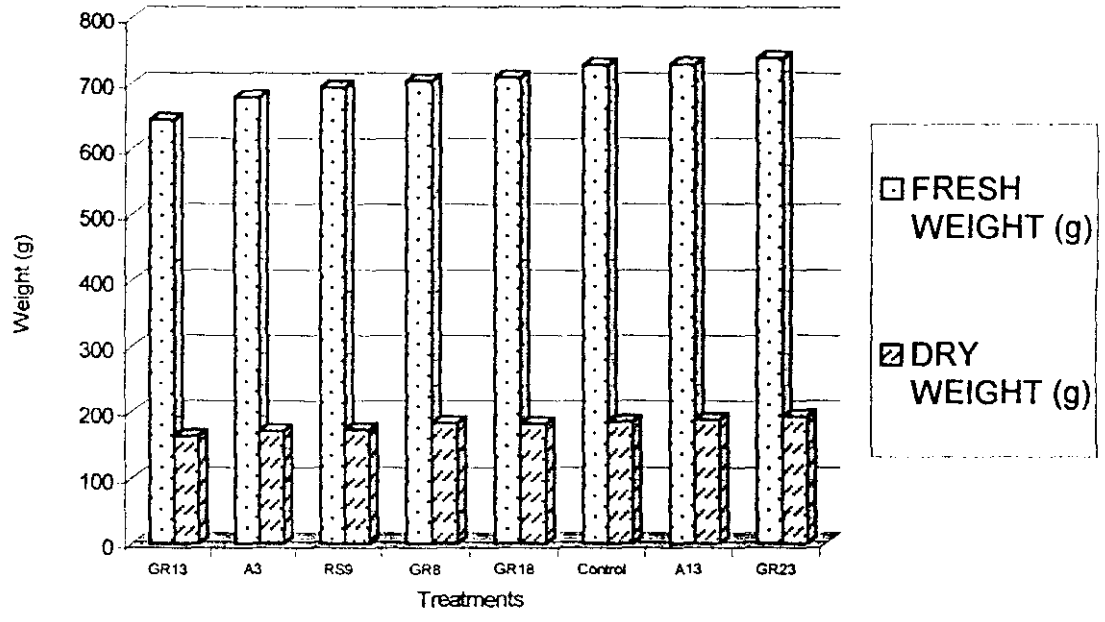


Fig 2

Effect of hypovirulent isolates of *R.solani* on fresh weight and dry weight of rice plants of variety,Jyothi



and biological control agents *viz Trichoderma harzianum* and *Pseudomonas fluorescens*. Observations regarding the lesion size, vertical and horizontal spread, and relative lesion height are presented in the Tables 12, 13 and 14 respectively. The relative disease intensity caused by various treatments on rice plants, graded according to scale proposed by Ahn *et al.* (1986) , is presented in the Table 15.

4.10.1 Lesion size

The average size of the lesions (cm^2) formed with in seven days of inoculation is presented in the Table 12

None of the hypovirulent isolates produced any symptom on rice plants till 20 days after transplanting, when challenge inoculated with the virulent isolate. After the second challenge inoculation 35 DAT, symptoms appeared. No symptoms were produced in treatment with isolate GR23 (Plate 10 b). The lesion length formed in treatments with the rest of the isolates were very small (Plate 11) with average length and breadth of 0.5 cm x 0.3 cm, while the lesion length of plants treated with virulent isolate was 4.0 cm x 0.4 cm (Plate 10 a) .The plants treated with *T. harzianum* and *P. fluorescens* produced lesions with average length 0.2cm x 0.1 cm and 0.26 cm x .1 cm respectively at 42 days after transplanting. The average dimensions of lesions produced in the control plants were 0.133 x 0.1 cm at 42 days after transplanting .

Table 12 : Effect of hypovirulent isolates of *R.solani* on lesion production by the virulent isolate,GR13

Treatments	Seven days after second challenge inoculation (42 DAT)			
	Lesion Length(cm)	Lesion Width(cm)	Lesion Area (cm^2)	
T1	0.30	0.27	0.08	(1.04)
T2	0.53	0.33	0.18	(1.09)
T3	0.53	0.40	0.21	(1.10)
T4	0.00	0.00	0.00	(1.00)
T5	0.57	0.40	0.23	(1.11)
T6	0.00	0.00	0.00	(1.00)
T7	4.30	0.43	1.86	(1.69)
T8	0.13	0.13	0.02	(1.01)
T9	0.10	0.13	0.01	(1.01)
T10	0.27	0.13	0.04	(1.02)
T11	0.13	0.13	0.02	(1.01)
CD (0.05)				0.089

* Where ,T1 =A3, T2=GR8, T3=RS9, T4=A13 ,T5=GR18, T6=GR23, T7=GR13, T8=CONTROL T9 =*T.harzianum* T10 =*P.fluorescens* and T11=Carbendazim

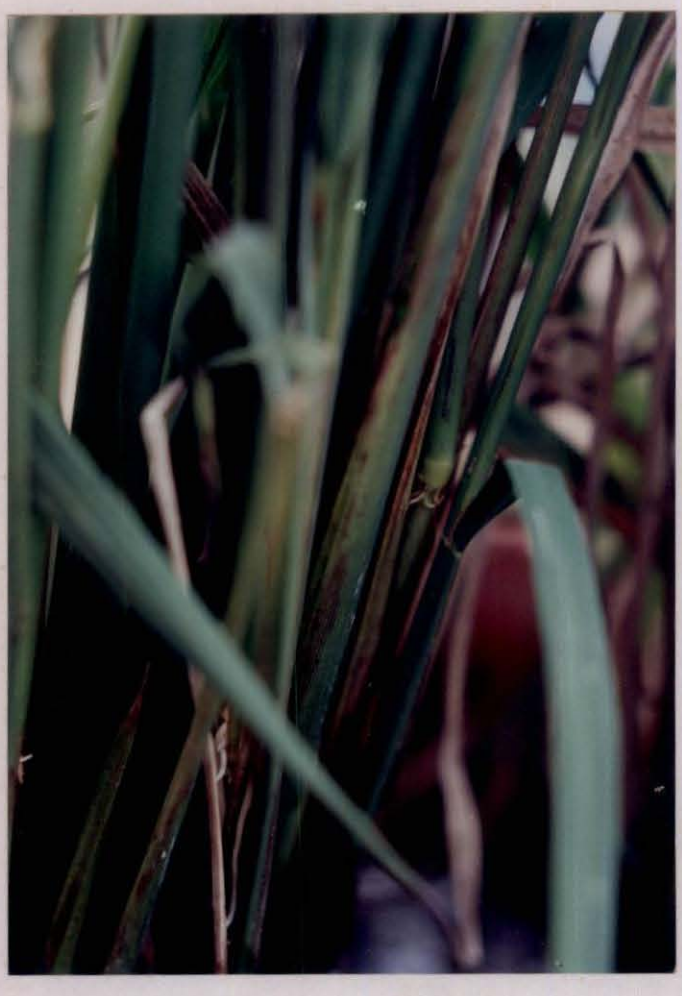
** figures in parentheses indicate square root transformed values

Plate 10. Effect of hypovirulent isolates of *Rhizoctonia solani* on suppression of sheath blight symptoms caused by virulent isolate

iR13 (Most virulent isolate)

10 b GR23 (Hypovirulent isolate)

Plate 11. Atypical symptoms of sheath blight caused by hypovirulent isolates of *R.solani* on rice.



4.10.2 Horizontal spread

The data on horizontal spread as measured by the progressive spread of symptom of sheath blight on the tillers in a hill recorded from 20 days after transplanting up to 90 days are presented in the Table 13.

The horizontal spread of sheath blight due to all hypovirulent isolates was significantly less than that of the virulent isolate at all intervals of observation (Plate 10). The rate of spread of the disease was slow in all the hypovirulent isolates when compared with that of the virulent isolate. The progress of disease was rapid in the treatment which received inoculation of virulent culture alone. In the plants treated with *P .fluorescens* and carbendazim, there was only slight increase in the horizontal spread of the disease caused by inoculation of virulent pathogen. However, these were statistically on par with the control. The horizontal spread of the disease in the treatments A13 and GR23 was significantly less than that of the untreated control and treatments of *T.harzianum* and *P. fluorescens* at all intervals of observation. Rate of horizontal spread of treatment of isolates A3,RS9,GR18 and GR8 at all intervals was higher than the control plants. The inoculation of hypovirulent isolates A13 and GR23 caused a reduction in the horizontal spread of sheath blight in comparison with the uninoculated check as well as the treatment receiving virulent pathogen alone and the biological and chemical treatments Though all the hypovirulent culture treated plants had a significantly lower spread than the virulent isolate treated plants, the horizontal spread of the treatments A3, GR8, RS9 and GR18 was found to be significantly higher than that of the untreated control plants (Fig 3).

Table 13 : Effect of hypovirulent isolates of *R.solani* on the suppression of vertical and horizontal spread of sheath blight

	Vertical spread (VS) and horizontal spread (HS) at different intervals							
	20 DAT		40 DAT		60 DAT		90 DAT	
	VS	HS	VS	HS	VS	HS	VS	HS
T1	0.00 (1)	0.00 (1)	2.00 (1.67)	2.00 (1.67)	3.67 (2.15)	2.33 (1.75)	11.33 (3.51)	4.73 (2.70)
T2	0.00 (1)	0.00 (1)	2.60 (1.89)	2.67 (1.91)	3.27 (2.06)	3.33 (2.31)	7.33 (2.88)	3.62 (2.88)
T3	0.00 (1)	0.00 (1)	3.07 (2.020)	3.33 (2.08)	3.73 (2.17)	3.33 (2.08)	7.33 (2.89)	3.69 (2.89)
T4	0.00 (1)	0.00 (1)	0.00 (1.000)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.67 (1.24)	1.34 (1.24)
T5	0.00 (1)	0.00 (1)	2.67 (1.91)	3.33 (2.08)	3.67 (2.13)	3.33 (2.08)	9.67 (3.27)	4.30 (3.05)
T6	0.00 (1)	0.00 (1)	0.00 (1.000)	0.00 (1.000)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.00 (1.00)
T7	6.33 (1)	4.67 (6.1)	16.00 (4.12)	7.67 (2.94)	24.33 (5.03)	8.33 (3.05)	34.33 (5.94)	8.46 (3.16)
T8	0.00 (1)	0.00 (1)	0.67 (1.24)	0.67 (1.24)	3.33 (2.08)	2.67 (1.90)	5.67 (2.58)	3.10 (1.90)
T9	0.00 (1)	0.00 (1)	0.67 (1.24)	0.67 (1.24)	2.67 (1.91)	0.67 (1.24)	6.00 (2.64)	3.26 (1.90)
T10	0.00 (1)	0.00 (1)	1.00 (1.33)	1.00 (1.33)	3.00 (2.00)	1.00 (1.33)	6.67 (2.77)	3.45 (1.79)
T11	0.00 (1)	0.00 (1)	0.67 (1.24)	0.67 (1.24)	1.33 (1.41)	0.67 (1.24)	6.93 (2.82)	3.50 (1.82)
CD(0.05)			0.576	0.57	0.631	0.42	0.307	0.42

* figures in parentheses indicate square root transformed values

** Where ,T1 =A3, T2=GR8, T3=RS9, T4=A13 ,T5=GR18, T6=GR23, T7=GR13, T8=Control,T9=*T.harzianum*,T10=*P.fluorescens*,T11=Carbendazim

Effect of hypovirulent isolates of *R.solani* on the suppression of horizontal spread of sheath blight

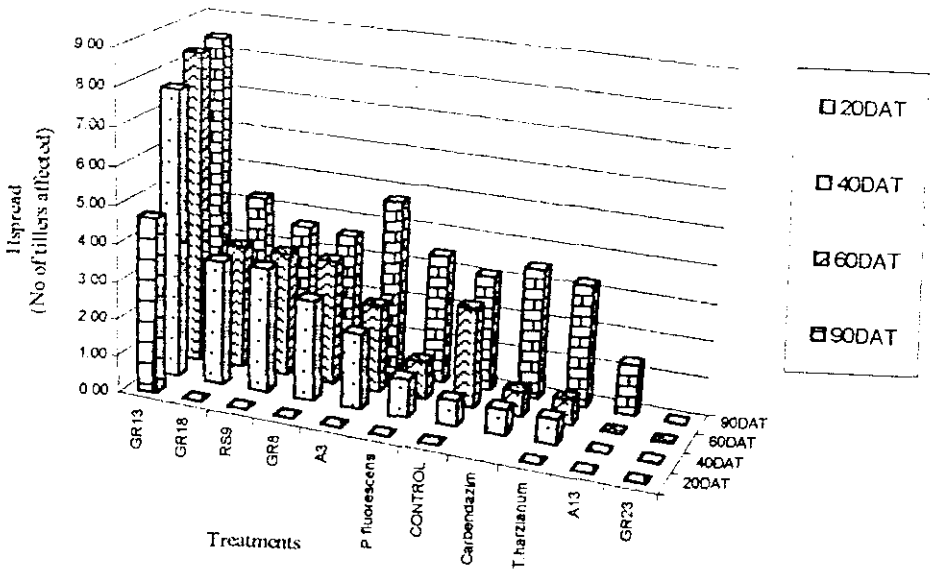
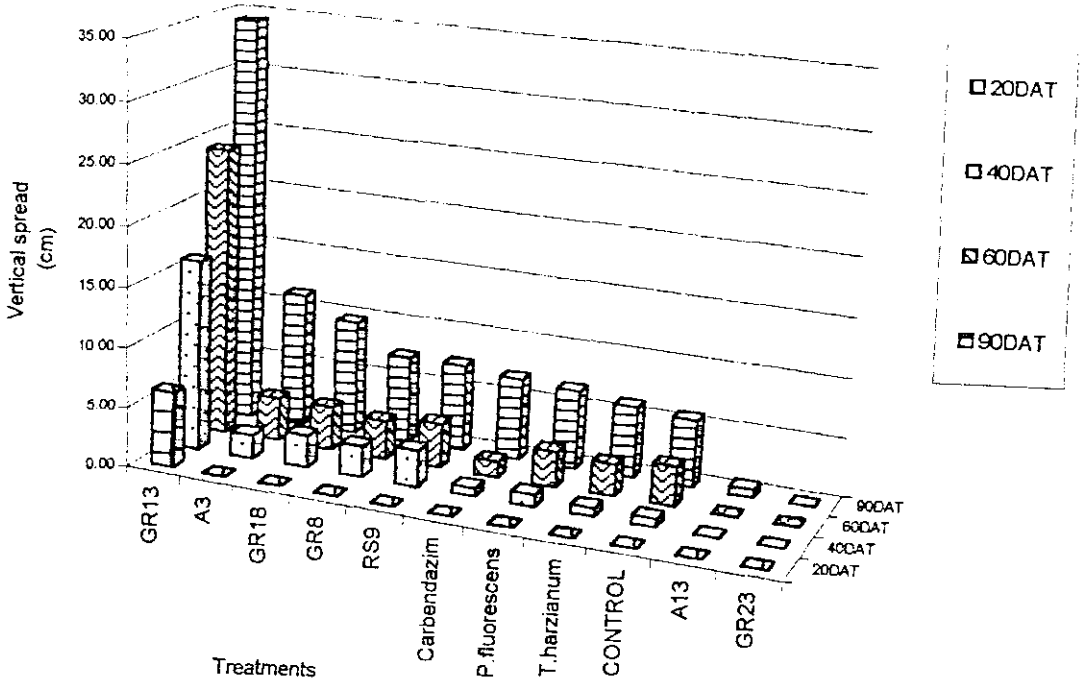


Fig 4

Effect of hypovirulent isolates of *R.solani* on the suppression of vertical spread of sheath blight



4.10.3 Vertical spread

The results of the vertical spread of the sheath blight on susceptible rice variety, Jyothi are presented in Table 13.

The prior inoculation of hypovirulent isolates of *R.solani* tended to offer protection to the rice plants from the virulent pathogen isolate on challenge inoculation. The treated plants were significantly different from those of plants inoculated with the virulent pathogen alone (Fig 4). Sixty days after transplanting, all the hypovirulent isolate treated plants were on par with the control. The vertical spread of the disease in the plants receiving inoculation of GR13, the virulent *R.solani* isolates, was 34.33 cm 90 days after transplanting. The plants treated with biocontrol agents like *T.harzianum* , *P. fluorescens* and the systemic chemical carbendazim produced 6.00, 6.67 and 6.93 cm respectively of vertical spread at the last observation (90 DAT), but these were statistically on par with the untreated control. The treatment effects of isolates GR23 and A13 were superior to all other treatments, including the chemical treatment and biological control checks. In the treatment with GR23, the hypovirulent isolate, the vertical spread was nil (Plate 10 b) whereas with A13, the vertical spread was the least, i.e.only 0.67 cm.

4.10.4 Relative lesion height (RLH)

This was recorded as the extent of plant height affected by the disease and was expressed as percentage (Table 14) (Fig 5). The maximum value of RLH was obtained for the plants treated with virulent *R.solani* alone.(Plate 12). Even at 20 DAT, the RLH was found to be 36.71 per cent and it further

Table 14 : Effect of hypovirulent isolates of *R.solani* on relative lesion height produced by inoculation of virulent isolate,GR13

Treatments	Relative lesion height (%)							
	20 DAT		40DAT		60DAT		90 DAT	
T1	0.00	(1)	6.67	(2.5)	7.42	(2.88)	21.47	(4.7)
T2	0.00	(1)	7.48	(2.9)	6.03	(2.64)	12.15	(3.6)
T3	0.00	(1)	9.41	(3.2)	7.70	(2.93)	12.63	(3.7)
T4	0.00	(1)	0.00	(1)	0.00	(1.00)	1.03	(1.3)
T5	0.00	(1)	8.78	(3.1)	7.95	(2.94)	17.50	(4.3)
T6	0.00	(1)	0.00	(1)	0.00	(1.00)	0.00	(1)
T7	36.71	(6.1)	54.52	(7.45)	54.83	(7.46)	70.70	(8.5)
T8	0.00	(1)	1.63	(1.5)	5.50	(2.56)	8.69	(3.1)
T9	0.00	(1)	2.78	(1.7)	4.60	(2.35)	10.95	(3.5)
T10	0.00	(1)	1.80	(1.5)	5.39	(2.52)	9.65	(3.3)
T11	0.00	(1)	1.75	(1.5)	3.47	(1.50)	11.26	(3.5)
CD(0.05)				1.20		0.68		0.47

* Figures in parentheses indicate square root transformed values

** Where ,T1 =A3, T2=GR8, T3=RS9, T4=A13 ,T5=GR18, T6=GR23,T7=GR13, T8=Control,T9=*T.harzianum*,T10=*P.fluorescens*,T11=Carbendazim

Fig 5 . Effect of hypovirulent isolates of *R.solani* on relative lesion height produced by inoculation of virulent isolate, GR13

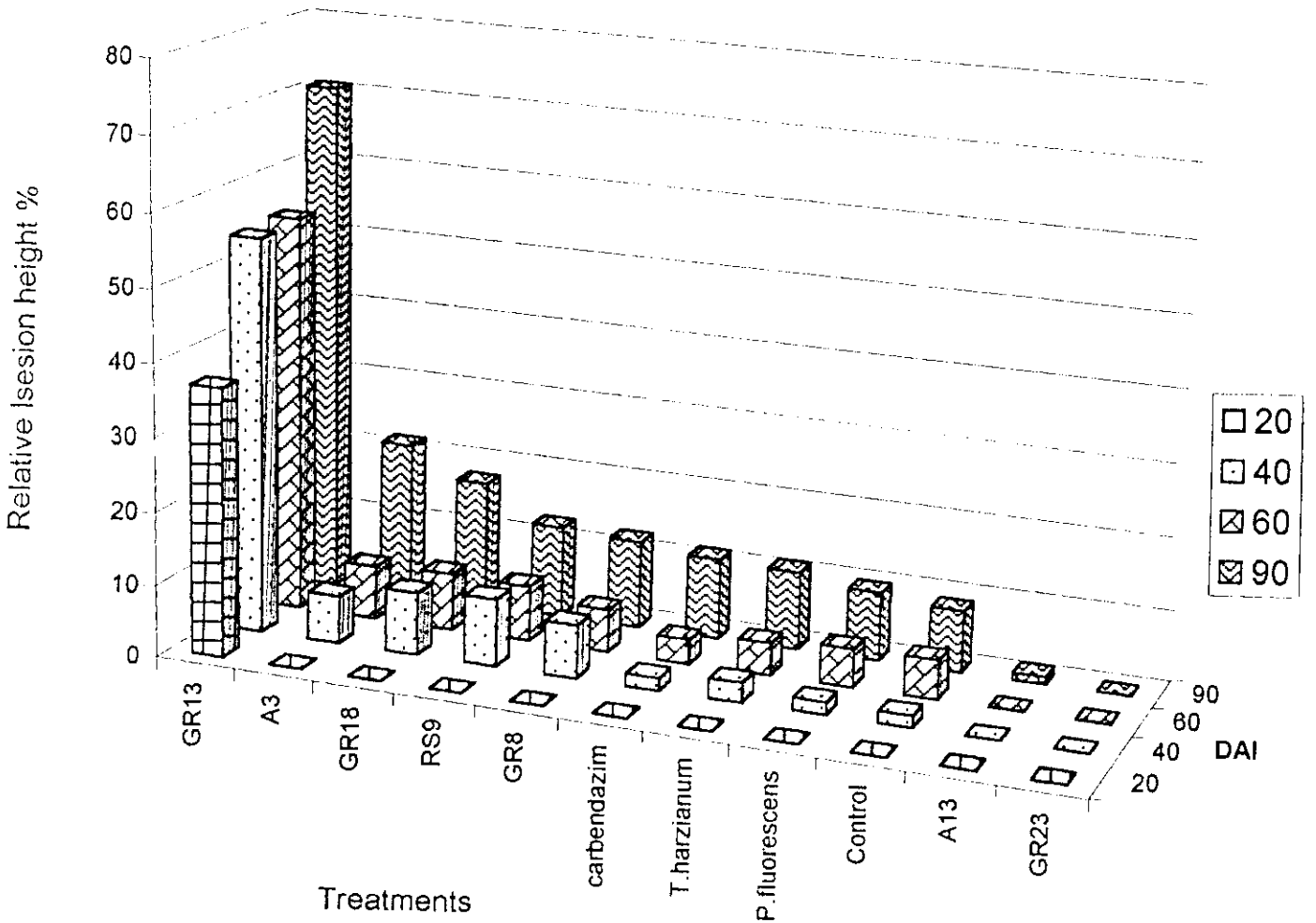


Plate 12 Effect of hypovirulent isolates of *R. solani* on the relative lesion height caused by virulent isolates

T6 GR23

T7 GR13

T8 CONTROL

T11 CARBEMDAZIM


T8
T11
T7
T6

progressed up to 70.7 per cent at 90 DAT. The hypovirulent isolate GR23 did not record any values of RLH. The RLH of the plants treated with isolate A13 also was found to be significantly less than that of the control and the different checks. The RLH of lesions on plants treated with isolates A3, GR8, RS9 and GR18 were significantly higher than that of the untreated plants but lesser than the effect of virulent isolate. But 60 days after transplanting all the treatments with hypovirulent isolates were either on par or had a significantly lower RLH than the control. Sixty DAT, the treatment effects of hypovirulent isolates GR23 and A13 were superior to all other treatments. Ninety DAT, the chemical treatment and treatment with biological agents like *T.harzianum* and *P. fluorescens* produced a RLH of 11.26, 9.65 and 10.95 per cent respectively, on challenge inoculation with virulent *R.solani* . These were statistically on par with the control. The effects of isolates GR23(0 %) and A13 (1.33 %) were significantly lower than all other treatments.

The relative disease intensity (90 DAT) for all the treatments was calculated and is presented in Table 15 . All the treatments with hypovirulent cultures showed a lower disease intensity than the treatment with virulent isolate alone indicating that susceptible plants were protected from virulent pathogen by the activity of the hypovirulent isolates. The hypovirulent isolate GR23 offered the best protection with showing a relative disease intensity of zero.

4.11 Effect of hypovirulent isolates on non-target plants in the rice ecosystem

Crop plants and weeds in the rice ecosystem were inoculated with the hypovirulent isolate GR23 which proved to be promising for biocontrol, to see if it produced any symptom development in the non-target crops. (Table 16).

Table 15 : Interaction of the virulent isolate and hypovirulent isolates of *R.solani* on the relative disease intensity

Treatment no	RLH (%)	Disease grade	Relative disease intensity
T1	21.47	3	5
T2	12.15	1	1
T3	12.63	1	1
T4	1.03	1	1
T5	17.5	1	1
T6	0	0	0
T7	70.7	9	100
T8	8.69	1	1
T9	9.65	1	1
T10	10.95	1	1
T11	11.26	1	1

Table 16 : Effect of inoculation of hypovirulent isolate (GR23) of *R.solani* on non-target flora in rice ecosystem

	Scientific names	Common /vernacular names	Lesion development
Crop plants			
1.	*Amaranthus tricolor* L.	Amaranthus	-
2.	*Amorphophallus companulatus* L.	Amorphophallus	-
3.	*Arachis hypogaea* L.	Groundnut	-
4.	*Colocasia esculenta* L.	Colocasia	-
5.	*Ipomoea batatus* (L.) Lam	Sweet potato	-
6.	*Sesamum indicum* L.	Sesamum	-
7.	*Vigna radiata* L.	Greengram	-
8.	*Vigna unguiculata* L (Walp.)	Cowpea	-
Weeds			
9.	*Ageratum conyzoides* L.	Appa	-
10.	*Amaranthus viridis* (L) Notrysag	Kuppacheera	-
11.	*Axonopus* spp.	Axonopus	-
12.	*Bulbostylis barbata* SW.	Sooryan	-
13.	*Cleome viscosa* L.	Kattukaduku	-
14.	*Commelina benghalensis* L.	Vazhappadathy	-
15.	*Cynodon dactylon (L.)*	Karuka	-
16.	*Cyperus rotundus* L.	Muthanga	-
17.	*Echinochloa colona* (L.)Link	Kavada	-
18.	*Hydrocotyl asiatica* Urban	Kudangal	-
19.	*Leucas aspera* Spreng	Thumba	-
20.	*Monochoria vaginalis* (Burm.f.) Presl	Neerthamara	-
21.	*Panicum distachyum*. Stapf	Paalpullu	-
22.	*Panicum repens* L.	Torpedo grass	-

Even after seven days of inoculation with GR23 , no symptom was noticed in any of the crops and weeds tested.

4.12 Changes in oxidative enzymes

Changes in oxidative enzymes were analysed to study whether there was any difference in the mode of action between hypovirulent and virulent isolates when these were inoculated on host plants. The enzymes analysed were peroxidase and phenol oxidase (Figure 6). Two hypovirulent isolates selected for enzyme extraction and assay were GR23 and GR8 – GR23 being the best hypovirulent isolate in terms of biological control and GR8 being intermediary based on the results obtained. Untreated plants served as control. The results are presented in Table 17

4.12.1 Effect on peroxidase

Changes in peroxidase activity recorded in plants by spectroscopic analysis after inoculation with the respective isolates at one, three and five days after inoculation and the results expressed as change in Optical density of the extract (ΔOD) per minute per gram fresh weight of the material are presented in Table 17.

There was significant difference between the treatments at all intervals. Peroxidase activity was the highest for virulent culture and the lowest for the control plants. The activity of peroxidase enzymes in plants treated with GR23 was lower than that of virulent culture and GR8. The peroxidase activity was highest for all treatments three days after inoculation.

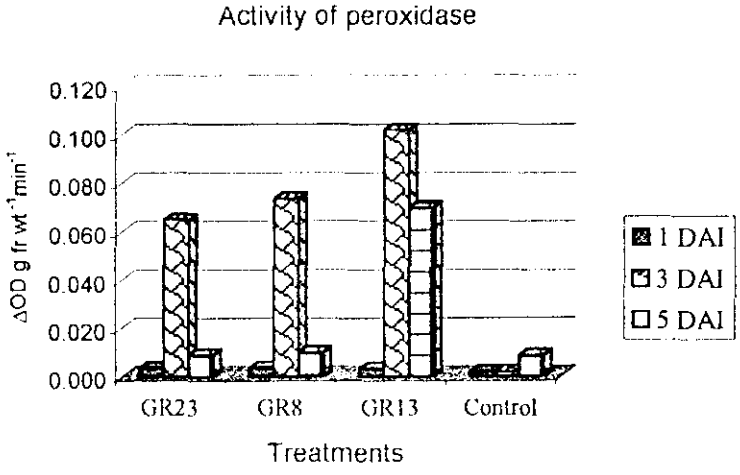
Table 17 : Oxidative enzyme activity of susceptible rice variety as affected by *R.solani* isolates Δ OD g^{-1} fr wt min^{-1}

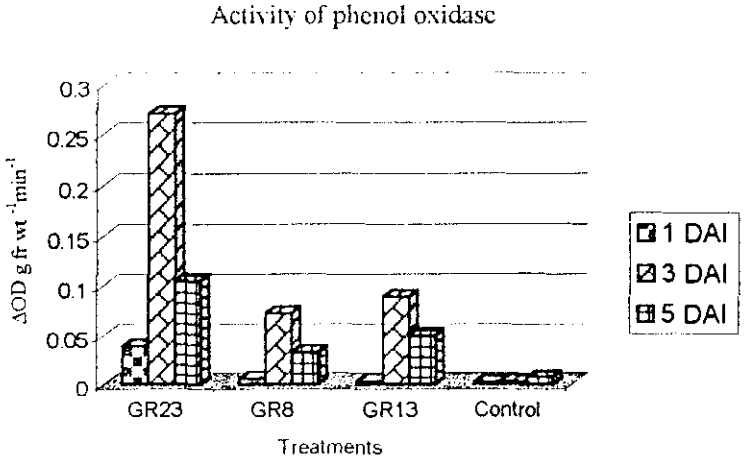
	Peroxidase			Polyphenol oxidase		
Treatments	1DAI	3DAI	5DAI	1DAI	3DAI	5DAI
T1	0.0390	0.2710	0.1050	0.0035	0.0650	0.0090
T2	0.0055	0.0725	0.0330	0.0035	0.0734	0.0100
T3	0.0030	0.0890	0.0500	0.0033	0.1020	0.0700
T4	0.0015	0.0027	0.0070	0.0020	0.0020	0.0088

DAI = Days after inoculation

*Where T1 =Hypovirulent isolate GR23, T2=Hypovirulent isolate GR8, T3 =Virulent isolate GR13, T4=Control

Fig 6. Oxidative enzyme activity of susceptible rice variety as affected by *R.solani isolates* (ΔOD g fr wt $^{-1}$min^{-1})





4.12.2 Effect on phenol oxidase

The activity of phenol oxidase also was measured on similar lines as mentioned under 4.12.1.The hypovirulent isolate GR23 showed higher activity than the virulent and control plants whereas the activity of GR8 was lesser than that of the virulent isolate. The activity was higher on the third day after inoculation for the virulent and hypovirulent isolates.

4.13 Screening of carrier materials

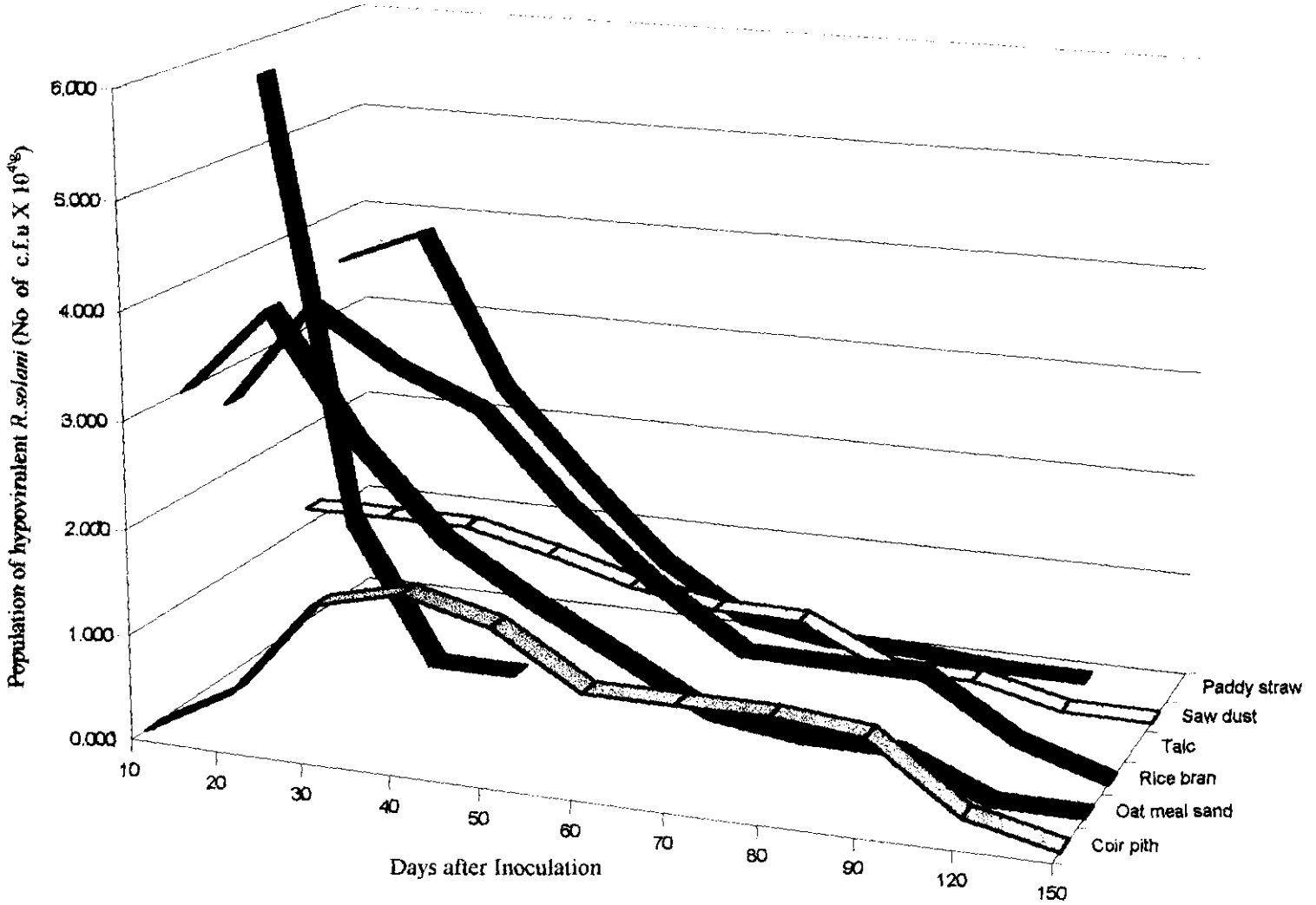
The substrates differed in their efficiency to support hypovirulent *R.solani* at different intervals of time. The number of colony forming units (cfu) per gram was highest for both oats meal sand medium and paddy straw for the first 20 days. Rice bran was the best carrier for longer use with the value for cfu being significantly higher than all other media during the remaining period. Though in talc, an initial high population was recorded, it was found to be ineffective as a carrier for hypovirulent *R.solani* , since cfu count decreased steadily with the lapse of time to the minimum after 20 days. The growth of *R. solani* was slow in coir pith and saw dust were slow initially. However the values for cfu of hypovirulent *R.solani* on coir pith 60 and 90 days after inoculation (DAI) were found to be on par with oats meal sand medium and rice bran respectively. One hundred and fifty DAI no colonies could be obtained from any of the substrates tested (Fig 7).

Table 18 : Survival of hypovirulent *R.solani* in different indigenous carrier materials

	Population of hypovirulent *R.solani*(X 10^4cfu\g material) at different days after inoculation (DAI)										
Carrier materials	10	20	30	40	50	60	70	80	90	120	150
Rice bran	2.667	3.667	3.167	2.833	2.000	1.333	0.800	0.783	0.783	0.267	0.000
Oats meal sand	3.000	3.833	2.667	1.833	1.333	0.917	0.483	0.367	0.003	0.000	0.000
Sawdust	1.167	1.167	1.167	0.967	0.733	0.600	0.583	0.200	0.200	0.003	0.000
Coirpith	0.002	0.460	1.400	1.600	1.400	0.900	0.900	0.900	0.820	0.200	0.000
Talc	5.667	1.333	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Paddystraw	3.500	3.833	2.333	1.483	0.700	0.200	0.070	0.068	0.006	0.000	0.000

CD(0.05) A (Career materials) – 0.19
B (DAI) – 0.155
Interaction A x B – 0.465

Fig 7 . Survival of hypovirulent *R.solani* in different indigenous carrier materials



DISCUSSION

5. DISCUSSION

Sheath blight of rice incited by *Rhizoctonia solani* Kühn, is a wide spread and destructive disease, the management strategies for which are quite inadequate. The pathogen is soil-borne and can infect a large number of hosts. The anamorph of this fungus exhibits heterogeneity and shows differences in virulence. The wide variability of *R. solani* prompted the present work on detection of isolates with reduced virulence with a view to develop a novel approach for biocontrol of sheath blight of rice.

Twenty-eight isolates of *Rhizoctonia* were obtained from leaf and sheath of rice and rhizosphere soil samples from different rice growing tracts of Kerala, which on screening yielded six hypovirulent isolates. Non-pathogenic *R.solani* has been reported to occur naturally. Ichielevich-Auster *et al.*, (1985 a) in his study found that 30 per cent of isolates collected from various ecological niches of Israel were non-pathogenic to the hosts tested. Several scientists have reported the occurrence of non-pathogenic *Rhizoctonia* spp. (Castanho and Butler, 1978 b, Burpee and Goulty, 1984,Ichielevic-Auster *et al.*, 1985 a ,Sneh *et al.*, 1986, Cardosso and Echandy 1987 a, Villajuan-Abagona *et al.*, 1996). Variations in pathogenicity of *R.solani* causing sheath blight of rice have been reported by several workers (Vijayan and Nair,1985, Kalaiselvi *et al.*, 1986, Girija, 1996)

The hypovirulent isolates selected should be distinguished from those with natural growth retardation. Ichielevich-Auster *et al.*1985 a, had stressed the importance of setting rigorous criteria for defining hypovirulence in order to avoid confusion between growth retardation and hypovirulence. They defined

hypovirulent strains as those that infected a wide variety of hosts but incurred no discernable damage to the host. Such strains should have no growth disadvantage and their growth rate should be similar to that of virulent strains. The selection of hypovirulent isolates for this experiment was based on these criteria.The growth rate of all the isolates obtained, whether virulent or hypovirulent, was similar, taking three to four days to completely cover a nine cm Petri dish.

Cultural characteristics and virulence assays have been used to predict the virulence of *R. solani* (Endo *et al.*, 1974, Shahjahan *et al.*, 1997). Colour of mycelium and sclerotial characters were reported to be correlated with virulence. Grente (1965) stated that pigmentation and sporulation were lower in hypovirulent strains than in the normal strains of *Cryphonectria parasitica*, the causal agent of chestnut blight. Atypical colony morphology was also noticed in hypovirulent isolates of *Sclerotinia homoeocarpa* (Zhou and Boland, 1997). In the present study, the cultural characteristics viz., the colour of the mycelium and the sclerotial characters were found to indicate correlation with hypovirulence. Two types of mycelial colour were noticed in the *R. solani* isolates i.e. light brown and tan colour, which had correlation with the difference in sclerotial production. All the isolates with light brown coloured mycelium produced sclerotia whereas the isolates with tan mycelium did not produce sclerotia. The isolates in the former group produced mycelium appressed to the surface of medium in Petri dishes and those in the latter showed fluffy growth. Similarly, differences were noticed for sclerotial characters. *R.solani* isolates with or without sclerotial production were

obtained. It has been earlier reported that the growth rate (Shahjahan *et al.*, 1997) and sclerotial production (Endo *et al.*, 1974) were related to virulence.

Pathogenicity of *R. solani* isolates was tested on cut stalks of susceptible rice variety, Jyothi. Eight isolates that did not produce typical lesions of sheath blight were the ones that produced tan coloured mycelia and without sclerotial production. Lack of sclerotia could be an indication of hypovirulence. Earlier workers have emphasized the role of sclerotia in the virulence of pathogen (Endo *et al.*, 1974). Castanho and Butler (1978 a) characterised hypovirulent cultures as having floccose, tan coloured mycelia, irregular in appearance with few or no sclerotia, and possessing poor growth. Ichielevich–Auster *et al.*, (1985 a) described that the selected hypovirulent cultures in his experiment were similar to virulent isolates except that the hypovirulent strains produced less melanin in mycelia, and, produced fewer sclerotia. The findings in this experiment are in conformity to most of the observations of Castanho and Butler (1978 a) and Ichielevich–Auster *et al.*, (1985 a), except for the debilitated state as suggested by Castanho and Butler (1978 a). However, Ichielevich-Auster *et al.*, (1985 a) stated that although less melanin and fewer sclerotia are indicative of hypovirulence there was no conclusive evidence.

A virulent isolate GR13 with the highest growth rate, the earliest sclerotial initiation and the highest values for sclerotial production and lesion length on inoculation in susceptible host was selected for comparison in further studies. The six hypovirulent isolates located *viz* A3, A13, GR8, GR18, GR23 and RS9, had however normal growth rate on artificial culturing

in potato dextrose agar. This appeared to be an added advantage in that they would be capable of quick colonisation, and thereafter capable of competing with the virulent pathogen. The absence of sclerotia and inability to produce lesions on rice stalks indicated their reduced virulence.

The exact cause of hypovirulence in *R. solani* has not been proved conclusively (Sneh, 1999). A cytoplasmically transmitted factor, a dsRNA has been suggested to be the cause of hypovirulence. It has been suggested to be transmitted by hyphal fusion or anastomosis as hypovirulence in *C. parasitica* (Castanho *et al.*,1978). It was proposed to study whether the character of hypovirulence could be transmitted from hypovirulent strain to virulent strain. The vegetative compatibility studies showed that the four out of six hypovirulent strains anastomosed with the virulent strain. Both perfect and contact fusion could be observed for these isolates. Two isolates did not show perfect fusion with the virulent isolate at any point of observation. Ichlievich – Auster *et al.*, (1985 a) have reported that hypovirulent strains could be isolated from all anastomosis groups, irrespective of geographic locations. The four isolates, which showed perfect fusion with the virulent isolate, could be of the same anastomosis group. The occurrence of perfect fusion between these hypovirulent isolates and the virulent strain could facilitate the transfer of hypovirulence between these strains. The two isolates, which did not show perfect fusion could belong to a different anastomosis group other than the virulent isolate. The isolates, A13 and GR23 were later found to have only two nuclei in the cells close to hyphal tip and had a hyphal width of 6μm typical of binucleate *Rhizoctonia* (BNR). The hyphae of binucleate *Rhizoctonia* usually measures less than 7μm and are thinner in comparison to

the multinucleate, virulent *R. solani* which measures between 7µm and 17µm (Sneh *et al.*, 1991).

The cultures GR23 and A13 were maintained for further pot culture experiments. Finkler *et al.* (1988) reported that cytoplasmic compatibility was not essential for inducing a protective effect on host plants since a similar effect was obtained between *R. zeae* and *R. solani.* Moreover there are numerous reports on protective activity of binucleate *Rhizoctonia spp* on *R.solani*- initiated diseases on host plants (Cardosso and Echandy 1987 b, Herr 1988, Hare *et al.*, 1994., Poromarto., 1998)

Dual culturing experiment could not conclusively prove any *in vitro* suppression of virulent pathogen by hypovirulent isolates. A reduction in growth of virulent culture on co-inoculation with the hypovirulent isolate was observed as compared to the control. While the virulent isolate showed 3.77 cm of growth radially in two days, the maximum growth in dual cultured plates was 3.1 cm. However, there was no lytic zone between the hypovirulent and virulent isolates. No indication of antibiosis or mycoparasitism was observed at the point of contact between the two isolates. Absence of hyperparasitism or antibiosis has been reported in the mode of biocontrol of hypovirulent *R.solani* (Sneh *et al.*, 1986, Cardosso and Echandy, 1987 a, Poromarto, 1998.) The two isolates maintained their original character, in spite of mutual contact, the virulent continuing to produce sclerotia and the hypovirulent ones maintaining their subdued virulence.

Serial subculturing of the portion of contact of the virulent and hypovirulent *R.solani* isolates, from different regions resulted in a mixture of

hypovirulent and virulent strains in an equal ratio. The method described by Zhou and Boland, 1997 for studying transmissible hypovirulence in *S. homoeocarpa*, offered a greater accuracy in detecting transmission of hypovirulence. In the present study, no hypovirulent strain was obtained by this method from the virulent isolate or, *vice versa*. This can be substantiated by the report of Finkler *et al.*, (1988) that hypovirulence in *R.solani* is not directly related to the presence of dsRNA, which is hypothesized to be the transmissible factor.

Castanho *et al.*,(1978) obtained virulent phenotypes from hypovirulent strains by their hyphal tip culture. Hyphal tip culture of any of the hypovirulent strains did not yield virulent strains, in their experiment. This also indicates the absence of a transmissible factor in the case of hypovirulence as suggested by Finkler *et al.*, (1988). But they could obtain hypovirulent strain from hyphal tips of virulent strain at a probability of 0.01. This suggested that the phenomenon of hypovirulence exhibited by the cultures might, probably not be controlled by a transmissible factor.

Hypovirulent *R.solani* isolates did not produce any deleterious effect on seed germination and seedling growth. A slight reduction in the height of seedlings treated with GR18 was observed at seedling maturity. Rice is susceptible to sheath blight any stage. (Roy, 1993) Since hypovirulent strains are suggested to come between avirulent and virulent strains in terms of virulence such an observation could be accommodated. However, it is to be stressed that no visible symptoms were produced by the hypovirulent isolate.

The hypovirulent isolates did not have any pronounced growth-promoting effect on rice plants. However, the mean height of plants treated with isolates GR23 and A13 was always greater than that of untreated control plants. So, a slight increase in growth rate may be suggested for rice plants by the application of the isolates GR23 and A13. These two isolates were earlier found to be binucleate. There are similar reports on the involvement of hypovirulent strains on stimulation of crop growth. Sneh *et al.*, (1989) reported that a hypovirulent isolate of *R.solani* induced plant growth responses in cotton. Khan *et al.*, (1992) found that treatment of soybean seeds with binucleate *R.solani* caused increased germination, better survival of plants and improvement in plant height. The hypovirulent strains were reported to produce lesser quantities of endopectinolyase II an enzyme that is released by the virulent isolates during the initial stages of infection process (Marcus *et al.* ,1986).

Inoculation of hypovirulent isolates on susceptible rice variety, Jyothi provided an insight into relationship between morphological characters, and subdued virulence to their capability as biocontrol agents against sheath blight of pathogen.

The hypovirulent isolates of *R. solani* provided protection against the virulent isolate of the pathogen. The performance of two isolates GR23 and A13 was especially significant. Even on multiple challenge inoculations with the virulent pathogen, no symptoms of sheath blight were produced on the plants previously treated with the hypovirulent isolate GR23. The relative lesion height of plants treated with hypovirulent isolates A13 and GR23 was

significantly lower than those of control and plants treated with other proven biocontrol agents as well as those that received application of the systemic fungicide. Further, the protection lasted through out the entire period of crop growth (110 days)

The horizontal spread of sheath blight, between tillers in a hill and vertically upwards the plants, recorded the minimum values in the treatment receiving inoculation of these hypovirulent isolates. The two most effective hypovirulent isolates GR23 and A13, were found to be of binucleate Rhizoctonia (BNR). BNR are promising biocontrol agents for management of soil borne pathogens on a variety of crops. (Herr, 1995,Poromarto, 1998)

The binucleate hypovirulent *R. solani* suppressed the pathogen from 20 DAT to 90 DAT, i.e. through out the crop growth. Disease suppression for extended period provides evidence that these isolates have potential for effective management of sheath blight. Zhou and Boland (1997) reported that a hypovirulent isolate of *S. homoeocarpa* reduced dollar spot of turf grass by up to 80 per cent and the effect persisted for one year after application of the mycelial suspension of the biocontrol agent.

Disease suppression by BNR has been attributed to the competition for infection sites or to induced systemic resistance (Cardosso and Echandi, 1987 b, Harris *et al.*, 1994). Induced systemic resistance (ISR) is a common response to non-pathogenic microorganism prior to or concomitant challenge inoculation with a pathogen (Kuc and Stobel, 1992). This is exhibited by a number of host responses such as production of defence related enzymes. In the present study, the inoculation of the hypovirulent *R. solani* isolates caused

increased induction of phenol oxidase, three days after inoculation. The activity of phenol oxidase was more pronounced in the hypovirulent isolate treated plants than in the virulent isolate treated plants. Phenol oxidases have been indicated to play a decisive role in the hypovirulent (defence) reaction and is accompanied by oxidation of phenolic compounds (Kiraly and Farkas, 1962, Simmons and Ross, 1970). The increase of peroxidase in the host tissue inoculated with virulent culture is in accordance with reports of increased enzyme activity in compatible and incompatible interactions (Gaspar *et al.*, 1991). Girija (1993) also reported increased peroxidase activity on inoculation of rice sheath with most virulent isolate than that with the least virulent isolate of *R.solani*.

The hypovirulent isolates found efficient in bio suppression of virulent *R. solani* of rice are not avirulent and therefore, could still cause disease in other host plants in the rice ecosystem. *R. solani* is an ubiquitous pathogen with very wide host range (Saikia and Roy,1975). So an understanding of the pathogenicity of the selected hypovirulent isolates on the other non-target flora in rice ecosystem was essential. However, the inoculation of hypovirulent isolates A13 and GR23 showed no symptom expression in any of the crop plants or the weed plants tested. Apart from clearing doubt on the possible ill effects of the selected isolates on non-target flora, these results also provide a scope for extending the spectrum of the selected isolates for management of *Rhizoctonia* induced disease in other crop plants. This finding is of special significance to Kerala with the wide range of components in the crop cafeteria, most of which are susceptible to this soil borne pathogen.

For field application of biocontrol agents, suitable delivery systems are essential which are cheap, easily available, eco-friendly and efficient. Out of the several indigenous materials tried, rice bran was found to be supporting the growth of biocontrol agent. Wheat bran has been reported to be an ideal carrier material for mass culture of *R.solani* (Sneh *et al.*, 1991). Coir pith could be a cheaper substitute since the survival of the hypovirulent isolate in this substrate at 60 and 90 days of inoculation was on par with that in rice bran. The shelf life of inoculum in the carrier material was estimated to be 120 days.

Another interesting reference is that the same non-pathogenic binucleate Rhizoctonia can afford protection to other soil borne pathogens. Cubeta and Echandy (1991) reported the biological control of *Rhizoctonia* and *Pythium* damping off of cucumber by using BNR. The scope of BNR could be extended for protection from an array of pathogens by further investigating the resistance response induced by them. Generation of hypovirulent/binucleate *Rhizoctonia* which can promote systemic or non-specific resistance in crop plants may be ideal for managing soil borne as well as foliar pathogens. Further improvement of these isolates through suitable biotechnological intervention could lead to the development of an effective, self-perpetuating, long lasting and broad-spectrum biocontrol agent.

SUMMARY

6. SUMMARY

The present study was aimed at detecting hypovirulent strains of *Rhizoctonia solani* and evaluating their efficiency as biocontrol agents against sheath blight disease of rice. The salient features of this study are given below.

1. *R.solani* was isolated from soil and plant samples from different rice growing tracts of Kerala. A total of twenty-eight isolates of the fungus were obtained and were screened for hypovirulence.
2. Screening was based on the cultural characteristics like mycelial growth and sclerotial production, and pathogenicity tests by *in vitro* cut stalk assay.
3. Among the twenty-eight isolates of *R.solani* obtained, six isolates were selected as hypovirulent. These were A3, GR8, RS9, A13, GR18 and GR23. The selected isolates had growth rate similar to the virulent isolate. However, they neither produced sclerotia nor induced typical symptoms of sheath blight when inoculated on cut stalks *in vitro* .
4. The isolate GR13 was selected as the most virulent isolate for comparative studies. The isolate produced sclerotia profusely after three days of inoculation. The maximum lesion length (4.3 cm) on inoculation in cut stalks was recorded for this isolate.
5. There was correlation between colour of mycelia and sclerotial production of isolates and hypovirulence. All hypovirulent isolates were tan in colour and produced no sclerotia.
6. Studies on anastomosis showed that the hypovirulent isolates A3, GR8, RS9 and GR18 had compatible hyphal fusion with the virulent isolate

. GR13 while two isolates A13 and GR23 had only contact fusion. Tip to side wall and side wall to side wall fusion were observed in the case of perfect fusion.

7. Neither antibiosis nor hyper-parasitism was observed when the hypovirulent isolates were dual cultured with the virulent isolate.
8. Excepting A13 and GR23, the six hypovirulent isolates and the virulent isolate selected were found to be multinucleate. The binucleate isolates were the ones which did not show perfect fusion with the virulent isolate.
9. The hyphal width of the binucleate *Rhizoctonia* (6.2-6.6µm) was found to be lower than that of the multinucleate isolates, which ranged from 9.2µm to 11.63 µm. The hyphal width of the hypovirulent isolates, in general, was found to be lower than that of the virulent isolate which had an average hyphal width of 11.63µm
10. Hypovirulence was not observed to be transmitted to the virulent isolate from the hypovirulent isolates, which showed perfect hyphal fusion. The cultures obtained through anastomosis continued to produce sclerotia.
11. The hypovirulent isolates had no appreciable effect on seed germination or seedling vigour of rice plants.
12. The hypovirulent isolates did not produce any visible symptoms of sheath blight on rice plants of susceptible variety, Jyothi, in a pot culture experiment.
13. Excepting two isolates A3 and RS9 , none of the hypovirulent isolates significantly affected the growth of the rice plants adversely. The

height, fresh weight and dry weight of the plants inoculated with hypovirulent isolate A13 and GR23 were found to be on par or superior to the uninoculated control plants at different intervals of observation.

14. The hypovirulent isolates were found to suppress the development of sheath blight by the virulent isolate. All the plant previously treated with hypovirulent isolates and later challenged by the virulent isolate of *R.solani* had lesser lesion size, horizontal spread and vertical spread of sheath blight and relative lesion height , than the plants inoculated with the virulent isolate alone. The hypovirulent isolates GR23 and A13 were found to be superior to all treatments including chemical and biological control treatments. The virulent isolate failed to produce any symptom in the plants treated with the hypovirulent isolate GR23, which gave protection to the plants for 90DAT.

15. Excepting one, all other treatments with hypovirulent isolates showed resistant reaction on plants. The treatment with RS9 showed moderate relative disease intensity. Plants treated with GR23 showed perfect immune reaction i.e., a relative disease intensity of zero, to the challenge inoculation with virulent isolate.

16. Activity of phenol oxidase was higher in the plants treated with hypovirulent isolate GR23 than in those treated with the virulent isolate of *R.solani.*

17. Activity of peroxidase was lower in the plants treated with the hypovirulent isolate than in the plants treated with the virulent isolate.

18. None of the non-target flora in the rice ecosystem tested was adversely affected by the selected hypovirulent isolate (GR23).

19. Rice bran was found to be the best carrier material for efficient hypovirulent *R.solani* . The survival rate of the fungus in oats meal sand medium was good 20 days after inoculation. The initial colonisation of hypovirulent *R.solani* was poor in coir pith. However, this medium supported fungal growth after 70 to 120 days of inoculation. This appeared to be an ideal indigenous and cheap carrier material for mass multiplication of hypovirulent *R.solani* .

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* Originals not seen

Appendices

APPENDIX – I

COMPOSITION OF DIFFERENT MEDIA AND STAINS

Potato dextrose agar

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

Potato dextrose broth

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

Martin's Rosebengal agar

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

King's B medium

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

Adjust pH to 7.2

Water Agar (2 per cent)

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

Semi Selective medium for *Rhizoctonia solani* (Ko and Hora,1971)

Gallic acid	- 0.4 g
Streptomycin sulphate	- 50 mg
Chloramphenicol	- 50 mg
Sodium Nitrate ($NaNO_3$)	- 2 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	- 1g
Magenium sulphate heptahydrate (MgSO4. 7H2O)	- 0.5 g
Potassium Chloride (KCl)	- 0.5 g
Ferrous sulphate (FeSo4. 7 H2O)	- 0.01 g
Tapwater	: 1L

0.5 % (w/v) potato starch diluted to 10 microgram /ml of active ingredient

Lactophenol cotton blue

Anhydrous lactophenol – 67.0 ml

Distilled water- 20 ml

Cotton blue – 0.1 g

Anhydrous lactophenol prepared by dissolving 20 g phenol in 16 ml lactic acid and 31 ml glycerol

Lactophenol Trypan blue

Anhydrous lactophenol – 67.0 ml

Distilled water- 20 ml

Trypan blue - 0.1 g

Anhydrous lactophenol prepared by dissolving 20 g phenol in 16 ml lactic acid and 31 ml glycerol

APPENDIX - II

Buffers for enzyme analysis

0.1 M Phosphate Buffer (pH 6.5)

Stock solutions

A : 0.2 M solution of Sodium dihydrogen orthophosphate (3.120g in 100 ml)

B : 0.2 M Disodium hydrogen orthophosphate (3.559g in 100 ml)

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

APPENDIX – III

Composition of carrier materials

Rice Bran

Rice Bran	70 ml
Water	30 ml

Oatmeal sand medium

Oats	20ml
Sand	75 ml
Water	5 ml

Coirpith

Coirpith	70 ml
Water	30 ml
Urea	0.5 g

Paddystraw

Paddy straw	75 ml
Water	25ml

Sawdust

Sawdust	70 ml
Water	30 ml

ABSTRACT

EXPLOITATION OF HYPOVIRULENCE IN *Rhizoctonia solani* Kühn FOR MANAGEMENT OF SHEATH BLIGHT IN RICE (*Oryza sativa* L.)

BY

RANJIT. A.

ABSTRACT OF THE THESIS
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VELLAYANI
THIRUVANANTHAPURAM

2000

ABSTRACT

A study was conducted at Department of Plant Pathology, College of Agriculture, Vellayani to explore the variability in *Rhizoctonia solani* with a view to detect hypovirulent strains of the fungus, and evaluate the efficacy of these isolates in management of sheath blight disease of rice. Six hypovirulent isolates were selected after screening 28 isolates obtained from different rice growing tracts of Kerala. Screening for hypovirulence was based on cultural characters like mycelial growth rate and sclerotial production under *in vitro* conditions, and pathogenicity tests through cut stalk assay. The hypovirulent isolates were non-sclerotial whereas the virulent isolate produced profuse sclerotia. The hypovirulent isolates produced tan mycelia appressed to the medium in Petri dishes. Antibiosis and hyperparasitism were absent in the interaction between the hypovirulent and virulent isolates *in vitro*. The character of hypovirulence could not be transmitted from hypovirulent to the virulent isolate. The hypovirulent isolates had no effect on the germination and seedling vigour of rice plants. They did not produce symptoms of sheath blight on susceptible variety. Two of the six hypovirulent isolates selected *viz* A13 and GR23 which were binucleate and did not anastomose with the selected virulent isolates, offered the maximum protection to the rice plants from sheath blight. The plants treated with GR23 showed immune reaction to challenge inoculation with virulent isolate, showing no symptom production on challenge inoculation with virulent isolate. The protective effect was found to be higher than that of chemical and biological control treatments. The phenol oxidase activity in the plants treated with the hypovirulent isolates was found to be significantly

higher than that of the plants treated with virulent isolate while the peroxidase activity was higher in the plants treated with virulent isolate. Among the different carrier materials tested for hypovirulent *R.solani* rice bran was found to be good for long term storage and coirpith was found to be a cheap indigenous material for its mass multiplication.