MOLECULAR POLYMORPHISM AND PATHOGENIC VARIABILITY IN RICE SHEATHBLIGHT FUNGUS *RH1ZOCTONIA SOLANI*

THESIS SUBMITTED TO THE UNIVERSITY OF HYDERABAD FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
C N NEERAJA

SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD - 500 046
INDIA

JULY, 1998

SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD - 500 046

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of **Dr.** N **P Sarma** Principal Scientist & Head, Department of Biotechnology, Directorate of Rice Research, Rajendranagar, Hyderabad and that this has not been submitted for a degree or diploma of any other University. All assistance and help received during the course of the investigation have been duly acknowledged.

C N Neeraja
Candidate

DR N P Sarma
Supervisor

CERTIFICATE

This is to certify that Ms C N Neeraja has carried out the research work embodied in the present thesis under my guidance and supervision for the full period prescribed under the Ph. D ordinance of the University of Hyderabad. 1 recommend her thesis entitled **"'MOLECULAR POLYMORPHISM AND PATHOGENIC VARIABILITY IN RICE SHEATHBLIGHT FUNGUS *RHIZOCTONIA SOLANI"*** for submission for the degree of Doctor of Philosophy in Plant Sciences of the University of Hyderabad.

Dr N P Sarma
Principal Scientist & Head
Department of BioTechnology
Directorate of Rice Research
Rajendranagar - Hyderabad.

24/7/08

Head - Department of Plant Sciences
University of Hyderabad - Hyderabad.

Dean - School of Life Sciences
University of Hyderabad - Hyderabad.

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Dedicated to
the fond memories of
an ever cheerful lady
my mother-in-law

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INTRODUCTION

Rice (*Oryza sativa)* is the **principal** food crop on which **two-thirds** of the world's population subsists wholly or partially. Human consumption accounts for 85% of the total production of rice, compared with 60% for wheat and 25% for maize (IRR1, Rice Almanac, 1993). Rice crop is grown under an extremely wide range of climatic conditions. Notwithstanding the high yielding cultivars generated that made several developing countries self-sufficient in food production, sustainable rice production is often seriously threatened by combination of stresses, both abiotic(such as drought, floods, low and high temperatures) and biotic(such as insects, pests, diseases and weeds). Rice crop is affected by several diseases **like** blast, sheath blight, bacterial blight and tungro which cause great damage to the crop.

Sheath blight disease **(ShB)** of rice, caused by the basidiomycetous fungus ***Rhizoctonia solani*** Kuhn has acquired alarming proportions both in temperate and tropical rice growing areas. This disease is aggravated since the introduction of high yielding varieties in **1960's,** as the high tillering capacity of improved cultivars, the greater density of plants, higher rates of Nitrogen fertiliser application and systems of minimal cultivation have resulted in greater incidence (Lee and Rush, 1983; Ou, 1985). The disease is considered second in importance after blast Various estimates of crop losses due to sheath blight have been made. They usually vary from negligible to 50% (Lee and Rush, 1983).

Current control of ShB in rice depends upon chemicals, cultural management and use of so-called tolerant cultivars, aU of which may be used in an integrated manner. Use of chemicals for control of this disease is hindered by high cost or potential damage to the environment. Cultural methods are not an ideal solution to the ShB control in rice as the measures such as crop rotation involve hidden losses that are unacceptable to specialist growers or those with few alternative crops. The use of resistant cultivars has been one of the most effective and economic means of combating several diseases in rice. But so far no commercially acceptable level of resistance to ShB pathogen has been found in rice germplasm. Though the knowledge of existing relative resistance in the available

rice germ plasm help in the efforts of breeding for resistance, a more appropriate approach for genetic enhancement of resistance to this disease is to engineer the resistance genetically , for which basic information on variability in sheath blight fungus is very important.

Isolates of *Rhizoctonia solani* show tremendous variation in characteristics such as morphology, pathogenecity and host specificity (Ogoshi, 1987). In spite of the economic importance of the fungus, very little is known about the genetic variation within the fungus. Information regarding the variability within the fungal population is important for better understanding of disease development and for prediction of future disease outbreak. The genetic variability of the pathogen complicates breeding for resistance and deploying available cultivars effectively. Knowledge of pathogen can contribute both to resistance breeding efforts and to the development of strategies for deployment of resistance. An improved understanding of pathogen population also facilitates researchers to determine which resistance gene or gene combinations will be most effective in protecting the crop from disease.

A method based on anastomosis group (AC) has been used for identification and classification of sheath blight isolates. Although AC concept correlates to some extent with pathogenecity, there is considerable reported variation among the isolates from the same AG (Vilgalys and Gonzalez, 1990). However, due to the lack of stable morphological or physiological markers in this fungus, there is no information by which this variation is generated. Lack of markers has also hindered the studies on the population dynamics and the epidemiology of the pathogen. Development of markers should be very useful for assessing the level of variation within the different groups and for identifying the mechanisms responsible for variation. Studies using markers like proteins, isozymes, RFLPs, RAPD-PCR etc., have revealed a hitherto unsuspected level of variation in a number of species of phytopathogenic fungi and the mechanisms of variation in those species (Michelmore and Hubert, 1987). Thus taking a holistic view based on the facts that (a) rice is the principal food crop in India (b) sheath blight is an important fungal disease that afflicts rice crop and (c) the knowledge of variability of the pathogen is essential for breeding / engineering resistance, present studies were undertaken with the following objectives.

i) to characterise pathogenic variability in rice sheath blight fungus *Rhizoctonia solani* at molecular level using proteins, isozyme polymorphism and RAPD-PCR profiles

ii) to understand the genetic relatedness among the different isolates of ShB.

and iii) to establish association, if any, of these variations with pathogenecity of rice sheath blight fungus.

The results of the experimental studies carried out towards these objectives and the conclusions drawn are presented in this thesis.

REVIEW

Rice is the principal food crop of more than half of mankind. It provides 20% of global human per capita energy and 15% of per capita protein (IRRI Rice Almanac, 1993). Global rice production needs to be raised by another 300 million tons in the next 30 years to meet the increasing food requirements. The area cultivated under rice is so large (about 56 million hectares world wide) that any marginal increase in yield would substantially influence the total rice production (Manibhushanrao, 1995). The grain yield in rice is influenced by a number of environmental factors like high and low temperatures, salinity etc., and biological factors like diseases, pests etc. Among the fungal diseases, sheathblight is considered as one of the major production constraints for rice. The principal components of the green revolution namely, intensive cultural practices, especially, close planting and heavier application of nitrogenous fertilisers on improved varieties, leading to the change of the microclimate of the crop favour the disease incidence. Further, the farmers also create an ideal situation for the spread of the disease by using a single glamorous variety and also by applying high doses of Nitrogen fertiliser (about 200 kg/ha and above). It is an excellent example to illustrate how a minor disease could become a major one due to the shifting in varietal pattern coupled with modification of agronomic practices.

In this chapter, an attempt has been made to review information on causal organism, disease development, pathogenic variability and characterization of rice sheathblight fungus.

CAUSAL ORGANISM

Rice sheathblight disease is caused by the basidiomycetous fungus anamorph or imperfect sclerotial stage *Rhizoctonia solani* with the teleomorph or perfect basidial stage *Thanatephorus cucumeris* (Frank.) Donk.(Parmeter, 1970). It infects all parts of rice plant (except roots in submerged condition) at all stages of growth (Ou, 1985). *R.solani* is both a pathogen and a saprophyte; it aggressively colonises organic debris and is one of the fastest growing fungi. The fungus is multinucleate and the cytoplasm is interconnected through a septal pore (doliopore) that

is characteristic of basidiomycetes. The fungus forms sclerotia and basidiospores. The basidiopores are white and powdery and are found on the host plants only around the booting stage of rice plants. The sclerotia form as soil borne propagules, which are knots of undifferentiated, intertwined, pigmented, moniloid cells.

ANASTOMOSIS GROUPING (AG)

On the basis of different anastomosis reactions, the isolates of *Rhizoctonia* are classified into 12 Anastomosis Groups (AGs) (designated as AG1 through AG 11 and AG B1 (Carling and Kuninaga, 1990). The grouping of isolates is based on their ability to anastomose. Hyphae of the isolates belonging to a common group make contact and their cell walls fuse and plasmogamy occurs. Plasmogamy between isolates belonging to different groups is followed by lysis of several cells on each side of the fusion, such that their contents are visibly vacuolated. Each Anastomosis Group (AG) represents a non-interbreeding population and a genetically independent entity. Characterisation and grouping of isolates using the AG system represents a valuable approach for understanding variations within the species as well as identifying characteristics of the various groups inciting plant diseases. Again within these AGs, by pathogenic variations, DNA-DNA homology, serological studies, fatty acid analysis, protein electrophoresis and nutrient utilisation, Intra Specific Groups(lSG) are recognised (Kuninaga and Yokosawa, 1982-85). A more recent concept has been to classify isolates on the basis of the pectic enzymes produced during growth on pectin (Sweetingham *et al,* 1986), according to which isolates are divided into pectic Zymogram Groups (ZG).

The sheathblight fungus belongs to AG I having 3 to 16 nuclei (Ahuja and Payak, 1985). Based on morphological and pathogenic characters, AG 1 can be divided into three Intra Specific Groups, viz., I A, IB and IC. The rice sheathblight pathogen was placed in sasaki type IA (Watanabe and Matsuda, 1966).

OCCURRENCE AND ECONOMIC IMPORTANCE

The disease now widespread in the East and South East Asian countries, was first recorded from Japan by **Miyake(1910). It** was first reported in India by Paracer and Chahal **(1963).** Besides the Oriental **countries,** sheathblight now occurs in Brazil, Venezuela, Surinam, Madagascar, USA , Fiji, Papua, New Guinea, **Sub-Saha** ran Africa, Nigeria and **Iran** (Ou, 1985),

The disease is considered second in importance after blast disease. Various estimates of crop losses due to sheathblight have been made and losses usually vary from negligible to 50% (Lee and Rush, 1983). The pathogen *R.solani* is a soil borne saprophyte with a wide host range and world wide distribution (Ogoshi, 1987). **Kozaka** (1965) recorded 188 species in 32 families from Japan. Dath (1990) listed plant species recorded as hosts from India. The pathogen invades all parts of the crop plants at all age levels in different climatic conditions (Baker, **1970).**

DISEASE DEVELOPMENT

The primary infection of ShB comes from sclerotia. Although basidiospores of this fungus have been reported from India, their role in pathogenesis is negligible (Singh and Pavgi, 1969). Basidiospores can initiate infection, but do not play important role in the epidemiology of this disease (Kozaka **1970, 1975;** Chin, 1976).

Two types of **mycelia-straight** and lobate are present, of which only the latter type is infectious. Lesion is covered by lobate mycelium while the straight type may extend beyond it without causing infection. **Infection** pegs develop from lobate mycelium forming infection cushion which penetrates through cuticle or stomata. Lesions in the initial stages appear as circular, oblong or ellipsoidal, greenish gray, water-soaked spots about 1 cm, which are later enlarged to 2 - 3 cm in length, with irregular dark brown margins, while the center is bleached to grayish white. Soon after the primary lesions are formed , mycelia raising from the lesions spread rapidly upwards or towards the lateral sides directly through air spaces or interstices between the sheaths and along the outer

surface of the plants (Yoshimura, 1954; Kozaka, 1961). Under favorable microclimatic conditions, the lesions coalesce and encompass the entire leaf sheaths and stems and reach to panicles (Ou *et al*, 1973). Severely diseased plants often snap at lesion sites on upper plant or lodge entirely on the ground.

Sheathblight is usually severe on cultivars that are short, high tillering and responsive to high fertiliser in comparison to tall cultivars with fewer tillers (Lee and Rush, 1983). The intensity of primary infection rests mostly on the number of sclerotia which are in contact with the plant at the water surface. Subsequent disease spread is governed by the favourable environmental conditions as well as susceptibility of the host plant. Under the conditions of high humidity(ca. 95% RH) and high temperature (25 - 32° C) runner hyphae spread rapidly to the upper parts of the plant and to adjacent plants. Kozaka (1961) reported two distinct phases of disease development, the vertical *i.e* upward spreading and the horizontal i.e., to the neighboring plants and fields. Both these developments occur rapidly under favorable conditions, while the vertical spread generally occurs only after heading stage. *R.solani* can attack rice plants at any growth stage right from seedling. Yoshimura and Nishizawa (1954) observed that maximum tillering stage is the optimum for varietal testing and Roy (1979) recorded active tillering as the most susceptible stage.

HOST - PLANT RESISTANCE

Studies on the mechanisms of host resistance are hindered due to the lack of rice cultivars resistant to tf.w/am(Marshall and Rush, 1980). C rill *et al* (1981) tested more than 20,000 selections from the IRRI rice germ plasm to evaluate their reaction to ShB disease and Tapoochoz, Bahagia and Laka were least affected and cultivar IR 1487-372-1-1 was considered as the most susceptible. Among 6000 rice cultivars collected from more than 40 countries of the world, no rice cultivar exhibited clear resistance to rice sheathblight (Hashiba, 1984). It has been shown that susceptibility of rice to this disease is greatly influenced by physiological, morphological and ecological factors.

Knowledge of the inherent level of resistance in rice cultivars to sheath blight disease is far from adequate. Resistance may be dominant and monogenic (Marshall and Rush, 1980; Ou, 1985), governed by two pairs of complementary genes and modified by epistasis, (Goita, 1985) or, most possibly and nearly universally, incompletely dominant due to multiple genes. Studies on the inheritance reactions with virulent isolate RH-9 using Tetep, IET 4699, Jawn no. 14 and Yedao as resistant donors and IR 9752-72-3-2 as susceptible parent revealed that the F1 of four combinations of moderately resistant donors and susceptible parent showed intermediate reactions to inoculation and the F2 distributions tend to be continuous implying that resistance to ShB is controlled by multiple genes (Sha and Zhu, 1989). Attempts have been made to locate ShB resistance in wild rice by Amante *et al* (1990). Accessions from *Oryza minuta*, *O.rufipogon* and *O.eichengeri* may serve as donors of ShB resistance for rice improvement. There is a great need for more work to identify sources of resistance genes from primary and secondary gene pool. Wax thickness is correlated with resistance (Ou, 1985).

Indica cultivars are usually more tolerant than Japonica cultivars (Roy, 1979). Short culmed and high tillering rice cultivars provide a favorable microclimate for disease development at early stages of plant growth than tall varieties with few tillers (Lee and Rush, 1983; Marchetti, 1983).

MANAGEMENT

Current control methods of sheathblight in rice depend upon chemicals, cultural management, biological control and use of so-called tolerant cultivars, all of which may be used in an integrated manner. Chemical control is the most effective counter measure against sheathblight. Organo-synthetic chemicals such as validamycin, mepronil, diclomezine *etc* are being applied. However, the use of chemicals is expensive and potentially damaging to the environment and over time chemicals become less efficient due to adaptation of the pathogen. Cultural manipulations are not an ideal solution to sheathblight control in rice as the measures such as crop rotation involve

hidden losses that are unacceptable to specialist growers or those with few alternative crops. Cultural operations such as destruction or burning of infected crop residue and destruction of collateral hosts were suggested as a part of integrated management Cultural methods can control the disease to some extent, but, as the only means of control, these methods are usually ineffective. Various fungi such as *Trichoderma sp.*, *Aspergillus sp.*, *Neurospora crassa* can inhibit mycelial growth, cause hyper-parasitism and lysis and exhaust substrates early and rapid competitive saprophytic colonization (Gangopadhyay and Chakrabarthi,1982 ; Mew and Rosales, 1984 ; Manibhushanrao *et al*, 1989). Vasanthadevi *et al* (1989) reported the loss of viability of sclerotia in soil by various bacteria like fluorescent and non fluorescent pseudomonads, enterobacteria like *Bacillus subtil is etc.*.Though the use of resistant cultivars has been one of the most effective and economic means of combating the disease, no commercially acceptable level of resistance to sheathblight pathogen has been identified in rice so far.

CHARACTERS ASSOCIATED WITH PATHOGENECITY

Although the AG concept correlates to some extent with pathogenecity, evidence from several studies suggests that there is considerable variation between isolates from the same AG and the pathogenecity of *R.solani* cannot be explained solely in terms of AG or ZG (Ogoshi, 1987; Jabaji-Hare *et al*, 1990; Vilgalys and Gonzalez, 1990). Size of sclerotia is related to pathogenecity. A positive correlation between number of sclerotia and disease severity was found, but not between plant density and sclerotia on soil surface (IRRI, 1986). Newton and Mayers (1935) were the first to detect a toxic principle in the culture filtrates of *R.solani*. The pathogen was reported to produce several low molecular non-enzymatic, extracellular, phytotoxic metabolites namely phenyl acetic acid (PAA) and its hydroxy (meta, ortho and para) derivatives (Ramalingam, 1981).Manibhushanrao and Ramalingam (1993) obtained a positive correlation between toxin production and the relative virulence of the ShB fungus isolates (Rl to R5). However, the quantitative correlation of the virulence of the isolates with toxin production is not easily interpreted since toxin production *invitro* is

influenced by the composition of the medium and the physical environment Pectin transeliminase (Lin, 1976) and Phosphatidase (Kuan and Kuo, 1974) have been implicated with pathogenesis. In studies with two *R.solani* isolates differing in virulence, Pectin lyase production was detected to take place earlier than Pectin Methyl esterase and was found to be greater in more virulent isolate (Ramalingam, 1983).

In studies on electrophoretic patterns of mycelial proteins and isozyme patterns of poly phenol oxidase, peroxidase, acetyl esterase and acid and alkaline phosphatase of differentially virulent isolates of *R.solani*, no relation could be obtained with enzyme patterns and virulence (Zuber and Manibhushanrao, 1982). Further, aminoacid composition of differentially virulent isolates of *R.solani* could not reveal any specific correlation with their relative virulence rating (ManibhushanRao *et al*, 1987).

Recent evidence shows that cytoplasmic determinant is required for virulence in *R.solani* (Finkler *et al*, 1985). All virulent isolates examined so far contained several double stranded (ds) RNA segments, at least some of which were encapsulated in isometric particles, whereas hypovirulent isolates had either no ds RNA or fewer ds RNA segments. The virulent factor could be transferred along with the ds RNA segments, from a virulent strain (resistant to benomyl, sensitive to BTN fungicide, melanin producing and with large sclerotia) to hypovirulent strain (sensitive to benomyl, resistant to BTN, no melanin produced, small hyaline sclerotia) by hyphal anastomosis, giving a virulent strain possessing all the nuclear markers of the hypovirulent strain. Hence virulence factor was reasoned to be cytoplasmic. Proof of the role of one or more specific segments of ds RNA in virulence will require infection and/or transformation of protoplasts with isolated ds RNA. In this respect it is noteworthy that Martini *et al* (1978) have suggested a DNA plasmid which may be required for the pathogenecity of *R.solani* and Hashiba *et al* (1984) reported the presence of a plasmid DNA designated as pRS64 in *R.solani* which was weakly pathogenic. These results do suggest that there is a possible relationship between extrachromosomal DNA and pathogenecity.

Three extra chromosomal elements were described that putatively diminish virulence in *R.solani.* The first was a double stranded RNA in AG 1, Castanho *et al* (1978) reported that a degenerative decline occurred in a severely diseased isolate *of R.solani* that has been associated with an assortment of double stranded (ds RNA) segments of three different sizes. The second was a **covalently** closed circular **DNA plasmid** (Martini *et al.,* 1978). The third was a linear **DNA plasmid** in AG 4. A double stranded DNA **plasmid,** designated pRS 64, was detected in three isolates of AG 4 by biophysical methods. Weakly pathogenic isolates contained the plasmids, but pathogenic isolates contained no detectable plasmid DNA (Hashiba *et al* **, 1984),** and the plasmids were **isolate-specific** and could not be transmitted to other isolates by hyphal anastomosis. Out of 114 field isolates **of *R.solani*,** 48 isolates showed plasmid like DNA. These 48 isolates were distributed among AGs and **ISGs** recognized among Japanese isolates of *R.solani.* Each isolate carried one, two or three plasmid DNAs identified by **gel** electrophoresis (Miyasaka *et al,* 1990).

PATHOGENIC VARIABILITY

The fungus is well known for its versatility as plant pathogen, a parasite, a symbiont or a facultative saprophyte and has a wide host range from semiarid plants to aquatic plants. **Isolates** of *R.solani* show tremendous variation in characteristics, such as morphology, pathogenecity and host specificity. There is need for more genetic information on the pathogen to provide a basis for understanding the high degree of pathogenic variability. The fungal isolates of sheath blight are highly variable in their aggressiveness **(Amaral** *et al,* 1979, Zuber and Manibhushanrao, 1982; Dath, **1985).** Many isolates from a wide variety of plants are pathogenic on rice. Chien and Chung (1963) grouped 300 isolates into 7 cultural types and 6 physiological races. Twenty strains were identified in Taiwan according to their pathogenecity (Ou and Bandong, 1976). Isolates differing in virulence were also reported by Tsai (1973) among 40 single spore cultures and by **Haque** (1975) among 25 field isolates.

Knowledge of pathogen population can **contribute** both to resistance breeding efforts and to the development of strategies for the deployment of resistance (Leung *et al,* 1993). An improved

understanding of pathogen populations also allows researchers to determine which resistance genes or gene combinations will be most effective in protecting the crop from pests or diseases, by reducing the destructive potential of pathogen evolution. Information regarding the variability within fungal populations is important for better understanding of disease outbreaks and for prediction of future disease development (Stakman and Christensen, 1953).

The genetic variability of the pathogen increases the difficulty of breeding for resistance and deploying available cultivars effectively. A quick method for characterizing isolates within the pathogen population would aid the researchers not only to provide uptodate information on the genetic diversity of the pathogen, but also to facilitate the study of the effect of various cultivars on the population structure of the pathogen. By regularly sampling diseased tissue at a number of fixed sites, researchers can follow the shifts in the genetic makeup of the pathogen population, which could provide a dynamic picture of the interactions between host and pathogen.

Development of suitable markers should be very useful for analysing the genetic structure of populations. In the absence of stable genetic markers it is very difficult to assess the level of variation within the different groups, and to identify the mechanisms responsible for this variation. For this, markers will need to be developed specifically for each 1SG. The markers often used to study pathogen variability are virulence, proteins, isozymes, Restriction Fragment Length Polymorphisms (RFLP),Random Amplified Polymorphic DNA(RAPD)-Polymerase Chain Reaction (PCR) etc.

VIRULENCE ANALYSIS

The most widely used method for characterizing pathogens is the determination of the virulence spectrum on a set of differential varieties carrying different resistant factors. This measure of genetic variability provides information which is of direct interest to farmers, breeders and pathologists. Differences in virulence have been widely used as phenotypic and genotypic markers (Al-Kherb *et al* 1987; Linde *et al*, 1990), but have not been fully investigated on all anastomosis groups of *R.solani* (Bolkan and Butler, 1974; Puhalla and Carter, 1976; Anderson and Stretton, 1978).

There are, however, several limitations to virulence analysis. Although it is technically simple, **pathotyping** is a laborious and time-consuming process. Scoring of disease phenotypes is influenced by environmental variation and the subjective judgment of the investigator. Even with well characterised differentials, new virulence in the pathogen population may not be detected immediately, simply because the available series cannot differentiate the new virulence. Perhaps the most serious disadvantage of relying on virulence data to infer population structure is that genes involved in host-specificity represent a very small fraction of genes in the pathogen and may be subjected to strong selection by the host (Leung *et al*, **1993).**

MOLECULAR ANALYSIS

Molecular approaches have been very fruitful for answering basic questions about genetic variation and systematic relations in many groups of phytopathogenic fungi. One direct approach for assessing genetic variation in fungi is by comparative study of molecular markers **(Michelmore** and **Hulbert,** 1987). A large number of genetic loci from the genome should be assayed so that genetic differences detected **will** reflect the variability of the genome as a whole. **Molecular** markers that are presumably selectively neutral satisfy such criteria. Many problems associated with studying differed levels of diversity in ***Rhizoctonia*** are best addressed through the use of molecular genetic markers.

PROTEINS

Gel electrophoresis of fungal proteins has been used as an adjunct to morphologic criteria for taxonomy. The use of electrophoresis in fungal taxonomy has been reviewed by Hall (1973) for fungi in general and by Snider (1973) for phytopathogenic fungi. Electrophoretic **poly pep** tide patterns of total soluble proteins have been found to be valuable in fungal taxonomy, because they express the genetic constitution of the cell. Clare *et al* **(1968)** demonstrated that total soluble protein patterns revealed on starch gel were variable within isolates **of *R.solani*** from crucifers and cereals and that those isolates could be grouped based on the similarity of their protein patterns. The results of

gel electrophoresis of soluble proteins of *R.solani* indicated that there was considerable variation in the banding patterns from isolate to isolate and this was interpreted as evidence that *R.solani* is a collective species. Welch (1976) surveyed the proteins from representative isolates of five AG and concluded that electrophoresis could prove useful in identifying the various groups. Reynolds *et al* (1983) were able to distinguish five different AGs (AGs 1 through 5) from each other based on distinct protein patterns. The total soluble protein patterns of AGs 1 to 7 and B1, were not only different among AGs but distinct patterns were recognisable among homologous groups within an AG (Kuninaga, 1986). Analysis of 117 AG identified isolates of East China, from 11 anastomosis groups showed that the same AG or sub AG shared the same bands even when they came from different areas or host plants or diverse virulence (Liu and Ge, 1988).

Comparative analyses of the soluble proteins from the mycelia of 45 isolates of 5 AG (AG I-AG 5) and subgroups of *Rhizoctonia* from Japan, USA and China using IEF electrophoresis showed that the protein patterns differed significantly. Groups AG 3, AG 4, AG 5 and subgroups AG 1-1 A, AG 1-1C, AG 2-1, and AG 2-2 had distinct protein patterns with most significant differences between subgroups AG 1 and AG 2 (Cehn *et al*, 1991).

ISOZYMES

The study of protein polymorphisms through isozyme analysis provides a powerful tool for assessing and characterizing the genetic variability in plant pathogen populations. The term isozyme describes different molecular forms of enzyme with the same substrate specificity. An isozyme is a direct expression of genotype and can be used as an indicator of genetic relationships within related populations (Ayala, 1983; Burdon and Marshall, 1983, Newton, 1987, Micales *et al*, 1988 b). They are usually coded by different alleles or separate genetic loci and appear on a gel as multiple bands due to their different electrophoretic mobilities (Micales *et al*, 1988 b). The technique

relies on the existence of genetic polymorphisms resulting in aminoacid substitutions that cause differences in relative mobilities among allelic forms on an electrophoretic gel (Lewontin, 1974).

Isozyme analysis has been used for several purposes, to clarify taxonomic relationships among organisms that are presumably closely related, but that are difficult or impossible to distinguish morphologically (Burdon and Marshall, 1981; Beakes and Ford, 1983; Leung and Williams, 1986; Micales *et al,* 1988 b), to study phylogenetic relationships among organisms (Bonde *et al,* 1984; Maghrabi and Kish, 1987; Micales *et al,* 1987) to study the genetics of organisms within the same biological species (Avise and Saunders, 1984, Old *et al,* 1984, Burdon and Roelfs, 1985, Tooley *et al,* 1985, Michaelis *et al,* 1987) and to develop a rapid electrophoretic technique for routine identification / diagnostic markers (Chen *et al,* 1992).

Isozyme electrophoresis data are increasingly used to describe inter or intraspecific variation in various fungal groups (May and Royse, 1988; Stasz *et al,* 1989, Oudemans and Coffey, 1991). Detailed studies have also shown that isozyme analysis can be a powerful method for genetic and population studies (Leuchtmann and Clay, 1989; Zambino and Harrington, 1989).

Isozyme analysis in taxonomy and genetics of fungi was reviewed by Micales *et al* (1988b). Species-specific isozyme banding patterns were reported for species of *Erysiphe* (Clark *et al,* 1989), *Perenosclerospora* (Bonde *et al,* 1984; Micales *et al,* 1988 a) and *Phytophthora* (Nygaard *et al,* 1989).

Leung and Williams (1986) used enzyme polymorphisms and genetic distance to examine the degree of similarity between isolates of *Magnaporthe grisea.* Lu and Groth (1987) used isozyme data to calculate the percentage similarity between isolates of the bean rust fungus. Linde *et al* (1990) constructed dendrograms with cluster analysis to examine relationships between isozyme phenotypes and virulence for *Uromyces appendiculatus.*

As sexual state is difficult to obtain in *R.solani*, hybridization methods of studying its genetic variability are not possible. Isozyme analysis provides an ideal method of examining the level of genetic variability. In *R.solani*, isozyme electrophoretic profiles provided a good indication of the genetic diversity among selected AGs and reconfirmed the genetic basis of the AG concept (Liu *et al.* 1990; Laroche *et al*, 1992). Zuber and Manibhushanrao (1982) studied electrophoretic patterns for five isozymes of five isolates of rice sheathblight fungus and no correlation was obtained with the enzyme patterns and virulence of the isolates examined.

Studies on genetic relationships among 14 isolates of *R.solani* anastomosis group AG 2 by evaluating electrophoretic data derived from 11 enzyme systems revealed variability across the isolates. Three groups were differentiated by isozyme analysis which were congruent with previous grouping of AG 2 isolates based on studies of anastomosis, morphology, physiology and DNA / DNA hybridization (Liu *et al*, 1990). Isozyme studies have also revealed evidence for at least five different ISG within AG 2 (Liu *et al*, 1990; Liu and Sinclair, 1992).

Laroche *et al* (1992) demonstrated the utility of isozyme data for discrimination between 48 isolates from potato fields belonging to AG 3 and AG 9. Based on variation in isozyme patterns of seven enzymes, three sub groups within AG 3 were identified indicating that AG 3 is a heterogeneous group. Based on isoenzyme profiles derived from 10 enzyme systems, several genetically distinct subgroups (6 within AG 1 and 5 within AG 2) were identified(Liu and Sinclair, 1993). Kaufman and Rothrock (1995) distinguished the isolates of AG 11 from Australia and Arkansas, USA through the studies of isozyme polymorphisms. The above studies demonstrate that isozyme electrophoresis is a promising means for studying relationships among groups and it has special merits and is well suited to the study of genetic diversity because isozymes normally show high levels of variation within species, they are encoded by numerous loci scattered over the genome and some of these loci could provide valuable genetic markers for population studies and the analysis is relatively easy to apply to large number of individuals.

Another technique which allows the detection of isozymes of specific enzymes such as pectic enzymes, has been found useful in identifying groups within ***R.solani.*** This method is based on the detection of various pectinases (e.g., pectin esterases and polygalacturonases) in pectic acid acrylamide gels by staining with ruthenium red **(Cruickshank** and Wade, 1986). Sweetingham *et al* (1986) separated **140** isolates of ***Rhizoctonia*** from Western Australia into distinct pectic zymogram pattern groups (ZGs) and further demonstrated that individual pectic zymograms contained cultural characteristics and pathogenic capabilities which are similar. Using the same technique, five distinct ZGs within AG 8 have been established (Neate ***et al,*** 1988, MacNish *et al,* 1993) that also corresponded with results based on random amplified polymorphic DNA **(RAPD)** markers (Duncan *et al,* **1993).** Schneider *et al* (1995) demonstrated the utility of pectic zymogram patterns for discriminating between AG 2 isolates pathogenic to tulips from AG 2-1 isolates, while Balali *et al,* **(1995)** separated field isolates of *R.solani* AG 3 into three distinct pectic zymograms and suggested that zymogram group variation is related to the morphology of tuber sclerotia and disease symptoms.

Other studies with members of *Rhizoctonia* complex from Australia have also shown that different ISGs can be differentiated on the basis of their pectic zymogram patterns (Sweetingham *et* ***al,*** 1986, Neate *et* ***al,*** 1988, MacNish *et al* 1993).

DNA / ONA HYBRIDIZATION

Molecular studies on DNA base sequence complementarity have provided convincing evidence for the possibility to demonstrate genetic relatedness between the species, by measuring the degree of reassociation (Hybridisation) between DNA molecules.

Genetic relatedness was estimated using spectrophotometric measurement of **DNA/DNA** hybridisation by **Kuninaga** and Vokosawa (1985) clearly demonstrated that different ISGs are genetically isolated. Vilgalys (1988) also demonstrated that genetic differences among **ISG** from four different AG using the measurement of the extent of hybridization based on isotope labeled DNA. The

relatively low DNA relatedness (47 - 87 %) between certain isolates within each of these AGs corresponded well with previously defined sub-groups based on cultural morphology and pathogenecity. Kinetic analyses of whole-cell DNA reassociation estimate a haploid genome size of *R.solani* to be around 2.8 X 10 nucleotide base pairs (Kuninaga, unpublished). As the evolution of fungi is considered to be accompanied by a progressive decrease in the G€ content of DNA, AG I - IA with its GC content of 49.1 moles of DNA could be considered as the most highly evolved. The percentage of DNA that hybridizes among AGs is typically around 15%. Thus the limited resolution afforded by DNA-DNA hybridization of the whole genome does not provide sufficient understanding of the evolutionary relationships in *R. solani* (Kuninaga, 1986).

RFLP

RFLP analyses in which mitochondrial, ribosomal or random cloned fragments were used as probes would result in DNA polymorphisms which in turn can be used as genetic markers. RFLP approach has been successful with other species of phytopathogenic fungi (Levy *et al.*, 1990; Manicom *et al.*, 1990).

Using Southern blot techniques with anonymous fragments of nuclear DNA cloned from an isolate of AG 3, Jabaji-Hare *et al.*, (1990) demonstrated that group-specific RFLP could be detected, in which cloned DNA probes hybridize only with members of their own ISC. RFLP of ribosomal DNA (rDNA) studies of 87 isolates of *R.solani* from 15 ISGs indicated divergent variations among isolates (Vilgalys and Gonzalez, 1990). Liu *et al* (1993) demonstrated based on restriction analysis of ITS sequences from a large sample of field isolates, patterns of r DNA variation revealed at least six ISGs within AG 1 and five ISGs within AG 2 (Liu and Sinclair, 1992; Liu and Sinclair, 1993). Evidence for the five ISG groupings detected within AG 2 is also supported by data from a portion of the mitochondrial rDNA gene (Liu and Sinclair, 1992) and also by isozyme data (Liu and Sinclair, 1992; Liu *et al*, 1993). Detailed restriction analyses of DNA fragments amplified by the PC R (Polymerase

Chain Reaction) provide even greater resolution of genetic differences within and among ISGs. Similar patterns of group specific hybridizations were seen in one of the three ISGs classified within AG 4 using random nuclear probes (Cubeta *et al*, 1991).

RFLP studies of isolates of *R. solani* from Japan and Australia, using clones of 18s ribosomal RNA (rRNA) gene or random cloned fragments of *R.solani* demonstrated that group specific patterns could not be identified for all groups with rRNA probe, whereas group specific patterns could be identified with random cloned DNA fragments (O'Brien, 1994). Development of a DNA probe specific to AG 8 showed that the variation occurs within AG 8 (Matthew *et al.*, 1995).

RAPD / PCR

PCR (Polymerase Chain Reaction) provides amplified portions of DNA of specific sequence, which are defined by primers that match the ends of DNA to be amplified. The method of RAPD (Random Amplified Polymorphic DNA), a modification of PCR procedure, employs a single primer (decanter) to amplify, under low stringency, any genomic region that happens to be flanked by a pair of 10 base priming sites in opposite orientations within 5000bp of each other, and yields fingerprint like patterns upon gel electrophoresis (Williams *et al*, 1990; Welsh and McClelland, 1990). The development of RAPD markers provide another powerful method for investigation of intraspecific genetic variation.

The presence of amplified DNA fragment in one individual and the absence of the same molecular weight fragment in another gives rise to polymorphism. The unique fragment constitutes a genetic marker. Ideally each band amplified by the use of random primers would represent a specific locus in the genome and alleles would produce easily identified bands of a different size. Polymorphisms may be because of fragment length or presence or absence of a band. Length polymorphisms are the result of insertion into or deletion from the amplified fragment between the primer binding sites, whereas presence-absence polymorphisms are caused by the destruction or

creation of one of a pair of the primer binding sites. Many areas in the genome contain a non-binding sequence that would, with a little alteration, serve as a primer-binding site. Mutation of, insertion into, or deletion from this site may convert the sequence into a valid primer-binding site. In this case, a new band would appear (if the criteria of proximity and suitable orientation of the corresponding primer-binding site were satisfied). Conversely, changes of sufficient magnitude to a functioning primer-binding site would lead to the disappearance of a band, because the fragment that has that site as one of its ends would no longer amplify exponentially (Guthrie *et al.*, 1992).

As long as a PCR amplified segment of a defined size produced by a particular primer represents a specific locus in the genome of the test species, RAPD markers have great potential for use in population analysis even when alleles cannot be identified. In fact 'null alleles' (i.e. the failure to detect a band at all) may be more common for changes at a specific locus.

ADVANTAGES OF RAPD / PCR

1. The amplified DNA fragments behave as simple Mendelian markers that can be used for characterising a population.

2. The main advantage of RAPD analysis is that no prior knowledge of DNA sequences is necessary to design the primers. Hence abundant genetic markers can be generated for any organism of interest.

3. Methods involving Southern Hybridisations and/or cloning are, however, relatively labour intensive and costly.

The reliability and reproducibility of RAPD techniques are at times questioned because results obtained from different labs were not always comparable. But the reproducibility of fingerprint patterns achieved in a standard reaction mixture with different thermocycler programs and in

different thermocyclers, suggests that the RAPD procedure can be reliable when the temperature profile was properly maintained (Tommerup *et al*, 1995).

RAPD-PCR has been successfully used to study populations of plant pathogenic fungi (Goodwin and Annis, 1991; Moller *et al*, 1995, Pipes *et al*, 1995). RAPD markers have been successfully used to differentiate among isolates of *Colletotrichum graminicola* (Guthrie *et al*, 1992), pathogenic races of *Fusarium oxysporum* (Grajal -Martin *et al*, 1993), to separate species within the genus *Hypoxylon* (Yoon and Glawe, 1993), to study intraspecific genetic diversity of *Chaunopycnis alba* (Moller *et al*., 1995), to characterize pathotype *of Fusarium oxysporum* f.sp. *ciceris* (Kelly *et al*., 1994). Duncan *et al* (1993) found that RAPD markers were able to discriminate between Australian 1SG in the *R.solani* complex, as well as between different strains with same ISG. Cubeta *et al*., (1991), were able to identify many AGs of binucleate *Rhizoctonia* species based on PCR amplification of region of DNA coding for the 25s rRNA. As RAPD-PCR is simple and easy to perform, careful studies using this method are very useful for characterising variation in rice sheath blight fungus *R.solani*.

It is a challenge for scientists to evolve strategies for resistance to diseases either by conventional breeding or genetic engineering techniques. Any such endeavour needs a thorough understanding of the variability of the pathogen. Though it is quite apparent from the preceding discussion that the progress has been made in understanding the molecular aspects of genetic variability of different anastomosis groups, several lacunae are still left to be removed. There are only a limited instances in which variability of AG 1 was investigated. The identification and characterization of the rice sheathblight fungal isolates can be useful in understanding the variability of the pathogen, thereby improving the effectiveness of breeding for resistance.

MATERIAL & METHODS

Experimental material employed in the present work have been detailed as under

3.1 RICE CULTIVARS USED IN THE STUDY

Four popular rice cultivars that include susceptible and tolerant were used in this study. Salient features of these four varieties are as follows.

Sl no	Cultivar	Maturity in days	Average tillers	yield t/ha
	IR 50	125	22	4.10
2	Kavya	125	20	4.15
3	Swarnadhan	150	10	5.00
4	Vikramarya	135	10	4.70

3.2 RICE SHEATHBLIGHT ISOLATES USED IN THE STUDY

The isolates of sheath blight fungus were collected from various agro-ecological regions of the country where rice is an important crop. The locations from where the isolates collected are listed below.

Sl no	Isolate	Location	State / U T
1	ADT	Aduthurai	Tamil Nadu
2	ALM	Almora	Uttar Pradesh
3	CNM	Chinsura	Orissa
4	FBZ	Faizabad	Uttar Pradesh
5	1AR	IAR1	U T of Delhi
6	KPL	Kapurthala	Punjab
7	MCP	Moncompu	Kerala
8	MPM	Machilipatnam	Andhra Pradesh
9	MTU	Maruteru	Andhra Pradesh
10	PAT	Patna	Bihar
11	PBR	Port Blair	U T of Andaman, Nicobar
12	PNT	Pantnagar	Uttar Pradesh
13	PTB	Pattambi	Kerala
14	RNR	Rajendranagar	Andhra Pradesh
15	RS5	Chennai	Tamilnadu
16	RSP	R S Pura	Jammu & Kashmir
17	TNL	Tenali	Andhra Pradesh
18	TTB	Titabar	Assam

3.3 LAB SUPPLIES

Experimental supplies employed in the present work were **procured** from **different sources as shown**

SI no	Item	Source
1.	Lab chemicals	Sigma chemicals company, StXouis, USA.
2.	Taq **polymerase**	Bangalore Genei Pvt Ltd., Bangalore, India.
3.	**RAPD** primers	Operon Technologies Inc., Alameda, USA.
4.	DNA 1 kb ladder	Stratagene, Heidelberg, Germany.

Experimental equipment used in the present work were as follows :

Sl no	ITEM	MODEL	MAKE
1	High speed **refrigerated** centrifuge	7820	**Kubota,** Tokyo, Japan
2	Refrigerated **Microfuge**	RMC - **14**	Sorval Dupont Co., Delaware, USA
3	Spectrophotometer	DU 650	Beckman, USA
4	**Thermocycler**	PTC -100 - 60	MJ Research **Inc.,** Watertown, USA
5	Vertical Gel Electrophoresis	Biorad Protean II IX	Hercules, USA
6	Vertical Gel Electrophoresis	**BRL V- 16 - 2**	**GIBCO** BRL Life Technologies, Gaithersburg
7	Submarine **Electrophoresis**	HE 100	**Hoeffer** Scientific **Instruments,** San Fransisco, USA
8	**Submarine** Flectrophoresis	BRL Horizon 11 : 14	GfBCO BRL Life Technologies, Gaithersburg
9	Photodocumentation System	MP-4	Polaroid Corporation, Cambridge, USA
10	**Transilluminator**	**FOTO UV 450**	New Berlin, USA

METHODS

3.5 COLLECTION OF RICE SHEATHBLIGHT ISOLATES, ISOLATION AND MAINTENANCE OF FUNGUS

3.5.1 Media

The following media were used for maintenance and growth of the fungus

i) **Water agar** (2%)

Agar	20 g
Dist. Water (DW)	**1000 mL**

Autoclaved before use.

ii) Potato Dextrose Broth (PDB)

Potato extract	500 **mL** (20 **g/1000 mL)**
Glucose	20 g
pH	7.0
DW	**1000 mL**

Autoclaved before use.

iii) Potato Dextrose Agar (PDA)

PDA was prepared by addition of agar **10 g/1000 mL** of PDB before autoclaving.

iv) Hayward's broth

Sucrose	20 g
Peptone	10 g
$MgSO_4, 7H_2O$	200 **mg**
Na_2HPO_4	50 mg
pH	7.0
DW	1000 **mL**

Autoclaved before use.

3.5.2 Procedure

All 18 fungal isolates were collected from different **ecogeographical** regions of India. **Individual** sheaths with **shea** th blight lesions obtained from the fields were washed with distilled water. The infected portions of sheath were cut into **2 x 2mM** pieces and plated on water agar. After 2 - 3 days of **growth,** hyphal tips were excised and transferred to fresh potato dextrose agar (PDA) plates to eliminate bacterial contamination. **Hyphae** from clean colonies were excised

again and maintained on PDA slants. After 7 days of growth, the isolates were stored in refrigerator. The cultures were grown on PDA petri-plates for experimental purposes (Fig. 1).

3.5.3 Culture methods for Protein extraction and ONA isolation

To obtain fresh mycelium for analysis, actively growing agar fungal culture plugs (5 mM in diameter) per isolate were inoculated in 100 mL flask containing 25 mL of PDB. Isolates were grown at 27° C for 7 days.

3.6 SCREENING OF HOST CULTIVARS

3.6.1 Preparation of plant material for screening

The seeds were surface sterilised and germinated under moist conditions. At the primary leaf stage, the seedlings were transplanted one seedling per pot in three replicated pots. The seeds were sown and seedlings were transplanted in such a way that there was a synchronized maximum tillering.

3.6.2 Preparation of inoculum for screening

Thoroughly washed sheath portions of Typha were cut into 3" long pieces and soaked in Hay ward's broth for 10 min. The conical flasks (500 mL) were filled up to two-thirds with typha pieces. The flasks were autoclaved and inoculated with one fungal isolate per flask in triplicate. The cultures were grown in dark, at 27 °C for 7 days.

3.6.3 Screening

As maximum tillering stage of the plant is more susceptible and optimum for differential screening (Roy, 1979), each hill was inoculated with fungus by inserting the fungus infected Typha pieces (8-10 pieces / plant) in between the tillers and ensuring that the tillers were tightly secured. The relative humidity in glass house was maintained between 70 and 80 % . After one week of inoculation, the observations were recorded. Triplicates for each isolate were scored for both tolerant and susceptible cultivars per isolate. Screening was repeated twice with all the isolates on tolerant and susceptible host varieties.

3.6.4 Screening Parameters

Two phases of disease development were distinguished :

Vertical Development, i.e. infection of the upper leaf sheaths which is expressed by the relative height of the uppermost lesions to the plant height and Horizontal Development, i.e. infection of neighboring culms which is expressed by the percentage of diseased hills (Kozaka, 1960).

i) Relative Lesion Height (RLH%)

$$\frac{\text{Maximum height at which lesion ended}}{\text{Plant height}} \times 100$$

ii) Disease Score

0-9 scale of the Standard Evaluation System **(IRRI,** 1986)

iii) **Disease** Index **(DI)**

$$\frac{\text{Actual Disease score}}{9\ \textbf{(Maximum}\ \text{Score)}} \times \text{Infected Tillers \%}$$

iv) Average Disease **Index** (ADI%)

Average of Disease index of three individual plants X 100

3.7 MORPHOLOGICAL CHARACTERISTICS OF FUNGAL ISOLATES *IN VIVO* STUDIED ON IR50

3.7.1. Number of sclerotia (NSC)

Total number of sclerotia per plant

3.7.2 **Sclerotial** diameter (SCD)

The diameter of sclerotia was measured in **mM** using rice grain measure instrument. Mean value of ten randomly chosen sclerotia per isolate were recorded and their average was taken.

3.8 PROTEIN ISOZYME STUDIES

3.8.1 Soluble protein extraction for Electrophoresis

Extraction Buffer

Tris - HC1 (100 **mM,** pH 7.0)
PVP (Poly Vinyl Pyrrolidine) 1%
β mercaptoethanol (10 **mM)**

For peroxidases **isozyme,** extraction buffer excluding **β-mercaptoethanol** was used. Mycelial mats were collected and washed thoroughly with cold distilled water to remove the traces of medium. The mats were blotted dry and homongenised at 4° C in a pre-chilled mortar and pestle with extraction **buffer (** 3 **mL/1** g).The **homogenate** was centrifuged at 10000 RPM for 20 min. at 4° C. The supernatant was stored at -20° C and used within one week.

3.8.2 Quantification of protein

The protein in the fungal samples was quantified by Micro **Lowry's** method (Lowry *et al.*, 1951).

3.8.3 Separation of proteins on Native PAGE

Electrophoretic separation was done under anionic conditions on native polyacrylamide gel eiectrophoresis (PAGE) (7.5%) as described by Davis (1964).

3.8.3.1 Solutions

1. Tris Glycine Electrode Buffer

Tris	3.0 g
Glycine	14.4 g
pH	8.3
DW	1000 **mL.**

2. Stock A

Acrylamide	30.0 g
NNNN Methyl Bis **Acrylamide**	0.8 **g**
DW	100 **mL**

Filtered and stored at **10° C** in dark bottle.

3. Stock B

Tris	18.15 g
pH	8.8
DW	**100 mL**

4. Stock C

Tris	6.05 g
pH	6.8
DW	**100 mL**

5. **Ammonium** Per Sulphate **(APS 10%**)

Ammonium Per Sulphate	0.1 g
DW	1.0 **mL**

made freshly.

6. TEMED - N, N, N', N'- Tetra methyl ethylenediamine

Commercially available for ready use.

7. Tracking Dye Solution

Bromophenol Blue	2.0 mg
Sucrose	1.0 g
DW	10 mL

3.8.3.2 Recipe for 7.5 % uniform concentration polyacrylamide native gel

Resolving Gel (7.5%)

Stock A	12.5 mL	
Stock B	12.5 mL	
DW	25.0	mL

The above solutions were taken in a side-arm flask and de-gassed for 5 min. 100 µl of APS solution and 50 µl of TEMED were added and thoroughly mixed. The mixture was poured into set gel mold plates steadily and topped with distilled water. After the polymerisation of the gel, water was drained off and stacking gel set on it.

Stacking Gel (6.0%)

Stock A	1.2 mL	
Stock C	2.5 mL	
DW	6.3 mL	
APS (10%)	10	µl
TEMED	5	µl

The above solutions were thoroughly mixed and poured onto the top of resolving gel. The comb was placed avoiding trapping of air bubbles in the wells.

3.8.3.3 Sample preparation

Sample volumes containing 400 ng of protein per isolate were loaded per well. Each sample was thoroughly mixed with tracking dye (6x) before loading.

3.8.3.4 Separation

Electrophoresis was done at constant current of 30 mA at 10°C. Three electrophoretic runs per enzyme were performed for all enzymes.

3.8.4 Staining of Proteins and Isozymes

After completion of **electrophoresis,** gels were stained overnight for total soluble proteins and for each of all 13 isozymes by selective protocols (Novacky and Hampton, 1968, Vallejos, 1983).

ENZYME SYSTEMS USED

ISOZYME	CODE	NOMENCLATURE
Acid phosphatase	ACP	3.1.3.2
Alcohol dehydrogenase	ADH	1.1.1.1
Aspartate **aminotransferase**	AAT	2.6.1.1
a-esterase	**α - EST**	3.1.1.1
β - esterase	β - EST	3.1.1.1
Glutamate dehydrogenase	GDH	1.4.1.3
Isocitrate dehydrogenase	**ICD**	1.1.1.42
Leucine aminopeptidase	LAP	3.4.11.1
Malate dehydrogenase	**MDH**	1.1.1.37
6 **Phosphogluconic** dehydrogenase	PGD	1.1.1.44
Phosphogluco isomerase	**PGI**	5.3.1.9
Peroxidase	POX	**1.11.1.7**
Polyphenoloxidase	PPO	1.10.3.2
Shikima te **dehyd** rogenase	SDH	1.1.1.25

3.8.4.1 Total Soluble Proteins

Staining solution

Coomasie blue	**1.25** g
Methanol	230 **mL**
DW	**230 mL**
Acetic acid	**40 mL**

Destaining solution

Methanol	**300 mL**
Acetic acid	**100 mL**
DW	600 **mL**

Gels were stained overnight and destained till the bands were clear.

3.8.4.2 Isozymes

1. Acid phosphatase

Sodium Acetate (50 raM, pH 5.0)	100 mL
$MgCl_2$ $6H_20$ (1M)	1 mL
Fast Black K Salt	100 mg
a - Naphthyl acid phosphate 1 %	3 mL

The diazonium salt was dissolved in the buffer and α-Naphthyl acid phosphate was added with continuous stirring. The gel was incubated in dark at 30° C for 1 hour.

2. Alcohol dehydrogenase

Tris (0.1 M, pH 7.5)	100 mL
Nicotinamide Adenine Dinucleotide(NAD)	30 mg
3-[4,5-Dimethyl thiazol-2 vl] 2,5-diphenyl tetrazolium bromide Thiazolyl blue(MTT)	20 mg
Phenazine Metho Sulfate(PMS)	4 mg
Ethanol	1 mL

Ethanol was added just prior to the gel placement in the stain and the gel was incubated at 30° C for 30 min.

3. Aspartate aminotransferase

Reagents A

Tris (0.1 M, pH8.5)	100 mL
a ketoglutaric acid	100 mg
Aspartic acid	200 mg

Reagents B

Pyridoxal 5 Phosphate	10 mg
Fast Blue BB salt	150 mg

Reagents in group A were mixed thoroughly and group B reagents were added just before use. The gels were incubated at 30° C for 2 hours.

4. Esterases

Phosphate buffer (0.05 M, pH 6.0)	250 mL
Fast Blue RR Salt	250 mg
a - Naphthyl acetate	75 mg
β - Naphthyl acetate	150 mg

Fast Blue RR salt was dissolved in phosphate buffer and filtered. Both a and βNaphthyl acetates were dissolved in 50% acetone and were added to the buffer and mixed thoroughly. The gel was incubated in the solution at 37° C for 1 hour and destained in 7% acetic acid.

5. Glucose phosphate isomerase

Tris(0.1 M, pH 7.5)	100 mL
$MgCl_2$ 6 H_2O (1M)	1 mL
Fructose 6 phosphate	80 mg
Nicotinamide Adenine Dinucleotide Phosphate(NADP)	15 mg
MTT	20 mg
PMS	4 mg
Glucose 6 phosphodehydrogenase	20 units

The solution was prepared just before use. The gel was incubated in the dark for 30 min. at 30° C.

6. Glutamate dehydrogenase

Tris (0.1 M, pH7.5)	100 ml
$CaCl_2$ (10 mM)	0.2 mL
Sodium Glutamate	800 mg
NAD	30 mg
MTT	20 mg
PMS	4 mg

The gel was incubated at 30°C for 30 min.

7. Isocitrate dehydrogenase

Tris (0.1 M, pH 7.5)	100 mL
$MnCl_2$ (1M)	1 mL
DL-Isocitrate	100 mg
NADP	15 mg
MTT	20 mg
PMS	4 mg

The gel was incubated in the solution at 30" C for 30 min.

8. Leucine aminopeptidase

Tris-maleate (0.2 M, pH 6.0)	100 mL
L-Leucyl-β-naphthylamide HC1	3 mL
Fast Garnet GBC salt	100 mg

The gel was incubated in the dark at 30° C for 1 hour.

9. Malate dehydrogenase

Tris (0.1 M, pH 7.5)	100 mL
DL-Malate (1 M, pH 7.5)	3 mL
NAD	30 mg
MTT	20 mg
PMS	4 mg

The gel was incubated in the solution at 30° C for 30 min.

10. Peroxidase

Sodium acetate (0.2 M, pH 5.0)	100 mL
Benzidine (1.3 mM)	1 mL
H_2O_2	1 mL

Dissolution of benzidine in the acetate buffer was aided by heat and constant stirring. Hydrogen peroxide(H_2O_2) was added to the benzidine solution just prior to placing the gels in the incubating solution. Peroxidase isozymes were visible after 30 min.

11. 6-Phosphogluconic dehydrogenase

Tris (0.1 M, pH7.S)	100 mL
$MgCl_2$	98 mg
6-Phosphogluconic acid	20 mg
NADP	15 mg
MTT	20 mg
PMS	4 mg

The solution was prepared just before use. The gel was incubated in the dark at 30° C for 30 min.

12. Poly phenol oxidase

Sodium phosphate (0.1 M, pH 6.8)	100 mL
Catechol 15	mg
Sulfanilic acid	50 mg

The gel was incubated in the solution at 30° C for 30 min.

13. Shikimate dehydrogenase

Tris (0.1 M, pH7.5)	100 mL
Shikimic acid	20 mg
NADP	15 mg
MTT	20 mg
PMS	4 mg

The solution was prepared just before use. The gel was incubated in the dark at 30°C for 30 min.

3.8.4.3 Fixation of the gels

The gels were rinsed with distilled water and were fixed in 7% acetic acid.

3.9. DNA STUDIES

3.9.1. Isolation of DNA

3.9.1.1 Solutions

1. Grinding buffer (5 mL/1 g of sample)

   Tris(100mM, pH 8.0)
   EDTA (100mM, pH 8.0)
   NaCl (250mM)

2. 10%Sarkosy! : N-Lauryl sarcosine 10% (w/v)

3. 7.5 M Ammonium acetate

4. Sodium acetate (3M, pH 5.2)

5. TE Buffer

   Tris (10 mM, pH 8.0)
   EDTA (I mM, pH 8.0)

6. Preparation of RNAse

   10 mg of RNAse was dissolved in 1 ml of Tris - HO (10 mM, pH 7.5) and NaCI (15 mM), boiled for 15 min and cooled slowly, dispensed into tubes (1 mL/tube) and stored at -20° C.

7. 5XTBE

   Tris-Borate (0.9M)
   EDTA (0.02M, pH 8.0)

3.9.1.2 Procedure :

DNA was isolated from 7 days old mycelial mats by using the modified protocol developed by Bennet and Nair(1993). One gram of hyphal mat was rinsed with distilled water and dried with blotting paper. The mat was crushed with liquid nitrogen into a fine powder to which 5 mL of grinding buffer and 1.15 mL of 10% sarkosyl were added. The whole mixture was allowed to incubate on ice for 2 h and centrifuged at 15,000 RPM at 4° C for 30 min.

To the filtrate, 150 µl of 7.5 M Ammonium acetate per mL was added and was incubated on ice for 30 min. To this, 0.6 volumes of isopropanol and 1/10 volume of 3 M Sodium acetate were added and left overnight at -20° C. The mixture was pelleted at 10000 RPM at 4° C for 20 min and the pellet was washed with 70% ethanol, dried and dissolved in TE. About 3.3 µl of RNase was added per 100 µl and incubated at 37°C for 15 min. Then equal volumes of phenol-chloroform-isoamyl alcohol were added and spun at 10,000 RPM at room temperature for 10 min. To the upper aqueous phase, equal volume of chloroform-isoamyl alcohol was added and spun at 10,000 RPM for 10 min. The supernatant was mixed with 2 volumes of cold ethanol and 1/10 volume of 3M Sodium acetate and precipitated overnight at -20°C. The DNA was pelleted at 10000 RPM for 20 min. and the pellet was washed with 70% ethanol, dried and dissolved in TE.

The DNA concentrations were determined spectrophotometrically at a wavelength of 260 nm. Depending on the concentration of DNA so determined, a stock solution with a concentration of 1µg/µl was prepared for each isolate. These were subsequently diluted to 5ng/µl before use in amplification.

3.9.2 RAPD

3.9.2.1 Primers

The decanter primers (5'-3') used in this study and their nucieotide sequence are listed below.

OPA06	GGTCCCTGAC
OPA10	GTGATCGCAG
OPB01	GTTTCGCTCC
OPC01	TTCGACGCAG
OPE20	AACGCTCACC
OPF06	GGGAATTCGG
OPH18	G AATCGGCC A
OPQ01	GGGACGATGG
OPR01	TGCGGGTCCT
OPZ20	ACTTTGGCGG

3.9.2.2 Amplification Conditions

The Polymerase Chain Reaction was conducted in a total reaction volume of 25 p.! per sample.

Each reaction mixture contained

dNTPs (1.25 mM each)	3.0 µl
sample DNA templates (5ng / µl)	4.0 µl
Random primer (5 ng / µl)	3.0 µl
Taq polymerase (3 U / µl)	1.0 µl
Taq polymerase buffer	2.5 µl
DW	11.5 µl

Assay buffer in 10X buffer

TAPS (100 mM, pH 8.8)
$MgCl_2$ (15 mM)
KCl (500mM)
Gelatin (0.1%)

Storage buffer

HEPES (20 mM, pH7.9)
KC1 (100 mM)
EDTA (0.1 mM)
2-mercaptoethanol (7 mM)
Tween (w/v) (0.5%)
Nonidet p-40 (v/v)
Glycerol (v/v) (50%).

The reaction mixture was overlaid with a drop of mineral oil.

The temperature profile consisted of

94° C : 10 sec
36° C : 60 sec
72°C : 150 sec
34 cycles

72° C : 6 min.

To reduce the possibility of cross contamination and variation in the amplification reactions, master mixes of the reactions were always used. In each set of amplification, a control was included with reaction mixture including Taq polymerase and primers but excluding any DNA.

3.9.2.3 Procedure

A 10 µl volume of each reaction was electrophoresed on 1.1% agarose gel run at constant voltage (6 V/cm) in 1X TBE and stained with Ethidium Bromide(10 mg/mL). The DNA marker used was 1 Kb ladder. The gels were photographed under UV light with Polaroid film 667.

3.10 DATA ANALYSES

3.10.1 Analysis of Variance (ANOVA)

Analysis of host variability was carried out by Analysis of Variance (ANOVA) following completely randomised design (CRD) with cultivars and isolates as sources of variation.

3.10.2 Simple Correlation Studies

Association between morphological characteristics and pathogenecity was tested by working out simple correlation coefficients.

3.10.3 Protein and Isozyme studies

Electrophoretic mobility or distance migration (in cm) from the origin was recorded for all isozymes. The relative intensity of bands was ignored. Although some isozyme alleles or loci could be interpreted for genetic background, because of the indirect evidence and lack of cross verification of the putative alleles and loci, a more conserved approach was taken. Since equal number of bands were not evident in all isolates for some enzymes and the genetic basis of these multiple bands is not known, the isozyme data were treated phenotypically. Each banding pattern (per enzyme and isolate) being considered a different phenotype as in previous studies (Leuchtmann and Clay, 1989). The most common allele at each locus was arbitrarily designated as 100, with additional alleles given numerical values according to their relative migration to the common allele.

3.10.4 Phenographic analyses

Both for RAPD and isozymes, a matrix of the presence and absence of bands was compiled from the electrophoretic data for each isolate. These data were used to calculate Simple Matching Co-efficients between all pairs of the isolate according to the equation

$$Sm = \frac{(++) + (--)}{(++) + (--) + (+-) + (-+)}$$

where ++ = positive matches (a band present in both isolates being compared)
-- = negative matches (a band present in another isolate but absent in both isolates being compared)
-+ = mismatch
+- = mismatch

Similarity values of both isozymes and RAPD were analyzed by Sequential, Agglomerative, Hierarchial and Nest (SAHN) clustering using Unweighted Pair Group with Arithmetic Averaging (UPGMA) (Sneath and Sokal, 1973). The results of the clustering algorithm were used to generate phenograms to display genetic relatedness between isolates.

RESULTS

As stated in Introduction (Chapter 1) the major objective of the present work was to characterize pathogenic variability of the fungal isolates using markers like isozyme polymorphism and RAPD profiles. The data obtained can be presented as variability of pathogenecity in the rice cultivars, variability of isolates at morphological and pathogenic level which was subsequently analysed by ANOVA and simple correlation studies, isozyme polymorphisms, RAPD profiles and phenographic analyses.

4.1 VARIABILITY OF PATHOGENECITY IN THE RICE CULTIVARS

The relative response of rice cultivars to pathogenecity of the fungal isolates at the maximum tillering stage was recorded by standard glass house screening as detailed under material and methods. When the three screening parameters Average Disease Index (ADI %), Relative Lesion Height (RLH%) and Disease Score (DS) were considered, out of the four cultivars screened against all eighteen fungal isolates, IR50 was the most susceptible with an ADI 73.02 and Swarnadhan was the least susceptible (tolerant) with an ADI 41.67. While Kavya was found to be moderately susceptible with an ADI 65.5, Vikramarya showed moderately tolerant reaction with an ADI 44.9. (Table 4.1)

Table 4.1 Cultivar response to inoculation with rice sheath blight fungal isolates (Means ± S.E.)

Cultivars	ADI %	RLH %	Disease Score
IR 50	73.02 ± 5.08	43.46 ± 3.87	6.86 ± 5.08
Kavya	65.50 ±4.88	41.87 ±3.91	5.92 ±1.42
Swarnadhan	41.67 + 4.28	32.36 ±3.55	3.80 ±1.17
Vikramarya	44.90 ± 14.22	34.72 ± 3.49	4.33 ± 1.21

4.2 MORPHOLOGICAL VARIATION OF THE FUNGAL ISOLATES IN *VIVO*

4.2.1 Number of Sclerotia

The number of sclerotia were counted per plant on the susceptible host IR50. Individual sclerotia were counted, but often the sclerotia coalesced to form large masses. Sclerotial number

varied from 5 to 208. Maximum number of sclerotia were recorded with ALM (208) and the least with the isolates IAR and PNT (5).

4.2.2 Sclerotial Size

Sclerotia were superficial, more or less globose with flattened bases. The sclerotial diameter ranged from 1mm (ALM) to 3.4 mm (MTU). (Table 4.2)

Table 4.2 Variability in *Rhizoctonia solani* isolates from different rice growing locations of India

Sl No	ISOLATE	LOCATION	NO. OF SCLEROTIA	SCLEROTIAL DIAMETER(mm)
1	ADT	Aduthurai, TN	15	2.5
2	ALM	Almora, UP	208	1.0
3	CNM	Chinsurah, WB	30	3.0
4	FBZ	Faizabad, UP	7	2.9
5	IAR	1AR1, Delhi	5	2.3
6	KPL	Kapurthala, PN	8	2.2
7	MCP	Moncompu, KE	39	2.8
8	MPM	Machilipatnam, AP	12	2.2
9	MTU	Maruteru, AP	42	3.4
10	PAT	Patna, BH	9	2.4
11	PBR	Port Blair, AN Islands	14	2.9
12	PNT	Pantnagar, UP	5	2.7
13	PTB	Pattambi, KE	12	2.4
14	RNR	Rajendranagar, AP	22	2.8
15	RS5	Chennai, TN	13	2.2
16	RSP	RS Pura, J&K	114	1.6
17	TNL	Tenali, AP	13	3.3
18	TTB	Titabar, AS	12	2.8

* Scored on susceptible cultivar IR50

4.3 VARIABILITY IN PATHOGENECITY

Variability in pathogenecity was evaluated based on Average Disease Index (ADI) on IR50 (susceptible) and Swarnadhan (tolerant) varieties.

All 18 fungal isolates, but for two viz. ALM and RSP, showed large and typical grayish lesions starting from disease initiation. The two isolates ALM and RSP showed small lesions with brown discoloration on both susceptible and tolerant cultivars.

Pathogenecity of isolates varied considerably with a range of 0% to 100% ADI, 0% to 55.8 % RLH and 0 to 9 disease score. The virulent isolates caused the blight symptoms even on the flag leaf and panicle resulting in lodging of the plant. Moderately virulent isolates showed lesions restricted to lower leaf sheaths.

The pathogenecity of the isolates were corresponding on both susceptible and tolerant cultivars, i.e. the scoring profiles were very parallel on both the susceptible and tolerant cultivars. The relationship is further confirmed by highly significant Spearmans rank correlation coefficient **0.813** (for ADI), 0.587 (for DS) and 0.618 (for RLH). Observations on vertical and horizontal spread of the disease are presented in Table 4.3 (Fig. 2).

Table 4.3 Variability in pathogenecity of *Rhizoctonia solani* isolates on susceptible and tolerant rice cultivars

Sl No	ISOLATE	AD1%		RLH%		DS	
		1R50	SD*	1R50	SD*	IR50	SD*
1	ADT	88.8	44.9	55.0	34.3	8.0	3.3
2	ALM	00.0	00.0	00.0	00.0	0.0	0.0
3	CNM	88.8	34.5	55.8	30.6	8.0	3.2
4	FBZ	87.4	38.9	51.7	30.3	8.0	4.0
5	IAR	68.2	30.9	39.3	34.0	6.3	3.5
6	KPL	62.4	32.7	40.1	32.0	6.4	3.1
7	MCP	76.9	51.8	45.7	35.1	7.0	4.6
8	MPM	81.4	46.4	43.0	36.0	7.3	5.0
9	MTU	100.0	59.2	42.9	40.6	9.0	5.3
10	PAT	55.5	47.6	52.5	35.2	7.3	4.6
11	PBR	81.4	51.8	41.4	29.5	7.3	4.6
12	PNT	81.4	26.8	48.9	25.6	7.3	3.6
13	PTB	77.7	42.1	43.4	33.3	7.0	4.0
14	RNR	85.1	40.7	49.1	30.9	7.3	4.0
15	RS5	71.3	48.9	42.1	39.8	6.6	4.6
16	RSP	1.7	1.3	12.7	9.0	1.0	1.0
17	TNL	88.8	50.3	54.3	33.6	8.0	4.6
18	TTB	81.4	34.4	45.2	34.7	7.3	4.0

*1R5O : (Susceptible); SD, SWARNADHAN (Tolerant)
Spearmans Rank Correlation Coefficient
0.813 (for **ADI),** 0.587 (for DS) and 0.618 (for RLH)

4.4 ANALYSIS OF VARIANCE FOR ADI, DS AND RLH OF THE 18 ISOLATES ON THE FOUR RICE CULTIVARS

The variation was studied for cultivars, isolates and their interaction through analysis of variance for ADI, DS and RLH. **All** these three parameters that contribute to variation in pathogenecity were highly **significant(**Table 4.4).

Table 4.4 Analysis of variance of Average Disease **I nd ex(A DI%), Relative** Lesion Height(RLH%) and Disease Score(DS) of the 18 isolates on rice cultivars

SOURCE	F	MEAN SUM OF SOUARES		
		ADI	RLH	DS
(**ultivar** ©	3	12137.60***	1678.31***	97.32***
Isolate (I)	17	4358.42***	1973.60***	35.77***
C X I	**51**	317.32***	54.81**	1.37***
ERROR	144	65.50	33.38	0.37
CV	-	14.35	15.16	**11.70**

** = Significant at 1 % level of probability by F test
*** = Significant at 0.1% level of probability by F test

4.5 SIMPLE CORRELATION STUDIES BETWEEN MORPHOLOGICAL CHARACTERS AND PATHOGENECITY IN THE 18 FUNGAL ISOLATES

Simple correlational studies between the morphological characters and pathogenecity parameters indicate a positive correlation between sclerotial size and pathogenecity (0.87 between SCD and ADI) and negative correlation between the number of **sclerotia** and pathogenecity (-0.88 between NSC and ADI). The three screening parameters ADI%, RLH% and DS showed high positive simple correlation coefficients ranging from 0.79 between SCD and RLH and 0.97 between DS and ADI (Table 4.5).

Table **4.5** Simple correlation coefficients between morphological characters and pathogenecity in the **18** sheathblight fungal isolates *

Parameter	ADI	RLH	DS	NSC	SCD
Average Disease Index (ADI)	1.00				
Relative Lesion Height (RLH)	0.90	1.00			
Disease Score (DS)	0.97	0.95	1.00		
No. of Sclerotia (NSC)	- 0.88	- 0.90	- 0.92	1.00	
Sclerotial Diameter (SCD)	0.87	0.79	*0.86*	-0.81	1.00

* = All correlation coefficients
are significant at 1% level of probability

4.6 GROUPING OF ISOLATES

The isolates from ALM and RSP are considered as avirulent with disease scores of 0 - 3. The isolates ADT, CNM, FBZ, MTU and TNL are considered as highly virulent and the rest of the isolates IAR, KPL, MCP, MPM, PAT, PBR, PNT, PTB, RNR, RS5 and TTB are considered to be moderately virulent isolates. (Table 4.6)

Table 4.6 Virulence pattern of rice sheath blight isolates

Disease Score on Susceptible Virulence (DS)	Average Disease Score	Isolates
Avinilent (0 - 3.9)	0.5	ALM,RSP
Moderately Virulent (4 - 7.9)	7.0	IAR,KPL,MCP,MPM,PAT, PBR,PNT,PTB,RNR,RS5,TTB
Virulent (8 - 9)	8.2	ADT,CNM,FBZ,MTU,TNL

4.7 TOTAL PROTEIN PATTERNS

The variation in the total protein banding patterns was very high. The number of bands ranged from 8 to 16 per isolate. There was variability in the patterns of minor bands as well as in the intensity of the bands from isolate to isolate (Fig. 3).

4.8 ISOZYMES

Out of the thirteen enzymes studied, twelve enzymes, ACP, ADH, a-EST, β-EST, GDH, ICD, LAP, MDH, PGD, PGI, POX and PPO produced well resolved and consistent electrophoretic banding patterns and strong staining activity.

No attempt was made to define the genetic composition from the isozyme data. When band was not detected for an enzyme, null allele was not proposed for that enzyme (although it could be a set of null alleles). The terms Relative mobility (Rm) and allele have been used for describing isozyme bands, depending upon genetic interpretation. The banding patterns were used as phenotypic

markers for the analysis of the eighteen isolates. The genetic basis of the isozyme phenotypes were inferred directly from the observed banding patterns.

4.8.1 Enzyme Banding Patterns

From the banding patterns of **isozymes,** a total of 153 electrophoretic phenotypes (EPs) were observed as shown in Table 4.7. All enzymes were polymorphic among the isolates with 7-18 distinct electromorphs. An Electrophoretic Phenotype (EP) is defined as a group of isolates having distinctive combinations of isozyme phenotypes in common. The method of Nygaard *et* ***al.,*** (1989) was used to produce diagrammatic representations of all EPs.

Table 4.7 Comparative Electrophoretic Phenotypes (EPs) of the 12 isozymes in the **18** sheath blight fungus isolates of India

	Electrophoretic phenotype for the enzyme											
	ACP	**ADH**	**α-EST**	p-EST	**GDH**	1CD	LAP	MDH	PGD	PG1	**POX**	**PPO**
ADT	1	1	1	1	1	1	1	1	1	1	1	1
ALM	2	2	2	2	2	2	2	2	2	2	2	2
CNM	3	1	3	3	1	3	3	3	3	3	3	3
FBZ	4	**1**	4	4	3	4	4	4	4	4	4	4
IAR	3	3	5	5	4	5	5	4	5	5	5	5
KPL	5	3	6	6	1	6	6	4	6	6	6	6
MCP	5	3	7	7	4	7	7	4	7	7	7	7
MPM	5	4	8	8	1	4	1	5	8	7	8	8
MTU	5	5	9	9	5	8	7	6	9	7	3	9
PAT	5	5	10	10	5	4	8	7	10	8	9	10
PBR	6	3	11	11	1	4	9	8	11	9	10	8
P1VT	5	3	12	12	4	9	1	9	12	9	10	10
PTB	5	1	12	13	1	4	10	10	13	10	11	8
RNR	5	6	13	14	1	3	11	11	14	11	12	10
RS5	5	3	14	15	1	10	12	12	15	7	6	**11**
RSP	7	3	15	16	6	11	13	13	**16**	12	2	12
TNL	3	7	16	17	7	10	6	14	17	13	13	10
TTB	5	3	17	18	1	10	14	15	18	14	13	10
Total EPs	7	7	17	18	7	11	14	15	18	14	13	12
Common EP	5	3	-	-	1	4		4	-	7	-	10

4.8.2 Isozyme Polymorphism

Acid Phosphatase (ACP)

Seven EPs were identified. Majority of the isolates (ten) showed EP 5 with double banded pattern and was present in ten isolates. All virulent isolates shared common isozyme band at Rm 0.22 which can be considered as the most common band (Fig. 4 & Zym. 1).

Alcohol Dehydrogenase (ADH)

Seven EPs were detected. EP 3 was the most common phenotype, characterised by a single banded pattern and present in eight isolates. The number of bands ranged from one to three. The band at Rm 0.17 was the most common band and was present in thirteen isolates (Zym. 2).

a - Esterase (a - EST)

Seventeen EPs were identified. Each isolate had unique EP except PNT and PTB sharing EP 12. The number of bands ranged from one to four. The most common band was the band with Rm 0.38. It was present in seven isolates (Fig. 5 & Zym. 3).

β - Esterase (β - EST)

Eighteen EPs were detected. Each of the isolates showed unique EP. The number of bands ranged from one to four. No common band was detected (Fig. 5 & Zym 4).

Glutamate Dehydrogenase (GDH)

Seven EPs were identified. EP 1 was shared by 50% of the isolates. Bands of virulent isolates ranged from one to three, whereas avirulent isolates (ALM and RSP) showed three to five bands. The band with Rm 0.2 was shared by all the virulent isolates (Zym. 5).

Isocitrate Dehydrogenase (ICD)

Eleven EPs were recorded in this enzyme and EP 4 occurred in five isolates. Mostly double banded pattern was observed. The band with Rm 0.14 was the most common band present in eleven isolates (Zym. 6).

Leucine Aminopeptidase (LAP)

Fourteen EPs were observed. EP 1 occurred in three isolates. The isolates showed two to five bands. All virulent isolates shared a common isozyme band with Rm 0.40. Avirulent isolates showed single banded pattern (Zym. 7).

Malate Dehydrogenase (MDH)

Fifteen EPs were observed. EP 4 was the most common phenotype and was seen in four isolates. The band at Rm 0.15 was common for sixteen isolates (Fig. 6 & Zym. 8).

6-Phosphogluconic Dehydrogenase (PGD)

Eighteen EPs were detected. Each of the isolates had several isozymes that were variable in both mobility and number. The isolates showed two to six bands. The band at Rm 0.16 was the most common band present in fifteen isolates (Zym. 9).

Phosphogluco Isomerase (PGI)

Fourteen EPs were detected. EP 7 was characterised by a single banded pattern and was present in four isolates. The number of bands ranged from one to four among the isolates. The band at Rm 0.30 was common for fifteen isolates (Zym. 10).

Peroxidase (POX)

In all, thirteen EPs were observed. The number of bands ranged from one to four. Avirulent isolates (ALM and RSP) had EP 13 in common, which was characterised by a single band at Rm 0.15 (Zym. 11).

Polyphenol Oxidase (PPO)

Twelve EPs were observed. The isolates showed one to four bands. The EP 10 was observed to be common in five isolates. The band at Rm 0.21 was the most common band and was present in ten isolates (Zym .12).

4.8.3 Isozyme Polymorphism and their Association with Pathogenecity

From the above observations on the isozyme phenotypes obtained, a summary statement on the association of the isozyme polymorphism with virulence / avirulence of the isolates is prepared as given below (Table 4.8).

Table 4.8 Isozyme Polymorphism in 18 Indian Isolates of Rice Sheath blight Fungus *Rhizoctonia Solani* and Association with Virulence (V) and Avirulence (AV)

ISOZYME	No Of Isozyme Phenotypes Observed	Polymorph differing between V & AV isolates	Utility of the Isozyme
Acid Phosphatase (ACP)	7	Rm 0.22	V / AV distinction
Alcohol Dehydrogenase (ADH)	7	0	None
a Esterase (a-EST)	17	0	Finger-Printing
β Esterase (β-EST)	18	0	Finger-Printing
Glutamate Dehydrogenase (GDH)	7	Rm 0.20	V / AV distinction
Isocitrate Dehydrogenase (ICD)	11	0	None
Leucine Aminopeptidase (LAP)	14	Rm 0.40	V / AV distinction
Malate Dehydrogenase (MDH)	15	0	None
6-Phosphogluconic Dehydrogenase PGD)	18	0	Finger-Printing
Phosphogluco Isomerase (PGI)	14	0	None
Peroxidase (POX)	13	Rm 0.15	V / AV distinction
Polyphenol Oxidase (PPO)	12	0	None

The utility of the above isozyme data for discriminating between virulence and avirulence of isolates was critically tested with a new isolate (RS 319), the virulence level of which is not known. For this purpose, two of the four isozymes namely Acid phosphatase and Leucine amino peptidase which showed virulence / avirulence distinction in the isolates were used. Based on the banding pattern obtained the new isolate was identified as virulent. Standard glass house screening technique later confirmed that isolate indeed as virulent.

4.9 DNA STUDIES

4.9.1 Random Amplified Polymorphic DNAs (RAPDs)

Out of the Polymerase Chain Reactions (PCRs) with ten primers, nine primers gave multiple products in 0.34 - 1.64 Kb range and no amplification products were observed for primer **OPA06.** The patterns of **the** bands obtained with primers used in this study were consistent. The number of amplified products was variable, depending upon the primer and isolate.

Table 4.9 RAPD Polymorphism in the 18 Indian Isolates of Rice Sheathblight Fungus ***Rhizoctonia solani***

Primer	Maximum Fragments per **Isolate** (no)	Average Fragments per **Isolate** (no)	% of Fragments Common to all the **Isolates**	**%** of overall Polymorphism detected
OPA10	**31**	22.0	6.4	83.8
OPB01	39	18.5	2.5	94.8
OPC01	30	13.0	0.0	100.0
OPE20	23	12.5	0.0	91.3
OPF06	27	10.3	11.1	81.4
OPH18	40	18.0	0.0	87.5
OPQ01	21	9.5	0.0	100.0
OPR01	27	12.6	0.0	88.8
0PZ20	31	13.5	3.2	93.5

The primers used in this study produced intragroup polymorphism indicating a high level of genomic variability within sheathblight fungus, ***R.solani*** albeit belonging to same Anastomosis Group(AG).

A total of 269 bands were scored. Some bands were common to all the isolates, whereas others were produced specifically by the two avirulent isolates, but not by the virulent isolates. Similarly, the bands produced by the virulent isolates, were not produced by avirulent isolates. When all the isolates were considered, average number of amplified DNA fragments per isolate varied from 9.5 to 22.0.

The maximum number of fragments per isolate per primer ranged from 21 to 40. The percentage of fragments common to all isolates ranged from 0.0 to 11.1. The percentage of polymorphism detected per primer isolate varied from 81.4 to 100.0 (Table 4.9).

Primer OPA10

The primer OPA10 yielded 31 bands. The number of bands ranged from 17-27 per isolate. Two bands were common to all isolates (611 bp and 1078 bp). A band at 457 bp was present in all virulent isolates only and could serve as molecular marker. Three unique bands were present in ADT, MCP and TTB isolates (Fig. 7).

Primer OPB01

The primer OPB01 amplified 39 bands. The number of bands per isolate was 14 to 23. Only one band at 1013 bp was common to all isolates. One band of 671 bp was present in all virulent isolates and on band of 700 bp was present in both avirulent isolates. One unique band was present in isolate from MPM (Fig. 8).

Primer OPC01

The primer OPC01 resulted in amplification of 30 bands. The number of bands varied from 8 to 18 per isolate. All bands were polymorphic (Fig. 9).

Primer OPE20

The primer OPE20 has produced 23 different fragments. The bands per isolate ranged from 8 to 14. One common band of 538 bp size was shared by all virulent isolates and one common band of 1472 bp size was present in both the avirulent isolates. One unique band was present in isolate RSP (Fig. 10).

Primer OPF06

The total number of bands were 27. The number of bands ranged from 7 to 17 per isolate. Three common bands of 2224 bp, 1466 bp and 1267 bp sizes were present in all isolates. Three bands

of 2351 bp, 1005 bp and 919 bp sizes were present in both avirulent isolates. Two unique bands were present in ALM and RSP (Fig. 11).

Primer OPH18

The primer OPH18 amplified 40 bands. The number of bands ranged from 14 to 22 per isolate. One common band of 1664 bp size was present in all virulent isolates. Four unique bands were present in the isolates, two in PAT and one each in ALM and CNM (Fig. 12).

Primer OPQ01

The primer OPQ01 produced 21 bands. The bands per isolate ranged from 5 to 14. Three common bands of 1793 bp, 1639 bp, and 858 bp sizes were present in all virulent isolates (Fig. 13).

Primer OPR01

The total number of bands amplified by this primer was 27. The number of bands per isolate varied from 9 to 15. Three bands of 1664 bp, 1400 bp and 1225 bp sizes were present in all virulent isolates and one common band of size 1220 bp was present in both avirulent isolates. Three unique bands were present, two of which were amplified in the isolate RSP and one was present in the isolate TTB(Fig. 14).

Primer OPZ20

The primer OPZ20 yielded 31 bands. About 9 to 18 bands were observed per isolate. One common band of 1010 bp size was shared by all the isolates. One common band of 749 bp size was common for the two avirulent isolates. One unique band was present in the isolate TTB (Fig. 15).

4.9.2 RAPD polymorphism and association with pathogenecity of Rice ShB Isolates

RAPD analysis provides a rapid method of differentiating among the isolates and between the pathogenic and non-pathogenic isolates. The utility of RAPD polymorphism in finger printing and association with pathogenecity was given in the Table 4.10. A rice ShB fungal isolate (RS 319) with

unknown level of virulence was subjected to RAPD analysis with OPAIO primer and the isolate was identified as virulent because of the presence of 457 bp molecular marker band. This was confirmed later by the standard glass house screening.

Table 4.10 Association of RAPD Polymorphism in Rice Sheath Blight Isolates with Virulence and Avirulence

Primer	Polymorphs Common for all 18 Isolates (bp)	Polymorphs Common for Virulent Isolates (bp)	Polymorphs Common for Avirulent Isolates (bp)
OPAIO	1078 611	457	—
OPB01	1013	671	700
OPC01	—	—	—
OPE20	—	538	1472
OPF06	2224 1466 1267	—	2351 1005 919
OPH18	—	1664	—
OPQ01	—	1793 1639 858	—
OPR01	—	1664 1400 1225	1220
OPZ20	1010	—	749

4.10 DATA ANALYSIS

4.10.1 Similarity Matrices

The similarity matrices derived from zymogram, RAPD and combined data were shown in Table 4.11, Table 4.12 and Table 4.13. Within the AG 1, the similarity coefficients were all higher than 40 %. The maximum amount of similarity between any two of the isolates (0.85) was recorded when the simUarities were calculated based on the RAPD profiles (between isolates IAR and KPL).

The maximum similarity in case of zymograms (0.84) was shared between MCP and MPM isolates, MPM and PTB isolates and PBR and PNT isolates. The maximum amount of similarity (0.82) was recorded in case of the combined data between IAR and KPL isolates.

Table 4.11 SIMILARITY MATRIX OF ISOZYME PATTERNS

	ADT	ALM	CNM	FBZ	IAR	KPL	MCP	MPM	MTU	PAT	PBR	PNT	PTB	RNR	RS5	RSP	TNL	TTB
ADT	1	0.7	0.76	0.78	0.80	0.76	0.75	0.79	0.74	0.76	0.75	0.78	0.80	0.76	0.77	0.678	0.76	0.78
ALM		1	0.70	0.71	0.71	0.70	0.72	0.71	0.74	0.72	0.72	0.72	0.69	0.73	0.75	0.736	0.73	0.76
CNM			1	0.80	0.74	0.70	0.77	0.78	0.76	0.75	0.75	0.76	0.79	0.78	0.75	0.697	0.75	0.79
FBZ				1	0.81	0.76	0.79	0.79	0.77	0.79	0.78	0.79	0.82	0.80	0.77	0.726	0.79	0.83
IAR					1	0.74	0.77	0.83	0.76	0.79	0.75	0.81	0.82	0.81	0.83	0.697	0.76	0.79
KPL						1	0.77	0.75	0.76	0.73	0.72	0.75	0.76	0.76	0.76	0.692	0.77	0.78
MCP							1	0.84	0.83	0.76	0.78	0.78	0.79	0.80	0.82	0.688	0.80	0.79
MPM								1	0.78	0.78	0.79	0.82	0.84	0.80	0.83	0.697	0.77	0.79
MTU									1	0.78	0.79	0.75	0.77	0.80	0.80	0.712	0.80	0.80
PAT										1	0.78	0.81	0.78	0.79	0.76	0.702	0.77	0.79
PBR											1	0.84	0.79	0.77	0.78	0.692	0.76	0.79
PNT												1	0.80	0.82	0.77	0.750	0.77	0.81
PTB													1	0.81	0.76	0.716	0.77	0.79
RNR														1	0.80	0.731	0.79	0.82
RS5															1	0.716	0.80	0.82
RSP																1	0.72	0.75
TNL																	1	0.84
TTB																		1

Table 4.12 SIMILARITY MATRIX OF RAPD PROFILES

	ADT	ALM	CNM	FBZ	IAR	KPL	MCP	MPM	MTU	PAT	PBR	PNT	PTB	RNR	RSS	RSP	TNL	TTB
ADT	1	0.49	0.73	0.69	0.74	0.70	0.72	0.71	0.76	0.69	0.70	0.72	0.74	0.73	0.75	0.47	0.66	0.67
ALM		1	**0.50**	0.51	0.48	0.47	0.48	0.49	0.51	0.50	0.47	0.48	0.51	0.52	0.52	0.65	0.45	0.47
CNM			1	0.70	0.70	0.69	0.79	0.73	0.74	0.69	0.76	0.74	0.74	0.75	0.72	0.47	0.65	0.71
FBZ				1	0.79	0.80	0.73	0.69	0.75	0.78	0.75	0.76	0.73	0.72	0.73	0.45	0.66	0.68
IAR					1	0.84	0.67	0.68	0.72	0.76	0.71	0.77	0.76	0.72	0.76	0.43	0.65	0.65
KPL						1	0.71	0.70	0.74	0.79	0.72	0.77	0.71	0.72	0.74	0.43	0.65	0.67
MCP							1	0.78	0.77	0.72	0.77	0.74	0.78	0.77	0.74	0.47	0.74	0.71
MPM								1	0.82	0.75	0.75	0.73	0.77	0.74	0.76	0.46	0.76	0.66
MTU									1	0.75	0.80	**0.72**	0.80	0.75	0.74	0.46	0.68	0.69
PAT										1	0.73	0.75	0.75	0.72	0.76	0.43	0.72	0.68
PBR											1	0.72	0.79	0.75	0.74	0.41	0.73	0.69
PNT												1	0.78	0.75	0.75	0.49	0.69	0.69
PTB													1	0.80	0.78	0.43	0.75	0.70
RNR														1	0.79	0.47	0.68	0.70
RSS															1	0.43	0.72	0.72
RSP																1	0.44	**0.42**
TNL																	1	0.73
TTB																		1

Table 4.13 SIMILARITY MATRLX OF 18 SHEATH BLIGHT ISOLATES
(combined data of zymograms **and** RAPDs)

	ADT	ALM	CNM	FBZ	IAR	KPL	MCP	MPM	MTU	PAT	PBR	PNT	PTB	RNR	RS5	RSP	TNL	TTB
ADT	1	0.58	0.74	0.73	0.76	0.72	0.73	0.74	0.75	0.72	0.72	0.75	0.76	0.74	0.76	0.55	0.71	0.72
ALM		1	0.58	0.59	0.58	0.57	0.58	0.58	0.60	rt.59	0.57	G.5S	0.58	0.60	0.61	0.68	**0.56**	0.59
CNM			1	0.74	0.72	0.70	0.78	0.75	0.75	0.72	0.75	0.74	0.76	0.76	0.73	0.56	0.69	0.74
FBZ				1	0.80	0.79	**0.75**	0.73	0.76	0.78	0.77	0.78	0.77	0.75	0.75	0.57	0.72	0.75
IAR					1	0.82	0.71	0.74	0.74	0.77	0.72	0.79	0.78	0.76	0.79	0.55	0.70	0.71
KPL						1	0.74	0.72	0.75	0.77	0.72	0.76	0.73	0.73	0.75	0.54	0.70	0.72
MCP							1	0.80	0.80	0.74	0.78	0.76	0.78	0.78	0.77	0.56	0.77	0.75 I
MPM								1	0.80	0.77	0.77	0.77	0.80	0.77	0.79	0.56	0.77	0.72
MTU									I	0.76	0.79	0.73	0.79	0.77	0.77	0.57	0.73	0.73
PAT										1	0.75	0.77	0.76	0.75	0.76	0.54	0.74	0.72
PBR											1	0.77	0.79	0.76	0.76	0.53	0.74	0.73
PNT												1	0.81	0.78	0.76	0.60	0.72	0.74
PTB													**l**	0.81	0.77	0.55	0.76	0.74
RNR														1	0.79	0.58	0.73	0.75
RSS															1	0.55	0.75	0.75
RSP																1	0.56	0.56
TNL																	1	0.78
TTB																		1

Similarly, the minimum amount of similarity between any two of the isolates (0.42) was observed when the similarity was calculated based on RAPD profiles (between RSP and TTB isolates), followed by that based on the combined data (0.53) of RAPD and zymogram profiles (between PBR and RSP isolates). The minimum amount of similarity (0.69) in case of zymograms was observed between KPL and RSP isolates and ALM and PTB isolates.

When the difference between the maximum and minimum similarities in a single set of data were considered, it was maximum (44%) in case of RAPDs and minimum (15%) in case of zymograms; the difference of 28% observed in the combined set fell very near to the average of RAPDs and isozyme sets (29%).

4.10.2 UPGMA Analysis

The total variability is summarised in the clustering pattern which were constructed from simple matching coefficients. The two sets of data were analysed individually, as well as combined. UPGMA analysis was done for protein data, RAPD data and combined data of both protein and RAPD data.

Isozyme Polymorphism

The UPGMA analysis of isozyme data showed two patterns of clustering. In both the patterns of clustering. The rooted tree showed two main clusters. The major cluster included all the sixteen virulent isolates and the minor cluster included the two avirulent isolates. The two clusters joined at 0.71 similarity. The two avirulent isolates were split at 0.74.

From the major cluster, at 0.75 similarity, KPL has separated from the other isolates. At 0.76 similarity CNM isolate has separated from the major cluster. At 0.77 similarity ADT isolate has also got separated from the rest of the isolates. Further the branching of clustering showed two patterns.

According to the pattern presented in Fig. 16, at 0.78 similarity PAT isolate was separated as a single isolate. The remaining major cluster was divided into 2 sub clusters. The minor sub cluster 0.79 similarity has divided into MTU and PBR isolates. The major sub cluster has divided two groups

at 0.79 similarity. The minor group consisted three isolates i.e. FBZ, TNL and TTB. The major group has divided into two sub groups at 0.80 similarity. One major group consisted four isolates from IAR, RSS, MCP and MPM and the minor group consisted of three isolates PNT, PTB and RNR.

According to the pattern presented in Fig. 17, the major cluster was separated into two sub clusters at 0.79 similarity. From the minor sub cluster, PAT has separated as a single isolate. At 0.81 similarity the isolate from PBR separated from the remaining two isolates PNT and PTB. From the major sub cluster at 0.79 similarity, MTU has separated out, leaving two major groups. One group consisted FBZ, TNL and RNR isolates and the other group consisted IAR, MCP, MPM and RS5 isolates.

RAPD Profiles

The UPGMA analysis of RAPDs separated the isolates into two clusters at .046 similarity (Fig. 18). Two avirulent isolates ALM and RSP constituted minor cluster. And the remaining virulent isolates formed a loose dissimilar second major cluster. The major group was again split at 0.69 similarity into two sub clusters. The two isolates TNL and TTB formed one minor sub cluster and the rest of the isolates formed major sub cluster in which two groups were linked at 0.73 similarity. The minor group consisted of five isolates viz. FBZ, IAR, KPL, PAT and PNT.

From the major group, ADT separated at 0.73 similarity. The major group again separated into two sub groups. The minor sub group consisted of CNM and MCP and the major sub group consisted MPM, MTU, PTB and RS5 isolates.

Combined analysis of Isozyme Polymorphism and RAPD profiles

In the phenogram obtained by combined data, three patterns of clustering resulted. The patterns shared 55.5% similarity where ten isolates were clustered in a similar pattern.

All the three patterns showed, two main clusters at 0.57 similarity. The major cluster included all the sixteen virulent isolates and the minor cluster included the two avirulent isolates. The two avirulent isolates ALM and RSP were split at 0.69 similarity.

From the major cluster, the isolates TNL and TTB got separated at 0.73 similarity level. At 0.74 similarity coefficient ADT was separated from the rest of the isolates. The remaining major cluster at 0.75 level was divided into two subclusters. From the minor subcluster again at 0.77 similarity level PAT isolate was separated leaving a group of three isolates comprising FBZ, IAR and KPL. From the major sub cluster 0.76 similarity level CNM isolate was singled out, leaving two groups at 0.77 similarity.

Further branching of the clustering showed three patterns. According to the pattern presented in Fig. 19, at 0.77 similarity level, two major groups were present. One group consisting isolates MCP, MPM, MTU and PBR, where PBR isolate separated at 0.78 similarity level from the rest of the three isolates. And the other group consisted PNT, PTB, RNR and RS 5, where at 0.77 similarity, RS 5 isolate was separated from the other three isolates.

According to the pattern presented in Fig. 20, at 0.77 similarity level, two groups were separated. The minor group consisted RNR and RS 5 which were separated at 0.79 similarity. The major group separated into two subgroups at 0.78 similarity. One subgroup consisted MCP, MPM and MTU isolated and other group consisted PBR, PNT and PTB isolates.

According to the pattern presented in Fig. 21, at 0.77 similarity level, two groups were separated out. From one group at 0.78 level RS 5 isolate got separated out, leaving FBZ, IAR and KPL at 0.79 similarity level. And from the other group at 0.78 level PBR isolate was separated out and MCP, MPM and MTU isolates were grouped at 0.80 similarity level.

Fig. 1. RICE SHEATH BLIGHT (*RHIZOCTONIA* ***SOLANI***) ISOLATES FROM DIFFERENT LOCATIONS SHOWING VARIABILITY IN CULTURAL CHARACTERSTICS AND SCLEROTIAL FORMATIONS

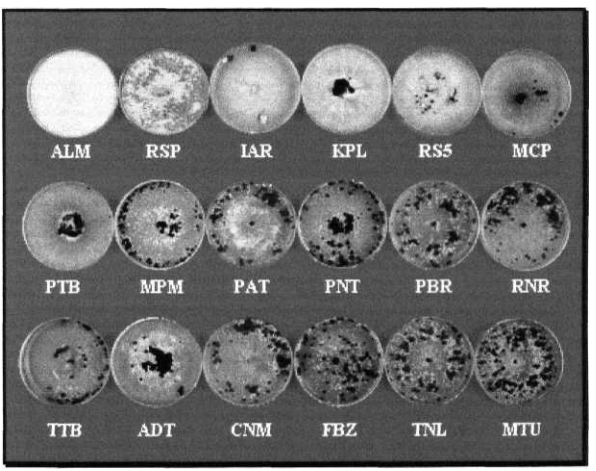
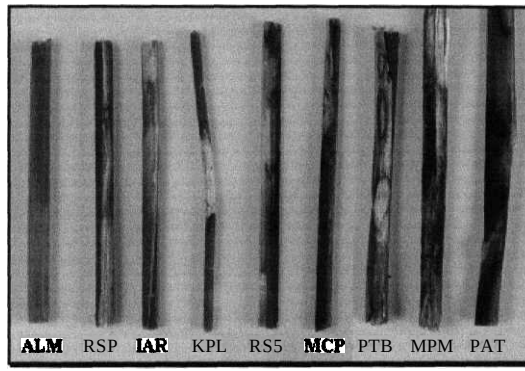


Fig. 2. SHEATHS INFECTED WITH DIFFERENT ISOLATES OF ShB FUNGUS SHOWS TYPICAL BLIGHT SYMPTOMS ON SUSCEPTIBLE IR50. ISOLATES FROM CNM, FBZ, TNL AND MTU SHOW SYMTOMS AND SCLEROTIAL FORMATION EVEN ON THE PANICLES.



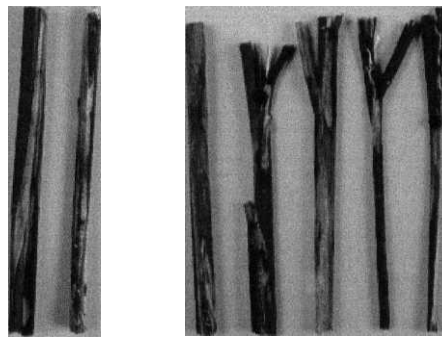


Fig. 3. ELECTROPHORETIC SEPARATION OF NATIVE SOLUBLE PROTEINS OF 18 **ShB** FUNGAL **ISOLATES.** LANE a(ADT) - LANE r(TTB)

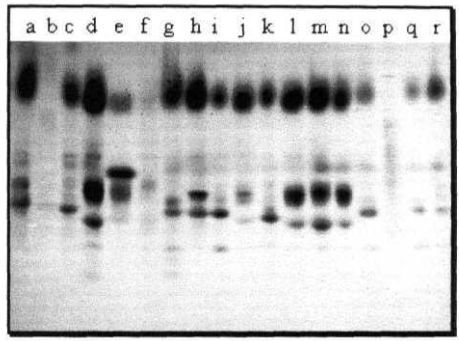


Fig. 4. ACID PHOSPHATASE BANDING PATTERNS OF ShB FUNGAL ISOLATES LANE a(ADT) - LANE **r(TTB)**

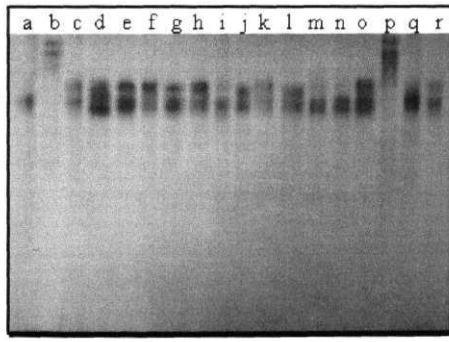


Fig. 5. ESTERASE ISOZYME BANDING PATTERNS OF ShB FUNGAL ISOLATES. LANE a (ADT) - LANE p (RSP), LANE *x* - TESTER ISOLATE, LANE q (TNL) AND LANE r (TTB)

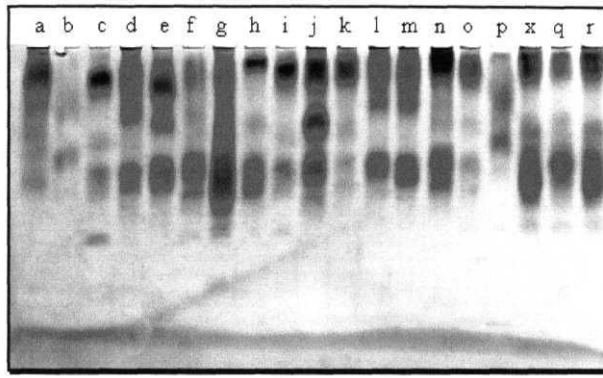
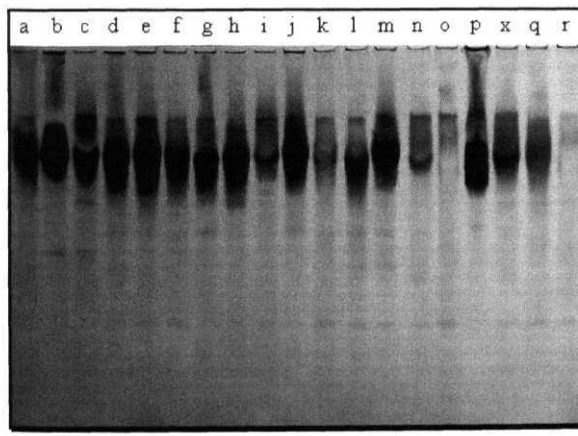


Fig. 6. MALATE DEHYDROGENASE ISOZYME BANDING PATTERNS OF ShB FUNGAL ISOLATES. LANE a **(ADT)** - LANE p (RSP), LANE x - TESTER ISOLATE, LANE q (TNL) AND LANE r (TTB)



ACID PHOSPHATASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.02																—		
0.03		—																
0.05		—																
0.06																—		
0.09		—														—		
0.15			—	—	—												—	
0.16						—	—	—	—	—		—	—	—	—			—
0.18											—							
0.19				—														
0.22	—		—	—	—	—	—	—	—	—	—	—	—	—	—		—	—

Zym. 1

ALCOHOL DEHYDROGENASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.17		—			—	—	—	—	—	—	—	—		—	—	—		—
0.19	—		—	—									—				—	
0.22		—							—	—				—			—	
0.25								—										
0.26									—	—								
0.27		—																
0.30								—										

Zym. 2

α ESTERASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.02																—	—	—
0.05								—						—				
0.06							—											
0.07									—	—	—							
0.09						—											—	—
0.10	—		—															
0.11							—											
0.12					—										—			
0.18																—		
0.20																		
0.28	—																	
0.36																	—	
0.37		—				—												
0.38					—			—			—	—	—		—		—	
0.41										—								
0.42				—		—												
0.43			—															
0.45					—													
0.48				—														
0.50		—				—			—									

Zym. 3

β ESTERASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.06												—						
0.07													—					
0.09				—														
0.19	—											—	—					
0.22		—				—												
0.23				—													—	
0.24										—				—	—			
0.25																		
0.28					—		—				—							—
0.29							—		—							—		
0.32			—															
0.35	—																	
0.38									—									
0.42			—															
0.45	—						—											
0.46													—					
0.48								—	—		—			—	—		—	
0.49			—															
0.51																		—
0.53																	—	
0.61							—										—	—
0.64						—	—											
0.81	—								—									

Zym. 4

GLUTAMATE DEHYDROGENASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.13		—														—		
0.14																		
0.17				—														
0.18																—		
0.20	—		—	—	—	—	—	—	—	—	—	—	—	—	—		—	—
0.23		—																
0.24																—	—	
0.27									—	—								
0.30	—		—			—		—	—	—	—		—	—	—		—	—
0.33		—														—		
0.36																—		
0.47				—														

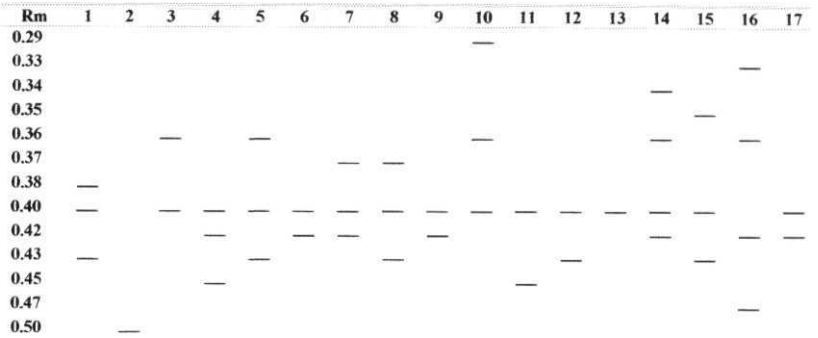
Zym. 5

ISOCITRATE DEHYDROGENASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.01		—					—											
0.11												—						
0.14			—	—	—	—	—	—	—	—	—		—	—				
0.26																—		
0.29									—									
0.30																—		
0.33						—	—											
0.35			—				—		—					—	—		—	—
0.38	—			—	—			—		—	—	—	—					
0.40						—												
0.45					—													

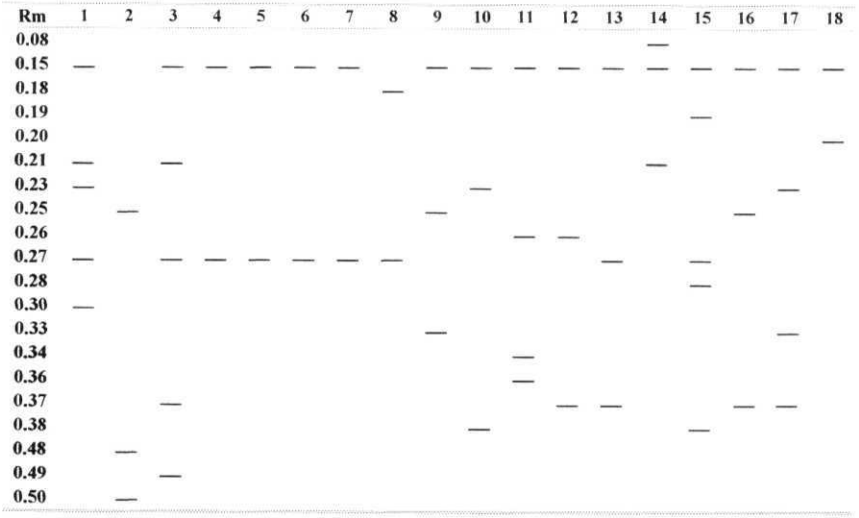
Zym. 6

LEUCINE AMINOPEPTIDASE



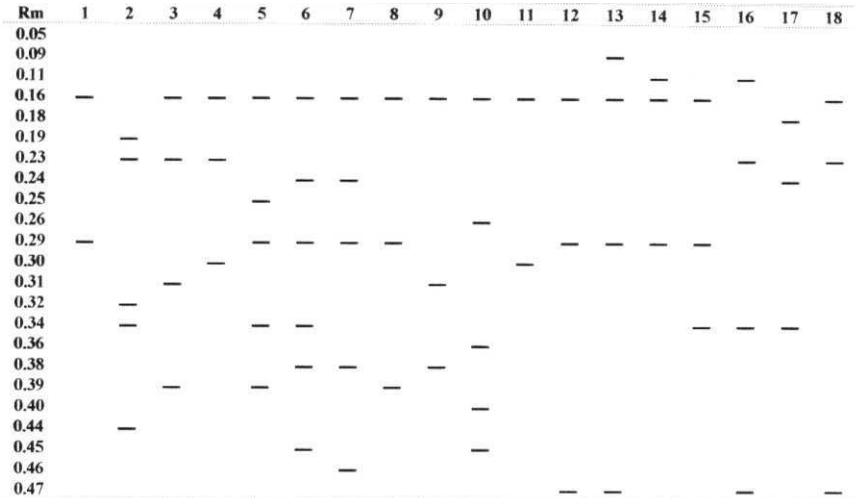
Zym. 7

MALATE DEHYDROGENASE



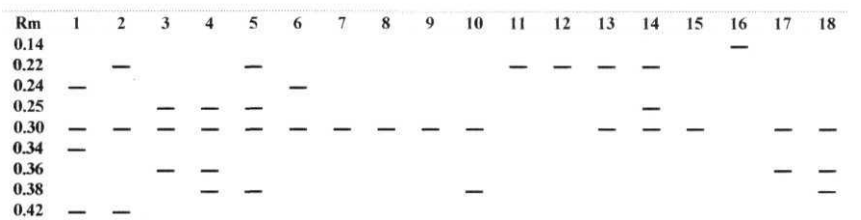
Zym. 8

6 PHOSPHO GLUCONIC DEHYDROGENASE



Zym. 9

PHOSPHOGLUCO ISOMERASE



Zym. 10

PEROXIDASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.15		—																
0.32	—															—		
0.34			—				—	—	—									
0.36														—			—	—
0.38					—	—									—			
0.40				—									—					
0.42										—	—	—						
0.60								—		—								
0.61							—				—	—	—					
0.64													—					
0.65					—													
0.66														—				
0.68													—	—				

Zym. 11

POLYPHENOL OXIDASE

Rm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0.13		—				—												
0.15							—											
0.16				—														
0.17					—													
0.18	—		—						—							—		
0.21			—				—	—		—	—	—	—	—			—	—
0.31																—		
0.38																—		
0.50							—											
0.52																		
0.64			—				—	—	—		—		—		—			

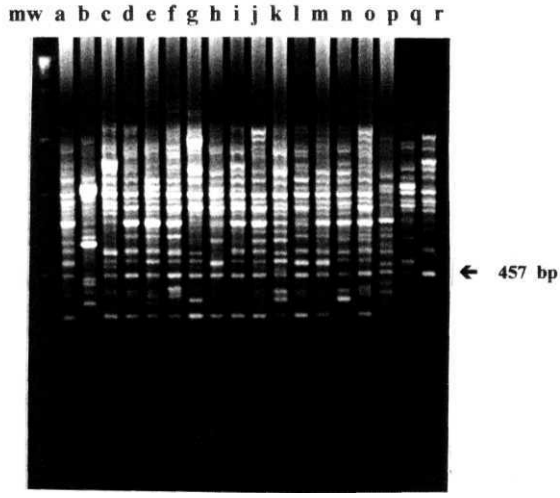
Zym. 12

The lanes shown in the Fig. 7 through Fig. 15 are as follows :

a - ADT	g - MCP	m - PTB
b - ALM	h - MPM	n - RNR
c - CNM	i - MTU	o - RS5
d - FBZ	j - PAT	p - RSP
e - IAR	k - PBR	q - TNL
f - KPL	1 - PNT	r - TTB

mw - DNA 1kb ladder (in all Figs except Fig. 14)
mw - 123 bp ladder (in Fig. 14)

Fig.7 RAPD banding patterns for isolates with primer OPA10



I Fig. 8 RAPD banding patterns for isolates with primer OPBOl

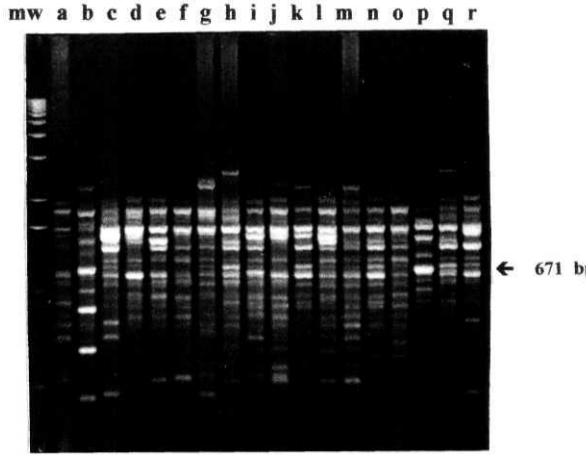
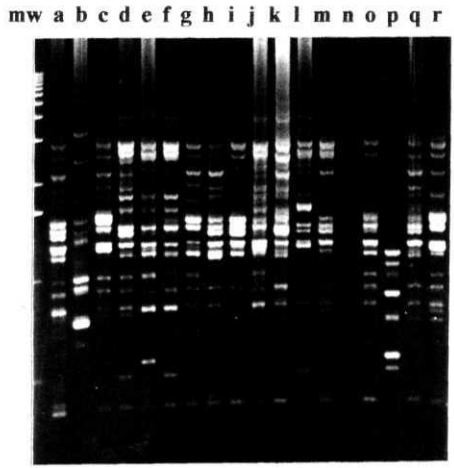
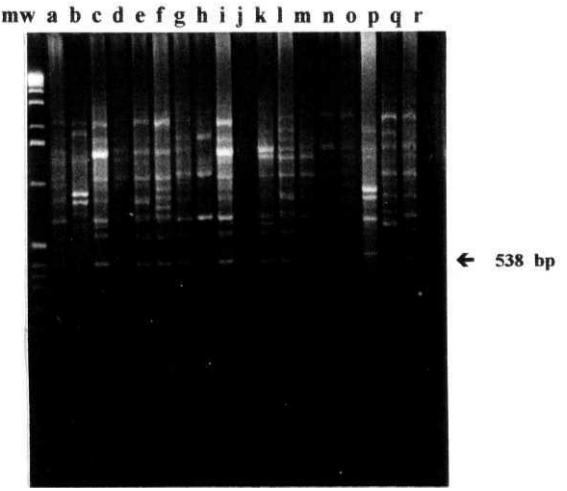


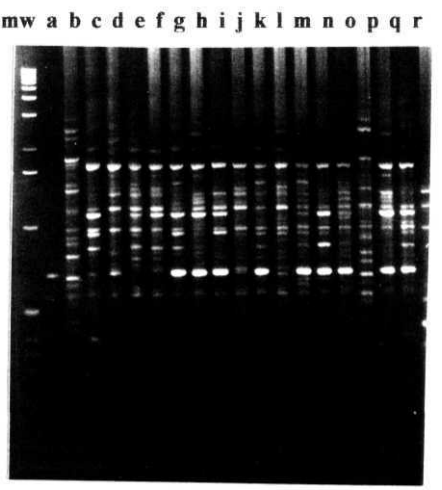
Fig. 9 RAPD banding patterns for isolates with primer OPC01



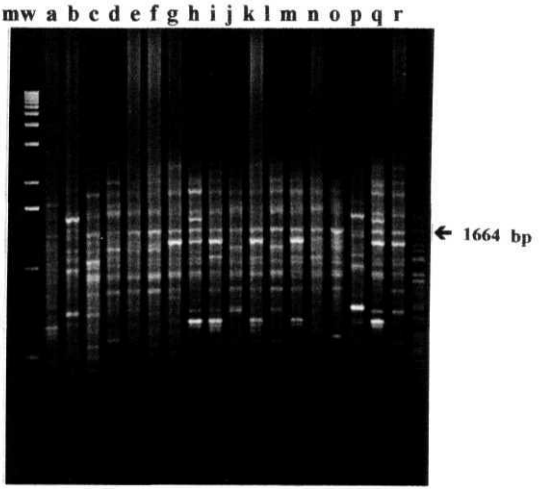
I Fig. 10 RAPD banding patterns for isolates with primer **OPE20** I



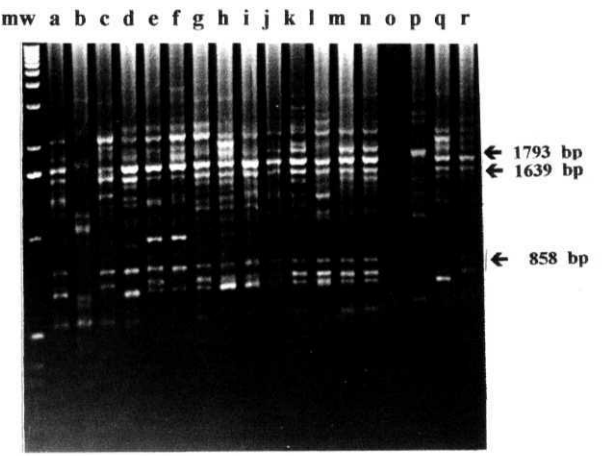
I Fig. **11** RAPD banding patterns for isolates with primer **OPF06** I



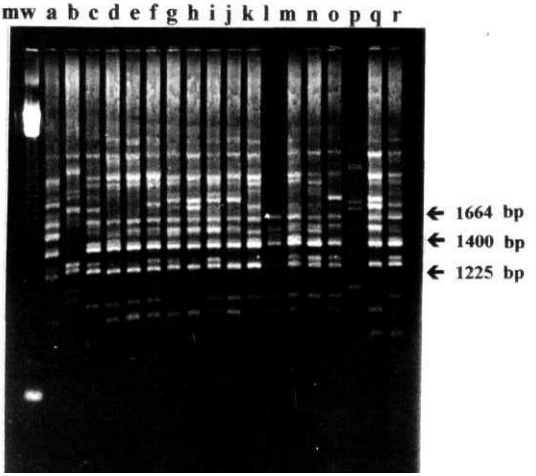
I Fig. 12 RAPD banding patterns for isolates with primer **OPH18 I**



I Fig. 13 RAPD banding patterns for isolates with primer **OPQ01 I**



I Fig. 14 RAPD banding patterns for isolates with primer OPROl J



J Fig. **15 RAPD** banding patterns for isolates with primer **OPZ20** J

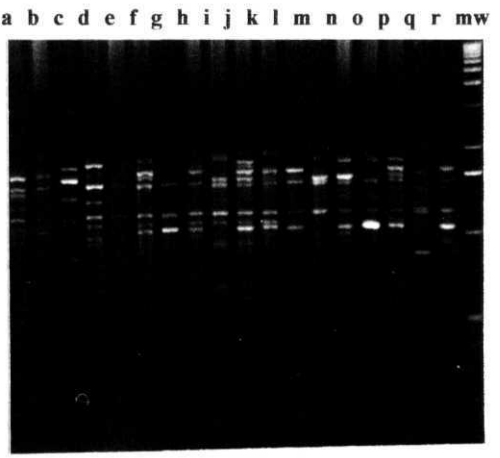


Fig. 16 CLUSTER DIAGRAM FOR 18 RICE SHEATHBLIGHT FUNGAL ISOLATES BASED ON ISOZYMES PATTERNS -1

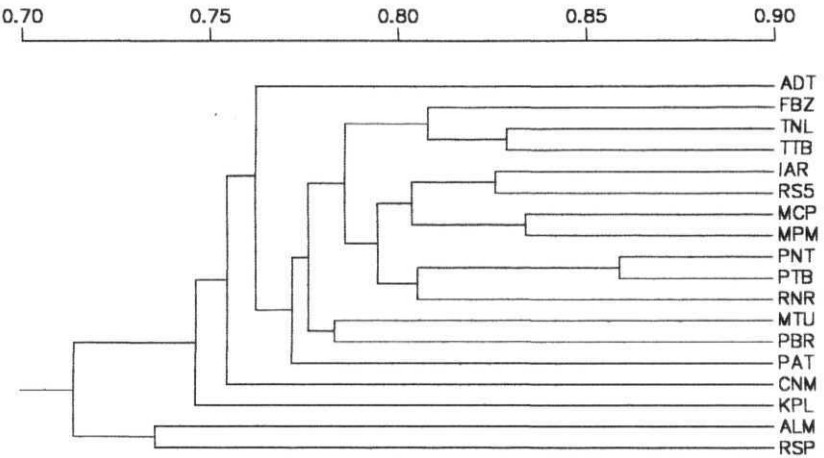


Fig. 17 CLUSTER DIAGRAM FOR 18 RICE SHEATHBLIGHT FUNGAL ISOLATES BASED ON ISOZYMES PATTERNS - II

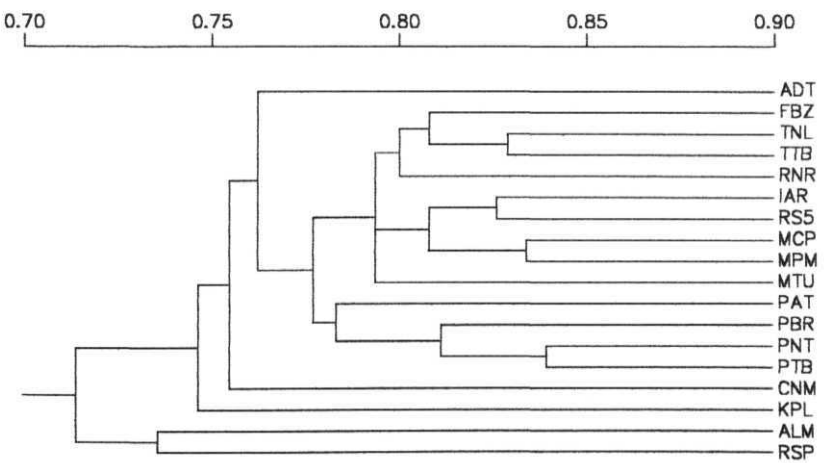


Fig. 18 CLUSTER DIAGRAM FOR 18 RICE SHEATHBLIGHT FUNGAL ISOLATES BASED ON RAPD PROFILES

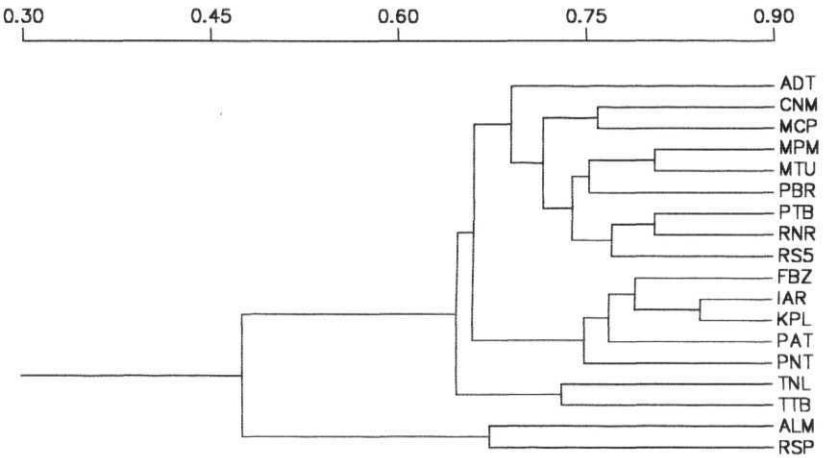


Fig. 19 CLUSTER DIAGRAM FOR 18 RICE SHEATHBLIGHT FUNGAL ISOLATES BASED ON ISOZYMES PATTERNS AND RAPD PROFILES - I

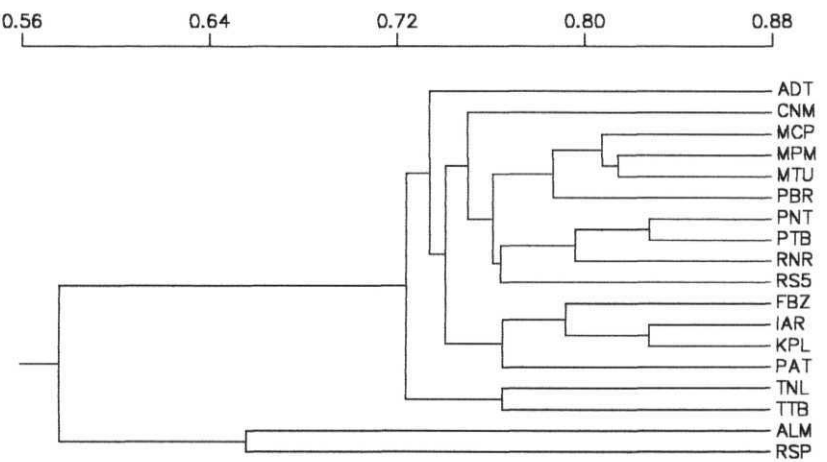


Fig. 20 CLUSTER DIAGRAM FOR 18 RICE SHEATHBLIGHT FUNGAL ISOLATES BASED ON ISOZYMES PATTERNS AND RAPD PROFILES - II

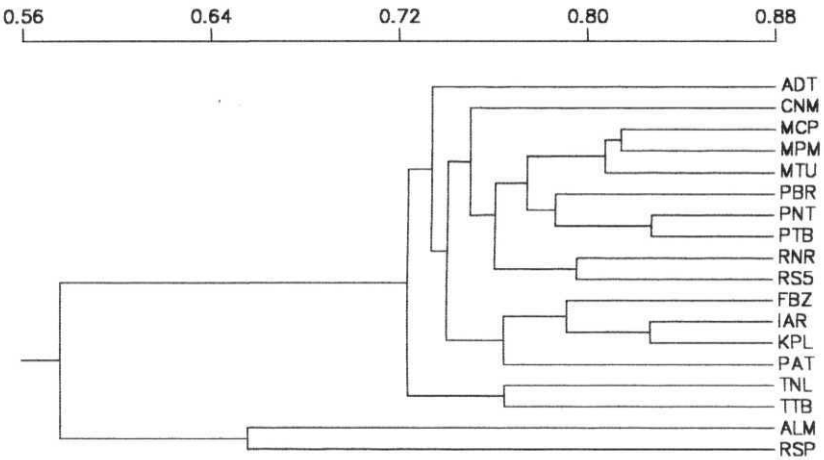
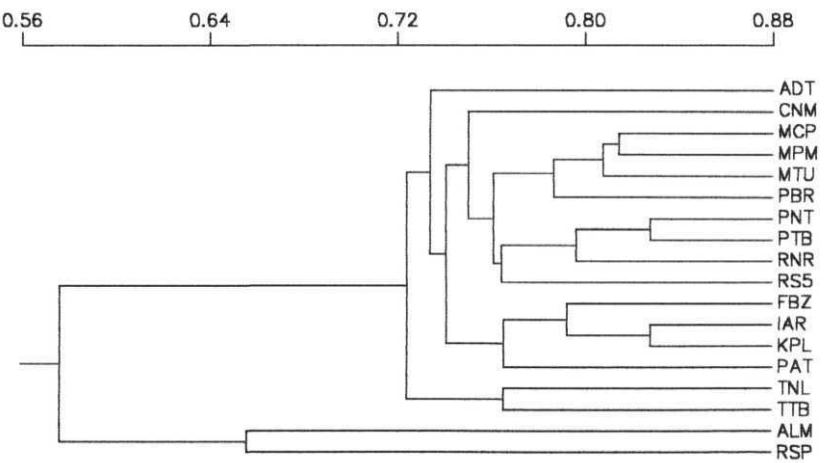


Fig. 21 CLUSTER DIAGRAM FOR 18 RICE SHEATHBLIGHT FUNGAL ISOLATES BASED ON ISOZYMES PATTERNS AND RAPD PROFILES - III



DISCUSSION

Sheathblight fungus *Rhizoctonia solani* has a wide host range (Adams, 1988) and isolates of *R.solani* are highly variable in aggressiveness (Tsai, 1973). Virtually all plant species are infected by *R.solani* and the fungus has world wide distribution (Ogoshi, 1987). The incidence of disease due to this pathogen has been increasing in recent years and there are no effective measures to control the spread of the pathogen (Anderson, 1982). Isolates show tremendous variation in their morphological and pathogenic characteristics. *R.solani* complex is a taxonomic entity composed of morphologically similar groups that share characteristics like multinucleate cells with doliopores, production of sclerotia and lack of conidia (Parmeter and Whitney, 1970). Identification *of R.solani* isolates is based on the ability of their hyphae to anastomose (Parmeter *et al.*, 1969 and Ogoshi, 1987). Each Anastamosis Group (AG) appears to have different physiological characteristics and is genetically isolated and diverse (Anderson, 1982, Ogoshi, 1987). Although the concept of anastomosis correlates to some extent with pathogenecity, evidence from several studies suggests that there is considerable variation among the isolates from the same AG and the pathogenecity cannot be explained solely in terms of AG (Ogoshi, 1987, Vilgalys, 1988, Jabaji-Hare *et al.*, 1990, Vilgalys and Gonzalez, 1990). However, due to a lack of stable genetic markers it is very difficult to assess the level of variation within the different groups in *R.solani*, and to identify the mechanisms responsible for this variation (Mordue *et aL*, 1989). Lack of markers has also hindered studies on population dynamics of the pathogen in the soil and on the epidemiology of the pathogen. In spite of the economic importance of *R.solani* very little is known about the genetic variation and systematic relationships within the fungus (Vilgalys and Cubeta, 1994). Useful methods are needed for recognising individuals in the anastamosis group and characterising their distributions.

Variability of Rice cultivars for pathogenecity :

Screening has rarely located high resistance among the cultivars, breeding lines or related wild species of *Oryza* (Dasgupta, 1992). In the absence of highly resistant cultivars against ShB pathogen, the term tolerance is considered to be the capacity to look susceptible but field resistant, *i.e.*, a cultivar

although diseased as severely as others, do not show a significant reduction in yield. Out of **7,614** lines screened **(IRRI,** 1987), 72 have been identified as resistant of which 48 belonged to upland , 17 to wild rice and only 3 among elite lines. Only a few lines have shown 0-2 disease scores, such as RP **1821** (Gangopadhyay and **Padmanabhan,** 1987), F 47 (CC147F - 112 - 18 - 4 - 106) and HIM 34 6 4 P **(Majumdar** *et aL,* 1989). **In** the present investigation, the variability of pathogenecity in rice cultivars has been assessed at maximum tillering stage using three scoring parameters, Average Disease Index **(ADI%),** Disease Score (DS) (0 - 9 scale of SES) and Relative Lesion Height (RLH%). Among the cultivars screened, Swarnadhan was found to be the most tolerant with an ADI 41.67% and IR50 as the most susceptible with an ADI 73.02% (Table 4.1).

From the present observations, the cultivar Swarnadhan could be taken as the tolerant check and **IR50** as the susceptible check, while screening the variability and pathogenecity of the fungal isolates. In general, tall genotypes with few tillers were found to show tolerant reaction relatively as compared to the improved high tillering rice cultivars (Lee and Rush, 1983, Marchetti, 1983). The above observations are in agreement with the present studies where Swarnadhan and Vikramarya with relatively fewer number of tillers (Average tillers of Swarnadhan : 10 and Average tillers of Vikramarya : 10) showed tolerant response compared to the cultivars Kavya and **IR50** (Average tillers of Kavya: 20 and Average tillers of IR50 : 22) with more number of tillers (Table 3.1). This differential response could be attributed to various factors which include closed canopy, congenial warm humid climate in the hills *etc*.. Yield loss differences between moderately resistant and moderately susceptible and highly susceptible cultivars can be substantial (Dasgupta, 1992). Thus cultivation of highly susceptible cultivars such as **TN1,** IR50, Kavya, Pusa2-21 can be discouraged and high yielding moderately resistant cultivars such as Jaya, Pankaj and Swarnadhan can be encouraged, at least in endemic areas.

Morphological variation:

The isolates selected for morphological studies in present investigation showed a continuum of variability in sclerotial dimensions and number (Table 4.2). There were no discrete morphological

differences among the isolates studied so as to categorize them into distinct groups. Nevertheless, a positive correlation was observed between the size of sclerotia and pathogenecity. Earlier studies at IRR1 also showed a positive relationship between sclerotial size and disease severity (IRRI, 1986).

Variability in Pathogenecity :

Because of inadequate varietal resistance against sheath blight disease, there is a need to understand more about the virulence pattern of the pathogen at various endemic locations. The variability in pathogenecity needs to be characterised carefully as the released variety could become susceptible to pathogen variants in farmer's fields but not at screening locations.

Pathogenecity of ShB isolates tested in the present study varied considerably showing a range of 0.00 (ALM) and 100% (MTU) disease index on susceptible IR 50 (Table 43). Isolates of *R.solani* vary considerably in the degree of aggressiveness (Tsai, 1973) and the disease severity is influenced by pathogenecity of the isolates. The highly significant values of all the pathogenecity parameters from ANOVA indicated that the three parameters studied *i.e.*, Average Disease Index (ADI%), Relative Lesion Height (RLH%) and Disease Score (DS) are effective for the study of the pathogenecity of the fungal isolates (Table 4.4). Each isolate showed an individual reaction. Similarly each cultivar showed a typical individual expression for each isolate. The reaction showed by each isolate and cultivar response were typical of their interaction. The pathogenecity of the isolates was corresponding on both susceptible and tolerant cultivars i.e., the scoring profiles were very parallel on both the susceptible and tolerant cultivars. The highly significant values of the pathogenecity parameters studied, coupled with highly significant Spearman rank correlation coefficients for the virulence parameters on susceptible and tolerant rice cultivars showed that the variation of the isolates was not a chance phenomena, but inherent property of the isolates(Table *43).* Previous studies on characterization of *R.solani* isolates showed that, morphologically, isolates were highly variable, with no consistent characters that related to origin, production system or geographic region. Pathogenecity testing on rice, soybean and a range of weed species showed little host specificity (Banniza, *et al.*, 1996). The

variability detected in pathogenecity of the isolates of *R.solani*, could be one of the principal reasons for the observed differences in reaction of rice varieties at different locations.

Simple Correlation Studies :

The present observations from simple correlation studies showed that the size of sclerotia was positively correlated with pathogenecity parameters, whereas their number was negatively correlated to pathogenecity. It was reported that a positive correlation was found between the sclerotia and pathogenecity for the isolates of *R.solani* from West Bengal, India. Isolates with large sclerotia were significantly more virulent than those with smaller sclerotia or without sclerotia (Basu and Gupta, 1992). The increased number of sclerotia of the two avirulent isolates (ALM 208 and RSP 114) would have contributed towards the negative correlation of the number of sclerotia with the pathogenecity (Table 4.5).

Grouping of Isolates:

Among the 18 isolates tested, the isolates RSP and ALM can be considered as avirulent indicating that these isolates are non pathogenic to rice cultivars. Five of the isolates ADT, CNM, FBZ, MTU and TNL can be considered as highly virulent, whereas rest of all the isolates come under category of moderately virulent Thus a continuum of variability was observed in the level of pathogenecity (Table 4.6).

In rice crop, grain is the metabolic sink that drain photosynthate from the rest of the plant, and this makes the plant more susceptible to disease. The greater the yield, the greater the sink, the greater the drain and the greater is the loss of resistance. There is sink-induced loss of resistance, the loss being related to the yield, this may be the reason that high yielding and high fertiliser responsive varieties are susceptible to the disease (Van der Plank, 1968). It is known that development of resistant cultivars is the best solution to control diseases, but so far, no cultivar with ShB resistance has been released for wide cultivation.

Total Soluble Proteins

The present study reveals a wide variation in the native soluble protein patterns of this group of sheath blight isolates and the banding patterns could not be used to reliably differentiate isolates (Fig. 2). None of the differences in protein patterns observed among the isolates could be related to their **virulence.** Similar investigations indicated that there was considerable variation in the soluble protein banding patterns from isolate to isolate of ***R.solani*** and this was interpreted as evidence that ***R.solani*** is a complex species (Clare *et* ***al,*** 1968). Reynolds *et al* (1983) reported that five different AGs (AG 1 through AG 5) could be distinguished from each other, where AG 1, AG 2 and AG 5 differ in protein patterns from each other and from the isolates of AG 3 and AG 4. However, the variability in the protein banding patterns of three isolates of AG 1 included in the study was quite high. Contrastingly, **Kuninaga** (1986) claimed that **distinct** patterns of total proteins (AG 1 through AG 7 and **BI)** were recognisable among homologous groups within an AG. Using same technique **Cehn** *et al* (1991) reported distinct protein patterns for groups AG 3, AG 4, AG 5 and subgroups AG **1-1** A, AG **1-1B,** AG **1-1C,** AG 2-1 and AG 2-2 with significant differences between subgroups of AG 1 and AG 2. Thus the above observations on the variability in total soluble protein patterns of isolates of AG 1 are in agreement with the observations of the soluble protein patterns of present study. The high level of variation observed in these isolates support the suggested heterogeneous nature of AG 1 (Adams, 1988). The differences in electrophoretic protein patterns could be attributed to the existing genomic differences among the isolates collected.

For analysing population structure, **single-locus** genetic markers such as isozymes and DNA based RFLP are probably most useful as they provide information on a locus-by-locus basis that can be analysed by standard population genetic methods. To identify genetically distinct strains (clones) in a population, methods that survey many loci at once such as RAPD or DNA **fingerprinting,** might provide a higher level of discrimination than single locus markers (Cubeta and Vilgalys, 1997). As each method has its own advantages and limitations, in the present study a combination of different methods is chosen. As a single locus specific marker for unambiguous assignment of genotypes of *R.solani* isolates, isozymes are selected as they are codominant and their inheritance can be easily

demonstrated through crossing studies. As for multilocus genetic markers, **RAPD** were studied, where high levels of variation can be obtained in order to assess genetic identity among isolates and RAPD profiling could be used in conjunction with hyphal anastomosis testing to assess the probable clonal structure in populations.

ISOZYMES :

Electrophoretic separation of enzymes, which exploits the polymorphism of detected isozyme forms, is another molecular technique that has been widely used to generate a large number of markers for the assessment of genetic diversity in fungi (Clark *et aL,* 1989, **Oudemans** and **Coffey,** 1991). **In** the present study, isozyme analysis of sheathblight (ShB) fungal isolates revealed considerable diversity, **confirming** the previous observations where electrophoretic patterns of isozymes provided a good indication of the genetic diversity among the anastomosis groups (AGs) and within subgroups of ***Rhizoctonia*** (Liu and Sinclair, 1992, 1993, Laroche *et aL,* 1992). High levels of intraspecific variation in isozymes also have been detected in other diverse fungi such as ***Trichoderma*** (Stasz *et aL,* 1989) and ***Rhyncosporium*** **(Mc** Dermott ***et al.*, 1989).** However, the level of intra specific variation in isozymes has often been reported low for phytopathogenic fungi (Newton, 1987). For example, rice infecting isolates of ***Magnaporthae grisea*** has almost no variation for isozymes **(Matsuyama** and **Kozaka,** 1971, Leung and Williams, 1986), even though this fungus is notoriously variable for pathogenic variability.

Out of 13 isozyme systems tested, 12 enzymes were selected because of their high resolution and reproducibility on polyacrylamide gel electrophoreis. From the banding patterns of isozymes, a total of 153 electrophoretic phenotypes (EPs) were observed in contrast to the modest number of EPs that were observed in earlier studies of *Rhizoctonia solani* (Table 4.7). Isozyme electrophoresis of AG 3 and AG 9 produced 76 EPs for 48 isolates, when screened with seven enzymes (Laroche *et aL,* 1992). Similarly, 50 isolates of binucleate *Rhizoctonia* were characterized by a total of 63 EPs with eight isozyme systems **(Damaj** *et al.*,**1993).** The screening of 27 isolates of *Uromyces* ***appendiculatus*** resulted in 15 EPs using 13 isozymes (Linde *et aL,* 1990). Isozyme analysis of 204 isolates of 10 ***Pythium*** species

produced 59 distinct £Ps (Chen *et al* ,1992). For **54** isolates *of A ltern aria solan i* and 96 isolates of *Alternaria alternata,* 35 EPs were detected for 10 enzyme systems **(Petrunak** and Christ, 1992).

In the present studies of isozyme polymorphism of all 18 isolates, a -esterase with 17 EPs, P-esterase with 18 EPs, 6 phosphogluconic acid dehydrogenase with 18 EPs produced the most polymorphic banding patterns. According to **La roc** he *et al* (1992) diaphorase was the most polymorphic isozyme followed by esterase and **malate** dehydrogenase among the isozyme studies of AG 3 and AG 9. Similarly, hexokinase and malate dehydrogenase displayed the most polymorphic banding patterns in the studies of isozyme polymorphism in binucleate *Rhizoctonia* **(Damaj** *et al.,* 1993). The isozyme polymorphisms of some enzymes *viz.,* esterases, 6 phosphogluconic dehydrogenase were variable to the extent that no EP was common in **all** the 18 isolates, thereby suggesting their utility in possible fingerprinting of the *R.solani* isolates. When an isolate **(RS319)** with unknown level of virulence was tested, the isolate was identified as the isolate from the location, Titabar with the help of esterase profile (Fig.6). It was reported that the blind isolate given for testing was collected from Titabar, Assam after two years of the original collection of isolates, which shows that the isozyme analysis data of the present investigation can be used for the identification of the individual isolates.

In the present study of isozyme polymorphism of 18 isolates, the isozymes acid phosphatase (ACP), glutamate dehydrogenase **(GDH),** leucine aminopeptidase (LAP) and peroxidase (POX) were able to distinguish between virulent and avirulent isolates of *R.solani* occurring on rice (Table 4.8). The virulent isolates produced a specific band with Relative mobility **(Rm)** 0.20, in case of ACP banding patterns, a band with Rm 0.20, in case of GDH and a band with Rm 4.0, in case of LAP. The avirulent isolates produced a band with **Rm 0.15,** in case of POX patterns. The identification of an isolate with unknown level of **pathogenecity** with the help of ACP, GDH and LAP isozymes as pathogenic , which was confirmed later by screening in the glass house, validates the credibility of the technique. Isozyme alleles for aconitase, esterase and phophoglucomutase were useful for distinguishing isolates in groups 2B and 2C (1MB and IV types) (Liu and Sinclair, 1992). Similarly isozyme alleles, aconitase, malate dehydrogenase and isocitrate dehydrogenase distinguished the two species of *Alternaria, A. solani* and *A. alternata* from one another. The isozyme alleles of the present

study *viz.*, ACP, EST, GDH, LAP and 6 PGD which were useful for distinguishing isolates among AG 1, could be used as biochemical markers for monitoring various populations and for an isozyme profile database/fingerprinting. Many enzymes are genetically polymorphic and shared bands are common within and among the related populations. Therefore, a single allele or locus should not be used for diagnostic purposes. The identification can be done by using more than one isozyme that can differentiate the isolates. As differential genotype tests can take at least three months to complete, it would be desirable to have a more rapid, cheaper and less labor intensive method of distinguishing the isolates, such as the isozyme profiling presented in this study (Table 4.8). An examination of the relationship between the virulence screening and isozyme profiles indicated virulence and isozyme analysis are complementary, and yet distinct methods of characterizing the pathogens. It is reassuring that the virulence analysis and isozyme analysis showed differentiation between the virulent and avirulent isolates, at least with two isozymes, if not all. However in *Phytophthora graminis. f. sp. tritici* of the eastern and central USA, no isozymic differences were detected between isolates of the same virulence race. Isozymic examination of 20 isolates of *Phytopthora* showed that isolates having the same virulence phenotype had the same isozyme phenotype (Burdon and Roelfs, 1985).

Based on isozyme profiles, 6 Intra Specific Groups (ISGs) within AG 1, 5 ISGs within AG 2 (Liu *et al.*, 1990, Liu and Sinclair, 1992, 1993) and 3 sub groups within AG 3 (Laroche *et al.*, 1992) were distinguished. AG specific banding patterns were also identified for AG 3 and AG 9 (Laroche *et al.*, 1992). Species specific banding were recognised for a number of *Phytophthora* species (Nygaard *et al.*, 1989) and in differentiating *Pythium* species (Chen *et aL,* 1991).Though the present findings suggested a specific banding pattern for all virulent isolates studied, these banding profiles cannot be specified to an ISG as there is no concrete evidence that all these isolates belong to one ISG.

From the present observations, inter-isolate variation could not be attributed to the location from which the isolates were originally obtained. However, Kaufman and Rothrock (1995) were able to distinguish between AG 11 isolates from Australia and Arkansas, USA.

ft is known that *R.solani* undergoes not only parasitic life cycle in association with host plants, but also a saprophytic cycle in the soil (Adams, 1988). A successful saprophyte must possess a diverse

array of metabolic enzymes to utilize various substrates under a wide range of soil conditions, such as pH, chemical composition, temperature, microbial antagonists *etc.* If this was the case, quite different selection pressures over many generations would be expected to cause an increase in genetic diversity within the fungus *R.solani*, which in turn will be responsible for the high level of the polymorphism observed in the present studies. It is possible to elucidate the role of chromosomal recombination by assessing genetic variation at loci at which selection is less intense and less variable between population than at loci for specific virulence. Typically, isozyme analysis, as used in this type of studies, offers a relatively independent and selectively neutral means of identifying genetic variation of pathogen populations (Markert, 1975).

It is apparent from the above observations that isozyme polymorphisms were useful for distinguishing virulent and avirulent isolates, suggesting their utility as molecular markers for monitoring the pathogen population and could provide information for the individual isolates.

RAPD :

Random amplified polymorphic DNA (RAPD) markers were used to study genetic variability among 18 rice sheath blight fungal isolates. RAPD markers have been used to differentiate fungal races in *Fusarium solani* f sp. *cucurbitae* (Crowhurst *et al.*, 1991), field populations in *Sclerotinia sclerotiorium* (Kohn *et al* , 1991) and species in *Metarhizium* (Cobb and Clarkson, 1993). An underlying assumption of RAPD marker analysis is that marker bands of the same size originate from the same genomic locus (Huff *et al.*, 1994). The present study revealed high level of genomic variability in the isolates. The total number of 269 bands observed in this study were distinguished as i) bands that were common to all isolates ii) bands that were common to the virulent isolates iii) bands that were present only in avirulent isolates and iv) bands that were unique to an isolate.

Out of the total bands present, only 3 % of the amplified fragments were shared by all the isolates tested corroborating the results of Duncan *et al* (1993), where, among the isolates of *R.solani* from Australia, the similarity coefficient of zero was observed which indicated no amplified DNA fragment was common to all the isolates (Table 4.9). A similar degree of genetic variability detected

by RAPD has been found in *Chaunopycnis alba*, where only 1 % of the amplified fragments were shared by all the strains (Moller *et al.*, 1995) and only 0.8% of the amplified fragments were shared among all the isolates of *Discula umbrinella* (Berk and Brooms , Mordet, M.) from various host groups (Haemmali *et al.*, 1992). The percentage of fragments common to all the isolates ranged from 0.0 to 11.1 per isolate per primer and the percentage of polymorphism detected per primer per isolate varied from 81.4 to 100 (Table 4.9). Thus amplification of primers OPC01 and OPQ01 with their polymorphism suggested their utility in the individual DNA fingerprinting of the 18 fungal isolates. The bands that were common to the virulent isolates produced by primers such as OPA10 , OPB01 *etc.*, could serve as molecular markers for the virulent isolates (Table 4.10). Association of RAPD profiles with pathogenecity, host specificity and morphological characters has been found for other pathosystems. Races of *Cochliobolus carborum* and *Gremmeniella abietina var abietina* were differentiated and their origins are traced using RAPD markers (Hamelin *et al.*, 1993, Jones and Dunkel, 1993). The present study indicated only two avirulent isolates; screening of a large number of isolates is required to identify some more markers for a virulence. Unique bands may not be taken for individual identification because of the limited number of isolates tested.

RAPD analysis provides a rapid method of differentiating among the isolates and distinguishing the pathogenic and non-pathogenic isolates. This was verified by subjecting an isolate with unknown level of virulence and comparing with profiles of OPA10 primer. This unknown isolate (RS 319) was identified as virulent and confirmed subsequently by its phenotypic reaction in standard glass house inoculation studies. The distinguishing bands were reproducible in repeated trials. In replicated experiments, variation in the intensity of some of the bands was observed, but the number of bands was, however, constant. The precision of identification of the isolates may be achieved with the inclusion of more primer sequences for testing. Thus primers that give clear differentiation may be identified. Greater specificity in identification might be possible by using more than one primer.

Studies carried out in the present investigation clearly demonstrate that RAPD markers have potential as a means of distinguishing the virulent isolates, characterising the rice sheathblight fungus and increasing our understanding of the ecology and biology of this fungus by providing genetic

relatedness and variation within and between fungal populations. Furthermore, the potential ability to correlate specific traits, such as **pathogenecity,** by using RAPDs may further enhance efforts to monitor and predict disease outbreaks in the future.

Similarity Matrices

To **quantify** the relationships among the isolates revealed by protein, isozyme and RAPD data, a similarity matrix using simple matching coefficients was produced from the data matrix. Within the AG 1 the similarity **co-efficients** were **all** higher than 40 %. **In** the construction of the matrix, it is assumed that corresponding bands arise by amplification of the same genetic locus. The maximum amount of similarity between any two of the isolates 0.848 was recorded (Table 4.12) based on RAPD **profiles.** The above observation is in agreement with the earlier findings of similarity co-efficients between the ***Ophiostoma ulmi*** EAN and NAN isolates with RAPD markers ranged from 0.50 to 0.73 (Pipes *et* ***al.,*** 1995). Similarly the maximum amount of similarity between any two of the isolates based on the protein and isozyme patterns was 0.84 (Table **4.11).** In bean rust fungus ***Uromyces appendiculatus,*** Linde *et* ***al*** (1990) found that even the most distinctly related isolates in the study approximately had 0.36 similarity. However, the highest difference between maximum and minimum similarities in case of RAPD sets (44%) and the least of such difference in case of protein and isozyme **patterns(15%)** indicate that the RAPDs are more efficient in discriminating similarities among the isolates than the isozymes. The differences in levels of similarity observed amongst the isolates in the phenograms based on isozymes compared to the ones based on RAPDs can be explained by the fact that the number of phenomorphs in RAPDs are much higher than in isozymes. When the difference between the maximum and minimum similarities in a single set of data was considered, the difference of 28 % as observed in the combined set of isozyme polymorphism and RAPD profiles (Fig. **4.13)** was very near to the average of RAPDs and isozyme sets ***i.e.*** 28 % which shows that the difference found was the average values from both isozymes and RAPD profiles.

UPGMA Analysis

The total variability is summarised in the clustering pattern that were constructed from protein data, RAPD data and combined data of both protein and RAPD data. The cluster analysis drawn from the studies of biochemical and molecular analysis also showed almost the same pattern. In the present study, large phenotype intragroup variation detected resulted in the placement of isolates into two separate and genetically distant groups in the phenogram. The UPGMA analysis of zymogram data showed two clusters at 0.71 (Fig. 16 and Fig. 17) similarity. The UPGMA analysis of RAPD profiles separated the isolates into two clusters at 0.46 similarity (Fig. 18) and the combined analysis of isozyme polymorphism and RAPD profiles separated at 0.54 similarity with three different patterns (Fig. 19, Fig. 20 & Fig. 21). The clustering derived from the electrophoretic data of isozyme polymorphism summarised the inter-isolate genetic variation among the eighteen isolates and demonstrated that two genetically distinct groups can be formed, among rice ShB fungal isolates. Isozyme electrophoresis of anastamosis groups 3 and 9 of *R.solani* produced phenograms with two major groups lineated 56 % similarity, with each cluster representing isolates of different AG (Laroche *et al.*, 1992). Similarly isozyme electrophoresis of binucleate *Rhizoctonia* resulted in four major groups designated as I, II, III and IV which are separated at 69 % similarity (Damaj *et al.*, 1993). Both of the above classifications were in accordance with anastomosis group suggesting the importance of isozyme electrophoresis in (he grouping of the isolates.

Jabaji-Hare *et al.*, (1990) reported AG 3 (ZG 7) isolates of *R.solani* from Canada, USA, Japan and Britain could be differentiated on the basis of the RFLP patterns obtained with a ribosomal DNA probe. And origin related genetic related ness was reported for the isolates from Australia (Duncan *et al.*, 1993). No such correlation between the geographic and genetic related ness were observed in case of the isolates in the present study.

The grouping of isolates into virulent and avirulent types using isozyme and RAPD profiles correlated with the disease inducing characteristics of isolates as previously determined by pathogenecity tests with differential host genotypes of rice. It is interesting to note that a close

association between pathotypic structure and molecular phylogeny in the present study suggests the utility of molecular typing for rapid pathotype diagnosis. The pathotypic structure is primarily detected by host reaction towards the isolate. Molecular phylogeny as inferred from neutral molecular markers reflects the natural relationships among the isolates. It is of practical interest to determine the relationship between the phylogenetic and pathotypic structures, which later could be applied to study the pathogen population (Leung *et al.*, 1993).

Both the isozyme and RAPD analysis showed a large amount of variation despite the isolate belonging to same AG *i.e.* AG 1. Adams (1988) reported that AG 2 type 1, BI, AG 3, AG 5 and AG 7 are each a genetically homogenous group of isolates whereas AG 1, AG 2 type 2, AG 4 and AG 6 are each a heterogeneous group of isolates. Apparently, each heterogeneous group contains isolates that are genetically somewhat divergent (Kuninaga and Yokosawa, 1982 through 1985). The diversity in AG 1 is well known, distinct cultural characteristics are correlated with distinctive ecological niches and host specificity. Because of its variability in anastomosis reaction, pathogenecity and cultural morphology, AG 1 has been further divided into three groups. Based on RFLPs, AG 1 isolates appeared to be composed of several genetically heterogeneous groups and all the three subgroups in AG 1 are highly variable. RFLPs within AG 1 varied considerably from isolate to isolate(Vilgalys and Gonzalez, 1990). The results of present studies are in good agreement with the above studies, as both isozyme and RAPD-PCR analysis showed a large amount of variation in the isolates collected. One possible explanation for intra group variation could be the evolutionary divergence as biological species (Anderson, 1982, Ogoshi, 1987). The reason for this heterogeneity is not known. It may be related to the frequency which may lead to gene transfer, and heterokaryon formation. Most AG 1 field isolates are heterokaryotic and some kind of obligate heterothallism operates in this group (Anderson, 1982). Many ecological aspects of *R.solani* have been well studied (growth, survival and saprophytic ability) (Papavizas *et aL,* 1975). However, little information is available on the genetic condition (heterokaryotic Vs homokaryotic) of field isolates and the nature of individual isolates. Given the variability of tuft formation as a sole criterion for heterokaryotization, development of suitable molecular markers should be very useful for addressing the genetic structure of pathogen

population. For this, markers will need to be developed specifically for each ISG. The isozyme polymorphisms and RAPD profiles studied in the present investigation can serve as molecular markers for the virulent isolates and may be applied for the ISG.

The present findings though broadly agree with above reports, the percentage of presence of RAPD-PCR bands common to all virulent isolates was more than the percentage of amplified fragments common to all isolates. The presence of bands only in virulent isolates can be explained by assuming that all the virulent isolates belong to one intra specific group (ISG). And the presence of those bands can be exploited further for molecular identification of ISG-1A of AG 1.

AG 1 of *R.solani* was divided into three ISGs by pathogenic variations and by DNA-DNA homology (Kuninaga and Yokosawa, 1982 through 85). The three ISGs comprise of IA (sasaki type), IB (web blight type) and IC (microsclerotial type). AG 1 - 1A causes true sheath blight, the web blight fungus IB produces different symptoms on rice, and the sugar beet and buck wheat fungus IC fuses well with LA and IB, but is not pathogenic on rice. The possibility of the two avirulent isolates from Almora and RS Pura belonging to ISG IC needs further investigation. The isolates belonging to the ISG IC though show anastomosis reaction with the other isolates of *R. solan i*, their pathogenecity on rice is relatively low. However, more intense studies are required on ISG of AG 1 before making any conclusion regarding their identification.

During the present studies on variability of the 18 sheath blight fungal isolates, one isolate from Almora (ALM) (one of the avirulent isolates) was later identified by IMI, UK as *Rhizoctonia oryzae-sativae* , one of the causal agents of *Rhizoctonia* sheath complex in rice, which produces sheathblight symptoms (Reddy, 1997). This was also born out by the fact that the pathogenecity parameters namely AD1(%), RLH(%) and DS were all recorded as 0.00 for this isolate. The separation of this isolate from the rest of the *R. solani* isolates by cluster analysis evidently support the credibility of the techniques of the present study. Further studies are needed to find out if there is any genetic relationship between the two avirulent isolates, *R.solani* and *R.oryza-sativae*.

Discussion

Although these results are based on a limited number of samples, nonetheless they suggest that a unique set of individuals may exist within most of the locations. If the sample sizes were to increase, however, the relative relationships would likely change because of corresponding fluctuations in genetic variation. A considerable amount of genetic variation is observed in the present study which is in contrast to some studies where it was suggested that asexually reproducing populations show little genetic variation. A plausible explanation is that asexual populations are unable to exchange genetic material, and therefore are restricted in genetic diversity. But this is in contrast to the results of the present investigation, in which a considerable amount of variation was found. One possibility is that genetic variation is generated through a parasexual cycle (anastamosis followed by asexual combination). The parasexual cycle has been reported for a wide range of pathogenic fungi (Tinline and MacNeill, 1969) and may be an important source of variation, particularly in those fungi which either lack or have a rare sexual phase. Given the apparent rarity of the perfect state for *R.solani*, it is possible that the variation observed for isolates is a result of parasexual combination. Bridging isolates also play a vital role in the evolution of *Rhizoctonia* by transferring genetic material from one isolate to another normally incompatible isolate that belongs to a different anastamosis group.

One explanation for the level of variation detected may be natural mutation and another possibility through which recombination might occur through asexual cycle. Multiple infections occurring within close proximity on the plant may allow for exchange of genetic material through asexual combination process.

The variation can also be explained as *R.solani* is often found as a saprophytic organism on a wide variety of plants. It is also possible that the saprophytic nature of *R.solani* may force it to be adaptable to more diverse range of hosts, which may subsequently lead to its being more diverse as a species. Exposure of geographically isolated populations to non-preferred hosts or a variable array of hosts would result in new selected pressures and add to genetic diversity (Butlin, 1987, McDermott *et aL*, 1989, Price 1977). This process would lead ultimately to fragmented or new species. Populations may also diverge in response to different host populations which would provide selection pressure that lead to genetic diversity among populations in different areas.

As *R.solani* has a wide host range and the differences among isolates were not obvious, breeding of varieties resistant to diseases caused by *Rhizoctonia* has not been extensively studied. However, with the concept of ISGs and AGs, the potential for breeding resistant varieties has improved; that is, the group causing a distinct disease of plant has been defined. Usually, even if several AGs or ISGs are isolated from a plant, the primary pathogen belongs to a specific AG or ISG and the target for breeding becomes clearer. For example, Yamaguchi *et al.*, (1977) have screened for varieties resistant to root rot of sugar beets. In this case, the challenged pathogen is *R.solani* AG 2 2 IV; only this group causes the serious root rot of sugar beets. The present studies identified a few isozyme and RAPD markers that can differentiate between the sheathblight isolates that arc pathogenic and non-pathogenic to rice crop.

The present investigation gives some insight to pathogen variation and its distribution which in turn allows more accurate assessment of either resistant germplasm or genetically engineered plants. Two major challenges for rice ShB disease control are, firstly to manage the rhizosphere pathogen propagules in the present intensive areas of cultivation and secondly, to prevent the spread of ShB (Manibhushanrao, 1995). Success in meeting these challenges will depend upon the clear understanding of genetic and molecular biology of the ShB fungus. In the present study, the fungal isolates from various locations were characterized using isozyme polymorphisms and RAPD profiles. These molecular data are likely to provide information for delimiting intraspecific groups in ShB fungus and aid in the development of species concepts by providing information about the limits of genetically isolated groups in relation to patterns of morphological variation and mating behavior.

SUMMARY & CONCLUSIONS

Rice is the principal food crop of India. The grain yield in this crop is severely affected due to various abiotic and biotic stress conditions. Among fungal pathogens that afflict rice crop, rice sheath blight fungus (*R.solani*) is considered as one of the major production constraints for rice in South East Asia where one third of the world's population live and depend on rice as major staple food. The introduction of IR-type semi-dwarfs and the advancement of production technology particularly adoption of intensive cultural practices have led to the failure of the crop against sheathblight (ShB) disease.

Rice sheathblight fungus, *R.solani* has a wide host range and is highly variable in pathogenecity. In the absence of suitable morphological and physiological characteristics, the identification of the isolates of *R.solani* has proved very difficult impeding breeding efforts and deployment strategies for resistance. Traditionally the isolates have been grouped into anastomosis groups(AG) and intra specific groups (ISG). Although the anastomosis grouping concept correlates to some extent with pathogenecity, evidence from several studies suggests that there is considerable variation among the isolates from the same AG and the pathogenecity cannot be explained solely in terms of AG. The major objective of the present study was to characterize pathogenic variability in rice sheathblight fungus *R.solani* using proteins, isozyme polymorphism and RAPD-PCR profiles and an attempt was made to assess the genetic diversity existing in ShB fungus. Towards characterization of variability, 18 isolates of ShB fungus were collected from different rice growing regions of India. The results emanating from the present study are summarised as follows.

1. The virulence spectrum of all 18 isolates was examined on susceptible IR 50 and tolerant Swarnadhan, based on which the isolates could be grouped broadly as avirulennt, moderately virulent and virulent. The highly significant values of the pathogenecity parameters studied, coupled with highly significant Spearman rank correlation coefficients showed that the variation of the isolates was not a chance phenomena, but inherent property of the isolates.

2. Simple correlation studies between the morphological characters and pathogenecity parameters indicate a positive correlation between sclerotial size and pathogenecity.
3. The variation in the total soluble protein banding patterns was very high and the banding patterns could not be used to differentiate the isolates.
4. Twelve, out of thirteen isozymes studied across the fungal isolates showed a total of 153 electrophoretic phenotypes. Isozyme polymorphs of acid phosphatase, glutamate dehydrogenase and leucine aminopeptidase were associated with virulence, whereas, a polymorph of the isozyme peroxidase was associated with avirulence.
5. The isozymes, 6-phosphogluconic dehydrogenase and esterases produced unique zymograms for each of the 18 isolates, there by indicating suitability of these isozymes for protein based fingerprinting of the Indian rice sheath blight fungal isolates.
6. When the utility of isozyme polymorphism was critically examined, one isolate RS 319 with unknown level of virulence was identified as a virulent isolate with the isozymes acid phosphatase and leucine aminopeptidase. Using the polymorphisms of esterases, the isolate was identified as the isolate from Titabar, Assam.
7. Studies based on RAPDs generated by using random decamer primers depicted DNA polymorphs ranging from 0.34-1.64 kb range. All the nine random primers produced polymorphism indicating high level of genomic variability within the isolates studied. Only 3% of the total amplified fragments were shared by all the isolates tested.
8. Some bands were unique for the virulent isolates, whereas some others were specific for avirulent isolates. With the help of amplification of OPA10 primer, an isolate with unknown level of virulence was identified as the virulent isolate, which was later confirmed by standard glass housing screening.
9. RAPDs generated by the primers OPC01 and OPQ01 showed distinct profiles for each of the 18 fungal isolates, thereby providing DNA fingerprinting patterns for the isolates of ShB fungus.
10. Within these 18 fungal isolates, the similarity coefficients were all higher than 40%.

11. Dendrograms generated using the combined similarity coefficients across the primers afforded distinct grouping of the avirulent isolates from the virulent isolates at similarity level of about 50%. Similar results were obtained when similarity coefficients from the isozyme and RAPD studies were pooled.
12. The clustering of 18 fungal isolates resulted in a major cluster, which included all sixteen virulent isolates and a minor cluster which included two avirulent isolates.

In the present study, an attempt was made to study diversity in the collected sheathblight fungal isolates using morphological and pathogenic variability, isozyme and DNA polymorphisms. The study indicated existence of variation among isolates of *R.solani*, which could be suitably used to find stable sources of resistance among cultivated rice and also moderately resistant varieties to ShB of rice. Though, the fungal isolates studied were representative of all important rice growing regions of India, numerical increase of isolates for identification and characterization is important for understanding 1SG and AG which in turn will give better appraisal of genetic diversity and distribution of pathogen.

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