Genetic engineering for resistance to bacterial leaf blight in indica rice cultivars

Thesis submitted in part fulfilment of the requirement for the degree of
Master of Science (Agriculture) in Plant Pathology
to the Tamil Nadu Agricultural University, Coimbatore

By

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1999

Certificate

This is to certify that the thesis entitled "**Genetic engineering for resistance to bacterial leaf blight in indica rice cultivars**" submitted in part fulfilment of the requirements for the degree of Master of Science (Agriculture) in Plant Pathology to the Tamil Nadu Agricultural University, Coimbatore, is a record of bona fide research work carried out by **Thiru M. Loganathan**, ID No. 97-614-004, under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, fellowship or other similar prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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Abstract

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Fourteen indica rice genotypes were screened for their resistance to *Xanthomonas oryzae* pv *oryzae* (*Xoo*), the bacterial leaf blight (BLB) pathogen. Among them, TKM9 showed a high level of resistance. Resistance of the genotypes to the pathogen was tested at different growth stages and W1263 was resistant to at least five isolates of *Xoo* at boot leaf stage. Seven *Xoo* isolates were grouped into six categories based on their virulence on the differentials. Two BLB-susceptible indica rice genotypes *viz*., ASD16 and ADT38 were transformed with *Xa21* gene cloned from *Oryza longistaminata*. Putative transgenic lines were regenerated and the transgene expression was confirmed by histochemical method and polymerase chain reaction. Two putative transgenic lines of ASD16 proved PCR-positive for two sequences from *Xa21* gene.

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Introduction

Chapter 1

Introduction

Rice (*Oryza sativa* L.) forms one of the major food crops of the world with its global output being 599 million tonnes in 1998 out of the total cereal output of 2,056 million tonnes. In India, rice is cultivated on an area of 42.30 million ha with an annual production of 122.22 million tonnes (FAO, 1999). Though India has the largest area under rice in the world, its annual production is lesser than that of China (192.97 million tonnes in 1998), which has the second largest area (31.85 million ha) under rice (FAO,1999). Rice production is limited by various biotic and abiotic constraints. Among them, diseases caused by fungi, bacteria and viruses are the major ones. Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings *et al.* 1990, (*Xoo*) is one of the serious diseases of rice. BLB is potentially an yield-limiting disease of rice (Mew, 1987) with an estimated loss varying from 2% to 74% (Reddy *et al.*, 1980). This disease occurred in an endemic form in Bihar during 1963 (Srivastava and Rao, 1963), Uttar Pradesh during 1973–1980 (Durgapal, 1985) and in Tamil Nadu during 1965 (Soumini *et al.*, 1969). Management of this disease in the past was limited to chemical application, mainly in the form of seed treatment (Tagami and Mizukami, 1962; Singh and Rao, 1982) and spraying (Mary and Mathew, 1983; Singh *et al.*, 1980) and no effective chemical management practice could be recommended for practical use (Ou, 1985; Reddy *et al.*, 1985).

Attempts were made to identify BLB resistance sources in the cultivated rice genotypes and their wild relatives (Seshu, 1989). Several BLB resistance genes namely, *Xa1, Xa2, Xa3, Xa4, xa5, Xa6, Xa7, xa8, xa9, Xa10, Xa11, Xa12, xa13, Xa14, xa15, Xa16, Xa17, Xa18, xa19* and *xa20* were reported (Ogawa and Khush, 1988; Ogawa, 1996). Unfortunately, none of the above *Xa/xa* genes appeared to possess complete resistance to all races of *Xoo*. However, *Xa21* gene identified in *Oryza longistaminata* by Khush *et al.*, (1989) which was subsequently cloned by Pamela Ronald of the University of California, Davis, USA (Ronald *et al.*, 1992; Song *et al.*, 1995; Song *et al.*, 1997; Wang *et al.*, 1998) appeared to confer resistance against all Indian and Filipino races of *Xoo* (Ikeda *et al.*, 1990; Khush *et al.*, 1990). From its deduced amino acid sequence, *Xa21* gene was found to be translated into a receptor kinase-like protein carrying leucine-rich repeats (LRR) in an extracellular domain, a single-pass transmembrane domain and a serine threonine kinase (STK) intracellular domain (Song *et al.*, 1995). Transformation of a susceptible rice cultivar with *Xa21* (an *R* gene) led to development of rice plants that were highly resistant to many isolates of *Xoo* (Wang *et al.*, 1996; Mourguez *et al.*, 1998). The molecular structure of *Xa21* supports a role for cellular signalling in plant disease resistance. R gene products from several plant pathogen interactions share some structural similarities namely, leucine-rich repeats which are probably involved in the perception of Avr signals and kinase domains or nucleotide-binding sites which may initiate signal transduction pathways. Hence, it became logical to clone this gene into cultivated rices to impart resistance to the deadly pathogen. Wang *et al.* (1996) transformed japonica rices with *Xa21* and transgenic indica rices expressing this gene have already been developed in at least two laboratories (Tu *et al.* 1998; Zhang *et al.*, 1998). In the present study, an attempt was made to incorporate *Xa21* into popular rice cultivars of Tamil Nadu besides optimizing a protocol for screening germplasm for resistance to BLB with a view to extending the protocol to identifying putative transformants for their enhanced resistance to the pathogen.

Therefore, the present investigation was taken up with the following objectives:

1. Screening available germplasm for resistance to BLB using appropriate differentials,
2. Transformation of a few indica rice genotypes with *Xa21* (Courtesy: Pam Ronald, University of California Davis, USA) using immature embryos as explants by Biolistic method.
3. Molecular analysis of transgene expression in the putative transgenic plants.

Review of Literature

Chapter 2

Review of literature

Occurrence

BLB has been reported to be more serious in Asia than in any other part of the world and is also one of the oldest recorded rice diseases that have been known for over a century (Mew, 1988). It was Japanese farmers who first reported the BLB on rice in 1884 in Fukuoka. Subsequently, it was reported in Korea (Takeuchi, 1930), Taiwan (Hashioka, 1951), Mexico (Dickson, 1956), China (Fang *et al.*, 1956), India (Srinivasan *et al.*, 1959), West Malaysia (Singh, 1969), Central America (Lozano, 1977) and in Pakistan (Mew and Majiid, 1977). In India, after its first report from Bombay (Srinivasan *et al.*, 1959) the disease was subsequently reported from Jammu and Kashmir (Kaul, 1959), Bihar (Srivastava and Rao, 1963), Punjab, Uttar Pradesh, Orissa, Madhya Pradesh, Andhra Pradesh (Srivastava and Rao, 1964), West Bengal (Mukherjee *et al.*, 1966), Goa and the Andamans (Patel, 1972). The disease caused heavy losses in Punjab, Haryana and Western Uttar Pradesh during severe outbreaks in 1979 and 1980 (Durgapal, 1985). In Tamil Nadu, BLB outbreak was first noticed when Taichung Native-1 cultivar was introduced (Soumini *et al.*, 1969).

BLB pathogen

Bokura (1911) first named the pathogen *Bacillus oryzae*. Ishiyama (1926) observed that the bacterium was rod-shaped and named it *Pseudomonas oryzae*. The pathogen was later renamed *Xanthomonas oryzae* based on the classification of Dowson (1949) and subsequently was reclassified and placed under *Xanthomonas campestris* as a pathovar (*X. campestris* pv *oryzae* (Ishiyama) Dye *et al.* (Dye *et al.* 1980) The name *X. oryzae* pv oryzae Swings *et al.* is currently followed after the revised naming of the bacterial pathogen (Swings *et al.*, 1990).

Losses caused by BLB pathogen

BLB has been responsible for severe yield losses in Asian countries. Suwa (1962) reported that when the disease intensity was moderate and above 30%, the grain yield was reduced by 20 to 30%. Losses in different varieties grown in India varied from 6 to 7% in IR20, 58% in TN1, and 74% in Bala (Rao and Kauffman, 1977). In crops raised during monsoon season, the yield losses were as high as 50% (Rangaswami and Rajagopalan, 1973). The number of chaffy grains were 32.2% more in infected tillers than in healthy tillers (Ray and Sengupta, 1970).

Losses caused by BLB pathogen varied from place to place; 30% loss in Japan (Suwa, 1962), 4.9 to 9.0% in China (Teng, 1986), less than 30% in Indonesia and Phillipines (Rao and Kauffman,1977), and 6 to 60% in India.(Srivastava, 1967). In India the loss varied with state to state, 23% loss in Maharashtra (Sulaiman and Ahamed, 1965), 72.7% in

Andhra Pradesh (Reddy *et al.*, 1978) 60 to 70% loss in Punjab (Raina *et al.*, 1981) and less than 50% loss in Tamil Nadu (Rangaswami and Rajagopalan, 1973).

Symptomatology

Leaf blight, *kresek* (wilt) and pale yellowing are the three distinct symptoms of BLB (Goto, 1964). Ishiyama (1926) described the symptoms of the disease as small water soaked lesions on the margins, which gradually enlarged to yellowish or whitish blotches or stripes. Occurrence of exudates made of clouded droplets is common. Pale yellowing of leaves were found on mature plants in the tropics, due to non availability of nutrients to the young leaves (Ou, 1985), Mew (1987) reported that the chlorophyll deficiency was due to malfunctioning of the photosynthetic system caused by toxin of the bacterium. The blight symptoms developed on leaf blades, sheath and sometimes on grains (Mizukami and Wakimoto, 1969; Mew, 1987). Small water-soaked spots appeared on the margins, extended rapidly and the entire leaf got blighted. Bacterial ooze was observed in the humid and warm conditions (Mew, 1988). 'Kresek' stage, the most serious BLB syndrome occurred usually one (or) two weeks after transplanting and showed complete withering of plants (Reddy, 1987). Mew (1987) reported that the pathogen entered through natural openings and ultimately reached xylem tissues, and then became systemic.

Methods of inoculation of *Xoo*

There are essentially three reported methods of the inoculation of *Xoo*. Ou (1985) reported that a pinprick method was found to produce lesions identical to natural infection and it was effective for large-scale screening. In 1979, dipping method was introduced by Mew and coworkers in which root and basal parts of the seedlings were dipped in a bacterial suspension before the transplanting (Mew *et al.*, 1979). This was done mainly for inducing *kresek* symptom. Kauffman *et al.* (1973) reported that clipping method, clipping off the leaf tip the inoculum was directly deposited in the infection court. Morinaka *et al.* (1978) who assessed the relative efficacy of the two methods concluded that there existed a correlation of 0.81 to 0.92 between clip and prick inoculation methods.

Assessment of disease development

Ezuka and Horino (1974) developed a scale with 8 classes (coded 0 – 7) to score prick-inoculated leaves ranging from symptomless to 100%. A standard evaluation system was developed at the International Rice Research Institute (IRRI, 1980) to score clip-inoculated leaves on booting or flowering plant ranging from symptomless to 100% disease leaf area coded as 0, 1, 3, 5, 7 and 9. The other parameters to assess BLB resistance are lesion length (Yoshimura *et al.*, 1984) or lesion area (Yamamoto *et al.*, 1977)

Varietal grouping

Srinivasan *et al.* (1959) identified the rice cultivars, BAM9 and MTV15 as the sources of BLB resistance. The cultivars, Sathi and N22 were highly resistant (Pavgi *et al.*, 1964), while W529 and TKM6 showed moderate resistance to the pathogen (Reddy, 1965; Purushothaman, 1973). Mahmood and Singh (1970) reported that TN1, T65 and Padma were highly susceptible, IR8, IR5, IR48 and CH10 were moderately susceptible and BR9, BR7and N136 were found resistant.

The response of rice plant to *Xoo* varied with different stages of crop growth in different genotypes. Adult plant resistance combined with seedling stage susceptibility was observed in Wase Aikoku3 (Qi and Mew, 1985). Mew (1987) observed differential resistance and apparent adult plant resistance at different growth stages in varieties such as, Zenith, Malagkit Sung Song, and their progeny lines IR1695 and IR944.

Variability of *Xoo* on rice cultivars

Differential interaction between rice and *Xoo* was reported (Mew 1987), when then resistant cultivar Asakaze became susceptible to BLB. Kozaka (1969) identified three bacterial groups based on a differential reaction of a set of rice cultivars. Sato et al. (1976) analysed *Xoo* on Kyushu area and identified two bacterial groups namely, the ones which attacked all differential cultivars including Wase Aikoku3 and other ones which did not.

Rice transformation

The cultivated varieties of rice are normally susceptible to a wide range of pests and diseases or lacking in agronomically important traits. The desirable traits are present in many wild species of rice. Attempts have been made to transfer such traits from wild type to cultivated species through normal breeding methods. However, the conventional methods are time-consuming. Hence, attempts were made to transfer the gene of interest directly to the cultivated varieties (Christou *et al.*, 1991; Fujimoto *et al.*,1993; Broglie *et al.*, 1989). Such attempts were made in rice also. To date several genes of interest including sheath blight resistance (Lin *et al.* 1995), BLB resistance (Song *et al.*, 1995; Zhang *et al.*, 1998) and insect resistance using *Bt* genes (Wunn *et al.*, 1996) have been developed.

Genetic engineering for rice disease management

A number of mechanisms are involved in plants to protect themselves from diseases. Some natural defense mechanisms have been identified which were controlled by variety of genes called defense genes, which were expressed constitutively. Such a single defense gene can strengthen plant defense against pathogens. So far, a few defense genes have been cloned into plants and still less in rice. Transformation of rice with a number of putative defense genes is being tried the world over. Different forms of chitinase genes from bean (Broglie *et. al.*, 1989), from rice (Huang *et. al.*, 1991) and from barley (Leah *et. al.*, 1991), and osmatin-like protein (AP24) from tobacco (Singh *et. al.*,

1989) have been cloned. Lin *et al.* (1995) reported genetic engineering of rice for resistance against sheath blight caused by *Rhizoctonia solani* using a rice chitinase gene. Several methods have been found to introduce a foreign gene into rice protoplast and intact cell. Polyethylene glycol (PEG) helps in fusing protoplasts and also promotes the uptake of genes by protoplast (Khush and Toenniessen,1991) through electroporation, the foreign DNA is allowed to penetrate the protoplast membrane (Nagata, 1989). The major limitations for these two methods were isolation of protoplast and regeneration of plants from rice protoplasts (Khush and Toenniessen,1991). , These methods are genotype-dependant and do not readily apply to all cereal species (Li *et al.*, 1993).

Agrobacterium mediated transformation has been well developed for dicotyledonous plant species, but not for monocotyledonous species except in few cases (Bytebier *et al.*, 1987). However, transformation methods based on the use of *Agrobacterium tumefaciens* are still preferred in many instances, as this does not require protoplast and in general, result in higher transformation efficiency and a more predictable pattern of foreign DNA integration rather than any other known transformation technique (Czernilofsky *et al.*, 1986; Chan *et al.*, 1993; Hiei *et al.*, 1994).

The biolistic method is best alternative method for cereal transformation (Li *et al.*, 1993) and is successfully used in maize, rice, sugarcane and wheat transformation (Fromm *et al.*, 1991; Bower and Birch, 1992; Vasil *et al* ., 1992). John Sanford of Cornell University invented the biolistic method (Klein et al .,1987). The major advantages of transformation through biolistic are that any kinds of cells or tissue can be used, false positive results arising from the growth *Agrobacterium* in host tissue is eliminated and transient expression can be examined within a few days (Hagio, 1998).

Biolistic transformation of rice

Rice transformation through biolistic method has been used for both japonica and indica rices. Transformation efficiency was observed to be about 25% in japonica rice (Fauquet *et al.*, 1996). Among the japonica rice, Taipei309 was used as model variety for transformation, using embryogenic cell suspension and/or embryogenic calli as the target tissue

Indica rice transformation has always been much more difficult to achieve. Previously, immature embryo (Rancé et al., 1994) and embryo cell suspensions (Zhang *et al.*, 1996) were used as target cells for transformation. Using the latter, variable success was achieved (Fauquet *et al.*, 1996). In indica rice, fertile lines were regenerated through bombarding immature embryos (Christou *et al.*, 1992) or electroporating embryogenic cells (Xu and Li 1994) or using suspension cells (Zhang *et al.*, 1995).

Transmission of transgenes to next progeny

Molecular analysis of transgenic plants by means of Southern blot hybridisation and/or polymerase chain reaction (PCR) is used extensively to demonstrate stable integration of foreign DNA into the genome of transgenic rice. Segregation pattern of the introduced genes into the progenies, has been used for genetic analysis of transgenic rice (Rashid *et al.*, 1996). Mendelian pattern of segregation confirms the stable integration of gene into the genome of transgenic rice (Hiei *et al.*, 1994; Rashid *et al.*,1996).

Genetic engineering for bacterial leaf blight resistance

Xa21 gene was identified from a wild African rice cultivar *Oryza longistaminata* (Khush *et. al.*, 1989). The *Xa21* gene was transferred from *O. longistaminata* to a susceptible cultivar, IR24 by conventional breeding and the variety was named IRBB21 (Khush *et. al.*, 1990). This line with *Xa21* conferred resistance to all the known races of *Xoo* in India and Philippines (Khush *et. al.*, 1990; Ikeda *et. al.*1990). From IRBB21, the gene was cloned using positional cloning (Ronald *et. al.*, 1992; Ronald, 1997). The molecular structure of *Xa21* represents the gene was found to be translated into a receptor kinase-like protein carrying leucine-rich repeats (LRR) in the putative extracellular domain, a single pass transmembrane domain and a serine threonine kinase (STK) intra-cellular domain (Song et.al.,1995).

Wang *et. al.* (1996) transformed japonica variety Taipei 309 with cloned *Xa21* gene. Recently, Tu *et. al.* (1998) studied the resistance spectrum and segregation pattern of *Xa21* in transgenic lines of an indica rice cultivar IR72.

Materials and Methods

Chapter 3

Materials and Methods

Isolation of *Xoo*

Rice leaves showing typical BLB symptoms were selected and cut into small bits of 3 mm in size. The leaf bits were surface-sterilized using 0.1% mercuric chloride for 30 sec and changed to sterile distilled water at least three times. The leaf bits were incubated on a modified Wakimoto's peptone sucrose agar (PSA) medium.

Modified Wakimoto's peptone sucrose agar medium (Durgapal, 1988)

Component	g per litre
Peptone	5.0
Sucrose	20.0
$Ca(NO_3)_2 . 4H_2O$	0.50
$FeSO_4 . 7H_2O$	0.50
$Na_2HPO_4 . 2H_2O$	2.0
Agar	20.0
Water	to one litre

After a lapse of 48 h, a yellowish bacterial growth was observed. The bacterium was purified by dilution plate technique (Waksman, 1952) using single colonies.

Xoo isolates

A total of seven isolates were used in the present study. The Aduthurai isolate was obtained from Tamil Nadu Rice Research Institute (TRRI), Aduthurai. The Coimbatore isolate was from the Paddy Breeding Station (PBS), Centre for Plant Breeding and

Genetics, Tamil Nadu Agricultural University, Coimbatore. Four isolates were received from the Central Rice Research Institute (CRRI), Cuttack (IAXO501, IAXO100, IAXO469 & IAXO34) and the Hyderabad isolate was received from the Directorate of Rice Research (DRR), Hyderabad.

The *Xoo* isolates were maintained on glycerol. The bacterium was inoculated into peptone sucrose broth and incubated for 48 h. The bacterial growth was mixed with sterile glycerol such that the final volume of stock contained 15% of glycerol. The glycerol stock was maintained at – 70 °C. For revival, a loopful of bacteria was streaked onto potato-sucrose agar plates and incubated at room temp.

Checking the pathogenicity of the isolates

The bacterial inoculum was prepared by suspending the actively growing 48h old pure culture in sterile distilled water. The inoculum density was adjusted with a spectrophotometer to about 10^9 colony forming units per ml (cfu. ml^{-1}). Taichung Native 1(TN1) plants were raised in pots of 22.5 cm diam containing 3 kg of wetland soil supplemented with recommended quantities of NPK. The 21 day-old rice plants were inoculated with *Xoo* inoculum by leaf clipping method (Kauffman *et al*, 1973). The inoculation was performed at late evenings and the plants were kept in a moist chamber to maintain a high RH in order to favour rapid development of lesions. The symptom development was observed in 7 to 10 d after inoculation and this was compared with control plants sprayed with sterile water. The bacterium from the lesions was reisolated in order to satisfy Koch's postulates.

Screening indica rice genotypes at seedling stage against *Xoo* isolates

Seeds of 14 varieties of diverse genetic background *viz.* TN1, ADT38, IR50, PTB18, Choorapoondi, TNAU831311, IR20, ASD20, PTB33, TKM9, W1263, ASD16, Co43 and NH56 were obtained from PBS, TNAU, Coimbatore. These genotypes are noted for their performance over several generations (Pers. Comm. Dr. P. Shanmugasundaram, Professor of Agricultural Botany, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore). These varieties were sown individually in pots filled with wetland soil. Twenty-one day-old seedlings were clip-inoculated with six isolates (IAXO 469, IAXO 34, IAXO 501, IAXO 100, isolates from Aduthurai and Coimbatore). The symptom development was observed 14 d after inoculation and scoring was done using the Standard Evaluation System followed in IRRI, The Philippines (IRRI, 1980) as follows:

Scale	Description
0	No lesion
1	1% diseased leaf area
3	1–5 % diseased leaf area
5	6–25% diseased leaf area
7	26–50% diseased leaf area
9	> 50% diseased leaf area

The per cent disease index (PDI) was also calculated using the formula of McKinney (1923).

$$\text{PDI} = \frac{\text{Sum of all individual ratings}}{\text{Total number of leaves graded}} \times \frac{100}{\text{Maximum grade}}$$

Based on the PDI and intensity (grade), the reaction of the plants to the disease was assessed and varieties were grouped (Kauffman *et al.*, 1973; Sahu and Parida, 1997) as follows:

Grade	Disease reaction
1	Resistant
3	Moderately resistant
5	Moderately susceptible
7	Susceptible
9	Highly susceptible

Screening the select genotypes against *Xoo* isolates during different growth stages

Six varieties were forwarded to screen at different growth stages *viz.*, seedling stage (21 d after sowing; DAS), maximum tillering stage (50 DAS) and boot leaf stage (70 DAS). The susceptible check (TN1) was also included in the experiments and the plants were inoculated with the six *Xoo* isolates. Observation was taken after 14 d after inoculation.

Grouping different *Xoo* isolates using differentials

A set of differentials, IR24 40179, IR8 40171, IR 20 40177, Cas209, DV85, IRBB21 and TN1, were obtained from International Rice Research Institute, Los Baños, Manila, the Philippines. The plants were raised and 21 day-old seedlings were clip-inoculated with seven isolates (IAXO 469, IAXO 34, IAXO 501, IAXO 100, Aduthurai, Coimbatore and Hyderabad). The observation was taken 21 d after inoculation.

Transformation of indica rice cultivars

Plasmid constructs used

pRQ6 is a pUC derived plant transformation vector of size 8.25 kb which harbours hygromycin phosphotransferase (*hph*) and β-glucuronidase (*gusA*) genes each with CaMV promoter and NOS terminator sequences (Courtesy: International Laboratory for Tropical Agricultural Biotechnology, The Scripps Research Institute, La Jolla, USA; Fig. 1). pWRG1515 harbours *hph* and *gusA* genes each with 35S CaMV promoter and NOS terminator sequences (Gift of the John Innes Centre, Norwich, UK; Fig. 2). pB822 carries *Xa21*, a BLB resistance gene, the size of the plasmid was 12.5 kb (Courtesy: Dr. Pamela C. Ronald, Department of Plant Pathology, University of California, Davis, USA; Fig. 3).

Preparation of competent cells

The *Escherichia coli* strain DH5α was grown on LB broth to an optical density of 0.5 to 0.6 at 600 nm.

LB broth (Sambrook *et al.*, 1989)

Component	g per litre
Tryptone	10.0
Yeast extract	5.0
NaCl	10.0
Water	to one litre

The cells were collected by centrifugation at 4000 rpm for 10 min at 4 °C. The cells were resuspended in 1/5 volume 100mM CaCl2 and incubated on ice for 10 min. The cell

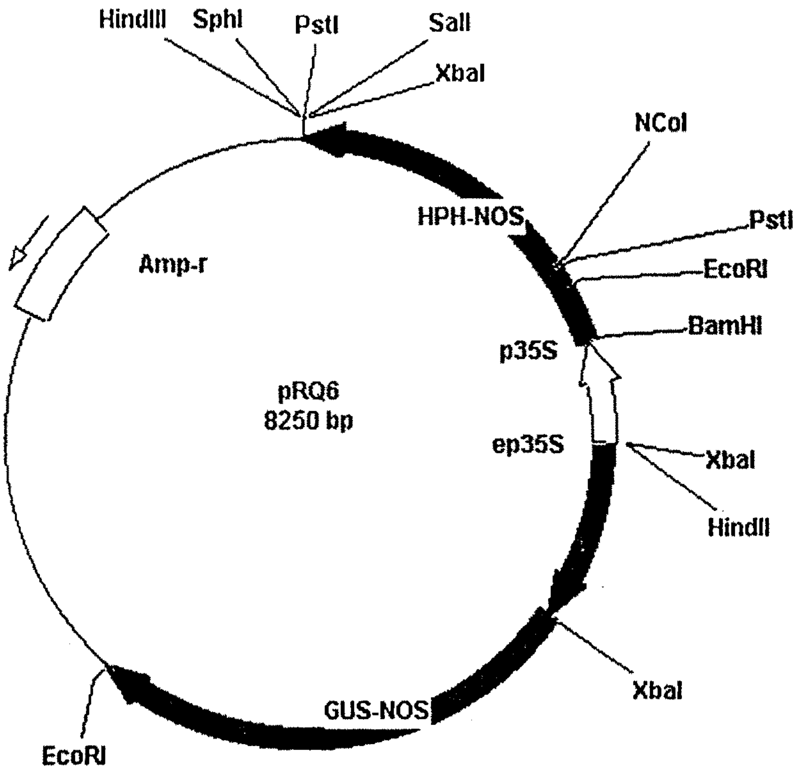


Fig. 1. Plasmid map of pRQ6

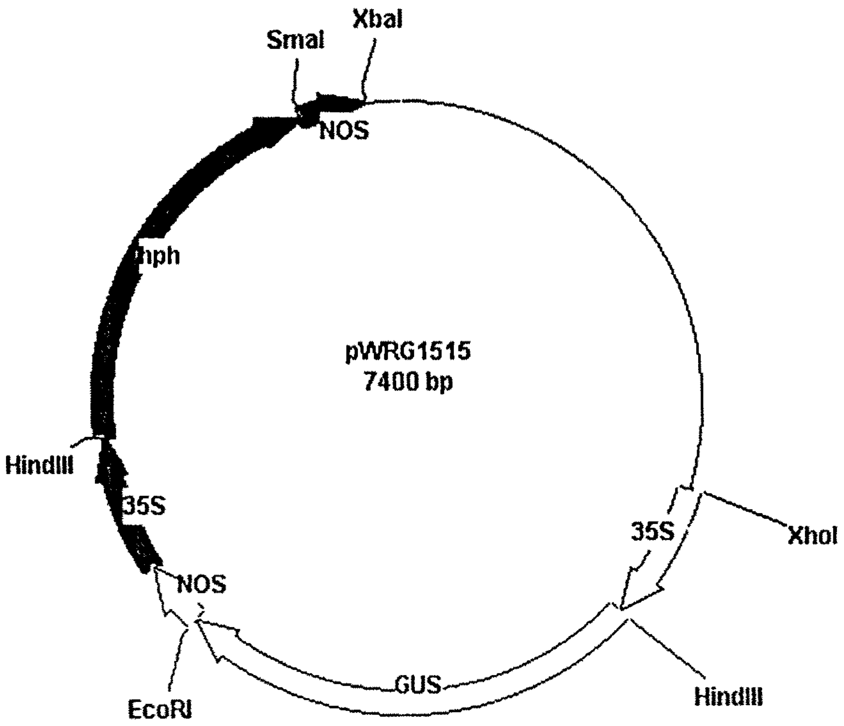


Fig. 2. Plasmid map of pWRG1515

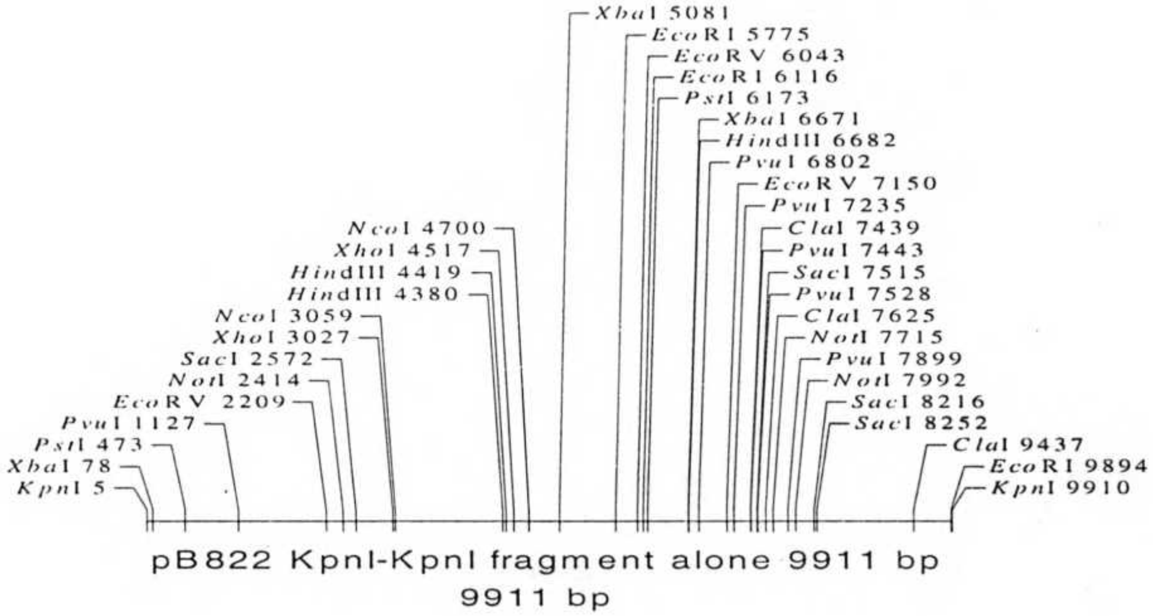


Fig. 3. Plasmid map of pB822; The *Kpn*I fragment is borne in a backbone replicon of pBluescript SK (+/-)

suspension was centrifuged at 4000 rpm for 10 min at 4 °C, the cells were again resuspended in 1/25 volume 50mM $CaCl_2$ and incubated on ice for 20 min, the cell suspension was centrifuged for 10 min at 5,000 rpm at 4 °C , the cells were resuspended in ice cold 100mM $CaCl_2$, the final content was mixed with 15 % v/v sterile glycerol and stored in – 80 °C for long time storage..

Bacterial transformation

To an aliquot of 100 µl competent cells, 25–50 ng purified plasmid DNA was added. The mixture was incubated on ice for 30 min and heat shock was given at 42 °C for 90 sec. The mixture was kept in ice for 5 – 10 min. Then, the transformed bacterial cells were multiplied by transferring the mixture to 3 ml LB broth and incubated at 37 °C for 1 h. After incubation, 50–100 µl of the cell suspension was plated on LB agar containing the appropriate antibiotic.(100µg ampicillin/ml of medium). The plate was incubated at 37 °C for 12 h and stored at 4 °C for further use.

Isolation of plasmid

A single transformed colony was picked from the selection plate and grown in LB broth with appropriate antibiotics for 16 h at 37 °C. The grown up bacterial culture was used for isolation of plasmid.

Isolation and purification of the plasmid was carried out using Wizard Plus Maxipreps DNA purification system (Promega, USA) by following the manufacturer's instructions.

Restriction digestion of Plasmids

One microgram plasmid DNA was restricted with the appropriate restriction enzymes (5 units per µg plasmid DNA). The reaction mixture contained plasmid DNA, appropriate assay buffer, restriction enzyme and water. The reaction mixture was incubated at 37 °C for 4 h.

Agarose gel electrophoresis

Required amount of agarose was weighed (0.8% w/v) and melted in 1x TAE buffer

Tris-acetate EDTA buffer (TAE; Sambrook *et al.*, 1989)

1X TAE contains 0.04M Tris acetate and 0.001M EDTA
(242g Tris base; 57.1 ml glacial acetic acid; 100 ml 0.5M EDTA; pH 8.0).

Ethidium bromide was added to the final concentration of 0.5µg/ml of gel. After cooling to 50–55 °C, the agarose solution was poured onto the preset template with appropriate comb. After solidification, the comb was removed and the gel with template was placed in an electrophoresis gel unit containing the running buffer (1x TAE).

To the DNA sample, required volume of gel loading buffer was added and the samples were loaded into the wells. Electrophoresis was carried out at 50 v (Sambrook *et al.*, 1989).

Gel loading buffer (Sambrook *et al.*, 1989)
6X buffer contains 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water
(stored at 4 °C)

Biolistic transformation of rice

Gene transformation system used in the study was Biolistic PDS–1000/He Particle Delivery System (BioRad, USA). The manufacturer's instructions were strictly followed for operating the instrument, coating the gold particle with plasmid DNA and bombardment.

Preparation of gold particles (microcarriers)

Six milligrammes gold particles (equal quantities of each of 1.0 µm dia size; BioRad, USA and 1.8–2.0 µm dia; Alfa, Germany) was weighed and suspended in 100 µl absolute alcohol. It was vortexed for 1–2 min, centrifuged for 10 sec at 10,000 rpm and the supernatant was removed. The pellet was suspended in 100 µl sterile double distilled water, vortexed for 1 min and recentrifuged at 10,000 rpm for 10 sec. The supernatant was removed and this was repeated three times. Finally the pellet was suspended in 100 µl of sterile double distilled water.

Coating gold particles with plasmid DNA

The construct harbouring *Xa21* gene, pB822 was bombarded with either construct harbouring marker genes (pRQ6/pWRG1515). The plasmids were mixed in a ratio of 5:1(pB822 and marker plasmid respectively). The ratio was based on the size of the plasmid.

Fifty microlitres gold suspension was taken in an Eppendorf tube and 5 µg plasmid DNA was added while vortexing at low speed. To the plasmid-gold mixture, 20 µl of 0.1M spermidine was added. To this, 50 µl of 2.5M $CaCl_2$ was added drop by drop while vortexing. The mixture was left for 10 min at room temp (25 °C). Then, the mixture was centrifuged at 10,000 rpm for 10 sec, supernatant was removed and the pellet was resuspended in 60 µl absolute ethanol.

Isolation of immature embryos

Milky to post-milky stage (15 d after flowering) panicles were collected and the developing grains were harvested from them. The seeds were removed from the grains and were surface-sterilised immediately after removing their lemma and palea with 70% ethanol for 5 min and in 0.1% $HgCl_2$ for 3 min. Subsequently, they were washed with sterile double distilled water 3 times. The immature embryos were extracted under a stereo binocular microscope. The isolated embryos were placed on CC proliferation medium and kept in dark at 25 ± 2 °C.

CC media (Potrykus *et al.*, 1979; Vain *et al.*, 1993)

Components	per litre	
	CC proliferation medium	CC osmoticum medium
CC macronutrients	100 ml	100 ml
CC micronutrients	10 ml	10 ml
Fe EDTA (4 g/l)	10 ml	10 ml
Sucrose	20 g	20 g
Mannitol	15 g	60 g
Sorbitol	15 g	-
2,4-D	2 mg	2 mg
Casein hydrolysate	1 g	1 g
Coconut water	100 ml	100 ml
Phytagel	4 g	4g
pH	5.8	5.8
CC vitamins sterilized, pH5.8	10 ml	10 ml

CC stock solutions

CC macronutrients	g per litre	Volume used per litre of medium
KNO_3	12.12	
NH_4NO_3	6.4	100 ml
$CaCl_2 . 2 H_2O$	5.88	
$MgSO_4 . 7H_2O$	2.47	
KH_2PO4	1.36	
CC micronutrients	mg per litre	Volume used per litre of medium
H_3BO_3	310	
$MnSO_4 . 7H_2O$	1115	
$ZnSO_4 . 7H_2O$	576	10 ml
KI	83	
$Na_2MoO_4\ 2H_2O$	24	
$CuSO_4 . 5H_2O$	2.5	
$CoCl_2 . 6H_2O$	2.8	
CC vitamins	mg per litre	Volume used per litre of medium
Nicotinic acid	600	
Thiamin HCl	850	
Pyridoxine HCl	100	10 ml
Glycine	200	
myo-inositol	100	

About 50-70 immature embryos were arranged at the centre of a 90 cm Petri plate with the proliferation medium containing osmoticum and incubated for 4 h.

Callus Induction

Four hours after bombardment the immature embryos were transferred to proliferation medium and incubated for one d at 25 ± 2 °C dark. After the lapse of 1 d, the embryos were transferred to selection medium containing hygromycin 50 mg/l and incubated in dark at 25 ± 2 °C. Calli obtained on hygromycin selection were subcultured on a fresh medium containing hygromycin. Four rounds of subculturing and selection were performed at 14 d interval on fresh medium with hygromycin.

GUS assay

GUS assay buffer was prepared by dissolving 5 mg of X-Gluc (5-bromo, 4–chloro, 3–indolyl β glucuronide, Sigma, USA), 359 mg sodium dihydrogen phosphate dihydrate and 0.01 ml Tween 20 in 20 ml double distilled sterile water, pH of the solution was adjusted to 7.0 using 1N NaOH and after filter sterilization, It was stored at 4 °C in a sterile container.

Transient GUS expression assay was carried out for 10 embryos randomly selected from 48 h after bombarded plates. The embryos were submerged into the GUS assay buffer placed in an Eppendorf tube and incubated at 37 °C overnight.

Stable GUS assay was performed on the calli selected on the fourth round of hygromycin selection.

Regeneration of putative transgenic plants

Calli selected on hygromycin for the fourth time were transferred to CC regeneration medium with hygromycin.

CC Regeneration medium (Potrykus *et al.*, 1979)

Components	per litre
CC macronutrients	100 ml
CC micronutrients	10 ml
Fe EDTA (4 g/l)	10 ml
Sucrose	20 g
Mannitol	15.0 g
Sorbitol	15.0 g
Casein hydrolysate	1 g
Coconut water	100 ml
pH	5.8
Phytagel	6 g
CC vitamins (sterilized) pH 5.8	10 ml

The calli were incubated in dark at 25±2 °C for 1 wk and subsequently to 16 h lighting alternated with 8 h darkness photoperiod cycles at 25±1°C using cool white fluorescent lamps (with light intensity of 110-130 mm/M2/S pAR; Philips F40/CW) until shoots developed. The plantlets were then transferred to Magenta boxes containing half strength MS basal medium with hygromycin for rooting.

MS Medium (Murashige and Skoog, 1962)

Chemical	Final Conc. per Litre of the Medium	Stock/100 ml	Quantity to be dispensed for 1 litre of MS medium
NH_4NO_3	1.65 g	16.5 g	10 ml
KNO_3	1.90 g	19.0 g	
$MgSO_4.7H_2O$	0.37 g	3.70 g	
KH_2PO_4	0.170 g	1.70 g	
$CaCl_2.2H_2O$	0.440 g	4.4 g	10 ml
$MnSO_4.4H_2O$	22.300 mg	2230 mg	1 ml
$ZnSO_4.7H_2O$	8.600 mg	860 mg	
H_3BO_3	6.200 mg	620 mg	
KI	0.830 mg	83 mg	
$Na_2MoO_4.2H_2O$	0.250 mg	25 mg	
$CuSO_4.5H_2O$	0.025 mg	2.5 mg	
$CoCl_2.6H_2O$	0.025 mg	2.5 mg	
Fe EDTA	11.25 µg	2.5 mg	0.450 ml
Nicotinic acid	0.5 mg	50	1 ml
Pyridoxine	0.5 mg	50	
Thiamin HCl	0.1 mg	10	
Glycine	2.0 mg	200	
Myo-Inositol	100 mg	added solid	100 mg
2,4 -D	2 mg	100 mg	2 ml
Sucrose	30 g	added solid	30 g

To prepare the medium, required amount of solution was taken from the respective stock solutions, to which the carbon source (sucrose or maltose) and other additives (in solid form) were added. The growth hormones, which can be co-autoclaved were also added as per the requirements of the different experiments. The pH of the medium was adjusted to 5.8 (using either 1N KOH or 1N HCl) before adding solidifying agent. After making up the final volume with distilled water, the medium was dispensed into flasks and covered the neck of the flasks with two layers of aluminum foil and then sterilized in an autoclave at 1.01 kg/cm^2 (15 lbs) at 121° C for 15-20 min.

When plants were 8–10 cm high (after 1–2 wk) they were transferred to Yoshida's culture solution (Yoshida *et al.*, 1976) for better root development.

Yoshida's Culture solution (Culture solution for hardening of tissue cultured plantlets)

Chemical	g/l in final solution	80x stock g/l	Quantity to be dispensed per litre of the medium
NH_4 NO_3	0.114	9.14	12.5 ml/l
$NaH_2PO_4.2H_2O$	0.050	4.03	
K_2SO_4	0.089	7.14	
$MgSO_4.7H_2O$	0.405	32.4	
$CaCl_2$	0.111	8.860	12.5 ml/l
$MnCl_2.4H_2O$	0.0018	0.1500	12.5 ml/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0000925	0.0074	
H_3BO_3	0.00117	0.0934	
$ZnSO_4$.$7H_2O$	0.0000437	0.0035	
$CuSO_4$.$5H_2O$	0.0000387	0.0031	
$FeCl_3$.$6H_2O$	0.009625	0.7700	

Stock solutions were stored at room temperature. To make one litre of culture solution, the required amounts of stocks were added and the volume was made up to 950 ml, the pH was adjusted to 6.0 using 1M KOH/NaOH. Final volume was one litre. The solution was autoclaved at 121° C for 15-20 min before use.

DNA isolation for PCR amplification

A small leaf bit (ca. 100 mg) was ground with 300 µl DNA extraction buffer.

DNA extraction buffer (Dr. K. Tang, John Innes Centre, Norwich, UK; Pers. Comm.)

200 mM Tris HCl (pH 7.5)
200 mM NaCl
25 mM EDTA
0.5% SDS

The homogenate was centrifuged at 12,000 rpm for 10 min. The supernatant was collected and to this 200 µl isopropanol was added and kept at –20° C for 10 min. This was centrifuged and the pellet was dissolved in 30 μl of TE buffer (10 ml Tris-Cl pH 8.0, 1mM EDTA, pH 8.0). For each PCR reaction 1 μl DNA was used.

Polymerase chain reaction

Polymerase chain reaction was performed as recommended by Sambrook *et. al.* (1989) with a few modifications. Twenty-five microlitres of reaction mixture containing 10x assay buffer (10μM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin; Bangalore Genie, India) , 200 μM each of dNTPs (Bangalore Genie, India), 1μM each of the following forward and reverse primers in each experiment and 1 unit of *Taq* polymerase (Bangalore Genie, India):

Experiment 1: Primer pairs based on *Xa21* intron/kinase region of *Xa21* gene
  Forward primer: U1 (5' CGA TCG GTA TAA CAG CAA AAC 3') and
  Reverse primer: 11 (5' ATA GCA ACT GAT TGC TTG G 3')
Experiment 2: Primer pairs based on the kinase specific region of *Xa21* gene
  Forward primer: K2F (5' GTG CTGG AAA TAG TAA CC 3') and
  Reverse primer: K2R (5' CCT GAG AGC AAG ACA ACC 3')
(Wang et. al., 1996; Primers supplied by Genemed Synthesis Inc., South San Francisco, USA)

The amplification was performed in a PTC-100 mini-cycler (MJ Research, U.S.A.) with the following conditions. Pre-incubation at 94 °C for 3 min, leading to 35 cycles of denaturation at 94 ºC for 1 min, annealing at 52 °C for 1 min and synthesis at 72 °C for 1 min followed by an extension at 72 °C for 6 min. After amplification, 10 μl reaction mixture was used for electrophoretic analysis in a 0.8 % agarose gel.

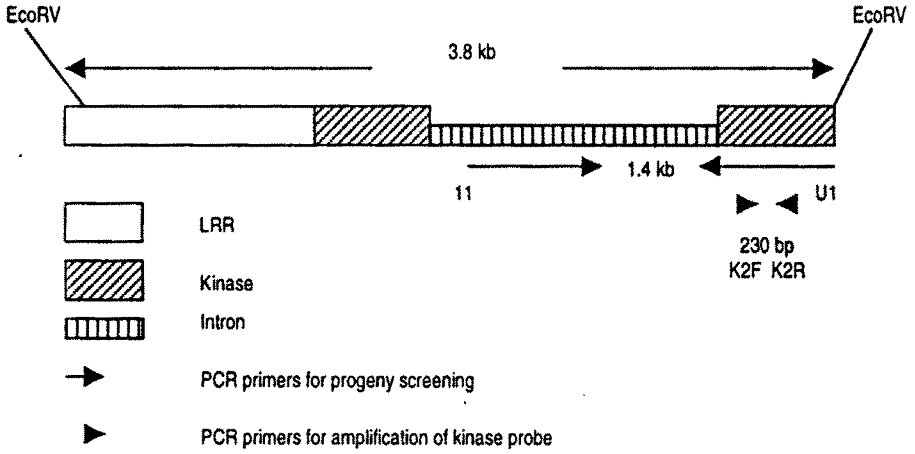


Fig. 4. Restriction map and primer combinations used in the analysis transgenic plant expressing the Xa21 gene. The primer pairs U1 and 11 amplify a 1.4 kb DNA fragment from the *Xa21* gene that was used in genotypic analysis of the T_0 progeny. The primer pairs, K2F and K2R amplify a 230 bp fragment which was also used a s a primer in PCR analysis of the T_0 lines (adapted from Wang *et al*, 1996).

Experimental Results

Chapter 4

Experimental Results

Screening indica rice genotypes at seedling stage against *Xoo* isolates

TKM9 was resistant to IAXO100 (PDI, 2.8) and the Coimbatore isolate (PDI, 3.65) while it was moderately resistant to IAXO34 (PDI, 9.62), IAXO469 and IAXO501 (PDI, 11.11). PTB18 registered a moderately resistant reaction to the Coimbatore isolate (PDI, 6.66), IAXO34 (PDI, 9.27), IAXO100 (PDI, 9.63), IAXO501 (PDI, 10.00) and the Aduthurai isolate (PDI, 11.11). W1263 was also moderately resistant to IAXO34 (PDI, 8.89) and IAXO100 (PDI, 11.48). NH56 registered a moderately resistant reaction only to the Aduthurai isolate (PDI, 8.71) and TNAU831311 was moderately resistant to the Aduthurai isolate (PDI, 9.27) and the Coimbatore isolate (PDI, 10.00) (Table 1; Fig. 5; Plate 1a-e, Plate 2). All the above six genotypes were forwarded to further screening experiments.

Screening the select genotypes against *Xoo* isolates during different growth stages

W1263 was resistant to IAXO501 (PDI, 4.10), IAXO34 (PDI, 4.12), the Coimbatore isolate (PDI, 4.60) and IAXO100 (PDI, 5.00) at boot leaf stage, while it was only moderately resistant to IAXO34 at maximum tillering stage (PDI, 6.00) and at seedling stage (PDI,

Table 1. Screening different rice varieties against *Xoo* isolates.

Isolate/ Varieties	PDI (%)						Disease Reaction					
	IAXO 469	Coimbatore	IAXO 34	IAXO 501	IAXO 100	Aduthurai	IAXO 469	Coimbatore	IAXO 34	IAXO 501	IAXO 100	Aduthurai
TN1	72.24 (58.20)^{a}	50.36 (45.20)^{c}	50.75 (45.42)^{c}	62.98 (52.52)^{a}	50.01 (45.00)^{c}	72.61 (58.44)^{a}	HS	HS	HS	HS	HS	HS
ADT38	44.82 (42.02)^{d}	41.12 (39.90)^{d}	54.09 (47.34)^{b}	57.78 (49.47)^{b}	57.05 (49.05)^{b}	24.07 (29.37)^{f}	S	S	HS	HS	HS	MS
IR50	48.90 (44.36)^{c}	55.94 (48.41)^{b}	65.20 (53.85)^{a}	39.64 (39.02)^{c}	29.16 (32.66)^{g}	40.75 (39.66)^{c}	S	HS	HS	S	S	S
PTB18	38.15 (38.14)^{e}	6.66 (14.92)^{i}	9.27 (17.71)^{i}	10.00 (18.42)^{i}	9.63 (18.06)^{j}	11.11 (19.47)^{h}	S	MR	MR	MR	MR	MR
Choorapoondi	66.69 (54.75)^{b}	30.00 (33.20)^{f}	32.60 (34.81)^{d}	64.46 (53.40)^{a}	30.37 (33.43)^{g}	20.00 (26.56)^{g}	HS	S	S	HS	S	MS
TNAU 831311	30.00 (33.20)^{gh}	9.99 (18.41)^{h}	25.90 (30.58)^{f}	20.00 (26.56)^{f}	40.34 (39.42)^{d}	9.27 (17.70)^{j}	S	MR	S	MS	S	MR
IR20	48.16 (43.94)^{c}	69.28 (56.34)^{a}	30.37 (33.43)^{e}	33.70 (35.48)^{d}	61.50 (51.64)^{a}	59.24 (50.32)^{b}	S	HS	S	S	HS	HS
ASD20	32.97 (35.04)^{f}	35.50 (36.56)^{e}	24.44 (29.62)^{fg}	33.71 (35.48)^{d}	36.30 (37.04)^{e}	37.04 (37.48)^{d}	S	S	MS	S	S	S
PTB33	29.63 (32.97)^{h}	50.75 (45.42)^{c}	28.89 (32.51)^{e}	18.15 (25.21)^{g}	39.27 (38.80)^{d}	27.41 (31.56)^{e}	S	HS	S	MS	S	S
TKM9	11.11 (19.45)^{i}	3.65 (10.94)^{j}	9.62 (18.05)^{i}	11.11 (19.45)^{i}	2.80 (9.52)^{k}	28.52 (32.27)^{e}	MR	R	MR	MR	R	S
W1263	33.71 (35.49)^{f}	50.18 (45 10)^{c}	8.89 (17.33)^{i}	26.67 (31.09)^{e}	11.48 (19.79)^{i}	23.71 (29.13)^{f}	S	MS	MR	S	MR	MS
ASD16	33.69 (35.47)^{f}	28.51 (32.27)^{f}	28.88 (32.50)^{e}	27.77 (31.79)^{e}	33.69 (35.47)^{f}	22.52 (28.32)^{f}	S	S	S	S	S	MS
Co43	29.92 (33.56)^{gh}	36.67 (37.25)^{e}	22.96 (28.62)^{g}	34.75 (36.11)^{d}	28.88 (32.50)^{g}	35.19 (36.44)^{d}	S	S	MS	S	S	S
NH56	31.85 (34.37)^{fg}	14.44 (22.32)^{g}	15.18 (22.92)^{h}	14.80 (22.39)^{h}	15.56 (23.22)^{h}	8.71 (17.13)^{j}	S	MS	MS	MS	MS	MR

In a column, means followed by the common alphabets are not significantly different at P=0.05 as per Duncan's multiple range test.
R, Resistant; MR, moderately resistant; MS, moderately susceptible; S, Susceptible; HS, highly susceptible

Fig. 5. Screening different rice varieties against *Xoo* isolates

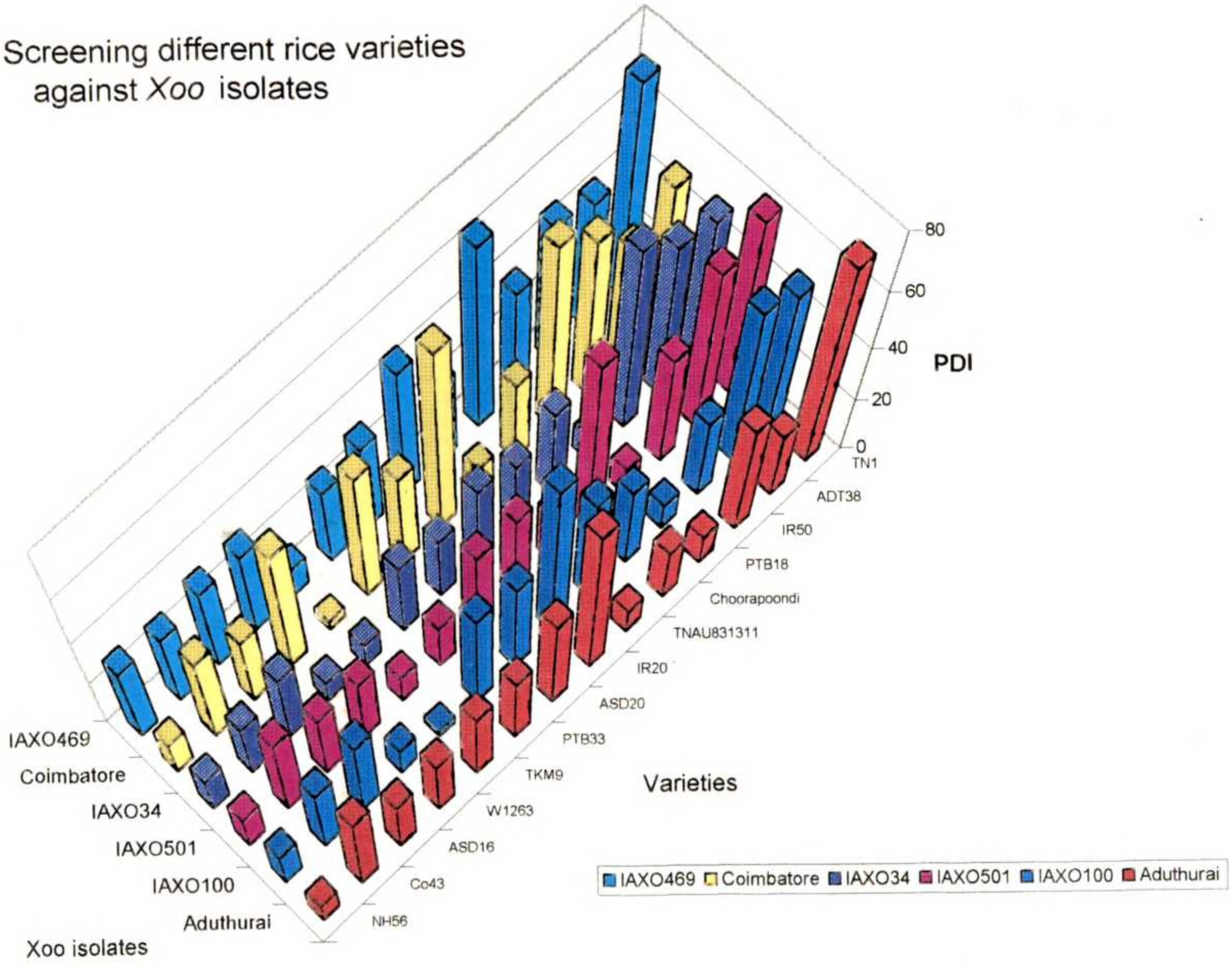


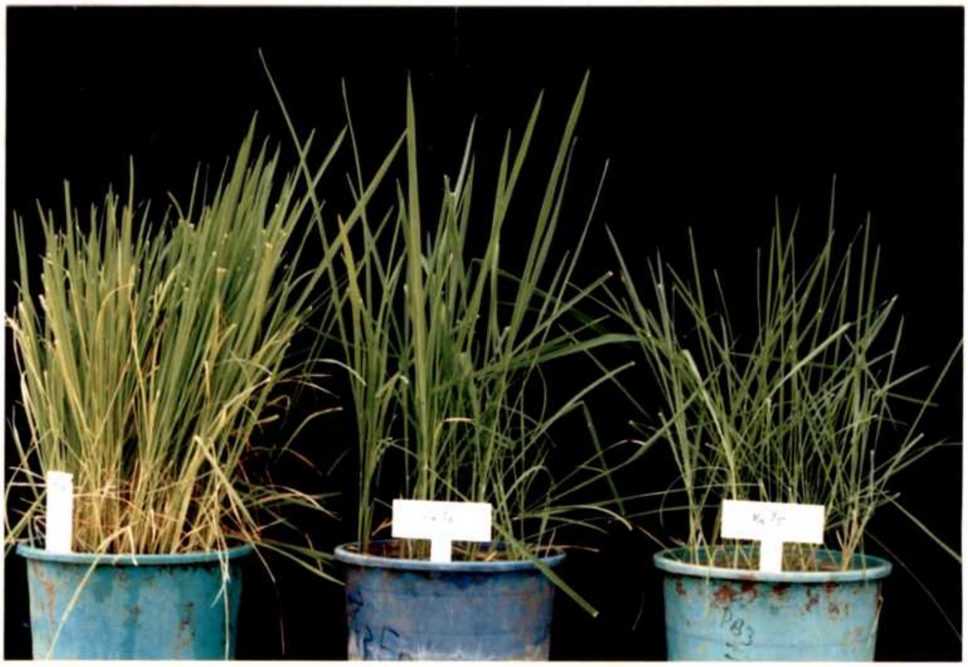
Plate 1a. Reaction of TN1, TKM9 and NH56 to IAXO100

1. TN1 (control)
2. TKM9
3. NH56

Plate 1b. Reaction of TN1, PTB18,TKM9 and NH56 to the coimbatore isolate.

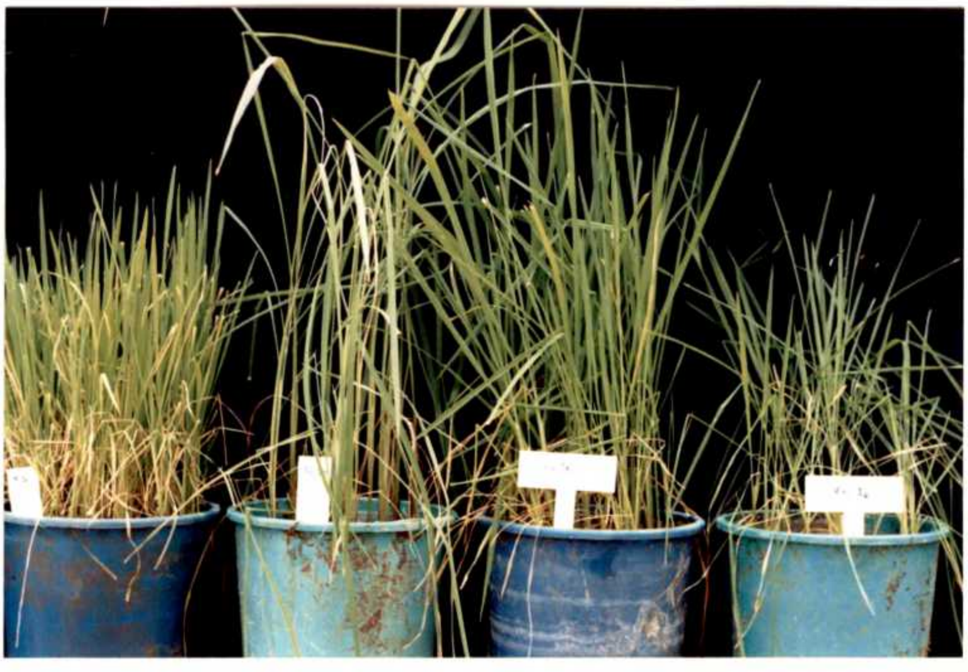
1. TN1 (control)
2. PTB18
3. TKM9
4. NH56

Plate 1a.



1 2 3

Plate 1b.



1 2 3 4

Plate 1c. . Reaction of TN1, TKM9, W1263 and NH56 to IAXO34

1. TN1 (control)
2. TKM9
3. W1263
4. NH56

Plate 1d. Reaction of TN1 and TKM9 to IAXO469

1. TN1 (control)
2. TKM9

Plate 1c.

Plate 1d.

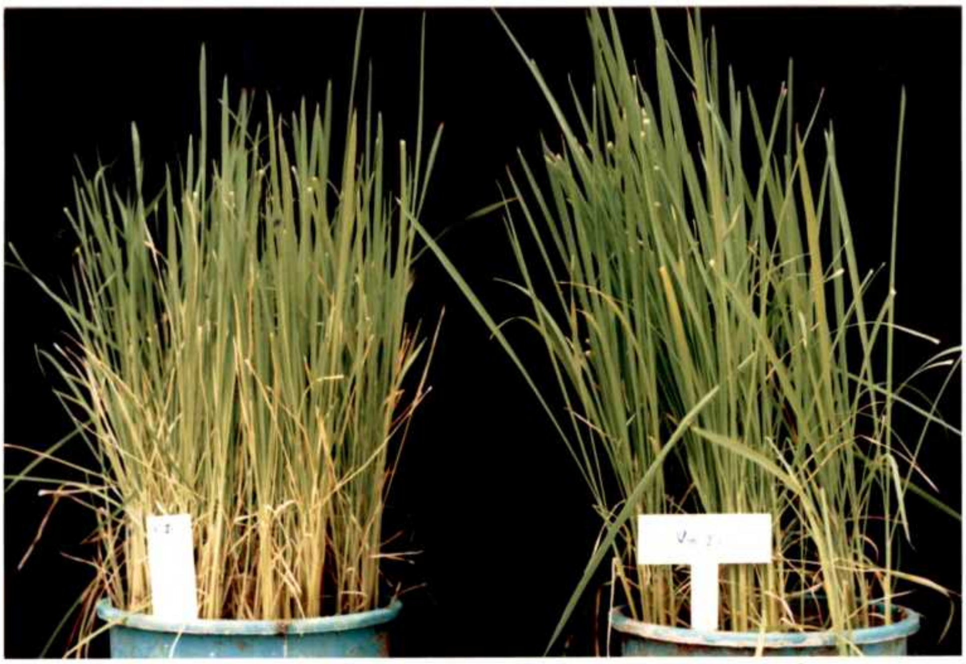


Plate 1e. Reaction of TN1, TKM9 and NH56 to IAXO501

1. TN1 (control)
2. TKM9
3. NH56

Plate 2. Reaction of TN1, PTB18 and NH56 to the Aduthurai isolate

1. TN1 (control)
2. PTB18
3. NH56

Plate 1e.

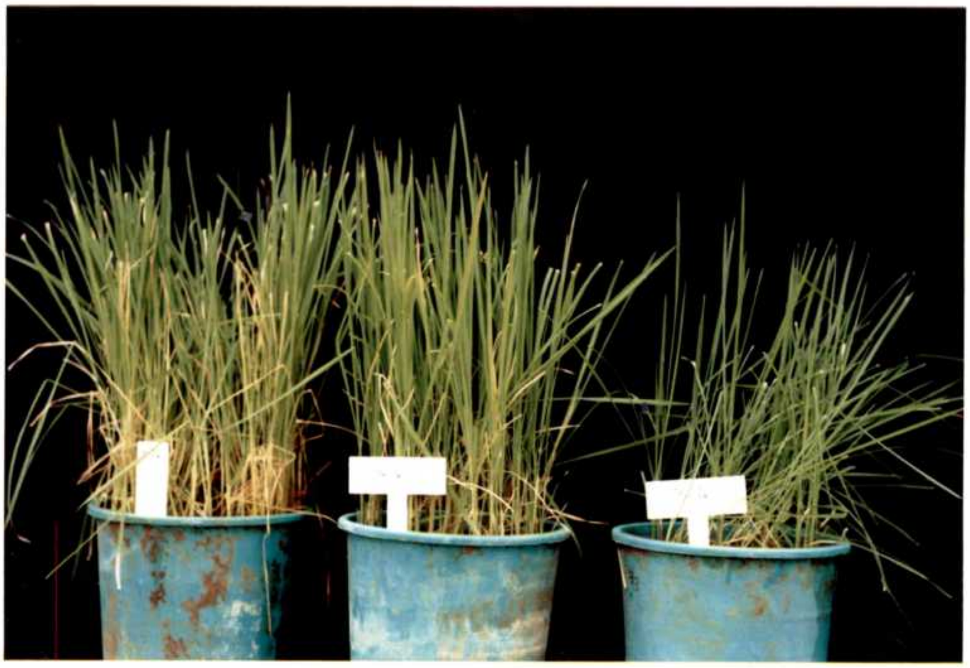


Plate 2.

8.89). PTB18 was moderately resistant to the Coimbatore isolate at seedling stage (PDI, 6.60) (Table 2; Fig. 6; Plate 3a,b; Plate 4).

Varietal grouping based on both PDI and BLB intensity, PTB18 and TNAU831311 were moderately resistant to the Coimbatore isolate, TKM9 to IAXO501 and NH56 to the Aduthurai isolate at all the three stages of growth. W1263 was moderately resistant to IAXO34 and IAXO100 during seedling and maximum tillering stages, while it became resistant to both the isolates at boot leaf stage. W1263 registered an increased resistance to IAXO501 with increasing age of the plant. However, a moderately resistant PTB18 (to IAXO100 and the Aduthurai isolate at the seedling stage) became susceptible when the plant aged (Table 3).

Grouping different *Xoo* isolates using differentials

Among the seven *Xoo* isolates, IAXO469 registered a resistance reaction with Cas209 (PDI, 2.25), DV85 (PDI, 3.7) and IR2040171 and IRBB21 (PDI, 4.6). IAXO34 registered a similar reaction with IR2040171 and DV85 (PDI, 2.7), IRBB21 (PDI, 3.7) and Cas209 (PDI, 4.6). The Coimbatore isolate and IAXO100 were also similar in that they exhibited a resistance reaction with Cas209, DV85 and IRBB21. IAXO501 registered such a resistance reaction only with IRBB21. None of the differentials showed any resistance to the Hyderabad isolate.

The *Xoo* isolates could arbitrarily be grouped into six virulence categories based on their disease reaction (Table 4; Fig. 7; Plate 5). IAXO469 and IAXO34 were assigned

Table 2. Screening select rice varieties against *Xoo* isolates during different growth stages

Isolates/varieties	PDI (%)																	
	Seedling stage						Maximum tillering stage						Boot leaf stage					
	IAXO 469	Coimbatore	IAXO 34	IAXO 501	IAXO 100	Aduthurai	IAXO 469	Coimbatore	IAXO 34	IAXO 501	IAXO 100	Aduthurai	IAXO 469	Coimbatore	IAXO 34	IAXO 501	IAXO 100	Aduthurai
TN1	72.2	50.3	50.7	62.9	50.0	72.6	74.9	58.2	64.7	71.2	77.7	73.1	44.9	55.0	51.8	60.1	68.9	74.9
	(58.18)^{a}	(45.17)^{a}	(45.40)^{a}	(52.47)^{a}	(45.00)^{a}	(58.44)^{a}	(59.94)^{a}	(49.72)^{a}	(53.55)^{a}	(57.54)^{a}	(61.82)^{a}	(58.76)^{a}	(42.07)^{a}	(47.87)^{a}	(46.03)^{a}	(50.83)^{a}	(56.10)^{a}	(59.94)^{a}
PTB18	38.1	6.6	17.1	31.8	9.6	11.1	26.2	11.0	21.2	23.1	15.7	19.4	28.4	10.1	17.5	21.2	25.9	12.9
	(38.11)^{b}	(14.88).^{e}	(24.42)^{c}	(34.32)^{b}	(18.04)^{e}	(19.44)^{d}	(30.78)^{c}	(19.32)^{c}	(27.41)^{d}	(28.72)^{c}	(23.33)^{e}	(26.12)^{d}	(32.20)^{b}	(18.51)^{c}	(24.72)^{c}	(27.41)$_{b}$	(30.58^{b}	(21.03)^{c}
TNAU831311	30.0	10.0	25.9	20.0	40.3	9.26	20.3	11.5	31.0	44.4	40.6	22.6	15.0	10.6	28.7	22.6	21.2	11.0
	(33.20)^{d}	(18.37)^{d}	(30.59)^{b}	(26.56)^{d}	(39.40)^{b}	(17.71)^{e}	(26.77)^{d}	(19.81)^{c}	(33.83)^{b}	(41.78)^{b}	(39.58)^{b}	(28.38)^{c}	(22.77)^{d}	(18.98)^{c}	(32.39)^{b}	(28.38)^{b}	(27.41)^{c}	(19.35)^{d}
TKM9	11.1	17.5	9.63	11.1	6.66	28.5	16.6	24.0	24.9	9.70	28.6	30.0	11.9	15.2	9.70	10..6	9.25	22.2
	(19.44)^{e}	(24.72)^{b}	(18.06)^{e}	(19.44)^{f}	(14.81)^{f}	(32.26)^{b}	(24.03)^{e}	(29.33)^{b}	(29.93)^{c}	(18.13)^{e}	(32.32)^{c}	(33.20)^{b}	(20.16)^{e}	(22.93)^{b}	(18.00)^{e}	(18.98)^{c}	(17.69)^{d}	(28.11)^{b}
W1263	33.7	15.1	8.89	26.6	11.4	23.7	18.9	11.5	6.00	14.3	11.1	12.7	15.2	4.62	4.12	4.10	5.0	10.6
	(35.48)^{c}	(22.86)^{c}	(17.34)^{e}	(31.04)^{c}	(19.73)^{d}	(29.12)^{c}	(25.76)^{d}	(19.82)^{c}	(14.17)^{e}	(22.21)^{d}	(19.46)^{f}	(20.87)^{e}	(22.93)^{d}	(12.38)$_{d}$	(11.65)^{f}	(11.68)^{d}	(12.92)^{e}	(18.98)^{d}
NH56	31.8	14.4	15.1	14.8	15.5	8.89	36.0	24.5	19.8	24.0	19.9	10.1	22.1	16.1	14.3	22.1	21.2	9.20
	(34.[illegible]2)^{cd}	(22.30)^{c}	(22.86)^{d}	(22.62)^{e}	(23.18)^{c}	(17.34)^{e}	(36.86)^{b}	(29.66)^{b}	(26.42)^{d}	(29.33)^{c}	(26.48)^{d}	(18.53)^{f}	(28.02)^{c}	(23.64)^{b}	(22.21)^{d}	(28.04)^{b}	(27.4[illegible])^{c}	(17.65)^{e}

In a column, means followed by the common alphabets are not significantly different at P=0.05 as per Duncan's multiple range test.

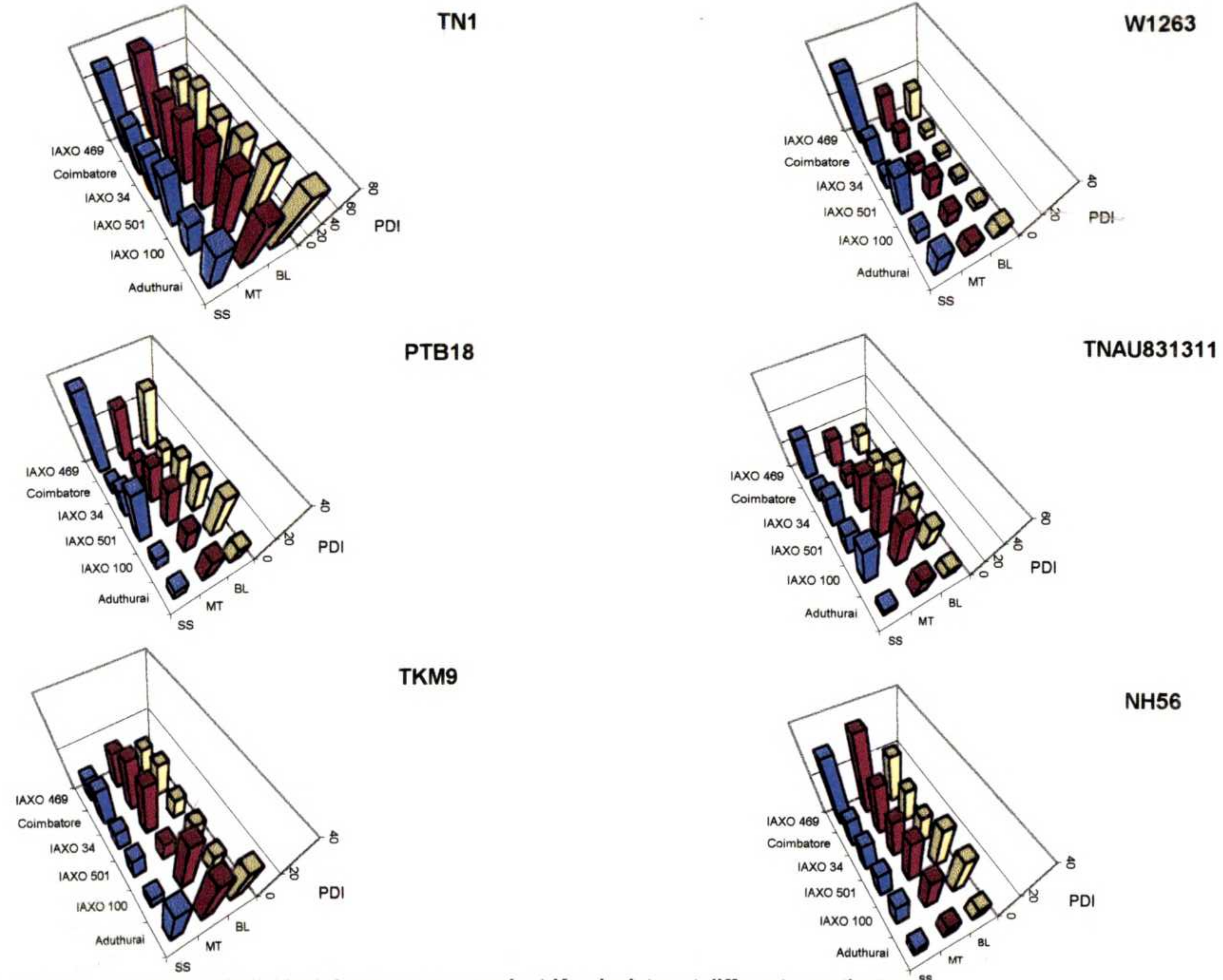


Fig. 6. A comparison of reaction of individual rice genotypes against *Xoo* isolates at different growth stages.
SS, seedling stage; MT, maximum tillering; BL, boot leaf stage

Plate 3a. Reaction of W1263 to IAXO501 and IAXO34 at boot leaf stage

1. TN1 (control)
2. W1263-inoculated with IAXO501
3. W1263-uninoculated
4. TN1 (control)
5. W1263-inoculated with IAXO34
6. W1263-uninoculated

Plate 3a.

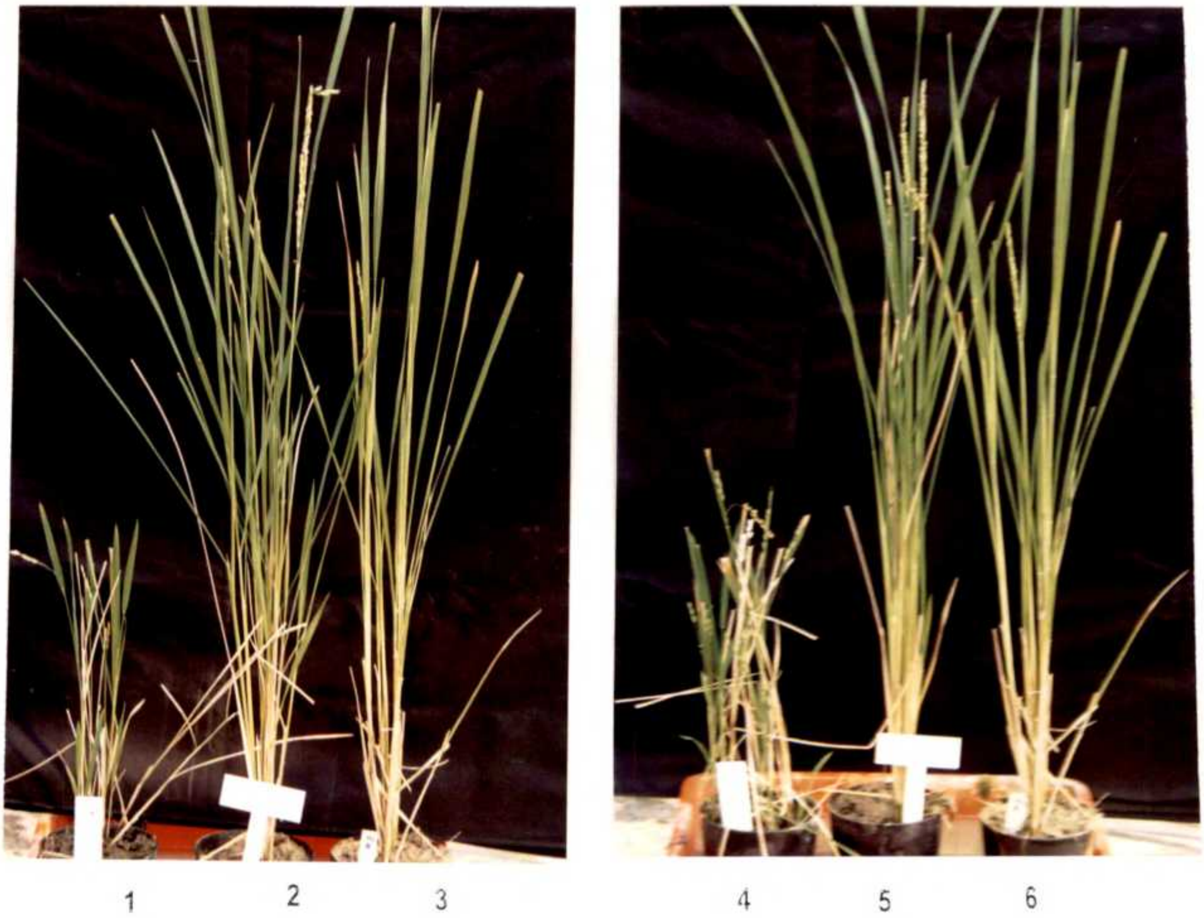


Plate 3b. Reaction of W1263 to coimbatore isolate and IAXO100 at boot leaf stage

1. TN1 (control)
2. W1263-inoculated with coimbatore isolate
3. W1263 uninoculated
4. TN1 (control)
5. W1263-inoculated with IAXO100
6. W1263 uninoculated

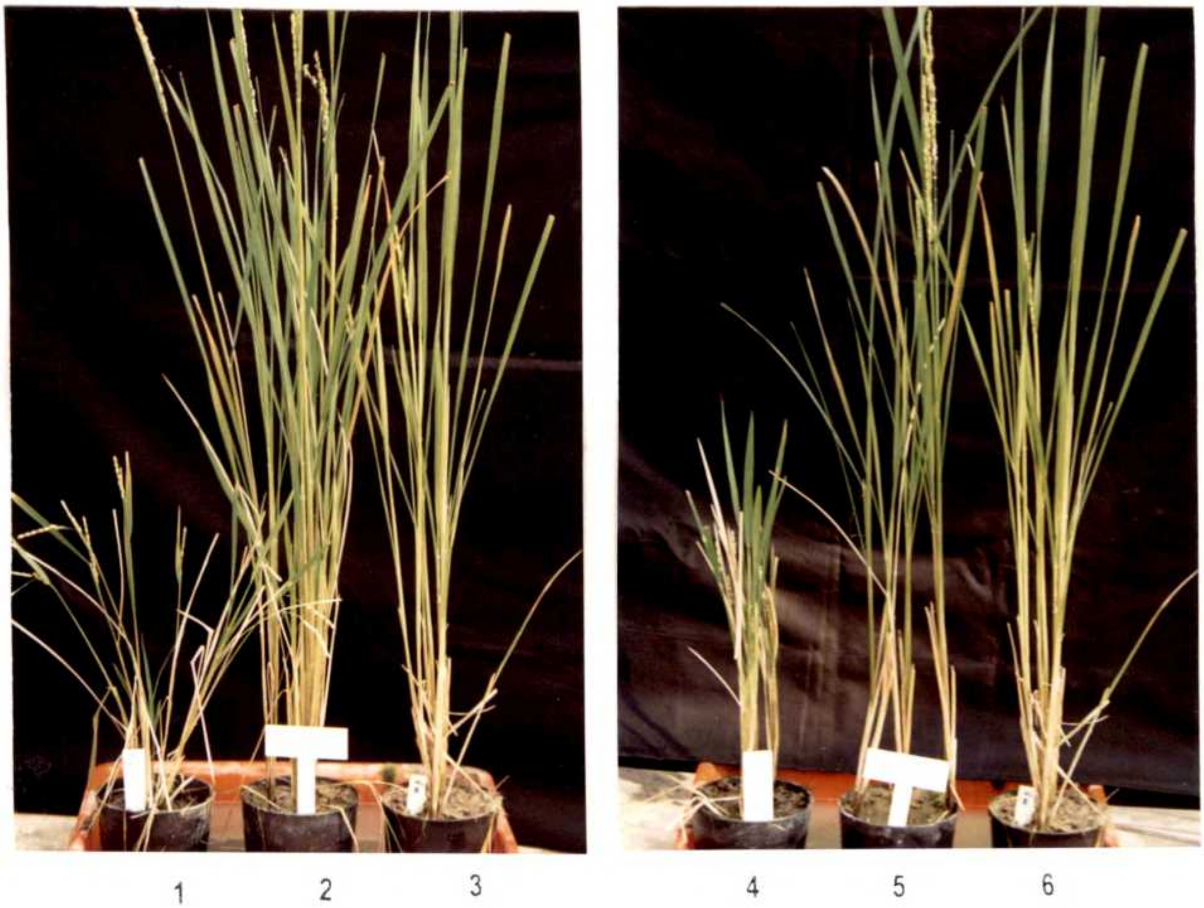


Plate 3b.

Plate 4a. Reaction of W1263 to IAXO34 at maximum tillering stage

1. TN1 (control)
2. W1263 inoculated with IAXO34
3. W1263 uninoculated

Plate 4b. Reaction of W1263 to IAXO34 at seedling stage

1. TN1 (control)
2. W1263 inoculated with IAXO34
3. W1263 uninoculated

Plate 4a.

Plate 4b.

Table 3. Comparison of reaction of rice genotypes against Xoo at different growth stages

Isolate/ variety	Reaction																	
	IAXO 469			Coimbatore			IAXO 34			IAXO 501			IAXO 100			Aduthurai		
	SS	MT	BL	SS	MT	BL	SS	MT	BL	SS	MT	BL	SS	MT	BL	SS	MT	BL
TN 1	HS	HS	S	HS	HS	HS	HS	HS	HS	HS	HS	HS	HS	HS	HS	HS	HS	HS
PTB 18	S	S	S	MR	MR	MR	MS	MS	MS	S	MS	MS	MR	MS	S	MR	MS	MS
TNAU 831311	S	MS	MS	MR	MR	MR	S	S	S	MS	S	MS	S	S	MS	MR	MS	MR
TKM 9	MR	MS	MR	MS	MS	MS	MR	MS	MR	MR	MR	MR	MR	S	MR	S	S	MS
W1263	S	MS	MS	MS	MR	R	MR	MR	R	S	MS	R	MR	MR	R	MS	MS	MR
NH 56	S	S	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MR	MR	MR

SS – Seedling stage MT – Maximum tillering stage BL – Boot leaf stage

Table 4. Grouping different BLB isolates using differentials

Differentials/ isolates	PDI (%)							Disease Reaction							
	IR 24 40179	TN1	IR8 40177	IR20 40171	CAS 209	DV85	IRBB 21	IR 24 40179	TN1	IR8 40177	IR20 40171	CAS 209	DV85	IRBB 21	Virulence category
IAXO 469	57.4 (49.25)^{c}	60.1 (50.82)^{cd}	12.9 (21.03)^{f}	4.6 (12.33)^{e}	2.25 (8.47)^{f}	3.7 (11.02)^{d}	4.6 (12.33)^{c}	HS	HS	MS	R	R	R	R	I
IAXO 34	53.7 (47.12)^{d}	62.9 (52.47)^{c}	19.4 (26.12)^{e}	2.7 (9.34)^{f}	4.6 (12.33)^{d}	2.7 (9.34)^{d}	3.7 (11.02)^{cd}	HS	HS	MS	R	R	R	R	I
Coimbatore	60.1 (50.82)^{bc}	57.4 (49.25)^{de}	14.8 (22.61)^{f}	9.2 (17.64)^{d}	2.7 (9.34)^{ef}	2.7 (9.34)^{d}	2.7 (9.34)^{d}	HS	HS	MS	MR	R	R	R	II
IAXO 100	59.2 (50.30)^{c}	67.5 (55.24)^{b}	31.4 (34.07)^{c}	29.6 (32.95)^{b}	3.7 (11.02)^{de}	3.7 (11.02)^{d}	4.6 (12.33)^{c}	HS	HS	S	S	R	R	R	III
IAXO 501	51.8 (46.03)^{d}	57.4 (49.25)^{de}	23.1 (28.72)^{d}	15.7 (23.33)^{c}	12.7 (20.86)^{c}	8.3 (16.72)^{c}	4.6 (12.33)^{c}	HS	HS	MS	MS	MS	MR	R	IV
Aduthurai	62.9 (52.47)^{b}	55.59 (48.15)^{e}	38.8 (38.52)^{b}	31.4 (34.07)^{b}	36.1 (36.92)^{b}	24.0 (29.33)^{b}	10.8 (19.17)^{b}	HS	HS	S	S	S	MS	MR	V
Hyderabad	71.2 (57.54)^{a}	70.3 (56.97)^{a}	51.8 (46.03)^{a}	50.0 (45.00)^{a}	53.7 (47.12)^{a}	31.4 (34.07)^{a}	15.7 (23.33)^{a}	HS	HS	HS	HS	HS	S	MS	VI

In a column, means followed by a common letter are not significantly different at 5%level by DMRT.

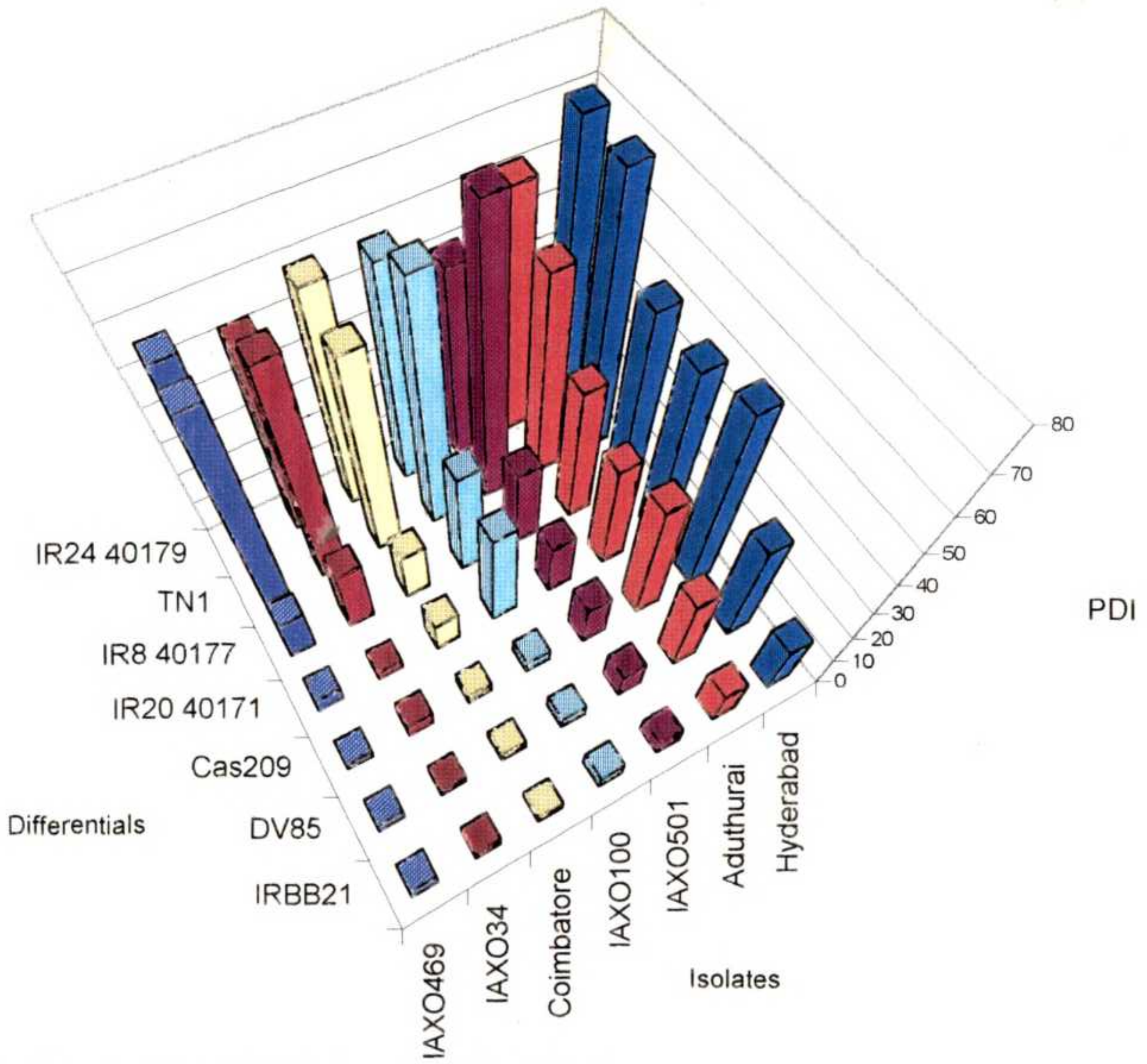


Fig. 7. Grouping *Xoo* isolates using differentials

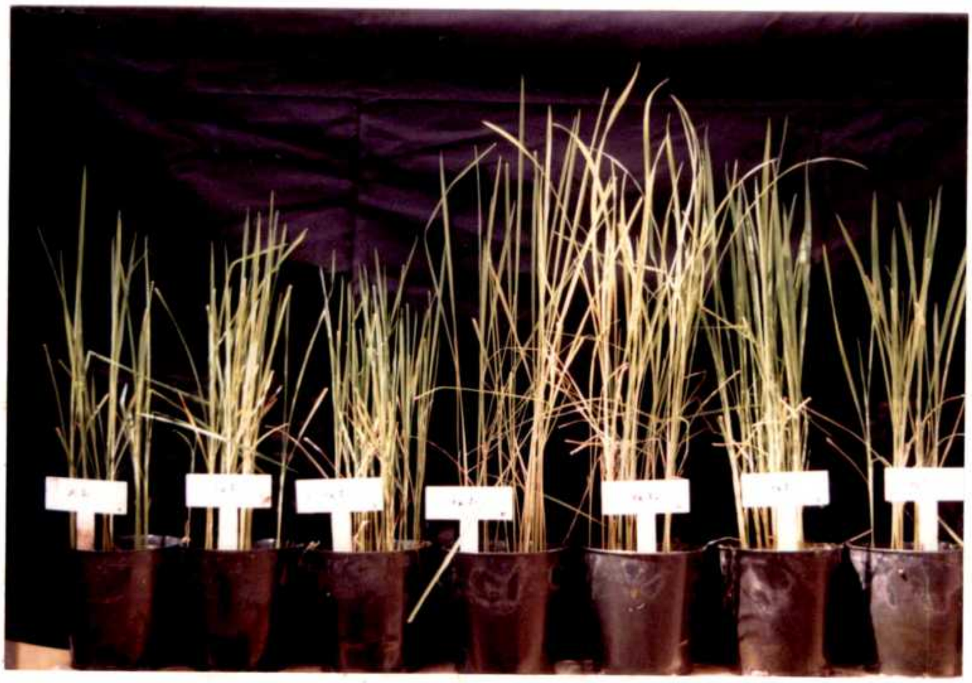
Plate 5a. Reaction of differentials to IAXO 469

1. IR2440179
2. TN1
3. IR840177
4. IR2040171
5. Cas209
6. DV85
7. IRBB21

Plate 5b.Reaction of differentials to IAXO 34

1. IR2440179
2. TN1
3. IR840177
4. IR2040171
5. Cas209
6. DV85
7. IRBB21

Plate 5a.



1 2 3 4 5 6 7

Plate 5b.

1 2 3 4 5 6 7

Category I, while the rest of the isolates were unique in their virulence and hence were assigned different category numbers (*viz.*, the Coimbatore isolate, Category II; IAXO100, Category III; IAXO501, Category IV; the Aduthurai isolate, Category V and the Hyderabad isolate, Category VI)

Transformation of indica rice cultivars

A restriction analysis of the plasmids employed in the present study was made with a view to checking the integrity of the plasmids (Fig. 8)

Among 48 individual bombardment events, transient GUS was randomly performed on two lots of immature embryos of either test-genotypes used in the present study (Table 5). Out of a sample of 10 immature embryos at the least two proved GUS-positive. At the end of the selection of putative transformed callus lines on hygromycin-containing CC medium, samples of proliferating calli (size, 10) drawn from each of those two lots were checked for the stable expression of the transgene using the histochemical assay method. Four of such calli proved GUS-positive. Regeneration frequency of the transformed calli was very low as it was only to an extent of 1-2.7%. The number of putative hygromycin-resistant lines arising out of individual bombardment events was between 1 to 4. Up till now, only 2 lines expressing the transgenes *viz., Xa21* (Fig. 9) have been regenerated. Those two lines are in the plantlet-stage and have been transferred to Yoshida's solution for further testing and screening for BLB resistance. Rest of the callus lines need to be

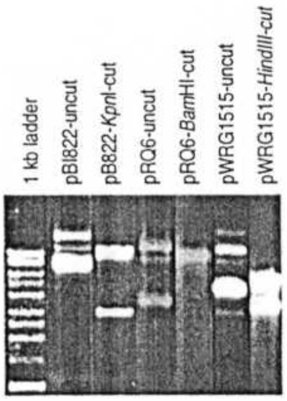


Fig. 8. Restriction analysis of plasmids used in the present study. Each plasmid was checked with a unique enzyme or a double cutter enzyme (pB822 with *Kpn*I; pRQ6 with *Bam*HI; pWRG1515 with *Hin*dIII) and the size of the fragments were ascertained. pB822 upon restriction with *Kpn*I gave rise to two bands of size, 9.6 kb and 2.9 kb. *Bam*HI linearised the plasmid, pRQ6. *Hin*dIII liberated 2 fragments of size 3 kb and 4.2 kb from pWRG1515.

Table 5. Co-transformation for evolving transgenic indica rice lines expressing *Xa21*.

Sl. No.	Variety	Plasmid combination	No. of immature embryos bombarded	Transient GUS-positive immature embryos	No. of putative hyg^r callus lines	No. of stable GUS-positive callus lines	No. of putative hyg^r regenerant lines	Regeneration efficiency (%)	No. of putative transformants expressing Xa21	Co-transformation efficiency
1.	ADT38	pB822/pWRG1515	70	4/10	10	6/10	1	1.4	yc	yc
2.	ADT38	pB822/pWRG1515	80	nd	4	nd	na	na	yc	yc
3.	ADT38	pB822/pWRG1515	120	nd	16	nd	2	1.7	yc	yc
4.	ADT38	pB822/pWRG1515	80	nd	8	nd	na	na	yc	yc
5.	ADT38	pB822/pRQ 6	150	nd	25	nd	4	2.7	yc	yc
6.	ADT38	pB822/pRQ 6	60	nd	8	nd	na	na	yc	yc
7.	ADT38	pB822/pRQ 6	70	nd	10	nd	1	1.4	yc	yc
8.	ADT38	pB822/pRQ 6	60	nd	7	nd	na	na	yc	yc
9.	ASD16	pB822/pWRG1515	80	nd	6	nd	na	na	yc	yc
10.	ASD16	pB822/pWRG1515	90	nd	8	nd	na	na	yc	yc
11.	ASD16	pB822/pWRG1515	80	nd	6	nd	na	na	yc	yc
12.	ASD16	pB822/pWRG1515	90	nd	7	nd	na	na	yc	yc
13.	ASD16	pB822/pWRG1515	70	nd	8	nd	na	na	yc	yc
14.	ASD16	pB822/pWRG1515	60	nd	5	nd	na	na	yc	yc
15.	ASD16	pB822/pWRG1515	100	nd	8	nd	na	na	yc	yc
16.	ASD16	pB822/pWRG1515	150	nd	12	nd	na	na	yc	yc
17.	ASD16	pB822/pWRG1515	80	nd	7	nd	na	na	yc	yc
18.	ASD16	pB822/pWRG1515	60	nd	5	nd	na	na	yc	yc
19.	ASD16	pB822/pWRG1515	70	nd	8	nd	1	1.4	yc	yc
20.	ASD16	pB822/pWRG1515	50	nd	3	nd	na	na	yc	yc
21.	ASD16	pB822/pWRG1515	100	nd	9	nd	1	1	yc	yc
22.	ASD16	pB822/pWRG1515	60	nd	4	nd	na	na	yc	yc
23.	ASD16	pB822/pWRG1515	80	nd	3	nd	na	na	yc	yc
24.	ASD16	pB822/pWRG1515	120	2/10	16	4/10	2	1.7	2	100
25.	ASD16	pB822/pWRG1515	130	nd	11	nd	na	na	yc	yc
26.	ASD16	pB822/pWRG1515	70	nd	8	nd	na	na	yc	yc
27.	ASD16	pB822/pWRG1515	150	nd	17	nd	na	na	yc	yc

28.	ASD16	pB822/pWRG1515	60	nd	6	nd	na	na	yc	yc
29.	ASD16	pB822/pWRG1515	60	nd	5	nd	na	na	yc	yc
30.	ASD16	pB822/pWRG1515	70	nd	8	nd	na	na	yc	yc
31.	ASD16	pB822/pWRG1515	70	nd	5	nd	na	na	yc	yc
32.	ASD16	pB822/pWRG1515	80	nd	7	nd	na	na	yc	yc
33.	ASD16	pB822/pWRG1515	80	nd	6	nd	na	na	yc	yc
34.	ASD16	pB822/pWRG1515	90	nd	7	nd	na	na	yc	yc
35.	ASD16	pB822/pWRG1515	80	nd	2	nd	na	na	yc	yc
36.	ASD16	pB822/pWRG1515	80	nd	2	nd	na	na	yc	yc
37.	ASD16	pB822/pWRG1515	90	nd	3	nd	na	na	yc	yc
38.	ASD16	pB822/pWRG1515	90	nd	8	nd	na	na	yc	yc
39.	ASD16	pB822/pWRG1515	70	nd	6	nd	na	na	yc	yc
40.	ASD16	pB822/pWRG1515	80	nd	7	nd	na	na	yc	yc
41.	ASD16	pB822/pWRG1515	60	nd	5	nd	na	na	yc	yc
42.	ASD16	pB822/pRQ 6	60	nd	7	nd	na	na	yc	yc
43.	ASD16	pB822/pRQ 6	80	nd	8	nd	na	na	yc	yc
44.	ASD16	pB822/pRQ 6	80	nd	12	nd	1	1.3	yc	yc
45.	ASD16	pB822/pRQ 6	120	nd	14	nd	2	1.7	yc	yc
46.	ASD16	pB822/pRQ 6	130	nd	16	nd	2	1.5	yc	yc
47.	ASD16	pB822/pRQ 6	120	nd	12	nd	na	na	yc	yc
48.	ASD16	pB822/pRQ 6	90	nd	8	nd	na	na	yc	yc

nd, not done; na, not arising; yc, yet to be confirmed

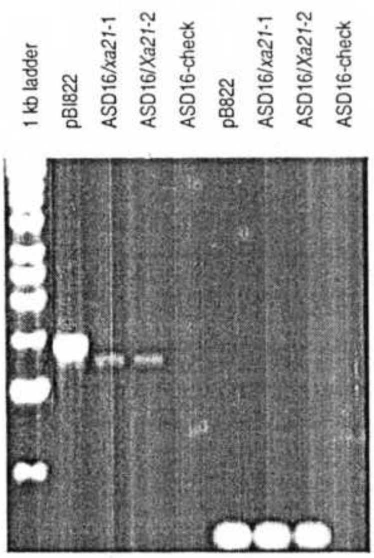


Fig. 9. PCR analysis for transgene expression using two sets of *Xa21* primers. Lane 1. 1 kb reference ladder. Lanes 2-5, PCR products using primers U1 and 11; the sequence flanked by the primers (1.4 kb) was amplified in pB822 (harbouring *Xa21* gene; Lane 2) and the two T_0 lines of ASD16 (Lane 3-4). There was no amplification in the non-transgenic ASD16 line (Lane 5). Lanes 6-9, PCR products using primers K2F and K2R spanning 230 bp in *Xa21* gene harboured by pB822 (Lane 6) and the two T_0 lines of ASD16 (Lane 7-8). No amplification of the band of similar size was found with non-transgenic ASD16 (Lane 9).

regenerated and assayed for the presence of transgenes and the experiments are continued (Plates 6-11).

Plate 6a. Transient GUS assay of ASD16 immature embryos bombarded with pB822/pWRG1515

Plate 6b. Transient GUS assay of ADT38 immature embryos bombarded with pB822/pWRG1515

Plate 6a.

Plate 6b.

Plate 7a. Selection of ASD16 embryogenic calli bombarded with pB822/pWRG1515 on hygromycin

Plate 7b. Selection of ADT38 embryogenic calli bombarded with pB822/pWRG1515 on hygromycin

Plate 7a.

Plate 7b.

Plate 8a. Stable GUS assay of ASD16 embryogenic callus bombarded with pB822/pWRG1515

Plate 8b. Stable GUS assay of ADT38 embryogenic callus bombarded with pB822/pWRG1515

Plate 8a.

Plate 8b.

Plate 9a. Putative transformed ASD16 embryogenic calli bombarded with pB822/pWRG1515 regenerating on CC medium with hygromycin

Plate 9b. Putative transformed ASD16 embryogenic calli bombarded with pB822/pRQ6 regenerating on CC medium with hygromycin

Plate 9a.

Plate 9b.

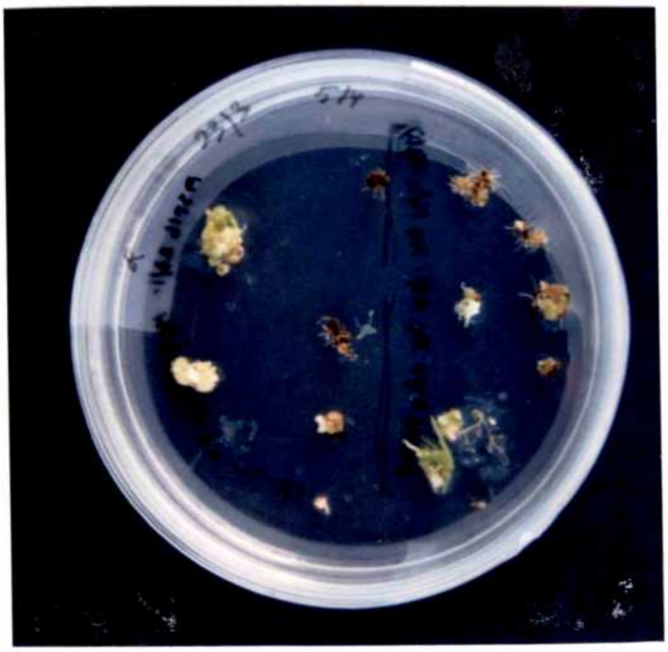


Plate 9c. Putative transformed ADT38 embryogenic calli bombarded with pB822/pWRG1515 regenerating on CC medium with hygromycin

Plate 9d. Putative transformed ADT38 embryogenic calli bombarded with pB822/pRQ6 regenerating on CC medium with hygromycin

Plate 9c.

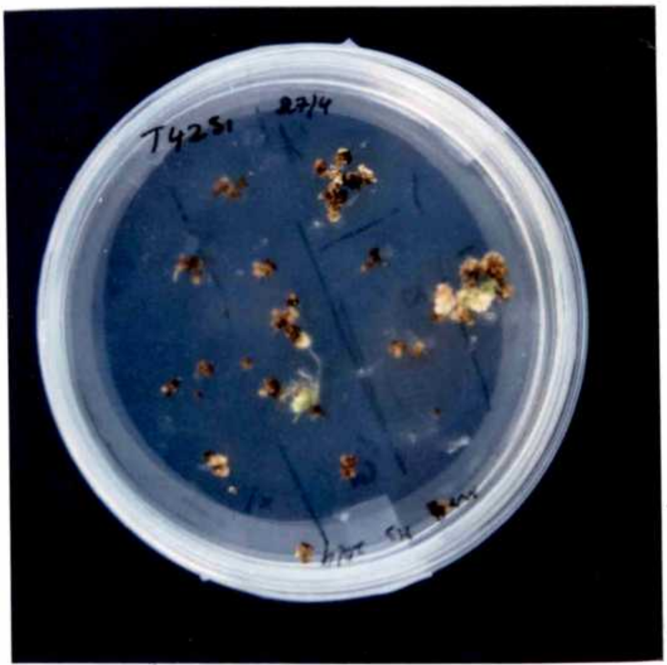


Plate 9d.

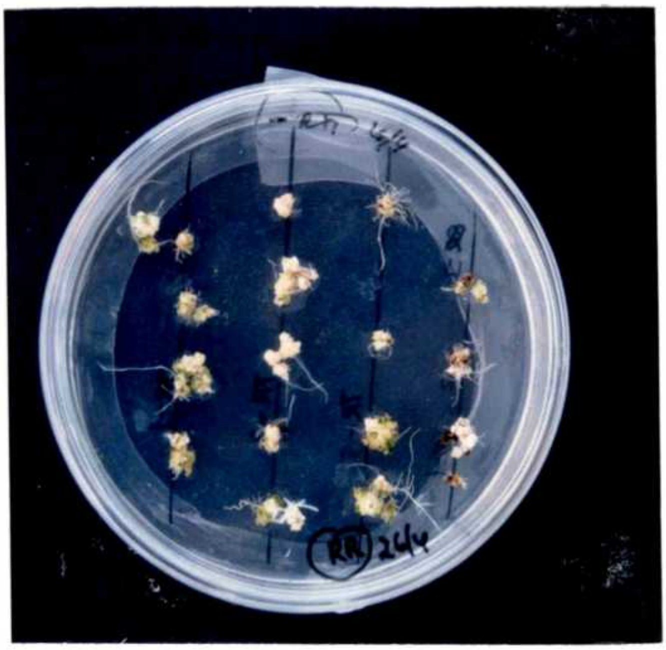


Plate 10a. Putative transgenic ASD16 plantlets on half MS from calli bombarded with pB822/pWRG1515

Plate 10b. Putative transgenic ASD16 plantlets on half MS from calli bombarded with pB822/pRQ6

Plate 10a.

Plate 10b.

Plate 10c. Putative transgenic ADT38 plantlets on half MS from calli bombarded with pB822/pWRG1515

Plate 10d. Putative transgenic ADT38 plantlets on half MS from calli bombarded with pB822/pRQ6

Plate 10c.

Plate 10d.

Plate 11. Putative transgenic ASD16 plants in Yoshida´s culture solution for hardening

Plate 11.

Discussion

Chapter 5

Discussion

A variety of genes involved in plant's natural defence mechanism has been identified. Several single defense genes or resistance genes have now been cloned (Martin *et al.*, 1993; Jones *et al.*, 1994; Whitham *et al.*, 1994; Song *et al.*, 1995). At least 21 loci conferring resistance to *Xoo* have hitherto been identified in rice and among them, *Xa21* located in *O. longistaminata* confers resistance to almost all the races/strains of the pathogen (Kinoshita, 1995; Lin *et al.*, 1995; Ronald, 1998; Sonti, 1998). An attempt was made in the present study to assess relative resistance/susceptibility of a few rice cultivars of proven performance using a set of differentials with a view to using them for engineering resistance for BLB resistance. Several isolates of *Xoo* obtained from all over India were used to screen the rice cultivars for their reaction. TKM9 was resistant to IAXO100 and the Coimbatore isolate while it was moderately resistant to IAXO34, IAXO469 and IAXO501. PTB18 and W1263 were moderately resistant to IAXO34 and IAXO100, while the former reacted similarly to the Coimbatore isolate, IAXO501 and the Aduthurai isolate too. NH56 and TNAU831311 registered a moderately resistant reaction to the Aduthurai isolate.

Ezuka *et al.* (1974) observed that resistance to *Xoo* varies with age of the crop and such resistance was not necessarily stable throughout the crop growth. However, Mew (1987) opined that such resistance was durable right from the seedling stage and it lasted till the plant reached maturity. In the present study, the relative resistance of a select five rice genotypes was assessed at different stages of crop growth and it was found that W1263 was resistant to at least five isolates of *Xoo* at boot leaf stage, while it was moderately resistant to only one isolate (IAXO34) at maximum tillering stage and at seedling stage. Varietal grouping based on both PDI and BLB intensity revealed that PTB18 and TNAU831311 were moderately resistant to the Coimbatore isolate, TKM9 to IAXO501 and NH56 to the Aduthurai isolate at all the three stages of growth. However, a moderately resistant PTB18 (to IAXO100 and the Aduthurai isolate at the seedling stage) became susceptible when the plant aged.

The two rice genotypes ADT38 and ASD16 which proved susceptible or highly susceptible to most *Xoo* isolates tested were selected for genetic engineering of resistance to BLB using *Xa21* gene. Tu *et al.* (1998) used a BLB-susceptible IR72 for incorporating *Xa21* by the biolistic method and evolved a few transgenic lines expressing resistance to 2 races of the pathogen. Zhang *et al.* (1998) were also successful in engineering resistance in four indica rice lines *viz.*, IR64, IR72, Minghui63 and BG90-2 and evolved six lines expressing resistance to *Xoo*. In the present study, 17 putative transgenic lines of ASD16 or ADT38 were evolved and the transgene (*Xa21*) expression was proved using PCR using appropriate primers in at least two lines. However, further transgene expression studies on rest of the lines evolved are necessary before they could be forwarded to testing their reaction to different *Xoo* isolates. Experiments in this line are continued.

Summary

Chapter 6

Summary

1. Fourteen indica rice genotypes with diverse genetic backgrounds were screened for their resistance to *Xoo*. Among them TKM 9 exhibited a high level of resistance to the pathogen.
2. When host resistance was tested at different growth stages *viz*., seedling stage, maximum tillering stage and boot leaf stage, W1263 was resistant to at least five isolates of *Xoo* at boot leaf stage, while it was moderately resistant to only one isolate (IAXO34) at maximum tillering stage and at seedling stage.
3. The seven Xoo isolates were grouped into six categories based on their degree of virulence based on the reaction of differentials to the pathogen.
4. BLB-susceptible ASD16 and ADT38 were biolistically transformed with a BLB resistance gene, *Xa21*. Putative transgenic lines were regenerated and the transgene expression was assayed by histochemical method and PCR analysis as well.

References

References

Bokura, U. 1911. Bacterial leaf blight of rice. **Teikoku nokaino 2:** 62-66.

Bower, R. and Birch, R. G. 1992. Transgenic sugarcane plants via micro projectile bombardment. **Plant J. 2:** 409-416.

Broglie, K., Biddle, P., Cressman, R. and Broglie, R. 1989. Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. **Plant Cell 1:** 599-607.

Bytebier, B., Deboek, F., Greve, H. D., van Montagu, M. and Hernalsteens, J. P. 1987. T-DNA organisation in tumour culture and transgenic plants of the monocotyledons *Asparagus officinalis.* **Proc. Natl. Acad. Sci. USA. 84:** 5345-5349.

Chan, M. T., Chan, H. H., Ho, S. L., Tong, W. F. and Yu, S. M. 1993. *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter-β-glucuronidase gene. **Plant Mol. Biol. 22:** 491-506.

Christou, P., Ford, T. L. and Kofron, M. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties *via* electric discharge particle acceleration of a exogenous DNA into immature embryo. **Bio/Technology 9:** 957-962..

Christou, P., Ford, T. L. and Kofron, M. 1992. Rice genetic engineering: A Review. **TIBTECH. 10**:239-246

Czerinilofsky, A.P., Rudiger, H., Baker, B. and Wirtz, U. 1986. Studies of the structure and functional organization of foreign DNA integrated into the genome of *Nicotiana tabacum*. **DNA 5**: 473-482.

Dickson, J.G. 1956. **Diseases of field crops**. McGraw Hill and Co., New York. 155p.

Dowson, W.J. 1949. **Manual of bacterial plant diseases**. Adam Charles Black, London. 132p.

Durgapal, J.C. 1985. High virulence of *Xanthomonas campestris* pv. *oryzae* a factor in the 1980 epiphytotic in the non-traditional rice growing region of North west India. **Indian J. Agric. Sci. 55**: 133-135.

Durgapal. J.C. 1988. Maintenance of virulence in *Xanthomonas campestris* pv. *oryzae* cultures. **IRRN. 13**: 13

Dye, D.W., Bradbury, J.F., Goto, M., Hayward, A.C. Lelliot, R.A. and Schroth, M.N. 1980. International standards for naming pathovars of phytopathogenic bacteria and list of pathovar names and pathotype strains. **Rev. Plant Pathol. 59**:153.

Ezuka, A. and Horino, O. 1974. Classification of rice varieties and *Xanthomonas oryzae* strains on the basis of their differential interactions. **Bull. Tokai-kinki Natl. Agric. Exp. Stn. 27**: 1-19.

Ezuka, A., Watanable, T. and Horino, O. 1974. Difference in resistance expression of *Xanthomonas oryzae* between seedling and adults to Wase Aikoku group of rice varieties. **Bull. Tokai-kiki-nat. Agric. Exp. Stn. 27**: 20-25

Fang, C. T., Liu, C. P. and Chu, C. L. 1956. A preliminary study on the disease cycle of the bacterial leaf blight of rice. **Acta. Phytopath. Sinica 2:** 173-285.

FAO, 1999. Production of rice, paddy- Fact file: India and the world Source: FAO STATS on the internet of FAO. The Hindu March. 1. pp 20

Fauquet, C. M., Zhang, S., Chen, L., Marmey, P., Dekochko, A. and Beachy, R. N. 1996. Biolistic transformation of rice : now efficient and routine for japonica and Indica rice. In: **Rice genetics III. Proc 3rd Int. Rice Gent. Symp., Manila, (Philippines)** (ed.,) Khush, G. S. pp. 153-165.

Fromm, M., Klein, T. M., Goff, S. A., Roth, B., Morrish, F. and Armstrong, C. 1991.Transient expression and stable transformation of maize using microprojectiles. **NATO ASI Ser. Ser. A. Life-Sci. NewYork 212 :** 219-224.

Fujimato, H., Itoh, K., Yamamato, M., Kuyozuka, J. and Shimamoto, K.1993. Insect resistant rice generated by introduction of a modified α endo toxin gene of *Bacillus thuringiensis*. **Bio/Technology 11:** 1151-1155..

Goto, M. 1964. `Kresek' and pale yellow leaf systemic symptoms of bacterial leaf blight of rice caused by *Xanthomonas oryzae* (Uyeda *et* Ishiyama) Dowson. **Plant Dis. Reptr. 48:** 858-861.

Hagio, T. 1998. Optimizing the particle bombardment methods for efficient genetic transformation. **JARQ 32:** 239-247.

Hashioka, Y. 1951. Bacterial leaf blight of rice and its control. **Agri. Hort. 26:** 644-648.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. **Plant J. 6:** 271-282.

Huang, J., Wen, L., Swegle, M., Tian, H., Thin, T. H., Naylor, H. M., Muthukrishnan, S. and Reeck, G. R. 1991.Nucleotide sequences of a rice genomic clone that encodes class II endo chitinase. **Plant Mol. Biol. 16:** 479-480.

Ikeda, R., Khush, G. S. and Tabien, R. E. 1990. A new resistance gene to bacterial blight derived from *O. longistaminata*. **Jpn. J. Breed. 40(Suppl. 1):** 280-281.

IRRI 1980. **Standard Evaluation System for rice** International Rice Research Institute Manila, The Philippines. 44p.

Ishiyama, S. 1926. Bacterial leaf blight of rice plant. **Proc. 3rd Pan-Pacific Sci. Congr. Tokyo. 2:** 2112.

Jones, D. A., Thomas, C. M., Hammond, K, E., Balint, P. J and Jones, J. D. G. 1994. Isolation of tomato *Cf9* gene for resistance to *Cladosporium fulvum* by transposon tagging. **Science 266:** 789-793.

Kauffman, H. E., Reddy, A. P. K., Hsieh, S. P. Y. and Merca, S. D. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. **Plant Dis. Reptr. 57:** 537-541.

Kaul, T.N. 1959. Out breaks and new records. **FAO. Pl. Prot. Bull. 7:** 54-55.

Khush, G.S., Bacalangco, E. and Ogawa,T. 1990. A new gene for resistance to bacterial blight from *O.longistaminata*. **Rice Genet. Newsletter 7:** 121-122.

Khush, G.S., Mackill, D.J. and Sidhu, G. S.1989. Breeding rice for resistance to bacterial blight. In: **Proceedings of the international workshop on bacterial blight of rice.** International Rice Research Institute, Los, Baños. The Phillipines. pp 207-217.

Khush, G.S. and Tonnissen, G.H. (eds.) 1991. **Rice biotechnology.** Wallingford Oxon, UK. 320p.

Kinoshita,T. 1995. Report of committee on gene symbolization, nomenclature linkage group. **Rice Genet. Newslett. 12:** 9-153.

Klein, T. M., Wolf, E. D., Wu, R.and Sanford, J. C. 1987. High velocity micro projectile for delivering nucleic acid into living cells. **Nature 327:** 70-73.

Kozaka, T. 1969. Control of rice disease with resistant varieties. **Agric. Hortic. 44:** 208-212.

Leah, R., Tommerup, H., Svensen, I. and Mundy, J. 1991.Biochemical and molecular characterization of three barley seed proteins with anti fungal properties. **J. Biol. Chem. 256:**1564-1573.

Li, L. C., Qu, R. D., deKochko, A., Fauquet, C. and Beachy, R. N. 1993. An improved rice transformation system using the biolistic methods. **Plant Cell Rep. 12:** 250-255.

Lin, W., Anuratha, C. S., Datta, K., Potrykus, I., Muthukrishnan, S. and Datta, S. K. 1995. Genetic engineering of rice for resistance to sheath blight. **Bio/Technology 13:** 686-691.

Lozano, J. O. 1977. Identification of bacterial leaf blight of rice caused by *Xanthomonas oryzae* in America. **Plant Dis. Reptr. 61:** 644-648.

Mahmood, M. and Singh, M. N. 1970. Investigation on bacterial blight of paddy caused by *Xanthomonas oryzae* (Uyeda and Ishiyama) Dowson. **Sci. Cult., 36:** 170-171.

Martin, C. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spvey, R., Wu, T., Earle, E. D. and Tanksley, S. D. 1993. Map based cloning of a protein kinase gene conferring disease resistance in tomato. **Science 262:**1432-1436.

Mary, C. A. and Mathew, J. 1983. Chemical control of bacterial leaf blight of rice using antibiotics. **Agric. Res. J. Kerala 21:** 17-20.

McKinney, H. H. 1923. Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. **J. Agric. Res. 26:** 195-217.

Mew, T. W. 1987. Current status and future prospects of research on bacterial blight of rice. **Annu. Rev. Phytopathol. 25:** 359-382.

Mew, T. W. 1988. An overview of world bacterial blight situation. **International workshop on bacterial blight of rice, 14-18 March, 1988. International Rice Research Institute,The Philippines.** 14p.

Mew, T. W. and Majiid, A. 1977. Bacterial blight of rice in Pakistan. **IRRN. 2:** 5.

Mew, T. W., Vera Cruz, C. M., Reyes, R. C. and Zaragoza, B. A. 1979. Study on kresek (wilt) of the rice bacterial blight syndrome. **IRRI. Res. Pap. Ser. 39:** 8

Mizukami, T. and Wakimoto, S. 1969. Epidemiology and control of bacterial leaf blight of rice. **Annu. Rev. Phytopathol. 7:** 51-72.

Morinaka, T. H., Kaku, H., Hori, M. and Kimura, T. 1978. Applying conditions of the clipping inoculation technique for evaluating resistance of rice to *Xanthomonas oryzae*. **Chugoku Agric. Exp. Stn., Ser. E. 13**: 1-16.

Mourguez, F., Brisset, M. N. and Cherreaue. 1998. Strategies to improve plant resistance to bacterial diseases through genetic engineering. **TIBTECH. 16**: 203-209.

Mukherjee, S. K., Das, C. R. and Sharma, B. D. 1966. Bacterial blight of paddy in West Bengal. **Indian Fmg. 2**: 11-12.

Murashige, T. and Skoog, F. A. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiol. Plant. 15**: 473-497.

Nagata, T. 1989. Cell biological aspect of gene delivery into plant protoplast by electroporation. **Int. Rev. Cytol. 116**: 229-255.

Ogawa, T. 1996. Monitoring race distribution and identification of genes for resistance to bacterial leaf blight In: **Rice genetics III. Proc. 3rd Int. Rice Genet. Symp., Manila, (Philippines)** (ed.) Khush, G. S. pp 456-460. International Rice Research Institute, The Philippines 1011 p.

Ogawa, T. and Khush, G. S. 1988. Major genes for resistance to bacterial blight in rice. In: **Bacterial blight of rice: Proc. of the Int. Workshop on bacterial blight of rice, (Philippines)** pp. 177-192. International Rice Research Institute, The Philippines 235 p.

Ou, S. H. 1985. **Rice diseases.** 2nd ed. Commonwealth Mycological Institute, Kew, England. 380p.

Patel, P. N. 1972. **Plant Bacteriology. Vol. I. Bacterial diseases of Plants in India.** Summer Institute of Plant Bacteriology. May 8- June 7, 1972, Indian Council of Agricultural Research, New Delhi.

Pavgi, M. S., Singh, R. A. and Dular, R. 1964. Bacterial leaf blight of rice in North India. **Sci.Cult.**. 30: 406-407.

Portykus, I., Harms, C. T. and Lorz, H. 1979. Callus formation from cell culture protoplasts of corn (*Zea may s* L). **Theo. Appl. Genet**. 54: 209-214.

Purushothaman, D. 1973. Screening or rice varieties for bacterial leaf blight disease caused by *Xanthomonas oryzae* (Uyeda *et* Ishiyama) Dowson, **Annamalai Univ. Agric. Res. Annu. 5**: 126-133.

Qi, Z. and Mew, T. W. 1985. Adult plant resistance of rice cultivars to bacterial blight. **Plant Dis. 69:** 896-898.

Raina, C. L., Sidhu, G. S. and Saini, P. K. 1981. Rice bacterial leaf blight status in the Punjab, India. **IRRN. 6:** 12.

Rancé, I., Tian, W., Mathews, H., Dekochko, A., Beachy, R. N. and Fauquet, C. M. 1994. Partial desiccation of mature embryos derived calli, a simple treatment that dramatically enhances the regeneration ability of Indica rice. **Plant Cell Rep. 13:** 647-651.

Rangaswamy, G. and Rajagopalan, S. 1973. **Bacterial plant pathology.** Tamil Nadu Agricultural University, Coimbatore, India. 291p.

Rangaswamy, G. and Rajagopalan, S. 1973. **Bacterial plant pathology.** Tamil Nadu Agricultural University, Coimbatore, India. 291p.

Rao, P. S. and Kauffman, H. E. 1977. Potential losses in dwarf rice varieties due to bacterial blight in India. **Phytopath. Z. 90:** 281-284.

Rashid, H., Yokoi, S., Toriyama, K. and Hinata, K. 1996. Transgenic plant production mediated by *Agrobacterium* in Indica rice. **Plant Cell Rep 15:** 727-730

Ray, Y. P. and Sengupta, T. K. 1970. A study of the extent of loss in yield of rice due to bacterial blight. **Indian Phytopath. 23:** 713-714.

Reddy, P. R .1987. Kresek syndrome of bacterial blight. **In: Advances in rice pathology.** (ed.) Kannaiyan, S. Tamil Nadu Agricultural University, Coimbatore, India.. 484p.

Reddy, A. P. K., Saxena, N. P. and Reddy, A. V. R. 1978. Analysis of loss in yield due to incidence of bacterial blight disease of rice. **Indian Phytopath. 31:** 444-447.

Reddy, A. P. K., Srinivasan, T. E., John, V. T. and Seetharaman, R. 1980. Breeding for bacterial leaf blight resistance in rice. **Proc. National seminar on disease resistance in crop plants,** Tamil Nadu Agricultural University, Coimbatore, India. pp. 5-13.

Reddy, A. P. K., Reddy, C. S. and Gosh, A. 1985. Recent trends in disease management in rice. In: **Integrated pest and disease management.** (ed.) Jayaraj, S. Centre for plant protection studies, Tamil Nadu Agricultural University, Coimbatore, India. pp. 86-99.

Reddy, P. R. 1965. Varietal susceptibility to the bacterial blight of rice. **Oryza 2:** 153-154.

Ronald, P.C. 1997. The molecular basis of disease resistance in rice. **Plant Mol. Biol. 35:**179-186.

Ronald, P. C. 1998. Resistance gene evolution. **Current Opinion In Plant Biology 1:** 294-298.

Ronald, P. C., Carland, F., Salmeron, J. and Staskawicz, B. 1992. The clone avirulence gene *avr pto* induces disease resistance in tomato cultivar containing the *pto* resistance gene. **J. Bacteriol. 174:** 1604-1611.

Sahu, A.K. and Parida, S. 1997. Screening of new semi-deep water rice selections for reaction to sheath rot and bacterial leaf blight. **J. Mycol. Plant Pathol. 27:** 83-85.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. **Molecular cloning. A laboratory manual** . 2nd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Sato, T., Choi, Y. S., Iwasaki, M. and Watanable, B. 1976. Distribution of races of *Xanthomonas oryzae* in Kyushu. **Ann. Phytopathol. Soc. Jpn. 17:** 42. (Abstr.).

Seshu, D.V. 1989. Salient findings from the multiplication evaluation of the international rice bacterial blight nursery. In: **Rice genetics III. Proc. III 3rd Int. Rice Gent. Symp., Manila, (Philippines)** (ed.,) Khush, G. S. pp 167-176.

Singh, K.G. 1969. Bacterial disease in west Malaysia. **FAO. Pl. Prot. Bull. 17:** 64-66.

Singh, N. K., Nelson, D. E., Kuhn, D., Hasegawa, P. M. and Bressan, R. A. 1989. Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. **Plant Physiol.** 90: 1096-1989.

Singh, R.A., Das, B., Ahmed, K.M. and Pal, V. 1980. Chemical control of bacterial leaf blight of rice. **Trop. Pest Manage. 26:** 21-26.

Singh, R. A. and Rao, M. H. S. 1982. Evaluation of several chemical treatments for eradicating *Xanthomonas oryzae* from rice seeds. **Seed Sci. Technol. 10:** 119-123.

Song, W. Y., Pi, L. Y., Wang,G. L., Gardener,J., Holesten, T. and Ronold, P. C. 1997. Evolution of rice Xa21 disease resistance gene family. **Plant Cell 9:** 1279-1287.

Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L.Y. Holsten, T., Gardner, J., Wang, B., Zhai, W. X., Zhu, L. H., Fauquet, C. and Ronald, P. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene *Xa21*. **Science 270:** 1804-1806.

Sonti, R.V. 1998. Bacterial leaf blight of rice: New insights from molecular genetics. **Curr. Sci. 74**:206-212.

Soumini, R., Padmanabhan, C., Natarajan, R. and Rao, A. V. 1969. Effect of hot water treatment on the control of primary seed infection in rice caused by *Xanthomonas oryzae*. **Madras Agric. J. 56:** 354-360.

Srinivasan, M. C., Thirumalachar, M. J. and Patel, M. K. 1959. Bacterial blight of rice. **Curr. Sci. 28:** 469-470.

Srivastava, D. N. and Rao, Y. P. 1963. Epidemic of bacterial blight disease of rice in North India. **Indian Phytopath. 16:** 393-394.

Srivastava, D. N. and Rao, Y. P. 1964. Paddy farmers should beware of bacterial blight – Measures for preventing the disease. **Indian Fmg. 14:** 32-33.

Sulaiman, N. and Ahamed, L. 1965. Bacterial blight of paddy (*Oryza sativa*) in the Maharashtra state. **Beitr. Trop. Sub Trop. Landwirtsch Tropenveteriner Medicin. 1:** 14-15.

Suwa, T. 1962. Studies on the culture media of *Xanthomonas oryzae* (Uyeda *et* Ishiyama) Dowson. **Ann. Phytopathol. Soc. Jpn. 27:** 165-171.

Swings, J., Mooter, M. V. D., Vauterin, L. M., Hoste, B., Gills, M., Mew, T.W. and Kersteris, K. 1990. Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris* pv. *oryzae*) and bacterial leaf streak (*Xanthomonas campestris* pv *oryzicola*) of rice as pathovars of *Xanthomonas oryzae* (*ex* Ishiyama 1922) sp. nov., nom. rev. **Int. J. Sys. Bacteriol. 40:** 309-311.

Tagami, Y. and Mizukami, T. 1962. Historical review of the researches on bacterial leaf blight of rice caused by *Xanthomonas oryzae* (Uyeda *et* Ishiyama) Dowson. **Spec. Rep. Plant Dis. Insect Pest forecasting serv. Minist. Agric. for Jpn. 10:** 1-112.

Takeuchi, H. 1930. On the occurrence of bacterial leaf blight of rice. **Bull. Chosen sotokuhu Agr. Expt. Sta. 5:** 62-64.

Teng, P. S. 1986. Crop loss appraisal in the tropics. **J. Plant Prot. Trop. 3:** 39-50.

Tu, J., Ona, I., Zhang, Q., Mew, T. W., Khush, G. S. and Datta, S. K. 1998. Transgenic rice variety IR72 with '*Xa21*' is resistant to bacterial blight. **Theor. Appl. Genet.. 97:** 31-36.

Vain, P., McMullen, M. D. and Finer, J. J. 1993. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. **Plant Cell Rep. 12:** 84-88

Vasil, V., Castilo, A., Fromm, M. and Vasil, K. 1992. Herbicide resistant fertile transgenic wheat plants obtained by micro projectile bombardment of regenerable embryogenic callus. **Bio/Technology 10:** 667-674.

Waksman, S.A. 1952. **Soil microbiology** John Wiley and sons Inc. New York . 356p.

Wang, G. L., Ruan, D. L., Song, W.Y., Chen, L.L., Pi, L.Y., Zhang, Z., Fauquet, C. and Gaut, B.S. 1998. Xa21D encodes a receptor-like molecular with a leucine-rich-repeat domain that determines race-specific recognition and is subject to adaptive evolution. **Plant Cell 10**: 765-779

Wang, G.L., Song, W.Y., Ruan, D.L., Sideris, S. and Ronald, P. 1996. The cloned gene. *Xa21* confers resistance to multiple *Xanthomonas oryzae* pv. *oryzae* isolates in transgenic plants. **Mol. Plant- Microbe Interact.** 9: 850-855.

Whitham, S., Dineshkumar,S. P., Choi, D., Hehl, R., Conc, C. and Baker, B. 1994. The product of tobacco mosaic virus resistance gene *N* : Similar to Toll and the interleukin-1 receptor. **Cell 78:** 1101-1105

Wunn, J., Kloti, A., Burkhardt, P. K., Ghosh, R., Biswas, G. C., Launisk, Lglesisas., V. A. and Potrykus, I. 1996. Transgenic indica rice breeding line IR 58 expressing a synthetic *Cry I(a), (b)* gene from *Bacillus thuringiensis* provides effective insect control. **Bio/Technology 14:** 171-176.

Xu, X. and Li, B. 1994. Fertile transgenic Indica rice plants obtained by electroporation of the seed embryo cells. **Plant Cell Rep.13:** 237-242.

Yamamoto, T., Machmud, M. and Hifni, H. R. 1977. Variation in pathogenicity of *Xanthomonas oryzae* and classification of rice varieties based on their reaction of rice to the pathogen. **Centr. Gen. Agric. Res. Stn., Bogor. Indonesia 8: 33-45**

Yoshimura, A., Mew, T. W., Khush, G. S. and Omura, T. 1984. Genetics of bacterial blight resistance in a breeding line of rice . **Phytopathology 74:** 773-777.

Yoshida, S., Forno, D. A., Cock, J. H. and Gomes, K. A. 1976. Laboratory manual for physiological studies of rice. pp. 61-66. International Rice Research Institute, The Philippines.

Zhang, Q., Gao, Y. J., Saghai-Maroof, M. A., Yang, S. H. and Li, J.X. 1995. Molecular divergence and hybrid performance in rice. **Mol. Breeding 1:** 133-14.

Zhang, S., Chen, L., Qu, R., Marmey, P., Beachy, R. N. and Fauquet, C. M. 1996. Regeneration of fertile transgenic Indica (group I) rice plantlets following micro projectile transformation of embryogenic suspension cultured cells. **Plant Cell Rep. 15:** 465-469.

Zhang, S., Song, W.Y., Chen, L., Ruan, D., Tayor, N., Ronald, P.C., Beachy, R. and Fauquet, C. M. 1998. Transgenic elite Indica rice varieties, resistant to *Xanthomonas oryzae* pv. *oryzae*. **Mol. Breeding** 4: 551-558.

Appendices

Appendix 1

Protocol for Preparing Gold Suspension

Weigh out 6 µg gold particles
↓
Add 100 µl of absolute ethanol
↓
Vortex for 1-2 min
↓
Centrifuge for 10 seconds at 10,000 rpm
↓
Pipet off the supernatant
↓
Add 100 µl of sterile double distilled water
↓
Vortex for 1-2 min
↓
Centrifuge for 10 seconds at 10,000 rpm
↓
Pipet off the supernatant
↓
Add 100 µl of sterile double distilled water
↓
Vortex for 1-2 min
↓
Centrifuge for 10 seconds at 10,000 rpm
↓
Pipet off the supernatant
↓
Resuspend in 100 µl of sterile double distilled water
↓
Divide into two (each 50 µl)

Appendix 2

Coating of Gold Particles

Take 50 µl of gold particle suspension in an eppendorf tube

↓

Add 5 µl of plasmid DNA (5 µl from the plasmid stock containing 1µg/ µl plasmid DNA) while vortexing continuously at low speed.

↓

Add 20 µl of 0.1 M spermidine, while vortexing continuously at low speed.

↓

Add slowly 50 µl of 2.5 M $CaCl_2$, drop by drop while vortexing continuously at low speed.

↓

Leave at room temperature (25° C) for 10 min

↓

Centrifuge for 10 seconds at 10,000 rpm

↓

Pipet off the supernatant

↓

Add 60 µl of absolute ethanol

↓

Mix well by pipetting up and down, avoided vortexing at this stage

↓

Distribute 7-8 µl of coated gold particle suspension onto macrocarriers

↓

Allow the macrocarrier to dry