CHANGES IN ENZYME ACTIVITIES AND METABOLITES ASSOCIATED WITH BACTERIAL BLIGHT OF RICE (*Oryza sativa*)

Thesis

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
in
BIOCHEMISTRY
(Minor Subject : Plant Breeding, Genetics and Biotechnology)

By
Chamandeep Kaur
(L-2005-BS-133-M)

Department of Biochemistry
College of Basic Sciences and Humanities
PUNJAB AGRICULTURAL UNIVERSITY
LUDHIANA-141 004

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CERTIFICATE-I

This is to certify that the thesis entitled, **"Changes in enzyme activities and metabolites associated with bacterial blight of rice (*Oryza sativa*)"** submitted for the degree of **M. Sc.,** in the subject of **Biochemistry** (Minor subject: **Plant Breeding, Genetics and Biotechnology**) of Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Chamandeep Kaur** (L-2005-BS-133-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled, **"Changes in enzyme activities and metabolites associated with bacterial blight of rice (*Oryza sativa*)"** submitted by **Chamandeep Kaur (L-2005-BS-133-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirement for the degree of **M.Sc.,** in the subject of **Biochemistry** (Minor subject: **Plant Breeding, Genetics and Biotechnology)** has been approved by the Student's Advisory Committee along with Head of the Department after an oral examination on the same.

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Head of the Department 6/11/07
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ACKNOWLEDGEMENT

In the most dedicated manner, I express my thanks to the 'Almighty' with whose blessings and consent, I have been able to fulfil this endeavour.

The sense of veneration and gratitude through words would hardly suffice my feelings for my revered advisor, Dr. (Ms.) Neerja Sharma, Assistant Biochemist, Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University, Ludhiana, for her adroit guidance, profound interest, constructive suggestions, critical evaluations and perpetual encouragement in the fruitful completion of this investigation. I consider myself very fortunate to be her disciple.

I feel great elation in expressing my sincere thanks to worthy members of my advisory committee, Dr. A.K. Gupta, Professor of Biochemistry, Department of Biochemistry, Dr. T.S. Bharaj, Senior Rice Breeder, Department of Plant Breeding, Genetics and Biotechnology and Dr. (Mrs.) Narinder Kaur, Professor and Head of Biochemistry, Department of Biochemistry (Dean, PG's Nominee) for their intellectual guidance, critical appraisal and for providing the necessary facilities for this study.

It is my prerogative to thank Dr. J.S. Lore, Assistant Plant Pathologist, Rice Section, Department of Plant Breeding, Genetics and Biotechnology and Dr. M.S. Hunjan, Assistant Plant Pathologist, Rice Section, Department of Plant Breeding, Genetics and Biotechnology for providing me technical help and useful suggestions throughout my study.

My parents are the greatest asset, words are too modest to express my feelings to my revered father S. Darshan Singh Panesar and mother Mrs. Jaswinder Kaur, whose supreme sacrifice, loving emotions, moral support and blessings were, are and will be the constant source of inspiration during my entire life.

It gives me immense pleasure to acknowledge heartily the love and encouraging moral support rendered by my loving brother Amandeep. God bless him with all the virtues of life.

Words never can; but for the very pleasure of it, I express my indebtness to my friends, Aman, Neerja, Rajwinder, Gurinder, Gurpreet, Harpreet, Jyoti, Puneet Rai, Rachna, Robin, Naveen, Shilpa, Khushi, Puneet, Navneet, Harinder Di, Rachna Di and Savita Di.

Mr. Deepak Kumar and Mr. Beer Bahadur (Ekta Computer Centre) deserve special thanks for bringing out this manuscript in its presentable form.

Deep in my heart, I appreciate the help of those whose name I might have missed.

Chamandeep Kaur

CHAMANDEEP KAUR

Title of the thesis	:	"Changes in enzyme activities and metabolites associated with bacterial blight of rice (*Oryza sativa*)"
Name of the Student and Admission No.	:	Chamandeep Kaur (L-2005-BS-133-M)
Name and Designation of Major Advisor	:	Dr. (Mrs.) Neerja Sharma Assistant Biochemist
Major Subject	:	Biochemistry
Minor Subject	:	Plant Breeding, Genetics and Biotechnology
Degree to be awarded	:	M.Sc.
Year of award of degree	:	2007
Total pages in thesis	:	132 + Vita
Name of University	:	Punjab Agricultural University Ludhiana

ABSTRACT

The present study was conducted to understand the biochemical basis of disease resistance in three lines of rice, one susceptible (TN1) and two resistant near isogenic lines IRBB13 and IRBB21 against bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae*. Rice leaves were infiltrated with *Xoo* and the observations were made at zero day (immediately after inoculation) 1,3,5 and 7 day stage and were compared with those made in uninoculated leaves. Peroxidase (PO), phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) were found to be significantly higher in the resistant line IRBB13 followed by another resistant line IRBB21 than the susceptible variety TN1. Total phenols, ortho-dihydroxyphenols and flavonols content also showed a similar trend as that of the enzymes. After bacterial infiltration a significant increase in the levels of total phenols, ortho-dihydroxyphenols, flavonols, peroxidase, phenylalanine ammonia lyase and tyrosine ammonia lyase was observed. However, this increase was comparatively rapid and higher in resistant near isogenic lines as compared to the susceptible line. Total sugars, reducing sugars and starch content was significantly higher in the leaves of the susceptible variety TN1 than in the resistant near isogenic lines IRBB13 and IRBB21. Differences were unnoticeable in the physicochemical characteristics of grains of three lines except head rice recovery, which was higher in the resistant near isogenic lines. From the observations made during the present studies, the higher expression of enzymes/phenolic constituents in the resistant lines than those in the susceptible line is suggestive of their significant role in development of resistance in the host against the pathogen.

Key Words : Rice, bacterial blight, enzymes, carbohydrates, phenolics.

Neerja Sharma 04/10/07
Signature of the major advisor

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Signature of the student

CONTENTS

ABBREVIATIONS

ASM	Acibenzolar S-methyl
BB	Bacterial blight
4CL	4-Coumarate CoA ligase
GT	Gelatinization temperature
HR	Hypersensitive reaction
ISR	Induced systemic resistance
PAL	Phenylalanine ammonia lyase
PCD	Programmed cell death
PO	Peroxidase
PPO	Polyphenol oxidase
PR	Pathogenesis-related proteins
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
TAL	Tyrosine ammonia lyase
Xoo	*Xanthomonas oryzae* pv. *oryzae*

Chapter - I

INTRODUCTION

Rice (*Oryza sativa*) occupies a pivotal place in Indian agriculture, as it forms the staple food for two-thirds of the population, besides providing 20-25% of the agricultural income (Anonymous 2001). India is the second largest producer of rice after China with a production of 86.30 million tonnes covering 44.97 million hectares of area (Anonymous 2002). In India, rice is cultivated under varying moisture regimes and diverse ecological situations, out of which about 42% of the area is under irrigated conditions. The irrigated rice in the rice belt of north-western India contributes a lion's share in the national rice pool (Anonymous 1999).

Punjab, once a non-traditional rice growing state, has now become a major rice producer, producing 101.93 lakh tonnes from 26.42 lakh hectares (Anonymous 2007). The average grain yield of rice was 3943 kg per hectare (1517 kg/acre). The average yield in terms of paddy in Punjab was 5914 kg per hectare (2275 kg/acre) (Anonymous 2006a). In 2005 the contribution of rice in Punjab was 37.2% to the central pool (Anonymous 2006b).

With the availability of improved crop practices and high yielding varieties, the productivity of this crop has shown a remarkable increase in last few years. However, the production is constrained by

diseases of fungal, bacterial and viral origin (Swings *et al* 1990). Due to higher application of nitrogenous fertilizers, rice crop is vulnerable to a large number of pests and diseases which cause losses to the tune of 30-40% (Siddiq 1993).

Bacterial blight (BB) of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the oldest and the most devastating diseases of rice (Jeung *et al* 2006). Plants are infected at the maximum tillering stage, resulting in significant decrease in crop yield of around 20-40% (Swamy *et al* 2006). *Xoo*, the causal organism of bacterial blight of rice, is widely distributed in all the major irrigated low land rice growing regions of the Asia (Gnanamanickam *et al* 1999). This disease caused 6-75% loss to the crop in different locations of Punjab during 1985, 1991 and 1992 (Brar 1992).

Bacterial blight is a vascular disease resulting in a systemic infection that produces tannish - grey to white lesions along the veins. Bacterial blight pathogen invades primarily the vascular tissues through multiplication of the bacteria in xylem elements and causes wilting of the leaf, especially young ones (Noda *et al* 1980). Symptoms are observed at the tillering stage and the disease incidence increases with plant growth, peaking at flowering stages (Mew *et al* 1993). Three distinct types of symptoms (i) leaf blight phase (ii) kresek phase (seedling blight or wilt phase of the syndrome) and (iii) pale yellow leaves are known in bacterial

blight of rice. Kresek phase causes more damage than leaf blight phase, and is the most destructive manifestation of the disease. In this phase the leaves of the entire plant turn pale yellow and wilt during the seedling to early tillering stage, resulting in a partial or total crop failure (Gnanamanickam *et al* 1999).

Diseases of rice are a major obstacle in achieving sustainable yield targets. This is inspite of multitude of approaches to build durable resistance of the rice plant to major diseases (Padmavati *et al* 1997). Chemical control of bacterial blight in the monsoon climate of Asia is impractical as well as environmentally unfavourable. Additionally, no effective bactericide is commercially available for disease control. So there is a need to develop strategies providing durable resistance, giving protection for a long time and over a broad geographic area for sustainable rice production in future. Therefore, the preferred strategy for disease management is through varietal resistance (Lee *et al* 2003). So far, more than 25 genes for resistance to *Xoo* have been identified from different sources and designated from *Xa*1 to *Xa*29 (Lee *et al* 2003, Gu *et al* 2004, Tan *et al* 2004).

The development of resistant varieties through field experiments is a laborious, time consuming and difficult process that takes number of years. The release of resistant varieties was initially successful in controlling this disease in Punjab and elsewhere in India, but there is a

continuous threat to the durability of the resistance against BB (Mew *et al* 1992). Thus, the quicker development of resistant varieties that can be suitably grown under north Indian conditions is very important. But this is possible only if we are fully acknowledged with biochemical basis of disease resistance involving the role of biological defense mechanism in plants.

Like many plant species, rice employs a diverse array of defenses that minimize losses during pathogen attack. Besides pre-existing physical and chemical barriers, a variety of defense mechanisms are activated upon pathogen attack (Chen *et al* 1999).

To understand the basic molecular interaction of *Xanthomonas oryzae* pv. *oryzae* with *Oryza sativa*, it is essential to know the basis of resistance conferred by these genes for resistance. The mechanisms of resistance in rice plants have been studied but no conclusive results unique to rice-*Xoo* association are available. Some work has been done to understand the physiological, histological and biochemical changes caused by infection with *Xoo* (Rao and Nayudu 1979b, Mohanty *et al* 1986, Saharan *et al* 1999, Padmaja *et al* 2004, Thipyapong *et al* 2004). Significant increase in peroxidase (PO) activity was observed in rice leaves one day after inoculation with *Xoo*. Three peroxidase isozymes (PO-1, PO-2 and PO-3) were induced after inoculation with *Xoo* (Velazhahan *et al* 2006).

Mohanty *et al* (1981) found higher phenylalanine ammonia lyase (PAL) activity in healthy rice leaves of resistant cultivars than in the

susceptible cultivars. Infection by *Xoo* enhanced PAL activity more in resistant one than in the susceptible ones. Phenolic compounds toxic to *Xoo* are also found in higher amounts in resistant varieties of rice (Sathyanathan and Vidhyasekaran 1981).

Because of the universal presence of phenols in vascular plants and their accumulation in both compatible and incompatible interactions, the relative contribution of any group or class of phenols to expression of resistance or the ultimate restriction of pathogen development in incompatible interactions remains to be investigated. Hence, there is a need for research which could address the time and concentration of phenols as well as their relationship to other putative defense responses.

Moses *et al* (1975) reported higher content of total and reducing sugars in susceptible variety than in resistant against bacterial blight of rice. Rao and Nayudu (1979a) found variations in total sugars, reducing sugars, and non-reducing sugars after inoculation with *Xoo*.

Seed is not believed to be an important source of infection, as the bacteria rapidly decrease in the month of June (Mizukami 1961) and in the course of seed soaking for sowing bacteria die in a few days (Tagami *et al* 1963). But Fang *et al* (1956) found that organism was present not only on the inside of the glumes but occasionally on the endosperm, so seed is also considered to be the source of infection. Srivastava and Rao (1964) found a high percentage of seed infection in India.

The thorough understanding of biochemistry of disease resistance would ultimately lead to the identification of certain chemical/biochemical markers thus providing milestones for screening resistant germplasm and enabling plant breeders to develop resistant varieties. Therefore, keeping in view the above facts, a study involving susceptible and near isogenic bacterial blight resistant lines of rice was planned to achieve the following objectives : -

- Investigation of the role of enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, tyrosine ammonia lyase in the mechanism of resistance.
- Identification of precise role of phenolic compounds and carbohydrates in the mechanism of resistance.
- Effect of bacterial blight on the physico-chemical quality of rice grain.

Chapter - II

REVIEW OF LITERATURE

Rice (*Oryza sativa* L.) is one of the most important cereal crop in the world, sustaining more than one-half of the global population (Poehlman 1983). In India rice is a staple food for more than 65 per cent of the population and is being grown over an area of 42.41 m ha with a production of 87.0 m tonnes (Anonymous 2004).

Bacterial blight (BB) is one of the most destructive diseases of rice plant in most rice growing countries especially in Asia (Adhikari *et al* 1995). It was first found in Japan in 1884-1885 and research on this disease was commenced as early as in 1901 (Ou 1985). Takaishi (1909) found that a bacterium caused this disease. In 1922 this bacteria was named *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) by Ishiyama. This disease was reported from India in 1959 (Srinivasan *et al* 1959).

During evolution plant pathogens have adopted diverse strategies to successfully colonize their hosts. Against such diverse pathogenesis strategies, plants have also evolved elaborate regulatory mechanisms to tailor their defense responses according to the nature of the invading pathogen. Because of the innate defense mechanisms of plants, they have been, and continue to be, widely exploited for the management of many important plant diseases. A better understanding of the disease

interaction and how the plant defense mechanisms operate is critical for the development of effective disease control measures via the use of breeding, genetic engineering, chemical, biological agents enhancing plant's disease resistance or a combination of these means (Ahn *et al* 2005).

Based on these observations, the literature in the present study has been reviewed under the following headings -

2.1 Host-pathogen interactions and disease resistance.

2.2 Infection and multiplication of *Xanthomonas oryzae* pv. *oryzae*.

2.3 Effect of pathogen attack on enzymatic pattern in plants -

(i) Peroxidase (PO) and Polyphenol oxidase (PPO)

(ii) Phenylalanine ammonia lyase (PAL) and Tyrosine ammonia lyase (TAL).

2.4 Changes in carbohydrate content

2.5 Phenolic compounds

2.6 Grain quality

2.1 HOST-PATHOGEN INTERACTIONS AND DISEASE RESISTANCE

It is well known that plants are endowed with various defense mechanisms against pathogens. However, in susceptible plants the defense mechanisms are not induced. For the induction of defense mechanisms, signals are needed. The defense mechanisms can be triggered even in susceptible cultivars by manipulating the signal transduction system (Vidhyasekaran 1997). Many defense mechanisms are triggered in plants in

response to infection by pathogens (M' Piga *et al* 1997), including phenolics, phytoalexins, callose, pathogenesis - related (PR) proteins and hydroxy proline rich glycoproteins (HRGP's) (Vidhyasekaran 1997, Vidhyasekaran *et al* 2001).

In the spectrum of plant - microbe interactions, disease is a rare outcome. In many interactions, complex and integrated mechanisms prevent infection and disease. A large number of physiological, biochemical and molecular changes have been observed that correlate with onset of pathogen attack (Klessing and Malamy 1994).

Pathogens require a pool of primary metabolites (proteins, carbohydrates and lipids) for their survival inside the host tissues. On the other hand, secondary metabolites e.g. phenolics, serve as antimetabolites to the pathogens for checking their spread inside the plant tissues. Carbohydrates, phenols and proteins have received considerable attention in relation to disease resistance. Metabolic pathways of these metabolites are interconnected, phenolics are the products of carbohydrate metabolism (Bahadur *et al* 2005).

Antibacterial compounds were extracted from rice leaves inoculated with incompatible strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). They inhibited bacterial multiplication in leaves and reached their maxima within 3 to 5 days after inoculation (Watanabe and Nakanishi 1977).

The induced resistance is first localised around the point of

pathogen infection. Subsequently, the resistance spreads systemically and develops in distal, uninfected parts of the plant thereby conferring an elevated level of protection (Sticher *et al* 1997). The use of induced resistance in plants is a promising environment friendly strategy for controlling plant diseases, including those caused by bacteria like bacterial blight of rice.

Disease development in plant involves altered rates of various metabolic activities. Enzymatic alterations triggered by infection are governed by resistance (Rao and Nayudu 1979b).

Resistance of plants to incompatible pathogens is manifested as biochemical and physiological responses, the hypersensitive reaction (HR) and the systemic acquired resistance (SAR). Xoo is a vascular pathogen so hypersensitive reaction is absent. SAR is a broad, physiological immunity that results from infection with a necrogenic pathogen. In addition, certain natural and synthetic chemical compounds can trigger similar plant responses (Kessmann *et al* 1994).

The mechanism of plant resistance to pathogens starts with specific recognition between molecules produced in the plant pathogen interaction and plant receptors. The interaction between elicitors and receptors triggers a complex response network aimed at determining resistance by stopping pathogen penetration into host tissue or inducing pathogen death (Mc Dowell and Dangl 2000). During incompatible plant -

pathogen interaction, recognition of potential pathogen often results in hypersensitive reaction, a localized activation of programmed cell death (PCD) which is cellular suicide mechanism, that generates a physical barrier restraining nutrient availability because of the rapid dehydration caused by tissue death. PCD requires the active participation of the host (Parker and Coleman 1997). Reactive oxygen species (ROS) produced at infection site, serve in the construction of a barrier against phytopathogens. Changes in reactive oxygen species in pathogen challenged plant tissue might also be affected by host-antioxidant systems (Vera - estrella *et al* 1994).

Neuenschueander *et al* (1996) made contention that both the expression of marker genes for systemic acquired resistance (SAR) and activation of SAR can be triggered by a number of viral, bacterial and fungal pathogens in a variety of dicotyledonous plants.

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance (Vallad and Goodman 2004). The enhanced state of resistance is effective against a broad spectrum of pathogens and parasites, including fungi, bacteria, viruses, nematodes and even insect herbivores.

Physical and chemical defenses may be either preformed or induced after pathogen penetration. Induced response include rapid production of reactive oxygen species (ROS), enhancement of preformed structural barriers, hypersensitive cell death, production of phytoalexins,

peroxidases and pathogenesis related (PR) proteins. The accumulation of PR-proteins upon infection with microbial pathogens is well documented in plants (Van Loon 1997).

Oxidative burst mediated by hydrogen peroxide has been recognized as a key component of plant defense response during incompatible interaction (Kachroo *et al* 2003). Hydrogen peroxide is used by peroxidase, which reinforces the cell wall and hinders pathogen penetration by catalyzing the cross-linking between the structural cell wall polymers and the oxidative polymerization of cinnamyl alcohol to lignin. Hydrogen peroxide is toxic as it inhibits calvin cycle enzymes, reducing photosynthetic CO_2 assimilation (Takeda *et al* 1995). The role of polyphenol oxidase (PPO) in the generation of ROS as well as quinones is evident from the large contribution PPO-based phenolic oxidation makes to H_2O_2 production in plant extract (Richard - Forget and Gauillard 1997).

Stopping the penetration of pathogens during plant infection is dependent on accurate time course of pathogen perception by plant host cells and the activation of networking systems resulting in induction of secondary metabolites and ROS (Kotchoni and Gachomo 2006). ROS results in reinforcement of cell wall through oxidative cross linking (Mellersh *et al* 2002) generally produced during aerobic phase of photosynthesis and photo respiration. ROS are known to mediate biochemical and physiological changes that occur under environmental stress conditions, which play a role

in disease resistance in plants (Kotchoni and Gachomo 2006). ROS which include superoxide radical, hydrogen peroxide and singlet oxygen are ubiquitous molecules produced as a consequence of normal cellular metabolism (Kotchoni 2004).

2.2 INFECTION AND MULTIPLICATION OF *XANTHOMONAS ORYZAE* pv. *ORYZAE*

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a vascular pathogen that enters vessels through wounds or through water pores, of the hydathodes (Huang and Cleene 1989). Hydathodes are natural openings by which phytopathogenic bacteria enter the host tissues. Hydathodes of rice consist of 10-20 water pores each and are densely distributed along the edge of the upper surface of the leaf (Mew *et al* 1984). After invasion of the water pore of a compatible host the pathogen multiplies in the epithem, a loose arrangement of parenchymatous cells and intercellular spaces beneath the water pores into which the xylem vessels open. After sufficient multiplication has taken place, bacteria enter the vascular system and block transpiration (Tabei 1967). Bacterial ooze, small, yellowish, spherical masses facilitates both ingress and egress of pathogen. Bacteria in both compatible and incompatible interactions, spread from the inoculation point, but spread was faster in the compatible interactions (Mew *et al* 1993). Lesions start at one or both margins of the leaf. As the disease advances lesions may cover the entire leaf blade, turn white and later become greyish

from growth of various saprophytic fungi (Ou 1985).

Reddy and Kauffman (1973) compared the multiplication and movement of Xoo in susceptible and resistant rice cultivars. In both resistant (BJ1) and susceptible (TN1) cultivars, the pathogen grew equally at the site of inoculation. In contrast, a lower rate of multiplication was observed in BJ1 than in TN1 at 1 cm away from the inoculation point.

Bacterial growth is also one parameter for host reaction type or resistance. Bacterial number in incompatible and compatible interactions in rice against *Xoo* increased equally until levels reached 10^7-10^8 colony forming units/leaf. Thereafter, bacterial growth slowed in the incompatible interactions as compared to the compatible ones (Barton - Willis *et al* 1989).

Leach *et al* (1989) monitored populations of *Xoo* over time to determine their growth kinetics in rice leaves. Both compatible and incompatible populations increased equally in rice leaves for about 2-4 days. Thereafter, in some rice cultivars, growth of incompatible bacteria slowed compared to that of compatible bacteria.

On the basis of the results of transmission electron microscopy, Horino and Kaku (1989) suggested that the production of fibrillar material in vessels might be a post infection defense mechanism.

Reimers and Leach (1991) reported a race-specific incompatible reaction when rice leaves were infiltrated with suspension of cells from different races of *Xoo*. The leaf tissue becomes dark greenish brown and the

rate of bacterial multiplication decreased compared to compatible interactions.

2.3 EFFECT OF PATHOGEN ATTACK ON ENZYMATIC PATTERN IN PLANTS

(i) Peroxidase (PO) and Polyphenol Oxidase (PPO)

Peroxidase catalyzes a redox reaction between H_2O_2 as electron acceptor and many kinds of substrates (phenolics, aromatic amines, ascorbic acid etc).

$$AH_2 + 2H_2O_2 \xrightarrow{\text{Peroxidase}} 3\,H_2O + \tfrac{1}{2}\,O_2 + A$$

The liberated oxygen oxidises a colourless compound (substrate) to a coloured compound (product). The first coloured reaction of biological material with guaiacol as substrate was noted in 1809, but the term peroxidase was used for the first time nearly a century later for an enzyme isolated from horseradish. Peroxidase is widely distributed both in animal and plant kingdom (Kawaoka *et al* 2003)

Peroxidases are heme - containing enzymes that catalyse one-electron oxidation of several substrates at the expense of H_2O_2 and are located in cell walls and vacoules. These locations are in accordance with their key role in determining the final cell wall architecture, especially regarding lignin deposition and the turnover of vacuolar phenolic metabolites (Ros-Barcelo *et al* 2003).

Peroxidase is thought to play an important role in modifying

cell wall during pathogen attack by polymerizing the lignin precursors and by cross linking cell wall proteins to polysaccharides (pectin and cellulose) and polyphenols (lignin) to form an impenetrable network around the plant cells in the vicinity of the infection site (Bradley *et al* 1992). It plays an important role in resistance to pathogens such as lignification and suberization (Quiroga *et al* 2000), xylem vessel thickening (Hilaire *et al* 2001), generation of ROS (Bolwell *et al* 1995), hydrogen peroxide scavenging (Kawoaka *et al* 2003).

Tuzun (2001) observed that plants have developed mechanisms to successfully co-exist in the presence of pathogenic organism. Induced systemic resistance depends on the timely accumulation of multiple gene products, such as hydrolytic enzymes, peroxidases.

Polyphenol oxidases (PPO) catalyze the oxidation of phenolic substances with molecular oxygen. The relatively high toxicity of the oxidation products to the plant pathogens have drawn attention in imputing the role of these enzymes in disease resistance (Manibhushanrao *et al* 1988). PPO activity may contribute to defense through production of oxidised forms of quinones. Ability of PPO to generate ROS as well as quinones - which possess a strong redox potential suggest that PPO may not only directly contribute to the defense response but could also provide a component to the process involved in amplification of wounding signals (Thipyapong *et al* 2004).

Peroxidase is the key enzyme in the biosynthesis of lignin and other oxidative phenols (Bruce and West 1989). Increased activity of cell wall bound peroxidases has been elicited in different plants viz. cucumber (Chen *et al* 2000), rice (Reimers *et al* 1992) due to pathogen infection.

Oxidation of phenols is mediated by PPO and PO. PPO is absent in rice plants so oxidation of phenols is mediated by peroxidase. Blast infected rice plants showed an enhanced peroxidase level over the healthy ones (Sridhar and Ou 1974).

Susceptible tomato plants showed a faster and higher increase in peroxidase activity over the resistant plants (Grezelinska and Sierakowska 1975). Da Graca and Van Lelyveld (1978 a,b) reported higher polyphenol and peroxidase activity and more isoenzymes in sunblotch infected avocado trees. Majumdar and Raychaudhuri (1978) reported high PPO and PO activities in *Fusarium* infected pear.

Akustu and Watanabe (1978) measured the PO activity of rice leaves infected by *Xoo* by a colorimetric method and found that the activity increased markedly as the lesions enlarged in susceptible leaves, and showed a value of 1.6 to 1.8 times higher than the initial value.

Sardhambal *et al* (1978) showed that in the susceptible rice variety TN1 infected by *Xoo,* peroxidase activity increased more than 6-fold after infection as compared to the IET 4141, the resistant one. Sathyanathan and Vidhyasekaran (1981) observed that PPO activity was almost equal in

both resistant and susceptible varieties of rice, but its activity increased several folds only in susceptible varieties along with that of peroxidase after inoculation.

Mohanty *et al* (1986) reported higher peroxidase activity in cowpea inoculated with the root-knot nematode *Meloidogyne incognita* than the uninoculated one. The increase was more in host showing incompatible reaction than the compatible one.

Peroxidases have been implicated in last enzymatic step of lignin biosynthesis (Gross 1980). Moerschbacher *et al* (1988) reported deposition of lignin and induction of enzymes associated with lignification i.e. PO, PAL, 4-coumarate CoA ligase in resistant race of wheat to *Puccinia graminis* after inoculation. Goy *et al* (1992) reported that resistance of the hybrid *Nicotiana glutinosa* X *Nicotiana debneyi* was associated with high levels of PO and PPO activity.

Reimers *et al* (1992) found increased activity of a cationic peroxidase associated with an incompatible interaction between rice and *Xoo*. In addition, it was correlated with the appearance of a 43-kilodalton peroxidase isoenzyme (Young *et al* 1995).

Ye *et al* (1996) studied PPO activity in rice leaves infected with *Xoo* and demonstrated that the activity was nearly the same as that in healthy leaves in some resistant cultivars and greater in others, but the activity decreased in susceptible cultivars. Chittoor *et al* (1997) reported expression

of POX 8.1, POX 5.1 and POX 22.3 genes in rice after infection with *Xoo* causing bacterial blight disease. Wounding also induced PO gene expression in rice (Hin *et al* 2000).

Saharan *et al* (1999) observed increase in PO and PPO activity with increase in disease severity in both resistant and susceptible genotypes of guar leaves. They reported that percent increase in activity of peroxidase was much higher in susceptible than in resistant genotypes. Peroxidase activity was higher in non-pathogen inoculated susceptible seedlings of pigeon pea (*Cajanus cajan*) than in resistant ones (Chakraborty and Gupta 2001).

In *Pseudomonas fluorescens* treated chilli plants infected with *Colletotrichum capsici* maximum PO activity was observed at fourth day after inoculation and activity was maintained at higher levels throughout the experimental period. Increased PPO activity was observed in plants challenged with pathogen (Ramamoorthy and Samiyappan 2001).

In tea plants infected with *Exobasidium vexans* activity of PO and PPO was more than in healthy ones. Five PO isozymes were reported in infected ones as compared to four in healthy ones (Chakraborty *et al* 2002). Li and Steffens (2002) showed the overexpression of PPO in tomato leads to significantly increased resistance to *Pseudomonas syringae* pv. tomato in compatible reaction.

Activities of PO, PPO increased in the tomato root tissues

challenged with *F. oxysporum* at one day after challenge. Maximum activity of PO and PPO were reported at 4th and 5th day respectively. PPO 1 and PPO 2 isoforms were expressed at higher levels in pathogen challenged tomato roots (Ramamoorthy *et al* 2002).

Sivakumar and Sharma (2003) studied biochemical changes in sheath blight affected maize plants caused by *Rhizoctonia solani* f. sp. *sasakii*. They reported accumulation of phenolic compounds and higher activity of PO, PPO and PAL in inoculated leaves than in uninoculated ones.

PPO activity in rice plants were reported to be maximum in resistant cultivar BJ1 at fifth day of inoculation with *Xoo* than in susceptible cultivar IR 50 (Seetharaman *et al* 2004).

Application of *Bacillus subtilis* increased defense related enzymes such as PO, PPO up to ten days after challenge inoculation with *C. cassicola* causing stem blight in *Phyllanthus amarus* (Mathiyazhagan *et al* 2004). In susceptible plants of cotton treated with biological inducer (*Pseudomonas fluorescens*) a unique isoform of peroxidase 'POX 4' was reported (Padmaja *et al* 2004).

Sasaki *et al* (2004) reported ten peroxidase genes in response to infection with blast fungus and multiple stresses in rice. Seven of the ten peroxidase genes were expressed at higher levels in the incompatible host than in the compatible host at 6-24 hr after infection.

Chakraborty *et al* (2005) reported the activity of peroxidase in

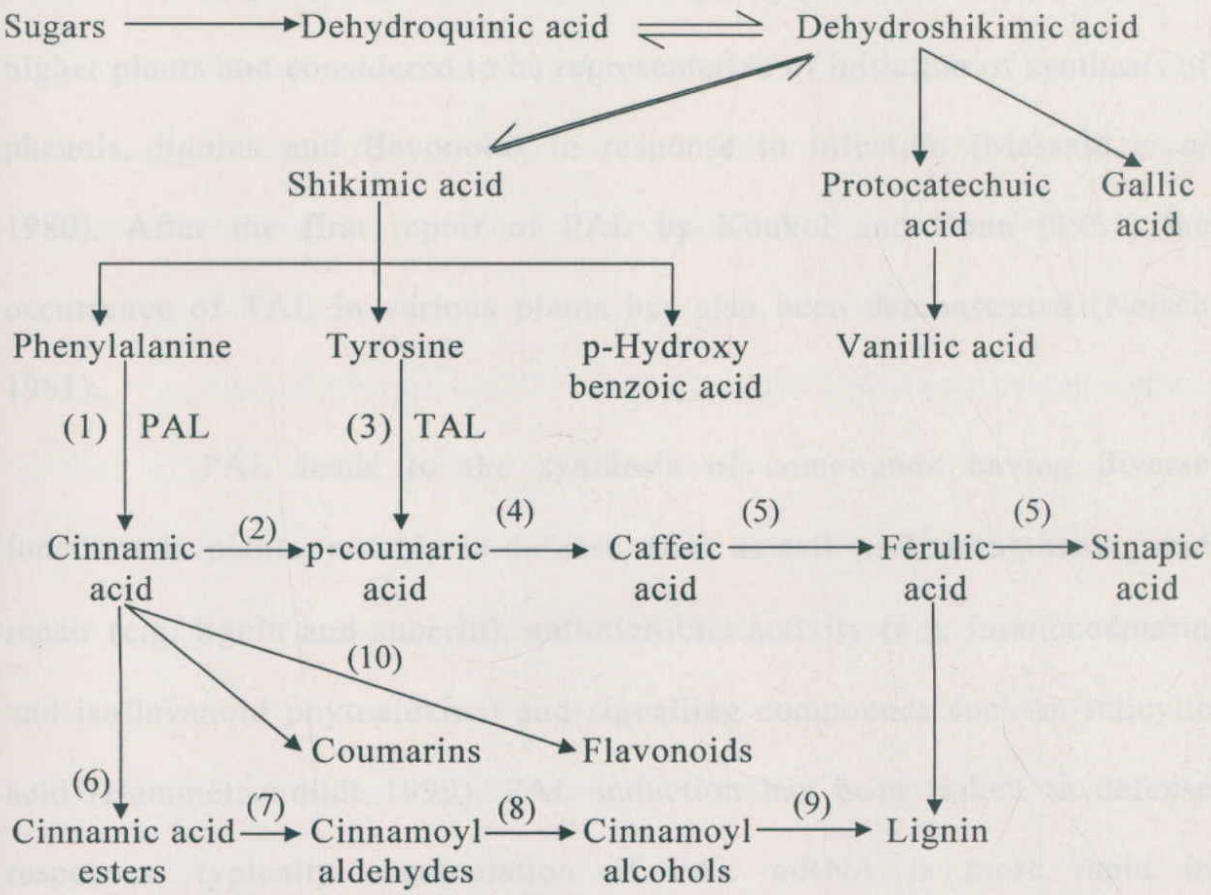
the tea plants against *Exobasidium vexans* and found a considerable increase in PO activity in plants showing resistant reaction.

Significant increase in PO activity was observed in rice leaves one day after inoculation with *Xoo*. Isozyme analysis indicated that three peroxidase isozymes (PO-1, PO-2 and PO-3) were induced after inoculation with *Xoo* (Velazhahan *et al* 2006).

Groundnut (*Arachis hypogea*) plants treated with biocontrol agent and challenge inoculated with *Alternaria alternata* recorded significantly increased activity of PO and PPO isozymes. Expression of POX 2, PPO 1 and PPO 2 isoforms were found in all plants but POX 1, PPO 3, PPO 4 and PPO 5 were found in challenge inoculated plants (Chitra *et al* 2006). Raj *et al* (2006) found that seedlings of resistant varieties of pearl millet had greater PPO activity than susceptible ones in response to downy mildew.

(ii) Phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL)

Phenylalanine ammonia lyase (PAL) catalyzes the deamination of L-phenylalanine into transcinnamic acid and tyrosine ammonia lyase (TAL) catalyzes the deamination of tyrosine into p-coumaric acid which in turn serves as a major substrate for eventual biosynthesis of phenolic acids.



1. Phenylalanine ammonia lyase (PAL)
2. Cinnamic acid-4-hydroxylase
3. Tyrosine ammonia lyase (TAL)
4. p-coumaric hydroxylase
5. o-methyl transferase
6. Cinnamate CoA ligase
7. Cinnamoyl CoA oxidoreductase
8. Cinnamoyl alcohol dehydrogenase
9. Peroxidase (PO)
10. Chalcone synthase (CHS)

Fig. 1 Role of PAL & TAL in synthesis of phenolic compounds (Mohanty *et al* 1982)

PAL is the first enzyme of phenylpropanoid metabolism in higher plants and considered to be representative of initiation of synthesis of phenols, lignins and flavonoids in response to infection (Massale *et al* 1980). After the first report of PAL by Koukol and Conn (1961) the occurrence of TAL in various plants has also been demonstrated (Neisch 1961).

PAL leads to the synthesis of compounds having diverse functions in plants, notably in defense, such as cell wall strengthening and repair (e.g. lignin and suberin), antimicrobial activity (e.g. furanocoumarin and isoflavanoid phytoalexins) and signalling compounds such as salicylic acid (Hammerschmidt 1999). PAL induction has been linked to defense responses, typically accumulation of PAL mRNA is more rapid in incompatible plant pathogen interactions (Cui *et al* 1996). Plant response to stresses is closely associated with the capacity of the antioxidant system (Mittler 2002).

Jaworski *et al* (1973) observed in carrot slices inoculated with *Ceratocystis fimbriata,* a six fold increase in PAL activity which went parallel with the accumulation of chlorogenic acid.

Potato inoculated with *Phytophthora infestans* showed increase in the activity of PAL accompanied by accumulation of lignin like polymer. Lignification was slower and diffused in infected areas of susceptible variety Majestic than in resistant variety Orion. PAL activity was more in resistant

variety Orion than the susceptible Majestic (Friend *et al* 1973).

Burrell and Rees (1974) reported that PAL activity in healthy leaves greatly exceeded that of TAL activity in both susceptible and resistant cultivars of rice. PAL activity doubled after inoculation of rice leaves with *Piricularia oryzae*.

Purushothaman (1974b) reported high PAL activity in resistant cultivar of rice TKM 6 than in susceptible cultivars Co13 and IR 8. Inoculation with *Xoo* increased PAL activity faster in TKM 6 than in other cultivars. The levels of phenylalanine and tyrosine decreased in TKM 6 but increased in Co13 and IR 8 cultivars after inoculation. Rao and Nayudu (1979b) reported that rice leaves infected by *Xoo* showed higher peroxidase and PAL activity than healthy ones.

Mohanty *et al* (1981) found higher PAL activity in healthy rice leaves of resistant cultivars than in the susceptible cultivars. Such difference was not observed in TAL. Infection by *Xoo* enhanced PAL activity more in resistant one than in the susceptible ones.

Levels of phenols in *Xoo* infected rice leaves increased compared to healthy leaves. Increase in PAL activity was due to infection by *Xoo*. The activity was more in resistant cultivar than the susceptible one (Mohanty *et al* 1982).

Zuber and Manibhushanrao (1984) studied PAL and TAL activity in resistant and susceptible rice cultivars to *R. solani* at different

stages of shealth blight disease and found higher activity of PAL and TAL in resistant cultivars.

Ralton *et al* (1988) found that in resistant variety Caloona of cow pea (*Vigna unguiculata*) infected with *Phytophythora vignae,* PAL activity doubled compared to pre-inoculation levels. But in Poona, the susceptible variety there is no significant increase above pre-inoculation levels. After 24 hr of inoculation PAL activity in resistant Caloona variety increased seven fold above pre-inoculation level.

Smith *et al* (1998) reported that cucumber (*Cucumis sativa*) leaves infiltrated with *Pseudomonas syringae* cells produced a mobile signal for systemic acquired resistance between 3 to 6 hr after inoculation. The production of a mobile signal by inoculated leaves were followed by a transient increase in PAL activity in petioles of inoculated leaves.

In case of cassava bacterial blight, PAL enzyme activity in the resistant interaction was significantly higher than in the susceptible interaction or the control. The transcripts of a partial PCR clone of a PAL gene were detected in leaves during the resistant interaction but not during the susceptible interaction (Pereira *et al* 1999). Chen *et al* (2000) reported that high levels of PAL were induced in cucumber roots inoculated with *Phythium aphanidermatum*.

Paul and Sharma (2002) reported a time dependent induction of activities of PAL, PO and phenolics in barley upon treatment with aqueous

neem leaf extract and found decrease in level of PAL, PO and total phenols in barley leaves with increasing length of elicitor treatment.

Gogoi *et al* (2000) reported behaviour of two defense related enzymes PPO and PAL in three wheat genotypes infected with *Neovossia indica* : HD 29 and DWL 5023 (resistant) and WL 711 (susceptible). The PPO and PAL enzyme activity increased much more in resistant genotypes than in susceptible ones.

PAL activity was high in *Pseudomonas fluorescens* treated chilli plants infected with *Colletotrichum capsici* than in untreated ones. Maximum activity was reported at 5th day and this remained at higher level throughout the experimental period of 10 days (Ramamoorthy and Samiyappan 2001).

Salicylic acid enhanced PAL activity in the leaves of rice inoculated by *Xoo*, three days following treatment (Wang *et al* 2002).

Chakraborty *et al* (2003) reported high PAL and PO activity in soybean infected by *Fusarium oxysporum*. PAL activity was significantly higher in infected roots of the resistant cultivars than in the susceptible cultivars. Sugar beet seedlings treated with cell wall protein factors from *Phythium oligandrum* showed enhanced activities of phenylalanine ammonia lyase and also showed significantly higher cell wall bound phenolic compounds compared with the control (Takenaka *et al* 2003).

Leaf extracts of *Datura metel* significantly reduced growth of *X.*

oryzae pv. *oryzae* (*Xoo*) and *Rhizoctonia solani* causing bacterial blight and sheath blight of rice by accumulation of pathogenesis - related (PR) proteins, phenylalanine ammonia lyase and defense related compounds (Kagale *et al* 2004).

In yeast elicited cassava suspension cells (*Manihot esculenta*) and leaves an oxidative burst, measured as hydrogen peroxide occurred. PAL activity was induced maximally at 15 hr and was preceded by PAL in mRNA accumulation which peaked at 9 hr (Vasquez *et al* 2004).

In alternaria blight infected cluster bean plants, activity of PPO, PAL, TAL and quantity of phenols, flavonols and lignin increased with increase in disease intensity, indicating thereby that these enzymes play important role in defense mechanism (Joshi *et al* 2004).

PAL activity in rice leaves increased significantly one day after inoculation with *Xoo* and maximum enzyme activity was observed two days after inoculation (Velazhahan *et al* 2006).

2.4 CHANGES IN CARBOHYDRATE CONTENT

Carbohydrates have received considerable attention in relation to resistance in plants against diseases. Increase in the carbohydrates content due to severity of the disease may serve as easily metabolized carbon source for the pathogen. Starch accumulation in wheat leaves infected with stripe rust has been reported (MacDonald and Strobel 1969).

Misawa and Miyazaki (1972) studied changes in the contents of

carbohydrates, nitrogeneous and phosphorous compounds in rice leaves infected with bacterial blight, three to seven days after inoculation. Reducing and non-reducing sugars, crude starch, total phosphorous content increased whereas total nitrogen and water insoluble protein nitrogen content decreased.

Moses *et al* (1975) reported that the content of total and reducing sugars were higher in the susceptible variety and susceptible F_2 progeny than in the resistant varieties and resistant F_2 progeny of rice to bacterial blight. Reddy and Sridhar (1975) revealed that susceptibility of rice leaves to bacterial blight was associated with higher amount of sugars and depressed levels of total phenols.

Healthy leaves of resistant cultivar of rice possessed more reducing sugars than the susceptible cultivar. Bacterial blight infection caused reduction in amount of reducing sugars 5 days after inoculation in resistant cultivar. Disease development favoured an accumulation of reducing sugars in the susceptible cultivar (Reddy *et al* 1977).

Total sugar content showed variation at all six stages i.e. 20 hr, 4th day, 6th day, 8th day, 12th day and 18th day after inoculation of rice leaves with *Xoo*. In healthy leaves, decrease was reported initially and then at the last two stages there was an increase in the total sugars content. Except at the 2nd and 4th stage the inoculated leaves have less amount of total sugars than the healthy ones. Reducing sugar content of inoculated

leaves was higher than that of healthy ones at all six stages of disease development. Healthy leaves showed an increase in reducing sugar content at initial stages and then decrease was reported at later stages. Non-reducing sugars in inoculated leaves decreased at 2nd and 3rd stage followed by variations over next three stages. Starch content in inoculated leaves was less at stages 1 to 4th and higher at 5th and 6th than in healthy ones. In healthy leaves starch content showed variation as it first decreased and then increased and again decreased at last stage (Rao and Nayudu 1979a).

Marimuthu (1981) reported that susceptible varieties of greengram contain higher reserve of total sugars, glucose and fructose than resistant ones. The higher levels of sugars may contribute in maintaining healthy flora of the saprophytic microbes that help to inhibit pathogens (Chahal 1986).

Parashar and Sindhan (1986) reported that a significant increase in disease intensity and decrease in phenolic content, total and reducing sugars was observed in plants supplied with nitrogen in both susceptible and resistant cultivars, of rice against bacterial blight, while a significant decrease in disease intensity and increase in phenolic contents, total sugars and reducing sugars was observed in rice plants supplied with potassium in resistant and susceptible cultivars. The reduction in phenolic and carbohydrate contents may be due to the reduction in phenol synthesis resulting from acceleration of protein synthesis with the application of

nitrogen, and subsequently decreased sugar contents of plants due to utilization of carbon in protein synthesis. The increase in concentration of phenolics and sugars may be due to the acceleration of activity of some enzymes involved in synthesis of sugars with the application of potassium and in turn increased phenolic contents.

Gupta *et al* (1987) reported a lower concentration of total and reducing sugars in resistant genotypes of *B. napus* than in susceptible ones. Vandana (1995) observed that in mustard plants following infection with *Alternaria brassicae* there was a gradual increase in total as well as reducing sugars in leaves of resistant cultivar RC-781 but converse was true for susceptible cultivar Varuna.

Carbohydrate content has been reported to be reduced after long term infection as compared to healthy plants of pea. Ndoumau *et al* (1996) reported that carbohydrates increased during the severity of infection which serve as easily available metabolites for the growth of fungal pathogens.

Sindhan and Parashar (1996) studied the changes in carbohydrate content in resistant and susceptible cultivars of groundnut infected with *Cereospora arachidicola*. The total and reducing sugars showed initial accumulation and then declined at maximum severity of infection and age of the plant.

The concentration of total as well as reducing sugars increased with age of the plant. In mustard, the resistant genotypes against alternaria

leaf blight registered considerably higher amount of total as well as reducing sugars in comparison to susceptible ones (Singh *et al* 1999, Yadav *et al* 2001).

The low levels of starch was reported in melon plants due to infection by cucumber mosaic virus (Shalliton and Wolf 2000). Kiran *et al* (2003) reported that total and reducing sugars in susceptible *B. juncea* cultivar Kranti was more than in resistant *B. napus* cultivar GSH-1 and *B. juncea* cultivar RH-781.

Atwal *et al* (2004) reported the accumulation of sugars in the lesion as well as lesion-free areas of the infected mustard leaves with the progress of infection. Higher levels of total sugars in chlorotic and green areas of infected leaves suggest that sugars are probably one of the factors responsible for resistance in *B. juncea* against alternaria leaf blight.

In cluster bean leaves, infected with *Alternaria cucumerina* var. *cyamopsidis* decrease in total sugars was reported in susceptible and resistant varieties. Increase in reducing sugars in diseased leaves as compared to healthy leaves was reported in resistant varieties. Non-reducing sugars in healthy as well as diseased leaves were more in resistant than in susceptible varieties. In infected plants non-reducing sugars decreased in all varieties except the moderately resistant HG 365 (Saharan and Saharan 2004).

Pea plants infected with *Erysiphe pisi* revealed that in different

parts of the plant the total carbohydrate content decreased in infected plants as compared to healthy ones. But carbohydrate content of both healthy and infected leaves increased with increase in the growth of plants (Bahadur *et al* 2005).

2.5 PHENOLIC COMPOUNDS

Plants produce thousands of compounds which contain one or more phenolic residues. The shikimate acid pathway gives rise to aromatic amino acids, which in turn may be directed towards either primary or secondary metabolism. The family of secondary metabolites derived from aromatic amino acids are known as phenolics, polyphenols or phenylpropane derivatives. The biosynthesis of most phenolics begins with the aromatic amino acids phenylalanine, tyrosine and tryptophan.

Phenolic compounds are considered as non-specific defense metabolites against the pathogen and resistant plants have the tendency to accumulate these metabolites in higher amount than susceptible ones following infection (Alam *et al* 1991). Such an increase in phenolics in resistant plants is due to high activity of β-glucosidase, which converts non-toxic phenolic glycosides to toxic phenolics, which are inhibitory to the pathogen. These phenolic compounds are possibly converted by increased, peroxidase activity to quinones in resistant cultivars. These quinones are reported to be more toxic to microorganisms (Sempio *et al* 1975).

The phenolics may accumulate as inducible low molecular

weight compounds called phytoalexins as a result of pathogen attack. The phenolic compounds particularly ortho-dihydroxyphenols are important in disease resistance (Yesuraja and Mariappan 1993, Sindhan and Parashar 1996). The onset of hypersensitive reaction may be accompanied by increased synthesis of phenolic compounds.

Perumalla and Heath (1991) suggested that accumulation of phenolases, an initial response to infection might reflect a general increase in host metabolism as well as accumulation of relatively non-toxic secondary metabolites which could ultimately serve as precursors for compounds essential to the expression of resistance.

Increase in phenolic content in both dicotyledonous and monocotyledonous plants has been correlated with resistance to pathogens (Vidhyasekaran 1997). It is well known that resistant plants accumulate phenols or produce polyphenols more rapidly than susceptible ones (Lyon and McGill 1988).

Phenolic compounds have been associated with defense mechanism because of their general accumulation near wounded and infected tissues and that phenols and their oxidation products are highly fungitoxic. Phenolics upon oxidation become highly reactive and are toxic to pathogens and pathogenic enzymes, thus inhibit the development of pathogen in the tissue (Sridhar and Ou 1974).

Phenols are formed in response to the ingress of pathogens and

their appearance is considered as a part of an active defense response. They accumulate in both compatible and incompatible interactions and play an important role in disease resistance. Due to early accumulation of phenolic compounds at the infection site, limited development of the pathogen occurs as a result of rapid cell death. Accumulation of polymerized phenols occurs as a rapid response to infection (Nicholson 1992).

Purushothaman (1971) reported the accumulation of total and o-dihydroxyphenols in the resistant variety of rice. The conversion of L-phenylalanine to t-cinnamic acid provides the phenylpropane skeleton for hydroxylated cinnamic acid derivatives such as caffeic acid, 'B' ring and 3-carbon bridge of flavonoid compounds. Hydroxylation of cinnamic acid leads to the formation of other phenolic acid like p-coumaric and ferulic acids.

Purushothaman (1974a) reported that the resistant TKM6 plants of rice inoculated with bacterial blight pathogen contained larger quantities of total and o-dihydroxyphenols than both the susceptible cultivars Co13 and IR8. Inoculation with the pathogen, in general, caused the accumulation of phenols in all the three cultivars, however the net increase was high in resistant TKM6 compared with less resistant cultivars. Reddy and Sridhar (1975) found that the levels of phenolic compounds decreased in diseased leaves of highly susceptible cultivar TN1 of rice, while that of the less susceptible IR8 showed an increase.

Total phenolic content in resistant variety was more than susceptible variety of cotton infected with bacterial blight (Jalali 1976). Total phenols increased in all varieties of cow pea after infection with *Xanthomonas vignicola* but increase was prominent in resistant varieties than in susceptible varieties. o-dihydroxyphenols were also more in resistant variety Co Pusa 4 than in V38 and CM 11, the moderately resistant and susceptible one respectively (Mohan *et al* 1978).

Resistant varieties of rice had more total and o-dihydroxyphenols than two susceptible varieties against brown spot disease. Phenols increased in all the four varieties after inoculation with *Helminthosporium oryzae* but increase was more in resistant varieties (Sathyanathan and Vidhyasekaran 1981).

Accumulation of phenolics in rice varieties due to infection by *X. campestris* pv. *oryzae* was studied by Valluvaparidasan and Mariappan (1983). They observed that the moderately resistant cultivar TNAV7124, contained more total phenols than moderately susceptible ASD5, susceptible Co40, and highly susceptible TN1. Total phenolic content of the four cultivars increased with inoculation and decreased with plant age.

Phenolics and sugars play an important role in disease resistance. Application of nitrogen increased the incidence of bacterial blight of rice caused by *X. oryzae* by reducing phenolic and sugar content of rice plants whereas application of potassium reduced incidence of disease by

increasing phenolic content of rice plants (Sindhan and Parashar 1986).

Gupta *et al* (1984) and Vandana (1995) reported a higher initial phenolic content in leaves of resistant cultivar RC-781 of *B. juncea* than susceptible cultivars Varuna and Prakash, the level of which further increased upon infection with *Alternaria brassicae*. Chattopadhyoy (1989) also observed that cultivars like Midas, RC-781 and YRT-3 of *Brassica juncea* resistant to Alternaria blight had slightly higher levels of phenolic compounds than the susceptible ones like cultivar Varuna.

Mahto *et al* (1987) reported highest total phenol content in resistant variety IR20 followed by Anand, the susceptible one against bacterial blight. Same trend was found in total and reducing sugars. They found that higher amounts of phenols and sugars keep lesion size smaller by generating a resistant reaction to the bacterial blight pathogen.

Singh and Singh (1989) found higher levels of total and o-dihydroxyphenols in leaves of chilli pepper after inoculation with cucumber mosaic virus. The content of flavonols was significantly low in different infected parts of leaves as compared to healthy leaves and this decrease was more prominent in necrotic area followed by chlorotic and green, therefore indicating less significant role of flavonols in the defense mechanism as compared to total and o-dihydroxyphenols.

Induced synthesis of phenolic compounds is associated with host-pathogen interaction and specific phenolics have been implicated in

host resistance. Ferulic and p-coumaric acids in bound form have been found to be involved in the resistance of wheat leaves to *Puccinia recondita* (Southerton and Deverall 1990).

Flavonols have been shown to accumulate in many plants under infection. However, there are few reports in cereal crops showing accumulation. In sorghum, the accumulation of 3-deoxyanthocyanidinis has been reported to be toxic to the pathogen *Coletotrichum graminicola* (Lo *et al* 1996). In addition an increase in flavan-4-ol content was reported in certain mould resistant sorghum lines (Jambunathan *et al* 1990).

Inoculation of parsley leaves with *P. megasperma* results in the accumulation of coumarin phytoalexins as well as esterification of phenylpropanoids. Such esters are thought to be involved in the formation of phenolic polymers by crosslinking (Nicholson 1992). In addition to this, lignin like polymers accumulate as a rapid response to infection and represent physical barrier to the pathogen.

Vandana (1995) reported a higher flavonol content in leaves of resistant cultivar RC-781 of *B. juncea* than susceptible cultivar Prakash. Flavanol content further increased in RC-781 leaves but decreased in Varuna upon infection with *Alternaria brassicae*.

Kumar *et al* (1995) studied the induction of resistance against *Xanthomonas campestris* pv. *oryzae* in high yielding rice variety Kranti susceptible to bacterial blight. Pretreatment of plants with killed bacteria did

not induce resistance to the disease and o-dihydroxyphenol level did not increase. They suggested that either elicitors were not present on the cell surface of bacteria or that they were destroyed by heat treatment.

Naringenin was detected in rice leaves exposed to UV irradiation and also after blast infection (Grayer *et al* 1996). Flavonols inhibit growth of rice pathogens and act as defense and survival compounds. Naringenin inhibit growth of all six strains of *X. oryzae* in rice. Naringenin and Kaempferol also inhibit spore germination of *Pyricularia oryzae* (Padmavati *et al* 1997).

Levels of total phenols and o-dihydroxyphenols were found to be higher in resistant pea cultivars than susceptible pea cultivars. Significant increase in the level of o-dihydroxyphenol content in powdery mildew resistant pea cultivars might be responsible for conferring resistance against *E. coli. polygoni* infection (Guleria *et al* 1998).

In *Pseudomonas fluorescens* treated chilli plants infected with *Colletotrichum capsici* accumulation of phenolics was observed. Maximum accumulation was reported at 5th day of inoculation. Phenolic compounds are fungitoxic in nature. They increase the physical and mechanical strength of host cell wall resulting in inhibition of fungal invasion (Ramamoorthy and Samiyappan 2001).

In apple rootstock infected with *Pythium ultimum* maximum amount of total and o-dihydroxyphenols and high activity of PAL, TAL,

PPO were detected in highly resistant rootstock and minimum in highly susceptible ones. However peroxidase activity was maximum in susceptible and minimum in resistant (Sharma 2003).

Exposure of potato tubers to *Phytophthora infestans*, the late blight pathogen, elicits multiple defense responses, including an oxidative burst, accumulation of phenylpropanoid compounds which are accompanied by *de novo* synthesis of the enzymes that produce them. These reactions are also induced in potato tubers treated with a crude elicitor prepared from mycelia of pathogen (Nakane *et al* 2003).

Acibenzolar - S- Methyl (ASM) treated rice plants when inoculated with *Xoo,* showed reduction in infection but there were disease symptoms in untreated control. Accumulation of phenolic compounds were observed in the ASM treated plants. Accumulation of phenolic compounds and PR (pathogenesis - related) proteins due to inoculation with *Xoo* in ASM- treated rice plants may be involved in the resistance of rice against *Xoo* (Babu *et al* 2003).

Total and o-dihydroxyphenols increased significantly with the increase in infection and age of plant in *B. juncea*. Total phenols, have protective role to restrict the growth, spread of disease and invasion of the pathogen by formation of lignin and lignin like substances which are more toxic to fungi (Atwal *et al* 2004).

Rice plants, when clip inoculated with *Xoo* showed the

maximum (65.5%) increase in amount of phenolics in the resistant cultivar BJ1 compared with the susceptible cultivar IR50 until 3 days of inoculation. o-dihydroxyphenols content was also higher in BJ1 cultivar (Seetharaman *et al* 2004).

Induced resistance in cucumber is largely correlated with rapid *de novo* biosynthesis of flavonoid phytoalexin compounds as when inhibitors of enzymes 4-coumarate CoA ligase (4CL) and chalcone synthase, involved in flavonoid biosynthesis were added there was a suppression in the induced resistance (Fofana *et al* 2005).

Xanthomonas oryzae pv. *oryzae* infection triggers accumulation of phenolics in rice leaves. The phenolic content in rice leaves increased significantly one day after inoculation and the maximum accumulation of phenols was observed two days after inoculation (Velazhahan *et al* 2006).

Rice plants inoculated with two rhizobial strains RRE6 and ANU843 and infected with *Rhizoctonia solani* showed more accumulation of phenolic acids i.e. gallic, tannic, ferulic and cinnamic acids than uninoculated plants (Mishra *et al* 2006).

2.6 GRAIN QUALITY

Amylose content has a major influence on the characteristics of cooked milled rice. It is considered to be one of the most important constitutional indices of rice cooking and processing behaviour as it determines hardness of cooked rice, gloss of final product and rice-water

ratio (Juliano 1971). It also correlates negatively with taste panel scores for cohesiveness, tenderness, colour and gloss of boiled rice. Rice varieties are grouped on the basis of their amylose content into waxy (1-2%), low amylose (8-20%), intermediate amylose (21-25%) and high amylose more than (25%) (Chakrabarthy *et al* 1972).

Waxy or glutinous rice that lacks in amylose does not expand in volume, cooks moist and sticky and remains firm when cooked. In contrast, non waxy or non-glutinous rice has intermediate amylose, cooks moist and tender and does not become hard upon cooling after cooking and are preferred in most rice growing areas of the world. High amylose content rice shows high volume expansion and high degree of flakiness. These grains cook dry, are less tender and become hard upon cooling. These differences clearly indicate the importance of amylose content as a selection criterion. (Denyer *et al* 2001, Kumar and Khush 1998).

Gelatinization temperature (GT) is the water temperature at which starch granules begin to swell irreversibly. In other words, the time required for cooking milled rice is determined by GT. Environmental conditions such as temperature during ripening, influence GT. A high temperature during development results in starch with a higher GT. An alkali test is used to measure GT. Alkali spreading values of 1-2 are indicators of high GT, 3 high intermediate, 4-5 intermediate (70-74°C) and 6-7 low (< 70°C) (Puri and Siddiq 1983).

The gelatinization temperature affects water uptake, volume expansion and linear kernel elongation after cooking. Rice varieties with intermediate gelatinization temperature are preferred all over the world as high gelatinization temperature rice becomes excessively soft when overcooked, elongates less and remains undercooked under standard cooking procedure and hence least preferred (Vanaja and Babu 2003).

Amylose content and gelatinization temperature are the prime determinants for excellent cooking qualities. Preference in the international market is for rices that possess intermediate to slightly high amylose content with intermediate gelatinization temperature (Bhattacharya 1989, Jennings *et al* 1979).

Gel consistency measures the tendency of the cooked rice to harden after cooling. Rices of similar amylose content can be differentiated according to tenderness measured through gel consistency. Gel consistency of rices is determined by heating a small quantity of rice in a dilute alkali. Rices are commonly classified into three groups - hard (20-40 mm), medium (41-60 mm) and soft gel consistency (61-100 mm) (Cagampang *et al* 1973). Rice with soft gel consistency cooks tender and remains soft even after cooling. Hard gel consistency is associated with hard cooked rice and this feature is particularly evident in high amylose rice. Hard cooked rice also tends to be less sticky. Rice with soft to medium gel consistency is preferred by most rice consumers (Sarkar *et al* 1994).

Tagami and Mizukami (1962) summarized several reports concerning the effects of bacterial blight on yield components and quality of rice crop. Total grain volume, straw weight, total grain weight and 1000-grain weight were reduced, husking ratio was lowered, sterile grains, empty grains, broken rice increased. Soluble non-nitrogenous substances decreased and crude protein increased. Straw was softened in quality. Grain number per panicle was not changed. Kaul and Sharma (1987) stated that all the components were affected adversely to varying degrees but grain filling was affected the most and this led to production of chaffy or unfilled grains. Fertile grain number is the major grain yield component, and is drastically reduced after bacterial blight infection.

Kirya and Kuhara (1962) studied the relation between yield loss, timing of disease appearance and disease severity and concluded that the earlier the disease appeared the more severe the disease was, the greater the decrease in straw weight, total grain weight, grain weight per panicle, and 1000-grain weight of hulled and unhulled rice, the greater the increase in empty grains. Tagami and Mizukami (1962) also indicated that the earlier the appearance of the disease, the worse were its effects.

Singh *et al* (1977) reported that rice cultivars with similar score to bacterial blight differed in losses in grain yield ranging from 14.7 to 81.3%. Grains from diseased crops were poor in water absorption, volume expansion and kernal elongation values. This effect also varied in different

cultivars depending on disease severity. Ho and Lin (1976) found that only the period of plant growth between maximum tillering stage and booting stage was sensitive to disease infection, as it affected the yield significantly in terms of filled grain weight per hill and total yield.

Degree of milling is a measure of the per cent bran removed from the brown rice kernel. It influences the amount of rice recovered and also the colour and cooking behaviour of rice. Unmilled brown rice absorbs water poorly and does not cook as quickly as milled rice. Milling yield and head rice recovery are the most important criteria of rice quality especially from marketing point of view (Mahmuda *et al* 2003).

Head rice percentage is the weight of head grain or whole kernels in the rice lot. Head rice normally includes broken kernels that are 75-80% of the whole kernel. High head rice yield is one of the most important criteria for measuring milled rice quality.

Preferences for grain length varies enormously from region to region. In tropical Asia, most rice is medium to long with some extra long types. Width and thickness, or shape, are less variable and less important than length, although the highest quality world markets usually demand a slender to medium width. Varieties having medium-long, slender and translucent grains give the best head rice yields (Khush *et al* 1979).

The weight per 1000 grains of unhulled rice is also reduced by bacterial blight as is the straw weight of affected plants. Percentage of

husked, sterile, unfilled grains show an increase in diseased plants (Kurita *et al* 1960).

Bacterial blight of rice causes significant reduction in 1000 grain weight in TN1, IR8, Jaya and IR24. Significant yield loss was also reported from all varieties of rice infected with *X. oryzae* as compared to the healthy ones. They found that the disease significantly reduced panicle number per m^2 and increased chaffiness of grains in all cultivars (Ahmed and Singh 1975).

Verma *et al* (1977) stated that losses due to bacterial blight were mainly due to loss in number of filled grains which subsequently resulted in a 20-38% weight reduction of diseased panicles.

Rao and Kauffmann (1977) evaluated yield losses due to bacterial blight disease and found that the grain losses were 56% in highly susceptible Karuna, 10% in moderately susceptible IR8 and very less in resistant cultivar IR22.

Reddy *et al* (2000) reported that rice seeds from sheath rot infected plants showed reduction in germination, protein content, sugar and starch contents which was more pronounced in susceptible than in resistant varieties. There was greater reduction in head rice recovery in the infected seeds than in uninfected seeds. Root length and shoot length was also reduced in inoculated seeds.

Crude protein content was less in leaves infected with

Alternaria blight of all resistant and susceptible varieties of cluster bean (*Cyamopsis tetragonoloba*). Crude protein content was more in healthy as well as diseased leaves in resistant varieties than in susceptible varieties. Decrease in crude protein content with increase in disease severity may be due to the utilization of protein by pathogen or due to increased activity of proteolytic enzymes (Saharan and Saharan 2001).

Sinha *et al* (1987) reported higher protein contents in resistant than susceptible varieties of rapeseed mustard infected by *A. brassicae*. Kiran *et al* (2003) reported that protein content of resistant cultivar RH-781 of *B. juncea* was higher than susceptible cultivar Kranti of *B. juncea* after challenge with *Alternaria brassicae*.

The amount of total protein of healthy pea plants infected with *Erysiphe pisi* varied significantly. Maximum accumulation was observed in both infected and healthy plants in samples of 25, 30, 35 and 40 day old plants. Infected leaves accumulated more protein at longer period of infection i.e. in 35 and 40 days old plant leaves in comparison to healthy ones (Bahadur *et al* 2005).

Chapter - III

MATERIALS AND METHODS

The present investigation "Changes in enzyme activities and metabolites associated with bacterial blight of rice" was carried out in the Department of Plant Breeding, Genetics and Biotechnology (Rice Section) and in Department of Biochemistry and Chemistry, Punjab Agricultural University, Ludhiana. The materials and methods used in the present study are described under the following headings -

3.1 Plant material

3.2 Chemicals

3.3 Extraction and estimation of enzymes from rice leaves

3.4 Estimation of total proteins

3.5 Extraction and estimation of carbohydrates from rice leaves

3.6 Extraction and estimation of phenolic constituents from rice leaves

3.7 Determination of physico-chemical characters of rice grain

3.7.1 Estimation of amylose and amylopectin

3.7.2 Determination of gelatinization temperature

3.7.3 Determination of gel consistency

3.7.4 Estimation of crude protein content

3.7.5 Extraction and estimation of carbohydrate content

3.7.6 Determination of length, breadth and shape of rice grain

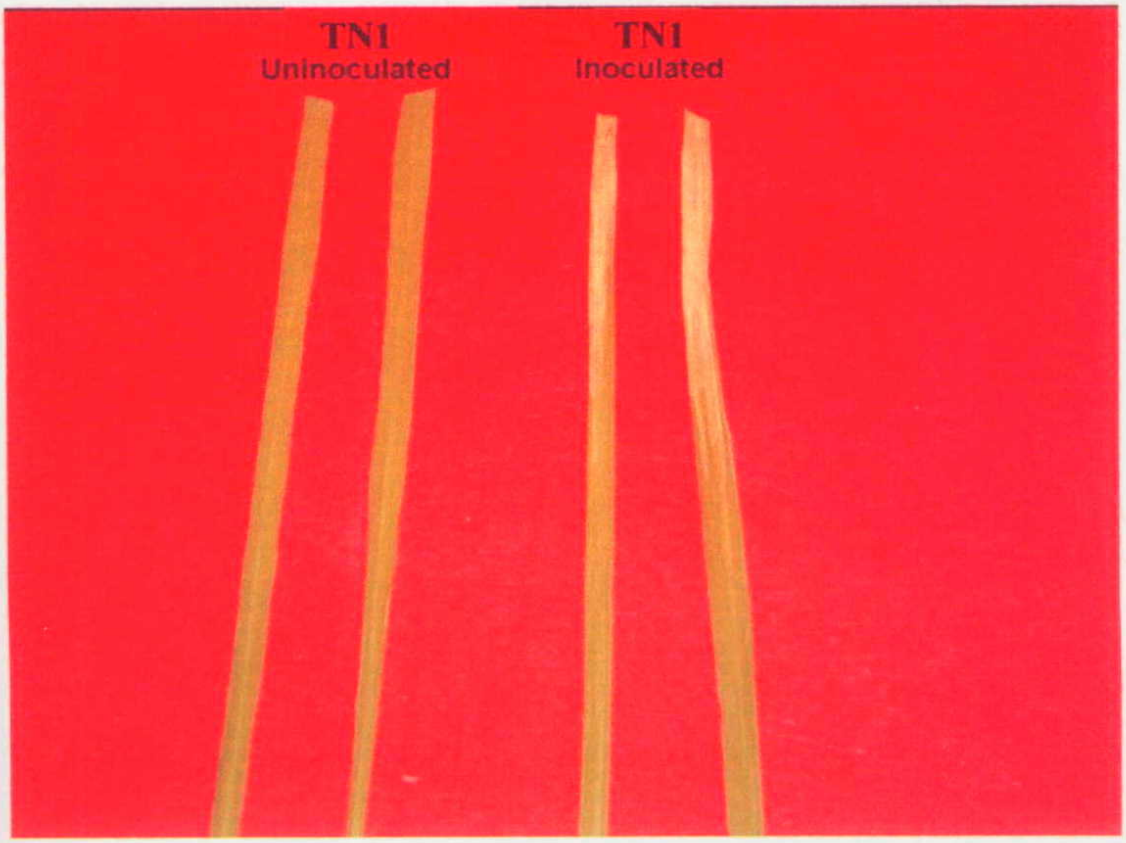


Fig. 2 : Leaves of the susceptible variety TN1 after inoculation with sterile distilled water (left; uninoculated) and virulent culture of *Xanthomonas oryzae* pv. *oryzae* (right inoculated) at 7 days after inoculation

3.8 Determination of milling quality of rice grain

3.9 Determination of seed germination and seedling growth

3.1 PLANT MATERIAL

3.1.1 Raising of rice plants

Nursery of bacterial blight resistant near isogenic lines viz. IRBB13 and IRBB21 and susceptible variety TN1 of rice (*Oryza sativa* L.) was raised in the experimental fields of Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University, Ludhiana. Thirty-day old seedlings of these rice lines were transplanted in six replications in a plot size of three rows with 50 plants in each row. Row to row and plant to plant spacing was 30x20 cm.

3.1.2 Inoculation

Xanthomonas oryzae pv. *oryzae* was isolated and grown on Waki Moto's medium. The composition of Waki Moto's medium is as follows -(di-sodium mercuric phosphate 2g, ferrous sulphate 0.5g, calcium nitrate 0.5g, peptone 5g, sucrose 15g, agar-agar 20g, distilled water 1lt). The pathotype was incubated for seventy-two hours to get bacterial suspension of approximately 10^9 cells/ml measured spectrophotometrically and inoculated by clip-inoculation technique (Kauffman *et al* 1973) on the three lines TN1, IRBB13 and IRBB21, 45 days after transplanting. Sterilized pair of scissors was dipped in the bacterial suspension and the tips of the leaves were cut about 2 cm from the top. Plants of each rice line in 3 replications were

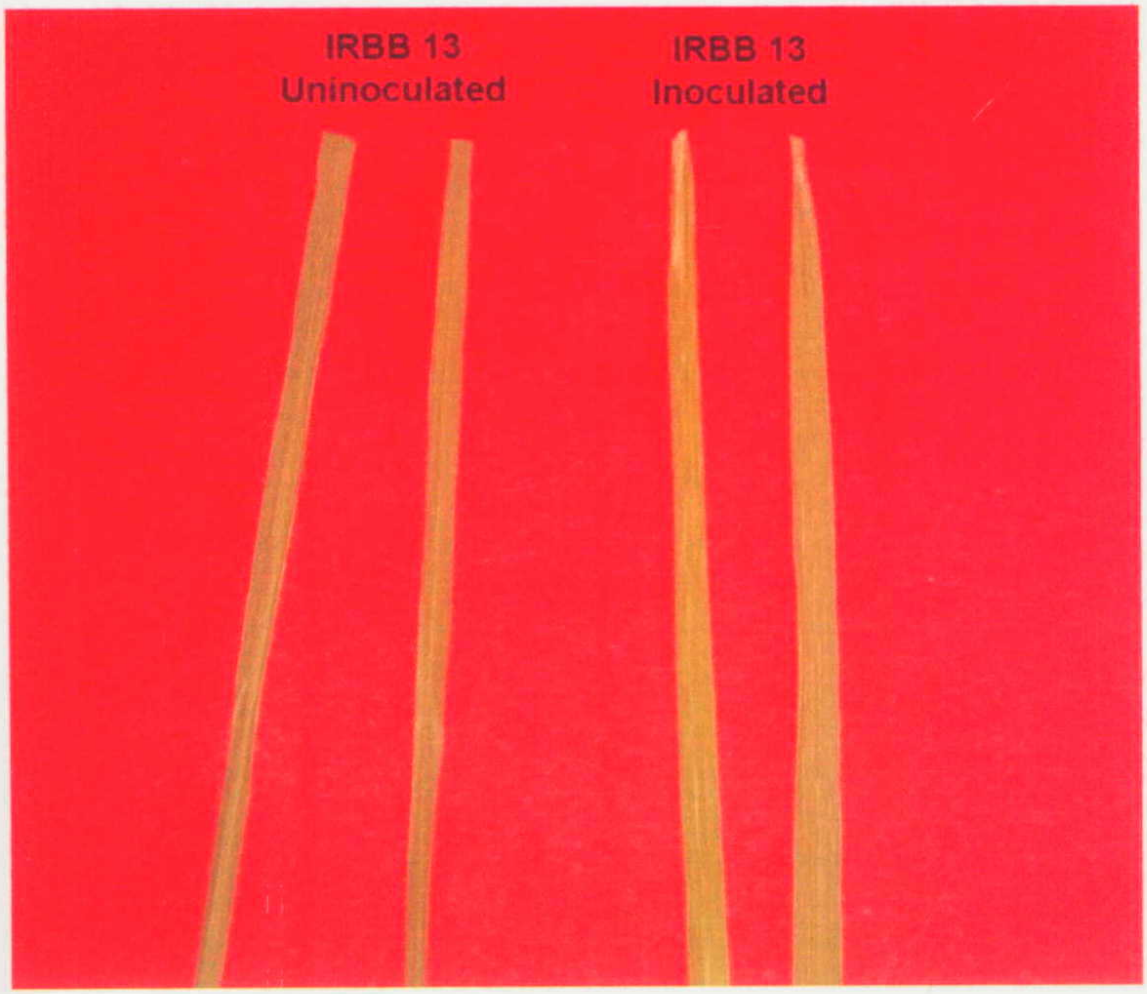


Fig. 3 : Leaves of the resistant line IRBB13 after inoculation with sterile distilled water (left; uninoculated) and virulent culture of *Xanthomonas oryzae* pv. *oryzae* (right inoculated) at 7 days after inoculation

inoculated with the virulent culture of *Xoo*. The other three replications were clip-inoculated with sterile distilled water and treated as control/ uninoculated plants.

3.1.3 Collection of samples

Leaf samples were collected at five different stages viz : zero (immediately after inoculation), 1, 3, 5 and 7 days after inoculation.

At every stage there were six types of leaf samples :

i. TN1 : Infiltrated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Inoculated) (Fig. 2).

ii. TN1 : Infiltrated with sterile distilled water (Uninoculated) (Fig. 2).

iii. IRBB13 : Infiltrated with *Xoo* (Inoculated) (Fig. 3).

iv. IRBB13:Infiltrated with sterile distilled water (Uninoculated) (Fig. 3).

v. IRBB21: Infiltrated with *Xoo* (Inoculated) (Fig. 4).

vi. IRBB21:Infiltrated with sterile distilled water (Uninoculated) (Fig. 4).

Leaves were cut 12 cm from the top with the help of a scissors, immediately put in properly labelled polyethylene bags and then placed in the ice box. Each sample was divided into three parts. One part was used immediately for the study of different enzymes. The second part was stored in 80% ethanol in a deep-freezer for the estimation of carbohydrate content while the third part was air dried and then dried to a constant weight in an oven at 50±2°C. This part of the sample was used subsequently for the estimation of total phenols, ortho-dihydroxyphenols and flavonols. The

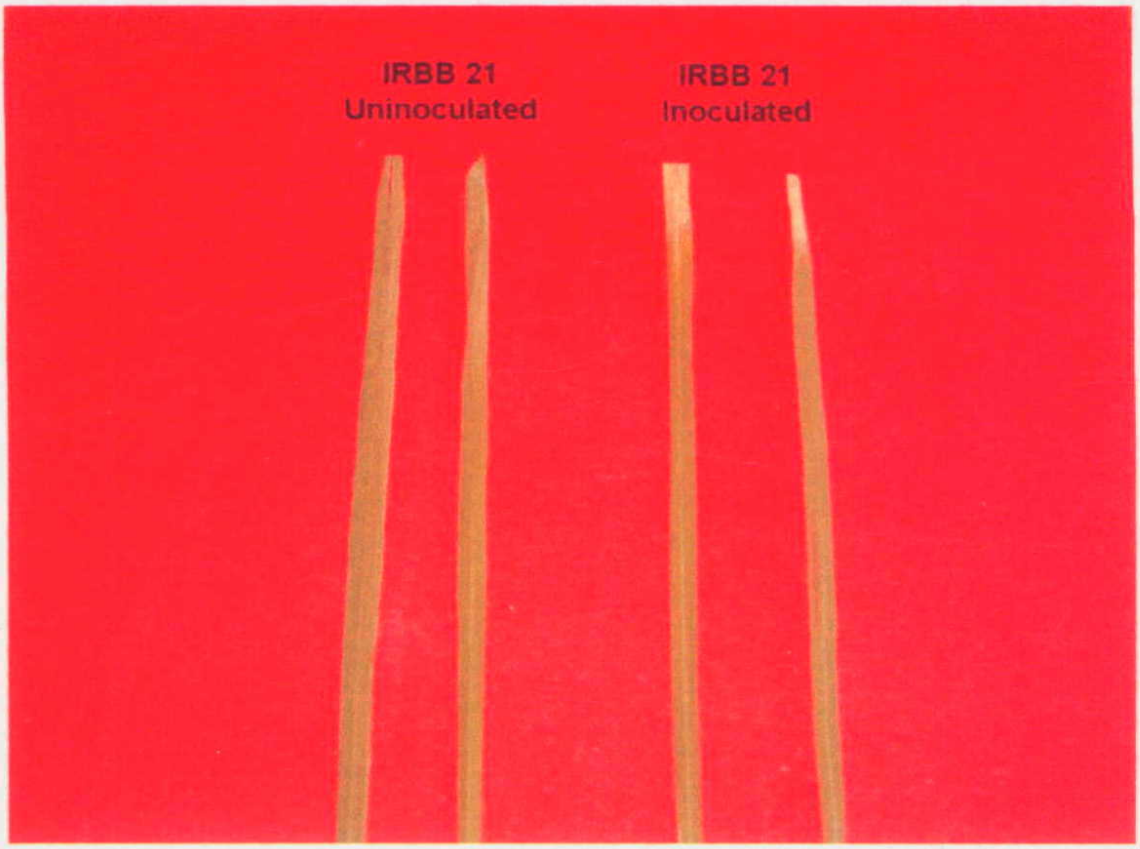


Fig. 4 : Leaves of the resistant line IRBB21 after inoculation with sterile distilled water (left; uninoculated) and virulent culture of *Xanthomonas oryzae* pv. *oryzae* (right inoculated) at 7 days after inoculation

paddy crop of the three lines viz. TN1, IRBB13 and IRBB21 was harvested at maturity. Paddy samples of inoculated and uninoculated plants of each line were collected and dried to 14 per cent moisture content and were stored in paper bags for carrying out the germination studies and for the estimation of physico-chemical characters of rice kernels.

3.2 CHEMICALS

All the chemicals used were procured from Sisco, India and were of analytical grade.

3.3 EXTRACTION AND ESTIMATION OF ENZYMES FROM RICE LEAVES

3.3.1 Extraction

250 mg of leaf sample was crushed in a prechilled pestle and mortar and enzymes were extracted with 5 ml of ice cold 0.1 M Tris HCl Buffer pH 7.5 containing 5 mM β-mercaptoethanol. The homogenate was centrifuged at 10,000g at 4°C for 25 minutes and the clear supernatant was used for estimating enzymes-peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and tyrosine ammonia lyase. Before estimating enzyme activities, all the buffers, substrate solution and other solutions if any except enzyme were kept at 37°C for 15 minutes and then enzyme activities were measured.

3.3.2 Peroxidase (Shannon *et al* 1966)

Reaction

Peroxidases detoxify H_2O_2 in the cytosolic part of the cell. They are nonspecific in utilizing electron donor for oxidation of H_2O_2

$$\text{Guaiacol} + H_2O_2 \xrightarrow{\text{Peroxidase}} H_2O + \text{Tetraguaiacol}$$

Enzyme Assay

A. Reagents

i. 0.05M guaiacol prepared in 0.1M potassium phosphate buffer (pH 6.5).

ii. 0.8M H_2O_2.

B. Procedure

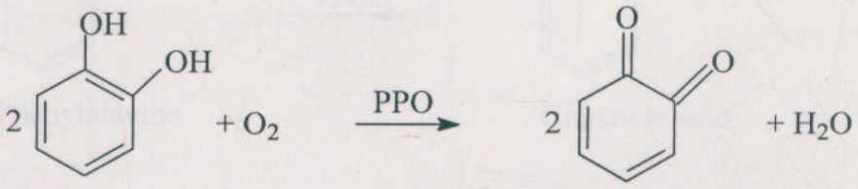
The enzyme was assayed by following the appearance of brown colouration resulting from guaiacol oxidation to tetraguaiacol in the presence of hydrogen peroxide.

Three ml of 0.05 M guaiacol prepared in 0.1 M potassium phosphate buffer (pH 6.5) and 0.05 ml of enzyme extract were added into spectrophotometric cuvette and the absorbance was set to zero. The reaction was started by adding 0.1 ml of 0.8 M H_2O_2 and the absorbance was recorded at 470 nm for 2 min at an interval of 15 seconds. The enzyme activity has been expressed as the change in absorbance at 470 nm min^{-1} g^{-1} of fresh tissue and min^{-1} mg^{-1} protein.

3.3.3 Polyphenol oxidase (Bastin and Unluer 1972)

Reaction

Polyphenol oxidase is an oxygen tranferring enzyme. It uses O_2 to catalyze the dehydrogenation of catechols to orthoquinones.



Catechol Orthoquinone

Reagents

0.01 M catechol in 0.1M phosphate buffer (pH 6.0).

Procedure

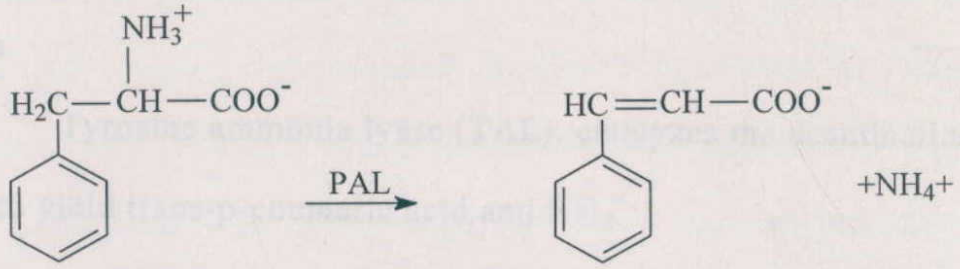
The enzyme was assayed according to the intensity of dark coloured polymeric compound formed from catechol. 2.5 ml of 0.01 M catechol (in 0.1 M phosphate buffer, pH 6.0) was taken in the cuvette and absorbance was set at zero. Then 0.2 ml of enzyme extract was added to catechol solution and change in absorbance was recorded at 495 nm after every 15 seconds upto 2 minutes. Enzyme activity was expressed as increase in absorbance min^{-1} g^{-1} fresh tissue and min^{-1} mg^{-1} protein.

3.3.4 Phenylalanine ammonia lyase (Burrell and Rees 1974)

Reaction

Phenylalanine ammonia lyase (PAL) catalyzes the deamination

of phenylalanine to yield cinnamic acid and NH_4^+.



Phenylalanine Cinnamic acid

Enzyme Assay

A. Reagents

i. 0.03M phenylalanine in 0.05M sodium borate buffer (pH 8.8).

ii. 5N HCl

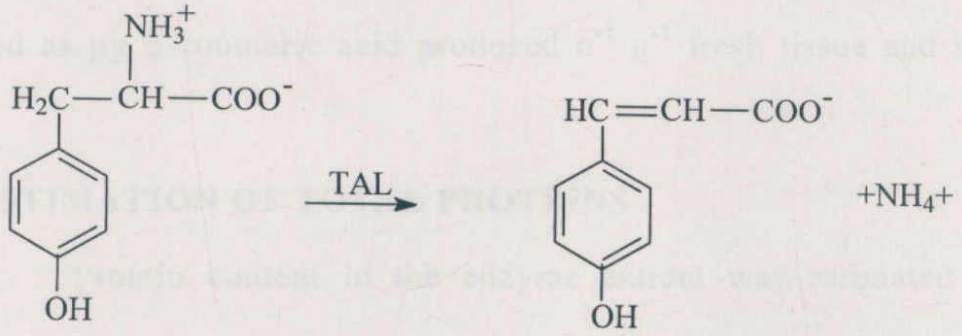
B. Procedure

The enzyme was assayed by following the appearance of trans-cinnamic acid resulting from deamination of L-phenylalanine. The reaction mixture contained 2.5 ml of 0.03M phenylalanine in 0.05M sodium borate buffer (pH 8.8) and 0.2 ml of enzyme extract. The reaction mixture was incubated at 37°C for 1 hour. The reaction was terminated by addition of 0.3 ml of 5N hydrochloric acid. The absorbance was recorded at 290 nm with the help of UV visible spectrophotometer (Hitachi – 2000, Japan). A reaction mixture in which the reaction was stopped at time zero served as a control. The concentration of trans-cinnamic acid was read from the standard curve prepared by using trans-cinnamic acid in the range of 5-40 µg. The enzyme activity was expressed as µg t-cinnamic acid produced h^{-1} g^{-1} fresh tissue and h^{-1} mg^{-1} protein.

3.3.5 Tyrosine ammonia lyase (Burrell and Rees 1974)

Reaction

Tyrosine ammonia lyase (TAL), catalyzes the deamination of L-tyrosine to yield trans-p-coumaric acid and NH_4^+.



Tyrosine p-coumaric acid

Enzyme Assay

A. Reagents

i. 33μM tyrosine prepared in 0.05M sodium borate buffer pH 8.8.

ii. 5N HCl

B. Procedure

The enzyme was assayed by following the appearance of trans p-coumaric acid resulting from the deamination of L-tyrosine. The reaction mixture contained 1.0 ml of 33μM tyrosine prepared in 0.05M sodium borate buffer, pH 8.8 and 0.4 ml of enzyme extract, and 1.0 ml of 0.05M sodium borate buffer, pH 8.8. The reaction mixture was incubated at 37°C for one hour. The reaction was terminated by the action of 0.1 ml of 5N

hydrochloric acid. The absorbance was recorded at 310nm with the help of UV visible spectrophotometer (Hitachi – 2000, Japan). The reaction mixture in which the reaction was stopped at time zero served as blank. The concentration of p-coumaric acid was read from the standard curve prepared by using p-coumaric acid in the range of 5-40µg. The enzyme activity was expressed as µg p-coumaric acid produced h^{-1} g^{-1} fresh tissue and h^{-1} mg^{-1} protein.

3.4 ESTIMATION OF TOTAL PROTEINS

Protein content in the enzyme extract was estimated by the method of Lowry *et al* (1951).

A. Reagents

a. Reagent A : 2% sodium carbonate in 0.1N sodium hydroxide.

b. Reagent B : 0.5% copper sulphate in 1% sodium potassium tartrate.

c. Reagent C : 50 ml of reagent A mixed with 1 ml of reagent B just before use.

d. Reagent D: Folin-ciocalteau's phenol reagent was diluted with distilled water in 1:1 ratio before use.

B. Procedure

To 0.5 ml of the protein sample in a test tube, 2.5 ml of reagent C was added. The contents were mixed well and allowed to stand for 10 min at room temperature. Then 0.25 ml of reagent D was added and mixed rapidly. The mixture was allowed to stand for 30 min at room temperature. The intensity of blue colour developed was read at 520nm. The amount of

protein in the samples was calculated from the standard curve prepared by taking different amounts of bovine serum albumin (20-100 µg).

3.5 EXTRACTION AND ESTIMATION OF CARBOHYDRATES FROM RICE LEAVES

3.5.1 Extraction of soluble sugars

A. Reagents

80% ethanol.

B. Procedure

Leaf samples (0.5-1g) preserved in 80 per cent ethanol were crushed and transferred into test tubes. The free sugars were extracted three times with 15 ml of 80 per cent ethanol. Water condensers were placed on top of the test tubes and kept in a water bath at 80-85°C for one hour. Supernatants from each extraction were filtered through Whatman No. 1 filter paper and pooled in a 50 ml beaker. Ethanol from the pooled extracts was evaporated on a water bath at 50-55°C until most of the alcohol is removed (total volume was reduced to about 3ml). The concentrated aqueous extracts so obtained were made upto a total volume of 10 ml with distilled water. This extract was used for the estimation of total and reducing sugars. The sugar free residue obtained after extraction of free sugars was dried and used for extraction and estimation of starch.

3.5.2 Estimation of total sugars (Dubois *et al* 1956)

A. Reagents

i. Concentrated sulphuric acid

ii. 5% phenol (w/v)

B. Procedure

To 1 ml of appropriately diluted sugar extract, containing 20-50 µg of sugars 1 ml of 5 per cent phenol was added followed by addition of 5 ml of concentrated sulphuric acid. The sulphuric acid was poured directly in the center of test tube to ensure proper mixing of the solutions. After 30 minutes, the absorbance was measured at 490 nm against a reagent blank. The concentration of total sugars (as glucose) was calculated from glucose standards (10-100 µg) run simultaneously.

3.5.3 Determination of reducing sugars (Nelson 1944)

A. Reagents

i. Reagent A - Alkaline copper tartrate reagent

It was prepared by dissolving 25g of anhydrous sodium carbonate, 20 g of sodium bicarbonate, 25g of potassium sodium tartrate and 200 g of anhydrous sodium sulphate in 800 ml of distilled water and making up the final volume to one litre.

ii. Reagent B - Copper sulphate reagent

It was prepared by dissolving 15g of copper sulphate in distilled water containing 2-3 drops of concentrated H_2SO_4. The final volume was

made to 100 ml with distilled water.

iii. Reagent C -

It was freshly prepared by mixing reagent A and regent B in the ratio 25:1 (v/v).

iv. Reagent D - Arsenomolybdate Reagent

To 25g of ammonium molybdate dissolved in 450 ml of distilled water, 21 ml of concentrated H_2SO_4 was added slowly while stirring. Separately, 3g of sodium arsenate was dissolved in 25 ml of distilled water and this solution was added dropwise to ammonium molybdate solution and volume was made to 500 ml with distilled water. The solution so prepared was incubated for 48 hr at 37°C and stored in an amber coloured bottle.

B. Procedure

To 1 ml of appropriately diluted sugar extract, 1 ml of reagent C was added. The tubes were covered with water condensors and kept in a boiling water bath for 20 minutes. After cooling the tubes to room temperature, 1ml of reagent D was added. The contents were shaken well and then 7ml of distilled water was added and the contents were mixed on a cyclomixer. The intensity of the colour was recorded at 510nm against a reagent blank. The concentration of reducing sugars (as glucose) was calculated from the glucose standard (10-100µg) run simultaneously.

3.5.4 Determination of non - reducing sugars

Non reducing sugars were calculated by subtracting reducing

sugars from the total sugars.

3.5.5 Extraction and estimation of starch from leaves

Extraction of starch

A. Reagents

i. 9.2 N perchloric acid

ii. 4.6N perchloric acid

B. Procedure

Dried the residue obtained after extraction of sugars in an oven at 80°C. Added 2 ml of distilled water to the test tube containing the dried residue. The tubes were put in a boiling water bath for 15 minutes and stirred occasionally. Allowed the tubes to cool and added 2 ml of 9.2 N $HClO_4$ while stirring constantly. Then stirred the solution occasionally for 15 minutes. Filtered it through whatman filter paper No. 1 and collected the filtrate in a test tube. Added 2 ml of 4.6 N $HClO_4$ to the residue while stirring constantly. Then stirred the solution occasionally for 15 minutes. Filtered it through Whatman filter paper No. 1 and pooled the filtrate and made volume upto 10 ml with distilled water. Starch content was estimated in this extract.

Estimation of starch

The reducing sugars were estimated in the above extract by the procedure of Nelson (1944). The amount of starch was calculated by multiplying the content of reducing sugars by a factor of 0.9.

3.6 EXTRACTION AND ESTIMATION OF PHENOLIC CONSTITUENTS FROM RICE LEAVES

3.6.1 Extraction

40 mg of ground dried leaf sample was refluxed with 5 ml of 80% aqueous methanol for 1hr at 60-80°C on water bath. The refluxed material was filtered through Whatman no. 1 filter paper and the volume was made to 10ml by washing with 80 per cent methanol. The extract thus prepared was used for the estimation of phenolic constituents viz. total phenols, o-dihydroxyphenols and flavonols.

3.6.2 Estimation of total phenols - (Swain and Hillis 1959)

A. Reagents

i. Folin phenol reagent (diluted 1:1 v/v with distilled water).

ii. Saturated solution of Na_2CO_3 (17.5g/50 ml of distilled water).

B. Procedure

In a test tube, 0.5ml of the methanolic extract was evaporated to dryness and the residue was dissolved in 6.5ml of distilled water. To this 0.5ml of Folin - phenol reagent was added and shaken thoroughly. After five minutes, 1ml of saturated solution of sodium carbonate was added. After one hour, absorbance of the blue colour developed was read at 760nm against the blank. Blank was prepared from water and reagents only. Concentration of total phenols was determined from the standard curve prepared by using gallic acid in the range of 10-50µg.

3.6.3. Estimation of o-dihydroxyphenols (Nair and Vaidyanathan 1964)

A. Reagents

i. 10% trichloroacetic acid

ii. 10% sodium tungstate

iii. 0.5% sodium nitrite

iv. 0.5N sodium hydroxide

v. 0.5N hydrochloric acid

B. Procedure

In a test tube, 3ml of methanolic extract was evaporated to dryness and the residue left behind was dissolved in 1 ml of distilled water. To this 0.3ml of 10% TCA, 1ml of 10 per cent sodium tungstate, 0.5ml of 0.5N HCl and 1ml of freshly prepared 0.5% sodium nitrite was added. A yellow colouration developed. After five minutes 2ml of 0.5 N NaOH was added. The light cherry colour developed was read after 15 minutes at 540 nm against the reagent blank. The concentration of ortho-dihydroxyphenols was read from the standard curve prepared by using catechol in the range of 6-40µg.

3.6.4 Estimation of flavonols (Balabaa *et al* 1974)

A. Reagents

i. 0.1M methanolic solution of aluminium chloride.

B. Procedure

In a test tube, 1ml of methanolic extract was evaporated to

dryness. The residue left behind was dissolved in 5ml of 0.1M methanolic solution of aluminium chloride. The yellow colour so developed was read at 420nm against the reagent blank. The concentration of the flavonols was read from the standard curve prepared by using rutin in the range of 40-200μg.

3.7 DETERMINATION OF PHYSICO-CHEMICAL CHARACTERS OF RICE GRAIN

3.7.1 Estimation of amylose and amylopectin

Estimation of amylose (Juliano 1971)

A. Reagents

i. 95% ethanol

ii. 1N acetic acid

iii. Iodine solution (0.2g of iodine + 2g of KI in 100ml of aqueous solution)

B. Procedure

Whole grain milled rice was ground to pass through a 100-mesh sieve. 100mg of the powdered sample was taken in a test tube. To this 1ml of ethanol and 9ml of 1N NaOH was added. The tubes were kept for 10 minutes in a boiling water bath to gelatinize starch. After cooling the tubes to room temperature total volume was made upto 10ml with distilled water 0.5ml of the above solution was pipetted into a 100ml volumetric flask. To this 1ml of 1N acetic acid and 2ml of iodine solution was added. The total

volume was made to 100ml with distilled water. The solution was shaken well and left undisturbed for 20 minutes. The colour developed was read at 620nm against a reagent blank. The amylose content was determined by a reference to a standard curve prepared by using potato amylose in the range of 0.4-2.0mg.

Estimation of amylopectin

Amylopectin content was calculated by subtracting the percentage of amylose present from 100.

3.7.2 Determination of gelatinization temperature (Little *et al* 1958)

A. Reagents

i. 1.7% potassium hydroxide.

B. Procedure

Selected duplicate sets of six milled kernels without cracks and placed them in small petridishes. Added 10ml of 1.7 per cent KOH to each petridish. Arranged kernels to provide enough space between them to allow for spreading. Covered the petri-dishes and allowed them to stand for 23 hrs at ambient temperature. Visually rated the appearance and disintegration of endosperm. A rating for spreading at 1-3 was classified as high, 4-5 as intermediate and 6-7 as low gelatinization temperature.

The numerical scale used for scoring gelatinization temperature of rice is given below :

Score	Spreading	Clearing
1.	Kernel not affected	Kernel chalky
2.	Kernel swollen	Kernel chalky, collar powdery
3.	Kernel swollen, collar complete or narrow	Kernel chalky, collar cottony or cloudy
4.	Kernel swollen, collar complete and wide	Centre cottony, Collar cloudy
5.	Kernel split or segregated collar complete and wide	Centre cottony, Collar clearing
6.	Kernel dispersed merging with collar	Centre cloudy, Collar clear
7.	Kernel completely dispersed and intermingled	Centre and collar clear

3.7.3 Determination of gel consistency (Cagampang *et al* 1973)

A. Reagents

i. 95% ethanol containing 0.025% thymol blue.

ii. 0.2 N KOH

B. Procedure

Whole grain milled rice was ground to pass through a 100 mesh sieve. Weighed 100 mg of the ground powder, in duplicate in culture tubes

(12 x 100 mm). Added 0.2 ml of 95 per cent ethanol containing 0.025% thymol blue and added 2 ml of 0.2N KOH and mixed on a cyclomixer. Covered the tubes with glass marbles and heated in a vigorously boiling water bath for 8 minutes, making sure that the tube contents reached 2/3rd of the height of the tube.

Removed the tubes from the water bath and allowed them to stand for 5 minutes. Cooled them in an ice water bath for 20 minutes. The tubes were laid horizontally over a graph paper on a table undisturbed. The total length of the gel (mm) from the bottom of the tube to the gel front was measured after one hour.

Sr. No.	Length of Gel (mm)	Gel consistency
1.	40 mm or less	Very flaky rices with hard gel consistency
2.	41-60 mm	Flaky rices with medium gel consistency
3.	More than 61 mm	Soft rices with soft consistency

3.7.4 Estimation of crude protein content (Mckenzie and Wallace 1954)

A. Reagents

i. Digestion mixture (1 part of copper sulphate and 9 parts of potassium sulphate).

ii. Concentrated sulphuric acid.

iii. 0.1N sodium hydroxide.

iv. Saturated sodium hydroxide (40%).

v. 0.1N hydrochloric acid.

vi. Methyl red indicator.

B. Procedure

To 1 g of powdered sample taken in a digestion flask added 1 teaspoon of digestion mixture (5-6 gm) and 25 ml of concentrated sulphuric acid. Digested the material on an electric heater till the mixture became a transparent solution (light green in colour). After cooling the mixture to room temperature added 350ml of water and 75ml of 40 per cent NaOH to it. Distilled the above solution into 25ml of 0.1N HCl using methyl red indicator. About 150ml of the distillate was collected, it was titrated it with 0.1N NaOH to a light yellow colour. Blank was also run. Nitrogen content was estimated by the above method and protein content was determined by multiplying nitrogen content with a factor of 5.95.

3.7.5 Extraction and estimation of carbohydrate content

Extraction and estimation of total sugars (Dubois *et al* 1956), reducing sugars (Nelson 1944), non-reducing sugars and starch was done by their standard methods as discussed in section 3.5.

3.7.6 Determination of length, breadth and shape of rice grain

Ten whole grains of brown rice were taken and their length and breadth was measured using a dial thickness gauge. The L/B ratio was determined by dividing the average length with the average breadth.

3.8 DETERMINATION OF MILLING QUALITY OF RICE GRAIN

3.8.1 Dehusking

The paddy samples (125g) were dehusked in Satake Laboratory Rubber Roll Sheller (Japan). The distance between the rolls was adjusted depending upon the shape and size of grain to get minimum breakage of the grain. The shelled rice was weighed to determine the percentage of brown rice and husk contents.

3.8.2 Milling/Polishing

Brown rice samples were milled/polished in the McGill Miller No. 1 (USA) for appropriate time adjusted so as to obtain a uniform 6 per cent degree of polish in all the samples. The milled rice was weighed to obtain the percentage of milled rice recovery from the paddy/rough rice.

3.8.3 Yield of head rice and brokens

The milled rice was separated into head and brokens using a laboratory model rice sizing device (Satake, Japan). The kernels with more than three-fourth length were also considered as head rice. The separated head rice and brokens were weighed to determine the head rice yield and broken yield.

3.8.4 Grain weight

One thousand kernels of paddy, brown rice and milled rice were counted randomly in triplicate and weighted to get the average value.

3.9 DETERMINATION OF SEED GERMINATION AND SEEDLING GROWTH

Surface sterilized grains of each near isogenic line were thoroughly washed with sterile distilled water and then soaked in seventy-two hour old bacterial suspension of *Xanthomonas oryzae* pv. *oryzae* of approximately 10^9cells/ml for 24 hr. Bacterial suspension treated grains were thoroughly washed with sterile distilled water. Grains soaked in sterile distilled water served as control. Grains were plated on moistened filter paper (15 seeds per petriplate) and incubated at 25°C. Per cent germination, shoot length and root length were recorded after five days of incubation.

Chapter - IV

RESULTS AND DISCUSSION

The results of the present study include comparison of enzymes, metabolites and rice grain quality parameters in resistant and susceptible lines of rice against bacterial blight. The results are discussed under the following headings -

4.1 Changes in enzyme activities in resistant and susceptible lines of rice against bacterial blight

4.2 Changes in carbohydrate content in resistant and susceptible lines of rice against bacterial blight

4.3 Comparison of phenolic constituents in resistant and susceptible lines of rice against bacterial blight

4.4 Comparison of physico-chemical characters of rice grain in resistant and susceptible lines against bacterial blight

4.1 CHANGES IN ENZYME ACTIVITIES IN RESISTANT AND SUSCEPTIBLE LINES OF RICE AGAINST BACTERIAL BLIGHT

The leaves of one susceptible (TN1) and two resistant (IRBB13 and IRBB21) near isogenic lines of rice (*Oryza sativa* L.), to bacterial blight after infiltration either with *Xoo* (inoculated) or sterile distilled water (uninoculated) were analyzed for enzymes viz. peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and tyrosine ammonia

lyase (TAL) at different stages after inoculation : 0(immediately after inoculation) 1, 3, 5 and 7th day. The results of analysis are described and discussed as follows:

Peroxidase activity was found to be significantly higher in the near isogenic resistant lines IRBB13 and IRBB21 than in the susceptible variety TN1 (Table 1). PO activity was higher in the inoculated leaves at all the 5 stages i.e. 0,1,3,5 and 7th day after inoculation in all the three lines under study but the final activity levels in TN1 were less than those observed in the resistant lines IRBB13 and IRBB21. Though statistically significant difference in the inoculated and uninoculated leaves of TN1 was observed only 5 days after inoculation, in both the resistant near isogenic lines IRBB13 and IRBB21, almost 2-3 times increase in PO activity was observed 24 hours after inoculation (Table 1). A similar trend was observed when specific activity of PO was estimated in the susceptible and resistant lines (Table 2). Velazhahan *et al* (2006) observed significant increase in PO activity in rice leaves one day after inoculation with *Xoo*. Isozyme analysis showed induction of three peroxidase isozymes (PO-1, PO-2 and PO-3) after inoculation. Harrach *et al* (2005) observed that PO activity increases in response to inoculation of powdery mildew in susceptible varieties of barley.

Peroxidases have been implicated in the last enzymic step of lignin biosynthesis, that is, the oxidation of hydroxy cinnamyl alcohols into free radical intermediates, which subsequently are coupled into the lignin

Table 1 **Activity pattern of peroxidase ($\Delta E\ min^{-1}\ g^{-1}$ fresh weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	6.43	6.00	9.98	11.87	15.57	9.97
	I	6.51	7.89	11.31	19.52	26.09	14.26
Mean		6.47	6.94	10.64	15.69	20.83	12.11
IRBB13	U	4.96	11.55	15.52	16.16	19.97	13.63
	I	7.04	20.19	60.51	61.01	65.06	42.76
Mean		6.00	15.87	38.01	38.58	42.52	28.19
IRBB21	U	5.36	7.55	13.33	14.56	15.63	11.32
	I	5.44	20.27	58.19	64.03	70.53	43.69
Mean		5.40	13.91	35.76	39.29	43.08	27.48

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of three replicates
CD (5%)

A (Varieties)	=	2.47	A x B	=	3.49
B (Treatment)	=	2.01	B x C	=	4.51
C (No. of days)	=	3.18	A x C	=	5.52
			A x B x C	=	7.80

Table 2 **Specific activity of peroxidase ($\Delta E\ min^{-1}\ mg^{-1}$ protein) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	0.22	0.31	0.38	0.42	0.46	0.36
	I	0.31	0.38	0.69	0.71	0.97	0.61
Mean		0.26	0.34	0.54	0.56	0.72	0.48
IRBB13	U	0.43	0.54	0.43	0.39	0.62	0.48
	I	0.44	0.92	1.39	1.09	1.52	1.07
Mean		0.44	0.73	0.91	0.74	1.07	0.77
IRBB21	U	0.44	0.39	0.40	0.34	0.45	0.40
	I	0.33	0.91	1.53	1.29	1.69	1.15
Mean		0.38	0.65	0.96	0.81	1.07	0.77

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of three replicates
CD (5%)

A (Varieties)	=	0.070	A x B	=	0.099
B (Treatment)	=	0.057	B x C	=	0.128
C (No. of days)	=	0.090	A x C	=	NS
			A x B x C	=	0.22

polymer (Gross 1980). Although a role for peroxidases in defense responses have not been clearly demonstrated, increases in peroxidase activity have been correlated with infection in many species, including cotton (Mellon and Lee 1985, Venere 1980), chilli (Ramamoorthy and Samiyappan 2001), cucurbits (Smith and Hammerschmidt 1988), rice (Sridhar and on 1974, Toyoda and Suzuki 1960), and wheat (Flott *et al* 1989, Moerschbacher *et al* 1988, Schweizer *et al* 1989). Bacteriostasis and the deposition of lignin like polymers have also been correlated with an increase in the activity of an extracellular peroxidase in seedling leaves of rice during incompatible interactions (Reimers *et al* 1992).

There are reports in the literature that the expression of peroxidase is induced following recognition of bacterial and fungal pathogens (Torres *et al* 2006). An increase in PO activity has been reported as an early response to different stresses and may provide cells with resistance against formation of H_2O_2 (Castillo 1992).

Because *Xoo* is found primarily in the vascular tissues and does not directly penetrate the host cell, it is unlikely that lignin itself serves as a physical barrier to pathogen spread. Perhaps, the lignin biosynthetic process, which involves PO activity, toxic phenolic compounds, and free radicals, is involved in the rice defense response against *Xoo* (Mew *et al* 1993). It has been proposed that PO isozymes catalyze the synthesis of lignin within rice leaves, generating a weakly bactericidal condition in uninfected host tissues.

At elevated levels, such as those observed early in the incompatible interaction, lignification and associated reactions escalate, producing highly bactericidal conditions that lead to the restriction of bacterial multiplication (Reimers and Leach 1991, Reimers *et al* 1992). Naidu *et al* (1979) also reported that PO activity in the leaves of inoculated plants of rice was higher than that of the healthy leaves at all stages except after 20 hours of inoculation.

Polyphenol oxidase activity could not be detected at any stage, either in uninoculated or infected leaves in all the three lines under study. There are reports in literature where PPO activity has not been detected in the leaves of rice cultivar TN1 either in the healthy or infected tissue following infection by the leaf blight pathogen, *Xoo* (Rao and Nayudu 1979b) and rice blast causing pathogen, *Pyricularia oryzae* (Sridhar and Ou 1974). The oxidation of phenolics is mediated by PPO and PO. In the absence of o-diphenol oxidase, oxidation of phenols is mediated indirectly by PO (Thomson 1964) and melanoid pigment forms due to polymerization of quinones.

The leaves of resistant line IRBB13 were found to contain higher (1.87 times) constitutive levels of PAL activity as compared to that of the susceptible leaves of TN1 at zero day of inoculation (Table 3). PAL activity was also numerically higher (about 1.35 times) in the healthy leaves of IRBB21 (resistant) than of TN1 (susceptible) at zero day stage, but it was not

found to be statistically significant in both the resistant lines. A similar trend is observed when PAL activity is expressed on per mg protein (specific activity) basis (Table 4).

In all the three lines under study, PAL activity increased till 3 days after inoculation, after which it declined in both the uninoculated and inoculated leaves (Table 3). The increase in activity after bacterial infiltration was much higher in the resistant near isogenic lines IRBB13 (2.8 and 3.2 times) and IRBB21 (2.5 and 2.6 times) as compared to the susceptible TN1 leaves (1.5 and 1.9 times), one and three days after inoculation, respectively. The decreased activity of PAL in TN1 might be due to the existence of a "lyase-inactivating system" in this line (Purushothaman 1974b). Several workers (Purushothaman 1974b, Rao and Nayudu 1979b and Mohanty *et al* 1982) have also reported an immediate and more pronounced increase in PAL activity in the resistant cultivar of rice after infiltration with *Xoo.* Foliar application of *B. subtilis* reduced the sheath blight disease of rice (*Oryza sativa*) cultivar IR 50 by the increased activity of PAL and accumulation of PR proteins in rice leaves. These responses restrict growth of *Rhizoctonia solani* which causes sheath blight of rice (Jayaraj *et al* 2004). Orczyk *et al* (1996) conducted RNA blot hybridization with barley PAL cDNA. They observed that the intensity of PAL transcript remained elevated for long time in sorghum after inoculation with a non-pathogen *Bipolaris maydis* and concluded that in response to invasion of pathogen, PAL is synthesized more

Table 3 **Activity pattern of phenylalanine ammonia lyase (µg-t-cinnamic acid formed/hr/g fresh weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	9.51	23.04	50.57	28.22	13.25	24.92
	I	10.25	34.56	96.08	47.97	15.82	40.94
Mean		9.88	28.80	73.32	38.09	14.54	32.93
IRBB13	U	17.00	44.68	83.74	65.44	45.83	51.34
	I	20.08	123.38	267.96	163.48	100.89	135.16
Mean		18.54	84.03	175.85	114.46	73.36	93.25
IRBB21	U	12.81	30.24	60.25	48.43	23.04	35.95
	I	13.91	76.51	155.22	115.74	48.38	81.95
Mean		13.36	53.38	107.73	82.08	35.71	58.95

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of three replicates
CD (5%)

A (Varieties)	=	4.56	A x B	=	6.45
B (Treatment)	=	3.72	B x C	=	8.33
C (No. of days)	=	5.89	A x C	=	10.2
			A x B x C	=	14.43

Table 4 **Specific activity of phenylalanine ammonia lyase (µg-t-cinnamic acid formed/hr/mg protein) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	0.75	0.87	1.59	0.95	0.45	0.92
	I	0.89	1.13	2.14	1.57	0.92	1.33
Mean		0.82	1.00	1.86	1.26	0.68	1.12
IRBB13	U	1.30	2.02	2.33	1.62	0.72	1.59
	I	1.50	3.28	4.35	2.90	2.83	2.97
Mean		1.40	2.65	3.34	2.26	1.77	2.28
IRBB21	U	1.09	1.59	1.98	1.12	0.65	1.28
	I	1.21	3.46	4.11	2.35	2.32	2.69
Mean		1.15	2.52	3.04	1.73	1.48	1.98

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of three replicates
CD (5%)

A (Varieties)	= 0.15	A x B	= 0.21
B (Treatment)	= 0.12	B x C	= 0.27
C (No. of days)	= 0.19	A x C	= 0.34
		A x B x C	= 0.48

rapidly in resistant host than in the susceptible ones.

Ralton *et al* (1988) reported that in the resistant variety of cowpea infected with *Phytophthora vignae*, PAL activity was significantly higher than the susceptible variety. Mote and Dasgupta (1979) observed that the activities of PAL were higher in root and shoot samples of tomato inoculated with *Meloidogyne incognita* than the uninoculated ones. The values for resistant variety were considerably higher than that for the corresponding susceptible ones. In yeast elicited cassava (*Manihot esculenta*) leaves and suspension cells, PAL activity was induced maximally at 15 hr and was preceded by PAL mRNA accumulation which peaked at 9 hr (Vasquez *et al* 2004). PAL is the key enzyme in the biosynthesis of phenylpropane unit which is a component of phenolic acids, flavonoids and lignins (Camm and Towers 1973). In addition to this, peroxidases have been implicated in the last enzymatic step of lignin biosynthesis, that is, the oxidation of hydroxycinnamyl alcohols into free radical intermediates, which are subsequently coupled to form lignin polymers (Gross 1980). The role of lignification as part of the hypersensitive responses in wheat to *Pucania graminis* has been presented by Moerschbacher *et al* (1988). The interaction of the resistant race and the host plant resulted in the deposition of lignin and the induction of enzymes associated with the lignification process like PAL, 4-coumarate CoA ligase, cinnamyl alcohol dehydrogenase and peroxidase. Inhibition of lignification by inhibitors of PAL and the lignin specific enzyme

cinnamyl alcohol dehydrogenase resulted in the inhibition of hypersensitive response (Moerschbacher *et al* 1990). In the present study, it seems that induction response of PO and PAL activity is almost qualitatively similar in both compatible and incompatible interactions, but is quantitatively to a lesser extent in the compatible interaction compared with the incompatible reaction. Higher induction of PO coincided with the induction of PAL in the resistant host-pathogen interaction. It might be that the accumulation of lignin precursors is related to the development of resistance to the pathogen and the susceptible interaction may reflect the absence of either lignin precursors or PO generated free radicals, that under normal conditions, are toxic to the plant cell itself (Ride 1978).

Reimers and Leach (1991) also showed that the race - specific resistance of rice carrying the Xa-10 gene to *Xoo* is correlated with the deposition of lignin at the site of infection. These workers identified lignin with a variety of histochemical stains at infection sites by both compatible and incompatible interactions. However, the deposition of lignin in the incompatible interaction occured within 18 to 24 hours after inoculation whereas increased accumulation of lignin was not seen in the compatible interaction until 96 hours after inoculation. Monitoring the population of bacteria in the host tissue further demonstrated a correlation between the initiation of the lignification process and lignin deposition with the cessation of bacterial multiplication in the resistant interaction. These results indicate

that lignin and toxic lignin precursors play a role in the active defense of rice to *Xoo*.

Activity pattern of TAL as given in Table 5 shows that the activity increased in the uninoculated leaves of both the susceptible and resistant lines till the third day stage and then started declining. A similar trend was followed in the bacteria infiltrated leaves but the increase is much higher in the resistant near isogenic lines IRBB13 and IRBB21 than the susceptible TN1 throughout the time course of the experiment. Zuber and Manibhushanrao (1984) studied PAL and TAL activity in resistant and susceptible rice cultivars to *R. solani* at different stages of sheath blight disease and found higher activity of PAL and TAL in the resistant cultivars.

Minamikawa and Uritani (1964) reported a marked increase in PAL and TAL activity in sweet potato roots in response to wounding or infection by *Ceratocystis fimibriata* and found a close relation of biosynthesis of polyphenols in the tissues with the remarkable rise in PAL activity in the wounded or infected root issues. The pattern of TAL activity was found to be quite similar to the PAL but enzyme activity has been reported to be very low. Similar results were obtained in our experiment.

PAL and TAL are reported to primarily play an important role in the conversion of amino acids phenylalanine and tyrosine to coumaric acid, which is a precursor of flavonoids, lignin and phenolic phenylpropane and thus in turn impart higher level of resistance (Mahadevan and Sridhar 1982).

Table 5 **Activity pattern of tyrosine ammonia lyase (µg-p-coumaric acid formed/hr/g fresh weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	5.86	13.93	22.84	12.17	6.13	12.17
	I	7.11	26.22	41.59	18.25	9.80	20.59
Mean		6.48	20.07	32.21	30.42	7.96	16.38
IRBB13	U	8.35	18.30	39.72	30.75	20.16	23.45
	I	10.69	63.68	90.66	68.08	62.39	59.10
Mean		9.52	40.99	65.19	49.42	41.27	41.27
IRBB21	U	5.94	11.81	32.26	18.66	10.21	15.77
	I	7.99	37.15	72.44	59.77	46.21	44.71
Mean		6.96	24.48	52.35	39.22	28.21	30.24

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of three replicates
CD (5%)

A (Varieties)	=	2.22	A x B	=	3.14
B (Treatment)	=	1.81	B x C	=	4.06
C (No. of days)	=	2.87	A x C	=	4.97
			A x B x C	=	7.03

The regulatory role of PAL and TAL in phenolic biosynthesis has also been reported by Creasy (1987) and McCallum and Walker (1990).

Matsuyama and Diamond (1973) reported changes in PAL and TAL activity in blast infected rice plants and found that these changes precede the alterations in phenolic levels suggesting that there are temporal and spatial differences between the increase in the amount of PAL that occur during infection and the change in the levels of phenolic compounds (Rathmell 1973).

Purushothaman (1974b) reported that the resistant TKM 6 variety of rice exhibited higher PAL activity, compared to the susceptible Co 13 and IR8 varieties, after inoculation with *Xoo*. It was observed that in TKM 6, the levels of the free amino acids phenylalanine and tyrosine decreased drastically after *Xoo* inoculation, while their levels increased in the susceptible IR8 and Co13 varieties. Sharma (2003) has also suggested that phenolics and their enzymes (PAL, TAL and PO) play an important role in apple tissues during pathogenesis by *P. ultimum*, since their levels and activities were more in resistant rootstocks and less in susceptible ones.

The specific activity pattern of TAL during the time course of the experiment (Table 6) follows a similar pattern as that of TAL activity expressed on per gram fresh weight basis.

Table 6 **Specific activity of tyrosine ammonia lyase (µg-p-coumaric acid formed/hr/mg protein) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	0.46	0.53	0.71	0.28	0.21	0.44
	I	0.42	1.05	1.44	0.82	0.85	0.92
Mean		0.44	0.79	1.07	0.55	0.53	0.68
IRBB13	U	0.57	0.85	1.11	0.76	0.62	0.78
	I	0.65	2.88	2.07	1.47	1.46	1.71
Mean		0.61	1.86	1.59	1.11	1.04	1.24
IRBB21	U	0.48	0.62	0.98	0.43	0.28	0.55
	I	0.52	1.67	1.91	1.21	1.10	1.28
Mean		0.50	1.14	1.44	0.82	0.69	0.92

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of three replicates
CD (5%)

A (Varieties)	=	0.08	A x B	=	0.12
B (Treatment)	=	0.07	B x C	=	0.16
C (No. of days)	=	0.11	A x C	=	0.19
			A x B x C	=	0.27

4.2 CHANGES IN CARBOHYDRATE CONTENT IN RESISTANT AND SUSCEPTIBLE LINES OF RICE AGAINST BACTERIAL BLIGHT

Total sugars, reducing sugars, non reducing sugars and starch content of inoculated and uninoculated leaves of three lines were analyzed.

The content of total sugars estimated in the susceptible line TN1 was found to be significantly more than that in the resistant near isogenic lines IRBB13 and IRBB21 (Table 7). After infection, the amount of total sugars increased from 0-5 days after infection after which a slight decrease was observed on the 7th day after inoculation in TN1. In the resistant line IRBB21, total sugars decreased from 0-7 days after inoculation while in IRBB13 also a decreasing trend was observed during the time course of the experiment except at 2nd stage, when the total sugars increased after infection (Table 7).

The reducing sugar content was also significantly more in the susceptible line TN1 than the resistant near isogenic lines IRBB13 and IRBB21 (Table 7). After bacterial infiltration, the reducing sugars content increased till three days after infection after which it started declining in TN1 while in the resistant near isogenic lines IRBB13 and IRBB21 after increasing initially at one day after infection, the reducing sugars content started decreasing from 3rd day after inoculation (Table 7). The constitutive level of total and reducing sugars was however higher in the resistant near isogenic lines IRBB13 and IRBB21 than in the susceptible line TN1 (Table 7 and 8).

Table 7 **Change in total sugars content (mg/g fresh weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	12.20	16.88	15.00	16.06	20.95	16.22
	I	11.06	16.57	19.69	20.01	19.77	17.42
Mean		11.63	16.72	17.34	18.03	20.36	16.82
IRBB13	U	14.37	13.44	14.57	13.12	11.88	13.47
	I	12.98	15.32	13.75	12.82	10.69	13.11
Mean		13.67	14.38	14.16	12.97	11.28	13.29
IRBB21	U	19.06	17.82	15.32	14.38	13.13	15.94
	I	19.52	16.25	11.88	11.56	10.38	13.91
Mean		19.94	17.03	13.60	12.97	11.75	14.92

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates
CD (5%)

A (Varieties)	=	0.64	A x B	=	0.90
B (Treatment)	=	NS	B x C	=	NS
C (No. of days)	=	0.82	A x C	=	1.43
			A x B x C	=	2.02

Table 8 **Change in reducing sugars content (mg/g fresh weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	1.30	2.25	3.43	2.98	2.32	2.46
	I	1.21	3.45	3.91	3.20	3.10	2.97
Mean		1.25	2.85	3.67	3.09	2.71	2.71
IRBB13	U	1.62	2.49	2.41	1.80	1.40	1.94
	I	1.49	3.28	2.64	1.93	1.67	2.20
Mean		1.55	2.88	2.53	1.86	1.53	2.07
IRBB21	U	2.41	2.59	2.40	2.18	2.13	2.34
	I	2.45	2.96	2.49	2.36	2.21	2.49
Mean		2.43	2.77	2.44	2.27	2.17	2.41

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates
CD (5%)

A (Varieties)	=	0.09	A x B	=	0.13
B (Treatment)	=	0.07	B x C	=	0.17
C (No. of days)	=	0.12	A x C	=	0.21
			A x B x C	=	NS

Reduction in sugar contents associated with simultaneous increase in phenolics in resistant near isogenic lines IRBB13 and IRBB21 in the present study (Tables 8 and 11) suggests that a major part of the sugars is shunted to polyphenol synthesis (Zuber and Manibhushanrao 1984).

The non-reducing sugar content was significantly higher in the susceptible TN1 line than both the resistant near isogenic lines IRBB13 and IRBB21 (Table 9). The non-reducing sugars increased steadily after inoculation till 5 days after inoculation and then decreased at 7 days after inoculation in TN1 but a reverse trend was observed in the resistant line IRBB21 where the non-reducing sugars decreased after inoculation and were the lowest at 7 days after inoculation (Table 9). In IRBB13 also the non-reducing sugars decreased after inoculation except one day after inoculation, when a slight increase was observed. The constitutive level of non-reducing sugars was however more in resistant lines.

The starch content was also higher in the susceptible TN1 than that in the resistant near isogenic lines IRBB13 and IRBB21 (Table 10). After bacterial infiltration the starch content decreased significantly in TN1 while in IRBB13 it increased but decreased significantly in IRBB21. No set trend could be discerned in the starch content either in the susceptible or in the resistant lines during the time course of study, both in the inoculated and uninoculated leaves (Table 10).

Table 9 **Change in non-reducing sugars content (mg/g fresh weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively**

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	10.90	14.63	11.57	13.08	18.63	13.76
	I	9.85	13.12	15.78	16.81	16.67	14.44
Mean		10.37	13.87	13.67	14.94	17.65	14.10
IRBB13	U	12.75	10.95	12.16	11.32	10.48	11.53
	I	11.49	12.04	11.11	10.89	9.02	10.91
Mean		12.12	11.49	11.64	11.10	9.75	11.22
IRBB21	U	16.65	15.23	12.92	12.20	11.00	13.60
	I	17.07	13.29	9.39	9.20	8.17	11.42
Mean		17.51	14.26	11.15	10.70	9.58	12.51

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates
CD (5%)

A (Varieties)	=	0.62	A x B	=	0.88
B (Treatment)	=	0.51	B x C	=	NS
C (No. of days)	=	0.81	A x C	=	1.40
			A x B x C	=	1.98

Table 10 Change in starch content (µg/g fresh weight) in rice leaves of susceptible and resistant varieties lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	43.00	54.00	72.00	55.49	61.42	57.18
	I	41.00	29.90	44.10	58.00	38.00	42.20
Mean		42.00	41.95	58.05	56.74	49.71	49.69
IRBB13	U	40.42	32.00	25.00	39.58	20.00	31.40
	I	37.80	47.00	65.00	52.00	29.00	46.16
Mean		39.11	39.50	45.00	55.39	24.50	38.78
IRBB21	U	47.00	41.25	55.00	43.75	12.30	39.86
	I	49.00	28.90	35.70	33.22	20.80	33.52
Mean		48.00	35.07	45.35	38.48	16.55	36.69

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates
CD (5%)

A (Varieties)	=	2.31	A x B	=	3.27
B (Treatment)	=	1.89	B x C	=	NS
C (No. of days)	=	2.99	A x C	=	5.18
			A x B x C	=	7.32

The accumulation of reducing sugars at whose expense phenols are synthesized also accounts for the low phenolic content of the diseased leaves of TN1 (Tables 8 and 11). It has been suggested that actual depletion of phenolic content helps in maintenance of greater reducing potential in the host tissue which is congenial for spread of the pathogen and enlargement of infected area in blight infected leaves of TN1 (Rao and Nayudu 1979a).

Misawa and Miyazaki (1972) reported that both reducing and non-reducing sugars and crude starch increase in early stages of infection in rice leaves infected with bacterial blight. Rice varieties resistant to bacterial blight were found to have a low proportion of reducing sugars to total nitrogen and high total sugar to total nitrogen as compared to susceptible ones (Srivastava 1972). Moses *et al* (1975) reported that the content of total and reducing sugars were higher in susceptible variety and susceptible F_2 progeny than in the resistant varieties and resistant F_2 progeny of rice to bacterial blight.

Reddy and Sridhar (1975) revealed that susceptibility of rice leaves to bacterial blight was associated with higher levels of sugars and depressed levels of total phenols.

Marimuthu (1981) reported that susceptible varieties of greengram contain higher reserve of total sugars, glucose and fructose than resistant ones. The total carbohydrate content was less in pea leaves infected with *Erysiphe pisi* (Bahadur *et al* 2005).

4.3 COMPARISON OF PHENOLIC CONSTITUENTS IN RESISTANT AND SUSCEPTIBLE LINES OF RICE AGAINST BACTERIAL BLIGHT

The total phenolic content in the leaves of all the three lines demonstrated an increasing trend from 0-5 days after which it declined (Table 11). However, the constitutive level of phenols in both the resistant near isogenic lines IRBB13 and IRBB21 was significantly higher than that of the susceptible TN1 cultivar. Maximum increase in the total phenolic content after bacterial infiltration was found in IRBB13 (41.5%) followed by IRBB21 (30.6%) and TN1 (25.2%). The change in total phenolic content can be correlated with the increase in the PAL, TAL and PO activities during the time course of the experiment, except that the enzymatic activities of PAL and TAL start declining after three days of inoculation, while the levels of total phenols, o-dihydroxyphenols and flavonols decline after 5 days of inoculation (Table 11-13).

Purushothaman (1974a) and Mahto *et al* (1987) have reported that total phenols occur in larger amounts in the bacterial blight resistant varieties as compared to the susceptible varieties of rice. Similar observations in rice- *X. translucens* f. sp. *oryzicola* were recorded by Reddy and Sridhar (1975).

Rice plants inoculated with two rhizobial strains RRE6 and ANU 843 and infected with *Rhizoctonia solani* showed more accumulation of phenolic acids i.e. gallic, tannic, ferulic and cinnamic acids than uninoculated

Table 11 Change in total phenols (mg/g dry weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	2.86	5.73	8.33	10.42	8.85	7.23
	I	3.04	8.07	9.63	17.19	10.42	9.67
Mean		2.95	6.90	8.98	13.80	9.64	8.45
IRBB13	U	4.42	7.29	10.68	12.76	10.93	9.22
	I	5.19	11.20	17.71	23.69	21.09	15.77
Mean		4.80	9.24	14.20	18.23	16.01	12.49
IRBB21	U	3.38	6.77	9.63	11.71	10.68	8.43
	I	3.98	9.63	12.76	20.05	14.32	12.14
Mean		3.68	8.20	11.19	15.88	12.50	10.28

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates
CD (5%)

A (Varieties)	=	0.32	A x B	=	0.45
B (Treatment)	=	0.26	B x C	=	0.58
C (No. of days)	=	0.41	A x C	=	0.71
			A x B x C	=	1.00

plants (Mishra *et al* 2006). Singh and Singh (1989) found higher levels of total and ortho-dihydroxyphenols in leaves of chilli pepper after inoculation with cucumber mosaic virus.

Phenolic compounds are considered as non-specific defense metabolites against the pathogen and resistant plants have the tendency to accumulate these metabolites in infection (Bell 1981, Alam *et al* 1991). These phenolic compounds are possibly converted by increased peroxidase activity to quinones in resistant cultivars. These quinones are reported to be more toxic to microorganisms (Clark and Lorbeer 1975, Sempio *et al* 1975, Vidhyasekaran 1988) and hence have been assigned a role in disease resistance. Reddy *et al* (1977) also reported that the resistant cultivar BJ1 of rice grown under normal light synthesized additional amounts of phenolic compounds in response to bacterial infection with *Xoo*.

Valluvaparidasan and Mariappan (1983) reported accumulation of phenolics in rice varieties due to infection by *X. campestris* pv. *oryzae*. They observed higher total phenolic content in moderately resistant cultivar TNAV7124, than the highly susceptible TN1. Total phenolic content of the cultivars increased with inoculation and decreased with plant age.

Similar to the case of total phenolic content, the ortho-dihydroxyphenol content exhibited an increasing trend from 0-5 days after inoculation in all the three lines under study, after which it started declining (Table 12). Ortho-dihydroxyphenols increased in all three lines after bacterial

Table 12 Change in ortho-dihydroxyphenols content (mg/g dry weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	0.78	0.95	1.19	1.67	1.67	1.25
	I	0.95	1.31	1.55	2.26	1.91	1.59
Mean		0.86	1.13	1.37	1.96	1.79	1.42
IRBB13	U	0.95	1.07	1.55	2.03	1.67	1.45
	I	1.07	1.55	2.26	2.97	2.50	2.07
Mean		1.01	1.31	1.90	2.49	2.08	1.76
IRBB21	U	0.71	1.07	1.55	1.91	1.55	1.36
	I	0.95	1.31	1.91	2.50	2.15	1.76
Mean		0.83	1.19	1.73	2.20	1.85	1.56

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates
CD (5%)

A (Varieties)	=	0.10	A x B	=	0.14
B (Treatment)	=	0.082	B x C	=	0.18
C (No. of days)	=	0.13	A x C	=	NS
			A x B x C	=	NS

infiltration but the increase was much higher in the resistant near isogenic lines IRBB13 and IRBB21 (Table 12). Our results are in agreement with those of Purushothaman (1974a) and Seetharaman *et al* (2004) who reported larger accumulation of ortho-dihydroxyphenols in bacterial blight resistant variety compared with that of the susceptible variety of rice after infection. Induced synthesis of phenolic compounds is associated with host - pathogen interaction and specific phenolics have been implicated in host resistance. Ferulic and p-coumaric acids in bound form have been found to be involved in the resistance of wheat leaves to *Puccinia recondita* (Southerton and Deverall 1990). Resistant varieties of rice had more total and ortho-dihydroxyphenols than two susceptible varieties against brown spot disease. Phenols increased in all the four varieties after inoculation with *Helminthosporium oryzae* but increase was more in the resistant varieties (Sathayanathan and Vidhyasekaran 1981). Inoculation of parsley leaves with *P. megasperma* results in the accumulation of coumarin phytoalexins as well as esterification of phenylpropanoids. Such esters are thought to be involved in the formation of phenolic polymers by crosslinking (Nicholson 1992). Higher levels of o-dihydroxyphenols in resistant cultivars of groundnut (Sindhan and Parashar 1996), apple rootstock (Sharma 2003), pea (Guleria *et al* 1998) and *B. juncea* (Atwal *et al* 2004) have also been reported.

The uninoculated leaves of resistant lines IRBB13 and IRBB21 were found to contain slightly more flavonols (23.4% and 9.9% respectively)

as compared to that in the susceptible leaves (Table 13). Flavonols content started increasing after bacterial infiltration in all the three lines from 0-5 days after inoculation and thereafter a decrease was observed on the 7th day after inoculation. But the total flavonol content was significantly higher in the resistant than the susceptible line (Table 13).

Similar results have also been reported by Voinilo (1975) in leaves of potato resistant to *H. rostochiensis,* Rich *et al* (1977) in limabeans infected with *Pratylenchus scribneri* and Essenberg *et al* (1982) in the interaction of *Xanthomonas campestris* pv. *malvacearum* with resistant variety of cotton. Their results demonstrated that flavonoids occured in a concentrated fashion in the cells immediately at the infection site such that greater than 90 per cent of the compounds were highly localized. Eventually these compounds accumulated to concentrations sufficient to account for bacteriostasis.

Flavonoids derived from both malonate and shikimate pathways, are emerging to be important, both as defense and survival compounds in plants (Lamb *et al* 1992, Wink 1988 and Dakora 1995). Flavonoids have been shown to accumulate in many plants under infection. However, there are few reports in cereal crops showing such accumulation. In sorghum, the accumulation of 3-deoxyanthocyanidins has been shown to be toxic to the pathogen *Colletotrichum graminicola* (Snyder *et al* 1990 and Lo *et al* 1996). In addition, an increase in the flavan - 4 - ol content was

Table 13 Change in flavonols content (mg/g dry weight) in rice leaves of susceptible and resistant lines against bacterial blight at different days after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		0 day	1 day	3 day	5 day	7 day	Mean
TNI	U	1.47	4.40	6.25	6.62	6.62	5.07
	I	1.68	5.51	7.35	11.38	7.72	6.72
Mean		1.57	4.95	6.80	9.00	7.17	5.89
IRBB13	U	1.84	5.52	7.72	9.19	8.82	6.62
	I	2.05	9.19	14.31	17.99	13.96	11.5
Mean		1.94	7.35	11.01	13.59	11.39	9.06
IRBB21	U	1.69	4.78	6.62	7.72	7.35	5.63
	I	1.87	6.62	9.18	13.95	10.66	8.45
Mean		1.78	5.70	7.90	10.84	9.00	7.04

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates
CD (5%)

A (Varieties)	=	0.42	A x B	=	0.60
B (Treatment)	=	0.34	B x C	=	0.77
C (No. of days)	=	0.54	A x C	=	0.95
			A x B x C	=	1.34

reported in certain mould resistant sorghum lines (Jambunathan *et al* 1990). Flavonols inhibit growth of rice pathogens and act as defense and survival compounds. Naringenin and Kaempferol inhibited spore germination of *Pyricularia oryzae* (Padmavati *et al* 1997). Naringenin (flavanone) was detected in rice leaves exposed to UV-B irradiation and also after blast infection (Grayer *et al* 1996). Besides, rice cultivars were shown to exhibit differential phytoalexin response to blast (Dillon *et al* 1997). Flavonols, both constitutive and induced, are reported to contribute to disease resistance (Kiran *et al* 2003, Vandana 1995).

4.4 COMPARISON OF PHYSICO-CHEMICAL CHARACTERS OF RICE GRAIN IN RESISTANT AND SUSCEPTIBLE LINES AGAINST BACTERIAL BLIGHT

The amylose content in the grain was estimated in the susceptible and resistant lines of rice (Table 14). Amylose content was numerically higher in IRBB13 and IRBB21 than TN1 but it was found to be statistically significant only in IRBB21. After bacterial infiltration, a significant decrease in amylose content was found only in TN1 (Table 14). The crude protein content was found to be significantly higher in the susceptible variety TNI than the near isogenic resistant lines IRBB13 and IRBB21 (Table 14). After inoculation with *Xoo,* crude protein content decreased significantly in all the three lines under study (Table 14). But percentage reduction was higher in the susceptible variety TN1 (22%) than in the resistant near isogenic lines IRBB13 (13%) and IRBB21 (12%).

Table 14 Chemical characteristics of rice grains of resistant and susceptible lines against bacterial blight of rice after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		Amylose (%)	Amylopectin (%)	Crude protein (%)	Gel consistency (mm)	Alkali spreading and clearing values
TNI	U	11.82	88.18	7.88	95.33	7,5.5
	I	9.19	90.81	6.14	93.33	7,5.5
Mean		10.50	89.49	7.01	94.33	7,5.5
IRBB13	U	11.12	88.88	6.81	97.33	6.0,5.0
	I	13.10	86.90	5.92	95.00	6.0,5.0
Mean		12.11	87.89	6.36	96.16	6.0,5.0
IRBB21	U	14.15	85.85	7.13	98.33	6.5,5.0
	I	16.65	83.38	6.30	95.33	6.5,5.0
Mean		15.40	84.60	6.71	96.83	6.5,5.0
U = Uninoculated (inoculated with sterile distilled water)						
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml						
Values are mean of two replicates						
CD (5%)						
A (Varieties)		0.07	0.12	0.093	NS	0.054
B (Treatment)		0.06	0.10	0.076	NS	NS
A x B		0.10	0.17	0.13	NS	NS

Gel consistency and alkali spreading and clearing value were found to remain unchanged in all the three lines and were not affected by bacterial infiltration (Table 14). The effect of amylose and protein content in the grain on the degree of disease incidence was studied in different mutant lines of rice by Singh and Rao (1971) but no clear trend was observed. An increase in protein content due to infection of rice has been reported for *Xoo* (Singh *et al* 1977, Tagami and Mizukami 1962) and Helminthosporiose (Vidhyasekaran *et al* 1973) infection.

Total sugar content was significantly less in the susceptible variety TN1 than in the resistant near isogenic lines IRBB13 and IRBB21. Bacterial infiltration caused a significant decrease in the total sugar content (Table 15). The three lines did not differ significantly in the content of reducing sugars, non-reducing sugars and starch (Table 15). Inoculation with bacterial blight causing microorganism *Xoo,* did not affect the reducing sugars and non-reducing sugar content while starch content decreased significantly in all the three lines (Table 15). Thus, resistance or susceptibility of the three lines of rice to *Xoo* was found to have no relationship with either total, reducing or non-reducing sugars, starch or amylose content of the grain (Tables 14 and 15).

The data in Table 16 indicates that bacterial infiltration did not cause significant milling losses in the three lines under study. However, the loss in the head rice recovery was significantly higher in TNI after infection. In the uninoculated TN1 grains also, the head rice recovery decreased significantly as

Table 15 Changes in carbohydrate content (%) in rice grains of resistant and susceptible lines against bacterial blight after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		Total sugars	Reducing sugars	Non-reducing sugars	Starch
TNI	U	0.32	0.09	0.23	81.37
	I	0.19	0.05	0.14	72.46
Mean		0.25	0.07	0.18	76.92
IRBB13	U	0.38	0.11	0.27	79.92
	I	0.31	0.09	0.22	73.51
Mean		0.34	0.10	0.24	76.72
IRBB21	U	0.37	0.10	0.27	78.51
	I	0.28	0.08	0.20	71.17
Mean		0.32	0.09	0.23	74.84
U = Uninoculated (inoculated with sterile distilled water)					
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml					
Values are mean of two replicates					
CD (5%)					
A (Varieties)		0.05	NS	NS	NS
B (Treatment)		0.04	NS	NS	2.51
A x B		NS	NS	NS	NS

Table 16 Milling quality of rice grains of resistant and susceptible lines against bacterial blight of rice after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		Brown rice (%)	Milled rice (%)	Head rice (%)
TNI	U	78.76	72.73	66.97
	I	77.26	71.08	56.38
Mean		78.01	71.91	61.66
IRBB13	U	78.22	72.39	68.34
	I	78.28	72.02	67.58
Mean		78.24	72.21	67.96
IRBB21	U	79.09	72.77	68.87
	I	78.55	72.27	68.00
Mean		78.82	72.52	68.43

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates

CD (5%)			
A (Varieties)	NS	NS	0.91
B (Treatment)	NS	NS	0.75
A x B	NS	NS	1.29

compared to that in IRBB13 and IRBB21. Similar results in respect of high percentage of broken rice due to BB infection has also been reported by Ou (1972) and Singh *et al* (1977).

The length, breadth and shape of the kernels were not affected due to the bacterial blight infection in either the susceptible variety TN1 or the resistant near isogenic lines IRBB13 and IRBB21 (Table 17). Significant reduction in 1000-grain weight was recorded in TN1 while it was not affected in IRBB13 and IRBB21. It has been reported that bacterial blight causes significantly reduced panicle number and 1000-grain weight (Kirya and Kuhara 1962). The quality of rice grains is known to depend on the metabolic state of rice leaves during grain filling phase (IRRI 1970). Altered metabolism in the leaves developed after BB infection and hence it is likely that such pathogen induced metabolic changes might affect not only the grain filling process but also the grain quality (Moses *et al* 1975).

The results of the effect of bacterial suspension of *Xoo* on germination of seed and seedling growth of the three lines is given in Table 18. Overall mean germination indicated that the per cent germination of the seeds of the three lines significantly differed from each other. Difference in germination between inoculated and uninoculated check was more in the susceptible variety TN1 (15%) than in the resistant near isogenic lines IRBB13 (5%) and IRBB21 (11%).

No significant difference in the root length of the three lines was observed (Table 18). Root length of bacterial suspension treated

Table 17 Physical characteristics of rice grains of resistant and susceptible lines against bacterial blight of rice after inoculation with *Xanthomonas oryzae* pv. *oryzae* and sterile distilled water respectively

		1000 grain wt. paddy (g)	1000 grain wt. brown rice (g)	1000 grain wt. milled rice (g)	Length of grain (mm)	Breadth of grain (mm)	Length/ breadth ratio
TNI	U	21.80	17.15	16.62	5.35	2.48	2.15
	I	18.22	15.56	14.94	5.38	2.52	2.13
Mean		20.01	16.35	15.78	5.36	2.50	2.14
IRBB13	U	20.68	17.89	16.64	6.33	2.12	2.98
	I	20.67	17.32	15.95	6.22	2.09	2.98
Mean		20.68	17.60	16.29	6.27	2.10	2.98
IRBB21	U	21.53	17.75	16.84	6.50	2.08	3.13
	I	20.69	17.84	16.15	6.40	2.05	3.12
Mean		21.11	17.79	16.49	6.45	2.06	3.125
CD (5%)							
A (Varieties)		NS	0.61	NS	0.108	0.067	0.096
B (Treatment)		0.76	0.50	0.94	NS	NS	NS
A x B		1.32	0.87	NS	NS	NS	NS

U = Uninoculated (inoculated with sterile distilled water)
I = Inoculated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of two replicates

Table 18 Effect of bacterial suspension of *Xanthomonas oryzae* on germination of seed and seedling growth of resistant and susceptible lines

		Per cent germination	Root length (cm)	Shoot length (cm)
TNI	Untreated	100	4.36	2.14
	Treated	86.6	2.76	1.86
Mean		93.33	3.56	2.00
IRBB13	Untreated	100	5.06	2.35
	Treated	94.6	3.58	2.05
Mean		97.3	4.32	2.20
IRBB21	Untreated	100	4.41	2.31
	Treated	90.7	3.41	2.03
Mean		95.35	3.91	2.17

Untreated = Treated with sterile distilled water
Treated = Treated with *Xanthomonas oryzae* of concentration 10^9 cells/ml
Values are mean of five replicates

CD (5%)			
A (Varieties)	0.652	NS	NS
B (Treatment)	0.532	0.558	NS
A x B	0.922	NS	NS

seedlings was significantly less than that of the untreated checks. Shoot length was not significantly affected in the bacterial suspension treated and untreated check (Table 18). Though a reduction in the shoot length after bacterial suspension treatment was observed in all the three lines, this reduction was not found to be statistically significant (Table 18).

Reduced germination and seedling growth affecting shoot length and root length as a result of infection with *Sarocladium oryzae* has been observed by Reddy *et al* (2000) and Velazhahan (1991). Reduction in seed germination and seedling growth could be due to the involvement of certain toxic substances produced by the pathogen (Velazhahan 1991).

SUMMARY

The present investigation was undertaken to understand the biochemical basis of disease resistance in rice plant against bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae*. A study involving three lines, one susceptible variety TN1 and two near isogenic resistant lines IRBB13 and IRBB21, was planned to identify the role of enzymes viz; peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and tyrosine ammonia lyase in the mechanism of resistance. Changes in carbohydrates and phenolic compounds were studied in resistant and susceptible rice lines. Effect of bacterial blight on the physico-chemical quality of rice grain of resistant and susceptible lines was also studied. The results obtained and conclusions derived are outlined as follows -

Peroxidase (PO) activity was significantly higher in the leaves of resistant line IRBB13 followed by another resistant line IRBB21 than the susceptible line TN1. After bacterial infiltration increase in peroxidase activity implies that this enzyme could be involved in the increased levels of lignins and quinones that from key barriers against the pathogen. The higher expression of this enzyme in resistant lines has been associated with development of resistance against bacterial blight.

Polyphenol oxidase (PPO) activity could not be detected in

leaves at any stage before or after inoculation in all the three lines.

Phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) activity was also higher in resistant near isogenic lines IRBB13 and IRBB21 before bacterial inoculation. Increase in activities of both the enzymes after bacterial infiltration was also higher in resistant lines which indicates that these enzymes may be involved in the synthesis of lignin precursors, flavonoids, phytoalexins, quinones and condensed tannins which are known to act as defense compounds.

The uninoculated leaves of resistant lines were found to contain higher levels of total phenols and ortho-dihydroxyphenols than those in the susceptible line before bacterial inoculation. However, after bacterial infiltration the observations revealed significantly higher increase in the levels of total phenols, ortho-dihydroxyphenols and flavonols in the resistant than the susceptible line. From this study, higher constitutive levels of total phenols and ortho-dihydroxyphenols were positively correlated with the development of resistance. Increased levels of total phenols, ortho-dihydroxyphenols and flavonols after bacterial infiltration by *X. oryzae* pv. *oryzae* appear to play an important role in protection of the host from the pathogen.

Total sugars, reducing sugars and starch content were significantly higher in the susceptible variety TN1 than in both the near isogenic resistant lines IRBB13 and IRBB21. Reduction in sugar content

associated with simultaneous increase in phenolics in resistant near isogenic lines IRBB13 and IRBB21 in the present study suggest that a major part of the sugars is shunted to polyphenol synthesis. The accumulation of reducing sugars at whose expense phenols are synthesized also accounts for the low phenolic content of the inoculated leaves of TN1.

Gel consistency and alkali spreading values were not affected by bacterial infiltration in the susceptible or resistant lines. Resistance or susceptibility of the three lines of rice under study to *Xoo* was found to have no relationship with either total, reducing, non-reducing sugars, starch or amylose content of the grain.

Constitutive levels of crude protein was higher in susceptible variety TN1. There was a significant decrease in the crude protein content after inoculation in all the three lines but per cent reduction was higher in the susceptible line.

Per cent brown rice and milled rice recovery did not differ in susceptible and resistant lines both before and after inoculation. But head rice recovery decreased significantly in all the three lines after inoculation with the maximum reduction in the variety TN1 (16%).

Length, breadth and shape of the grain was not affected after inoculation, in all the three lines, while 1000 grain weight of paddy, brown rice and milled rice decreased significantly in the susceptible line TN1.

Per cent germination decreased significantly in all the three

lines after treatment with the bacterial suspension of *Xanthomonas oryzae* pv. *oryzae*, with maximum reduction in the susceptible variety TN1. Root length and shoot length did not vary significantly in the three lines after treatment with bacterial suspension of *X. oryzae*.

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