CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Identification of Bacterial Leaf Blight Resistance Genes in Diverse Local Rice (*Oryza sativa*) Germplasm

by

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A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2025

I would like to dedicate this thesis to Allah Almighty, my parents, my husband, my brothers, sisters, family, friends and teachers

CERTIFICATE OF APPROVAL

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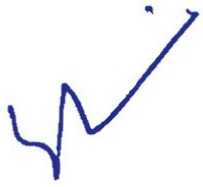

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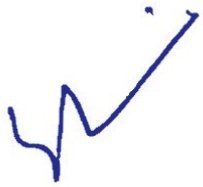
Acknowledgement

Alhamdulillah! I'm extremely thankful to Almighty Allah the most merciful and most magnificent. I offer my gratitude to Holy Prophet (PBUH) who preached us to seek knowledge for the betterment of mankind. I would like to express my gratitude to my family including my father Abdul Rauf, my mother Razia Begum, my husband Muhammad Munir, my brother Rahat Mehmood and Shafqat Mehmood, my sisters, all family and friends. I'm thankful to my supervisor Dr. Sohail Ahmed Jan for continuous support throughout my work. A lot of people assisted and helped me in this work. I would like to give special thanks to my friend Hira Tasqeen. I would like to acknowledge all of them, and wish good luck ahead. My thanks and appreciation also goes to my friends Zarafshan Kaneez and Zainab Azad.



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Abstract

Rice (*Oryza sativa*) is the most consumable food all over the world. Asia is the top Rice producing continent. Pakistan is tenth Rice producing as well as fourth Rice exporting country. Among many biotic as well as abiotic factors affecting Rice crop yield, Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzae* (*Xoo*) is most serious threat to rice production. Inner genetic resistance of rice plants to BLB is most useful way to overcome the disease being more economical and ecofriendly approach as compared to other approaches. To date no comprehensive study is available on local Rice germplasm. Therefore the current study is conducted to identify and characterize locally collected Rice genotypes for BLB resistance.

Total 23 rice genotypes were collected from KPK and Punjab regions and being subjected to genetic analysis for presence of *Xa5* and *Xa7* resistance genes. Doyle and Doyle method with minor modification was used For DNA extraction. The extracted DNA further subjected to PCR amplification using *Xa5* and *Xa7* linked primers.

The results revealed that *Xa5* and *Xa7* were present in all genotypes except UD-205 and UD-201. UD-205 lacked *Xa5* but had *Xa7* gene whereas UD-201 possessed *Xa5* but lacked *Xa7* gene. Genetic similarity analysis on basis of genes present, divided the local germplasms in three main clusters showing genetic diversity in rice germplasm. Further Dendrogram analysis using NTSYS pc 2.1 software, revealed strong genetic relationship among rice germplasms. For further confirmation, 2D and 3D plot being used. The findings emphasize the importance of incorporating both known and novel resistance genes in breeding efforts. The study highlights the potential of utilizing molecular markers to enhance BLB resistance in Rice. This research provides valuable insights into the genetic diversity of BLB resistance in local rice germplasm and contributes to the development of more resistant and climate-resilient rice cultivars, improving the sustainability of rice production system.

Key Words: Bacterial leaf blight, Pakistan, *Xanthomonas oryzae*, Rice, *Xa5*, *Xa7*, R genes

Contents

List of Figures

List of Tables

Abbreviations

BLS	Bacterial Leaf Streak
BLB	Bacterial Leaf Blight
EPS	Extracellular Polysaccharides
FACE	Free Air Carbon dioxide Enrichment
FATI	Free Air Temperature Increment
FSB	False Smut Balls
LRR-RLK	Leucine-rich repeat receptor-like kinases
MAS	Marker Assisted Breeding
RBSDV	Rice Black Streaked Dwarf Virus
RFS	Rice False Smut
RSNV	Rice Stripe Necrosis Virus
RYMV	Rice Yellow Mottle Virus
SRBSDV	Southern Rice Black-Streaked Dwarf Virus
SWEET	Sugar Will Eventually Be Exported Transporter
TGT	Temperature Gradient Tunnels
Xoo	*Xanthomonas oryzae* (bacteria)

Chapter 1

Introduction

For more than 3.5 billion people all over the world, *(Oryza sativa)* rice serves as a staple food. Asia is ranked as number one in production and consumption of rice. Due to the rapid growth of the human population in the world, about a 70% increase in rice demand is seen. Pakistan gives 1.7% of the production of rice in Asia, becoming the tenth rice-producing country and fourth in exportation [1]. A report by USDA's Rice Outlook in May 2024 reported 527.6 million tons of global rice production, mainly contributed by China, India, and Bangladesh. The most recent report by USDA for September 2024 gives 527.3 million tons, in which China and India remain at the top [2].

Pakistan contributes 2% of global rice production, with an estimated 9.87 million metric tons of production. The most famous premium Basmati Rice is exported mainly to Europe, the Middle East, and Africa. Punjab and Sindh majorly produce rice as they are irrigated by the Indus River. Pakistan is an important competitor in the global rice market due to its high quality and aroma [3]. According to USDA, Pakistan's milled rice production will reach 9 million metric tons with a 64% increase every year, so soon, it will become the second-largest crop for Pakistan. But it demands good planting conditions as well as more profit [4].

A large number of factors like soil, water availability, seed quality, land, weeds, pests, disease-causing microbes, etc., affect the cultivation of the rice crop. Rice production and yield are significantly lost due to diseases leading to food insecurity

in the Asian continent. Loss in rice yield every year mainly occurs because of biotic factors such as weeds, insects, pests, and pathogens. A disease known as bacterial leaf blight is caused in rice by a bacterium named *Xanthomonas oryzae pv. oryzae (Xoo)*. BLB is considered a very important rice disease, as it may affect the rice plant at any stage [5].

About half of the world's population consumes rice. Among all continents, Asia grows 90% of the world's rice. In the exportation of rice, especially Basmati rice, Pakistan ranks fifth. Due to the lack of tolerant genes, rice becomes vulnerable to many stresses, including biotic and abiotic. The most threatening biotic stress to rice crops is a disease called Bacterial Leaf Blight. The agent responsible for BLB is a bacterium named *Xanthomonas oryzae pv. oryzae*. Among all diseases affecting rice, BLB is the most dangerous, especially in lowland and irrigated fields [6].

Xoo is a rod-shaped bacterium causing BLB. In South Asian lowland areas and irrigated fields, BLB is the most severe disease due to repeated cultivation and conducive environmental conditions. Crops defenseless against the disease have been exposed by close spacing and high nitrogen levels with the management of many highly productive and improved varieties. This disease has been a devastating and serious threat to rice production. The reasons for this disease include increased humidity, more rain, and wind, which encourage disease development. At all stages of rice, water-filled yellow-colored stripes along the leaf sides appear. It leads to a loss in productivity that typically ranges about 30% but can be as high as 80% [6].

Rice provides food to over half of the world's population and is an important source of energy for urban and rural people. Rice production is lost due to many fungal, bacterial, and viral diseases. BLB, caused by *Xoo*, is the most harmful disease, affecting entire rice acreages. It causes yield losses of up to 80%, depending on cultivar susceptibility, crop stage, and environmental conditions [7]. In 1884 BLB was first time reported in Japan. There was a belief that acidic soil causes it. In 1909 acidic dew drops of infected rice leaves were used to take large number of bacteria. These dewdrops were then used to infect the healthy leaves. Since then,

it confirmed that it's a bacterial disease. The agent responsible for the disease then isolated and named as *Bacillus oryzae*. The bacteria was again classified as *Pseudomonas oryzae*, then *Xanthomonas oryzae*. The bacterium again was classified in 1978 as *X. campestris pv. oryzae*. The bacteria belong to family *Xanthomonadaceae* in the Gammaproteobacteria [7].

A large number of diseases including bacterial, viral and fungal threatening production of rice along with food security. About 70 diseases are known at present that affect rice. Among these 11 are caused by bacteria. In these bacterial diseases, Bacterial Leaf Blight is the fourth major disease. It is also one of oldest disease in rice. As it has high pathogenicity so it is assumed the main hindrance for achieving sustainable rice production [1].

Xoo that is a gram-negative bacterium is responsible for BLB disease. In Japan 1884 Fukuoka, Kyushu, it was first reported. In Southeast Asia it became wide spread during the 1960s when new high yield producing varieties were introduced. There is 50% loss in yield which is of about 16% of crop yield loss throughout world caused by bacterial blight disease [8]. Conventional control methods like chemical sprays are unable to overcome high spreading of BLB. Inner genetic resistance of rice plant against the disease is most economical and ecofriendly way to control BLB. As resistant varieties are developed, the bacteria also continually evolve itself. So, it becomes necessary to find out more resistant varieties. As compared to chemical control method, deploying resistant varieties is less costly and ecofriendly approach. Today rice cultivars and wild varieties having 35 resistance genes are being identified [6].

Although different type of strategies like chemical or traditional have been tried for BLB control. But they are not considered much due to more time consumption and duration. The most easy and reliable way controlling BLB is development of resistant varieties. This may also have limited impact as resistance in not much long lasting and diminishes over time. It is necessary to explore the germplasm of rice to get complete genes details to find out the variety within different rice lines. To get the details and disease development symptoms, or screening germplasm many artificial techniques are being used [1].

Out of most advanced technology to find out resistant genes for BLB in rice is use of DNA markers. Using this technology up to present day, approximately 34 genes are being identified for resistance to BLB. 23 of them are dominant and 16 are recessive. Now by the use of this advanced technology various rice cultivars are introduced via specific resistant genes against various *Xoo* races. *Xa4, Xa5, Xa7, Xa13* and *Xa21* genes are most used against *Xoo* [1].

Till now, chemical methods are not much effective. To minimize loss of rice production and control BLB a lot of techniques are applied but they did not showed much success because pathogens change their sensitivity and response towards these approaches. So only the method which is effective and ecofriendly is introducing new resistant verities of rice. In rice there are about 46 genes present which are resistant to BLB. *Xa1, Xa7* and others including *Xa21* which is a broad-spectrum resistant gene against six BLB races are reported [9].

There are about 45 resistance genes against BLB being identified. 11 of these genes are cloned and their function is assessed. These include *Xa1, Xa3, Xa26, Xa4, Xa5, Xa13, Xa21, Xa23, Xa25, Xa27* and *Xa41*. On the basis of proteins which these genes encode, they are classified in four groups. SWEET genes, RLK genes, EXECUTOR genes and others. All these genes including cloned as well as un-cloned genes specifically those having multiple resistance, are used extensively is different rice breeding programs. Nowadays wild and local species of rice are thought as containing those genes which are unexplored and more valuable to BLB disease so all researchers are focusing more on those germplasms finding new resistant cultivars [8].

Gene *Xa21* which is a *LRR-RLK* is first resistance gene being cloned. Its origin was *Oryzae longi staminata* which is a wild rice species. *Xa21* is believed to have resistance that is broadspectrumin range to many races of *Xoo.* The resistance conferred by *Xa21* begins from two leaf young stage which becomes maximum at adult stage. If *Xa21* is expressed more, it will confer resistant at all stages to rice plants. A study in China revealed that gene *Xa26* and *Xa3* are same and both produce *Xa3/Xa26* mediated resistance. *Xa4* was introduced in rice commercial varieties in 1970. It encodes cell wall associated kinase. It is involved in race

specific resistance against *Xoo*. In breeding programs, it is extensively used. It makes cell wall of plant stronger to protect from *Xoo* attack. It provides strength to the stem of plant [10].

To identify those cultivars having multiple disease resistance genes, conventional method becomes less effective because of epistasis as well as masking effect of genes.So the accepted and highly usable way for searching resistance genes is molecular method.It is most efficient and easy way for identifying resistance genes to BLB.*Xa5, Xa8, Xa21, Xa13* and *Xa23* are those genes which are considered as the most effective in giving resistance many different type of *Xoo* strains [8].

Xa13, Xa25, and *Xa41* code for clade III SWEET proteins. These three are recessive genes. SWEET proteins have very important role in pollen nutrition, aging, completion of seeds and plant interactions with pathogen. 22 SWEETS gene are found in rice. For specific resistance to race 6 of *Xoo*, *Xa13* gene is involved.

The *Xa13* protein plays contribution in copper distribution that is a significant element in pesticide control. From Minghui 63, three genes *Xa3, Xa26, Xa25* were isolated. These give resistance to PXO339 strain of *Xoo* at embryo and mature stage. PXO339 induces dominant *Xa25*. Then *Xa41* is considered as multi-spectrum resistance gene against 50 % *Xoo* strains from various Asian and African countries [10].

Although 480 genes including *NLR* and nucleotide binding domain are being reported but only *Xa1* was isolated. By identifying many *TAL* effectors, it provides resistance to *Xoo*. *Xa5* a recessive gene commonly occurring in Aus Boro varieties in Bangladesh, is also a broad-spectrum gene in action. *Rxo1* which is a non-host resistant gene, is highly resistive to *Xoo*. Although it has control over other pathogens resistance like Ropogonis. Some other genes like *Xa7* has long lasting resistance and important in different breeding programs of rice.

In DV85, it was first reported present on chromosome 6. One significant feature of *Xa7* is that as compared to other resistance genes, it is most effective at greater temperature. *Xo1*, whose characteristics are not much known, found in American

heirloom rice, only gives resistance to African strains [10].

All these cloned genes, along with some un-cloned genes like *Xa7*, are extensively used in rice programs of breeding, particularly in efforts to pyramid multiple resistance genes for developing BLB rice varieties. However, wild species and collections of germplasm are recognized as valuable reservoirs of essential genes. As a result, investigating germplasm collections to identify resistant cultivars has become a key research priority [8].

1.1 Problem Statement

Biotic stress including *Xoo* negatively affects both morpho biochemical and molecular processes of economically important rice genotypes by causing BLB of rice. It negatively effects both its qualitative and quantitative traits. As rice is a globally important crop but due to BLB there is much loss in its yield. In Pakistan loss to rice production is a serious matter of concern as it is a staple food for most people.

1.2 Aim and Objectives

1.2.1 Aim

To identify Bacterial blight resistant diverse local rice genotypes using molecular markers.

1.2.2 Objectives

1. To characterize diverse locally collected Rice genotypes for Bacterial Leaf Blight resistance genes.
2. To identify promising bacterial blight resistant rice genotypes.

1.3 Scope

By employing a combination of traditional screening methods and modern molecular techniques, this study seeks to uncover the genetic mechanisms that enable certain rice varieties to withstand Xoo infection. The ultimate goal is to provide breeders with the tools and knowledge necessary to enhance the bacterial blight resistance of rice cultivars, thereby securing the crop against this devastating disease and supporting the livelihoods of rice farmers worldwide. Identifying resistant genotypes will improve livelihood of local rice farmers as well. Use of resistant genes in host plant gives a more economical and environment friendly way to get control over plant disease and reduce the losses in yield.

Chapter 2

Literature Review

2.1 Global Production of Rice

In Global rice production, China and India come at the top as they contribute more than half of the global rice output. Some other countries such as Bangladesh, Vietnam, Indonesia, Thailand and Philippines also give a significant contribution. As rice is a basic food specifically in Asia so these countries contribute much in global food security. About 10,000 years ago, it was originated in Asia but now it has become basic food source in all over the world. It is a vital energy source which contains high content of Carbohydrates having high nutrition value and versatility. Along with its major contribution to global food security, rice farming is very crucial in economy of many nations [3]. During 2018-19 consumption of rice throughout the world was 486.62 million metric tons. Globally in production of all crops, rice production is at the top. Rice is considered as a basic food and an earning crop. The number of verities which are sowed and sold are only 48. A loss of about 3.3 million was recorded in 2019.This loss was due to two major diseases of rice called brown spot and leaf blast. A pest named as Hispa also contributed to a greater loss in rice crop yield [2].

USDA foreign Agriculture Service reported 1% increase in annual rice production across the world. Asia is at top in production and exportation of rice. 521.52 million metric tons rice production recorded in 2023 and 2024.

The top 10 countries producing rice include China, Bangladesh, India, Pakistan, Japan, Indonesia, Burma, Thailand, Vietnam and Philippines. BLB was first seen in Japan in 1884. Since the disease has become more severe due to high cultivation. The disease affects the plants during tillering and cause 10% to 50% yield losses [3].

Around the world Rice is a well-known crop. After wheat and maize, it is second largest cereal which is grown on 149,000,000 ha. Along with 380000000 tons production, it is ranked as third cereal crop. Only in Asia production is 643 million tons while 24.8 million tons production comes from Africa. There are a lot of biotic as well as abiotic factors which limit rice production. Among biotic factors diseases such as rice yellow mottle, rice blast, and bacterial leaf blight mainly affect rice production. *Xoo* causes two very important rice diseases; BLB and BLS. *Xoo* is causing agent of BLB and *Xoo* causes bacterial leaf streak (BLS). Studies conducted in South and Southeast Asia show that effect of bacterial disease affecting vascular tissues is less. The reason may be selected host resistant plant lines which has decreased yield losses [7].

2.2 Local Significance of Rice

In Pakistan, BLB disease was first reported in Kala Shah Kaku, Rice Research Institute, and nearby fields in 1977, after that the disease was reported in a cultivar of rice IRRI 6, Paalman and Basmati 1998. An increase in the incidence of the BLB has been found in fields of various farmers, viz., 11-16, 16-22 and 21- 26%, in Sindh, Punjab and Khyber Pakhtun Khuwa, respectively. Incidence of BLB increased up to 41-50% in Fakanda-Abad Dhing fields. In some nearby villages fields 71-81% and 90-95% disease spread was recorded. In most areas of the Punjab Mean incidence of disease recorded was 64, 63, 43, 36, 34, 28, 41, 55, 45, 55, and 48% in Hafizabad, Sheikhupura, Sargodha, Gujranwala, Gujrat, Sialkot, Lahore, Qasoor and Okara. In September 1999 in Hafizabad, Sheikhupura, Gujranwala and Gujrat areas, the average occurrence of bacterial leaf blight disease was recorded as 25, 28,15, and 29% [8].

In KPK 0-90% disease incidence rate was recorded in Malakand, 0-100% in Lower Dir and 0-0% in Mansehra. Similarly, severity of the disease measured as 0.2-6 in Sawat, 0.3-7 % in Malakand Agency and 0.4-9% in Lower Dir and Mansehra. In Sindh, mean incidence of BLB was reported as 0.00 % in Dado, 0.01-5.0 % in Shikarpur,5.0 % in Larkana, 1.0-2.0 % in Nawabshah and 0.1-5.0 % in Thatta. As far as severity of the disease is concerned, it was 0.01 % in Larkana, 0.1-1.0 % in Dadu,1.1 % in Nawabshah and 0.1 % in Shikarpur and 1.0 % in Thatta. In Baluchistan 0.0-6.0 % mean disease incidence was recorded. However, BLB was not present in Jammu and Kashmir [8].

2.3 Biotic Stresses Affecting Rice Crop

2.3.1 *Xanthomonas oryzae* Bacteria and its Types

Xoo is from c division of Gram-negative proteo-bacteria. It is causative agent of BLB. It attacks the vascular system of plant and grows there extensively clogging vessels with bacterial cells as well as polysaccharides which are extracellular. There are many races of *Xoo*. Each race has specific effector which it secretes in the xylem vessels, activating individual response which in turn leads to infection. A virulence factors are also released by BLB pathogen. These factors bind as well as activate genes transcription producing resistance response. These genes are called resistance genes [9]. *Xoo* and *Xoc* are the causal agents of BLB and BLS in rice respectively. BLB is one of the most dangerous rice diseases, while BLS is gaining significance as an emerging threat. Rice serves as both a staple food worldwide and a model organism for cereal biology. Both *Xoo* and *Xoc* are crucial from a food security perspective and as models for studying bacterial interactions with plants. Despite their genetic similarity, these pathogens infect rice through distinct mechanisms, and the genetic basis of resistance to BLB and BLS differs accordingly.

Moreover, both pathogens display considerable diversity between isolates, and resistance genes against them exhibit remarkable structural and functional variation.

This article provides a comprehensive review of findings from both applied and fundamental research conducted over the past century on these pathogens and the diseases they cause. It also discusses challenges in enhancing BLB and BLS control, as well as the opportunities these pathogen-host interactions present for advancing knowledge in plant pathology, microbial biology, and innate immunity, including its relevance to animal systems [9].

2.3.2 Discovery of BLB

BLB was first documented in 1884 in Fukuoka Prefecture, Japan, where it was initially thought to result from acidic soil conditions. In 1909, bacterial masses were isolated from the turbid, dew drops of acidic nature on infected leaves of rice, and the disease was successfully produced again by infecting healthy leaves with these drops. This led to the identification of the disease as bacterial in origin, with the pathogen initially classified as *Bacillus oryzae*, later renamed *Pseudomonas oryzae*, and eventually *Xanthomonas oryzae*. In 1978, it was classified again as *X. campestris* pv. *oryzae* [9]. BLS was first seen in the Philippines in 1918 under the name "Bacterial Stripe," though it was mistakenly referred to as bacterial blight for many years. In 1957, research conducted in southern China distinguished BLS as a separate disease from BLB, leading to its formal naming as bacterial leaf streak. The causal pathogen was initially identified as *Xanthomonas oryzicola* but underwent multiple reclassifications, including *X. translucens* f. sp. *oryzae*, *X. translucens* f. sp. *oryzicola*, and *X. campestris* pv. *oryzicola*. In 1990, both BLB and BLS pathogens were formally designated as separate species under their current names: *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola*. These species belong to the family *Xanthomonadaceae* within the class *Gammaproteobacteria* [10].

2.3.3 Morphology and Physiology of *Xanthomonas oryzae*

Xoo belongs to gram negative bacteria having rod shape and round ends. The length of individual cells ranges between 0.7 to 2.0 µm and the width is between

0.4 to 0.7 μm. Each cell has a single polar flagellum for movement (Fig 2.1 and 2.2). When growing on solid media with glucose, the colony appears as convex, round, and mucoid. It shows yellow color that is due to xanthomonadin, pigment. Capsular extracellular polysaccharide (EPS) is produced by the cells. EPS is helpful in dispersal by wind and air as well as prevent from desiccation by forming bacterial strands from infected leaves. *Xoo* usually grows between 25 and 30 °C. It is an obligatory aerobic bacterium. It is weak in producing acids from carbohydrates. It is also not reducer of nitrate. *Xoo* also has catalase positive activity [10].

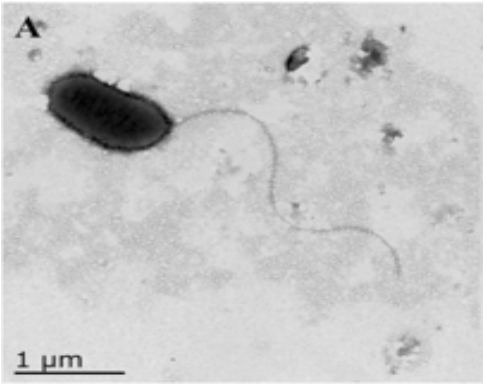

FIGURE 2.1: Microscopic image of *Xanthomonas oryzae* single cell [11].

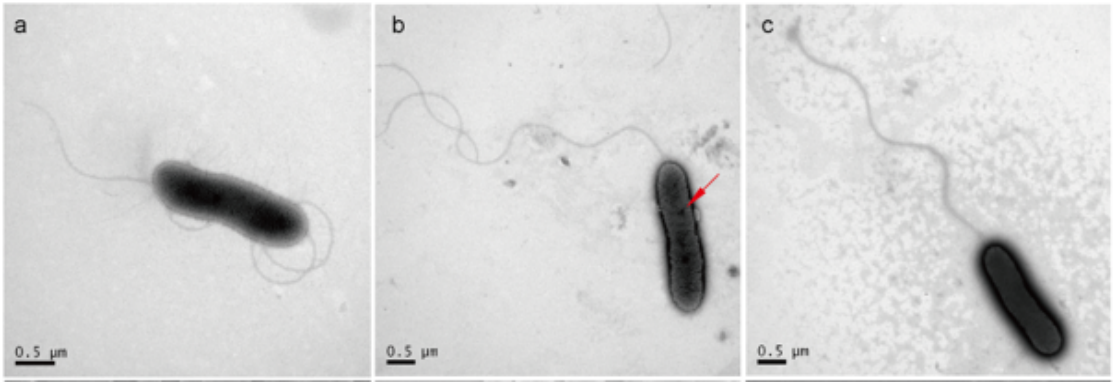

FIGURE 2.2: Cell morphology of *Xoo* observed with TEM [12].

Xoo is an obligate aerobe that does not produce spores, with an optimal growth temperature of 25–30 °C. It is catalase-positive, incapable of reducing nitrate, and exhibits weak acid production from carbohydrates. The two pathovars, *Xoo* and (Xoc), can be differentiated based on several characteristics: (a) acetoin production (negative in *Xoo*, positive in *Xoc*), (b) ability to utilize L-alanine as the sole carbon source (negative in *Xoo*, positive in *Xoc*), (c) growth on 0.2% vitamin-free

casamino acids (negative in *Xoo*, positive in *Xoc*), and (d) resistance to 0.001% $Cu(NO_3)_2$ (positive in *Xoo*, negative in *Xoc*) [10].

2.4 Viral Diseases

Asia is the continent which has most number and occurrence of viral diseases of rice. About 15 viruses are being reported in rice in Asian continent. Before green revolution in Japan, 3 viral diseases of rice were being reported caused by rice stripe virus (RSV), rice dwarf virus (RDV) and rice black streaked dwarf virus (RBSDV). In 1883, the first reported virus in rice was RDV which caused rice dwarf disease. The symptoms in plants being infected by RDV include restricted growth, very short roots, and low-quality grains. Hence rice yield is reduced. The disease occurrence was very high 1889 to 1979 in Japan and in 1969–1973 in China. RSV was also reported first time in Japan in 1897. In 1963, RSV outbreak severely affected rice production in Jiangsu–Zhejiang–Shanghai (JZS) district of China. In 2002-2004 RSV spread in Yangtze River region causing about 40% loss in rice yield in Eastern China [13].

RBSDV as a newly arising pathogen causing dwarf associated disease in rice was reported in 1952 in Japan. Three most important crops; maize, wheat and rice are affected by it. This disease affected mainly China, Japan and Korea. The reason for its occurrence was upgradation of old rice varieties as well as more cultivation of wheat. Another virus rice grassy stunt virus (RGSV) was reported in Philippines. This disease affected more than half of rice crops. At present it is found in China, Japan and Indonesia. In Indonesia from 1974 to 1977, loss of about 3.3 million tons of rice occurred due to viruses [13]. During 1963 in South East Asian countries, a disease of rice named as Rice Tungro Disease which was caused by two viruses called Rice Tungro Spherical Virus (RTSV) and Rice Tungro Bacilliform Virus (RTBV) was observed. About 1.5 billion dollars loss occurred due to this disease. Rice Yellow Stunt Virus (RYSV) was first time reported in Taiwan and Gwangdong in China in 1965. Then Rice Bunchy Stunt Virus (RBSV) was also reported causing disease in rice in China in 1907s. A member of genus

Phyto Reovirus named as Rice Gall Dwarf Virus (RGDV) occurred in Thailand in 1979. Rice Necrosis Mosaic Virus (RNMV) which is transmitted by fungus was seen in Japan and India. It also affected other crops economically important like *Ludwigia perennis* and *Corchorus olitorius* causing over growth in shoots and leaves. These reported viruses caused many outbreaks in rice growing regions [13].

Rice is one of the most important staple foods globally. However, a significant challenge to rice production is the persistent yield losses caused by plant diseases, including rice blast, sheath blight, bacterial blight, and, notably, various vector-borne viral diseases. Since the late 19th century, 19 rice virus species have been documented across rice-growing regions worldwide, each causing varying degrees of damage to rice production. Among these, Southern Rice Black-Streaked Dwarf Virus (SRBSDV) and rice black-streaked dwarf virus (RBSDV) in Asia, Rice Yellow Mottle Virus (RYMV) in Africa, and Rice Stripe Necrosis Virus (RSNV) in America currently pose serious threats to rice yields [14].

Over the past five decades, advancements in rice productivity have significantly helped mitigate food crisis resulting from population growth, environmental stresses, diseases, and pests. However, as rice breeding has progressed, diverse vector-borne viral pathogens have increasingly emerged in many rice-producing regions, endangering both yield and quality. Three primary endemic regions for rice viral diseases are Asia, Africa, and America. Most of these viruses are prevalent in Asia, while only two rice yellow mottle virus (RYMV) in Africa and Rice Hoja Blanca Virus (RHBV) in America have been reported recurrently in their respective regions [14].

2.5 Fungal Diseases

Ustilaginoidea virens is a highly destructive fungal pathogen that affects rice cultivation globally. It is responsible for causing Rice False Smut (RFS), a disease that leads to significant yield losses and deterioration in grain quality. Additionally, the mycotoxins produced by this pathogen pose serious food safety risks, impacting both human and animal health. Recent research has focused on unravelling the

life cycle of *U. virens*, its infection mechanisms, pathogenic genes, genome structure, and associated bio-molecules. *Ustilaginoidea virens Takahashi*, is ascomycete fungus that produce RFS. Once considered a highlighting issue in rice growing regions, RFS has now emerged as a major threat, affecting rice crops in multiple countries. Between 2015 and 2017, the disease impacted Chinese approximately 2.4 Million Hectares , with high prevalence in provinces such as Hunan, Hubei, Jiangsu and Zhejiang. Its incidence is also rising on a global scale [15]. The disease is characterized by the formation of false smut balls (FSB), which initially appear yellowish-orange or green before turning greenish-black (Fig 2.3 and 2.4). Rice false smut was first documented in 1878 in the Tirunelveli district of Tamil Nadu, India, and later reported in North-Eastern Arkansas counties in 1997.

FIGURE 2.3: Rice False Smut disease [16].

The pathogen infects rice flowers, colonizing their inner tissues with mycelia that develop into smut balls covered in powdery chlamydospores. These smut balls, ranging in colour from yellowish-brown to greenish-black, may also form sclerotia when exposed to significant temperature fluctuations between day and night. FSB reduces grain filling, increases sterility in nearby spikelets, and ultimately leads to yield losses.

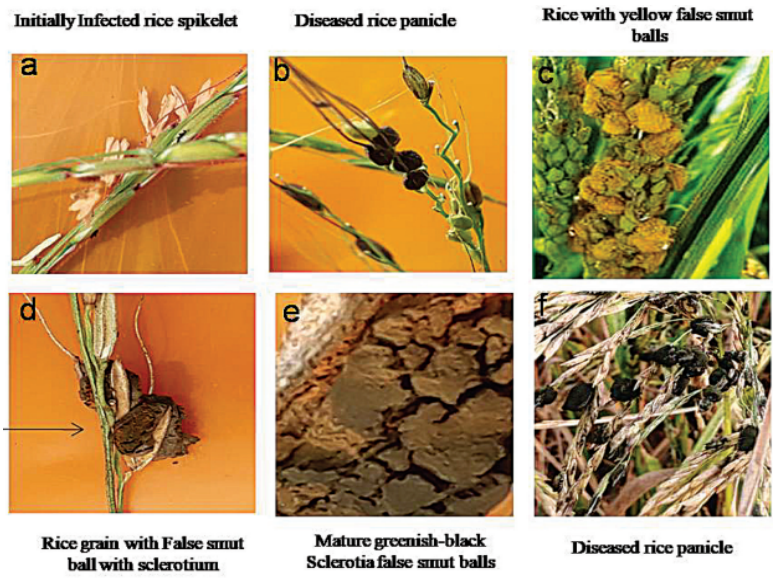

FIGURE 2.4: The disease and symptoms caused by *Ustilaginoidea virens* in rice. (a) Infected rice spikelet with the *U. virens* and false smut ball development, (b) false smut balls produced by *U. virens* in rice panicles, (c) a typical diseased panicles of rice showing yellow colour false smut balls, (d) rice panicle with greenish-black balls of false smut in a rice grains, (e) mature false smut ball with a sclerotium covered with greenish-black chlamydospores, (f) heavy infected paddy field by *U. virens* [17].

Infected grains contain mycotoxins that pose severe health risks to humans and animals. Symptoms typically appear post-flowering, with affected spikelets developing large, orange-to-green smut balls. Given its increasing prevalence and global spread, RFS has become an escalating concern for rice production worldwide [15].

2.6 Other Abiotic Stresses Affecting Rice Crop

Agricultural output across all over the world is severely affected by Global warming and changing climate so the production of many crops including rice is decreasing alarmingly. Many abiotic stresses like heat, drought, submergence, and deficiency of nutrients etc. affect germination, vegetative growth, grain filling, initiation of flowers as well as productivity. These characteristics put in danger many characteristics of plants like growth, grain quality and production. A few abiotic factors promote the development and infection of disease-causing agents, increasing the losses in grain quality and production [10].

Rice is one of the most vital cereal crops all over the world, as a basic food source for half population of world. It nourishes more people than any other crop. About 90% of rice production of world take place in Asia with both irrigated and rainfed rice farming systems being central to food security. Rice farming in flooded conditions is notably good, producing fewer negative environmental impacts than other agricultural ways, such as reduced soil erosion and increased organic matter of soil, despite the release of methane [18]. However, challenges such as land and water scarcity, coupled with increasing demand, have driven rice cultivation beyond traditional monsoon seasons with optimal growing conditions into warmer summer seasons, where high temperatures pose significant constraints. Over the next century, climate change due to human activities is projected to raise surface air temperatures to 1.8 to 4.0°C, along with greater variation. As a result, rice crops will increasingly face hotter growing conditions, both now and in the future [15].

Abiotic stress is described as the destroying effects of nonliving factors of environment on living organisms in a particular ecosystem. It is an unavoidable challenge that significantly impacts plant growth and crop yield, making it a leading factor in global agricultural productivity concerns. Studies have also shown that abiotic stresses are particularly damaging when they occur simultaneously, combining various environmental factors. To cope with these harsh conditions, plants must adapt by developing specialized mechanisms that enable them to survive under stress. Global research efforts have been focused on understanding how rice responds to these stresses, as gaining a deeper understanding of the physiological and biomolecular processes behind stress resilience can aid in the development of more resistant rice varieties. Climate change, however, poses an even greater threat to crop production, potentially jeopardizing the food supply needed to sustain future generations, especially in regions like Asia. Changes in temperature, patterns of rainfall, and the increasing extreme weather phenomena are already affecting agriculture. Yet, there remain significant gaps in our understanding of how these climate changes will affect agricultural systems in both the short and long term, as well as their potential consequences for rural livelihoods, particularly for the most vulnerable populations [15].

2.6.1 Drought Stress

Due to climate change gravity and occurrence of hydrological events is disturbed greatly putting crop production and food security at risk. Latin America, South Asia, South East Asia, and Sub Saharan Africa are mainly affected by drought stress. About 8 million ha of highland area of rice and 34 million ha of lowland rice area in Asian continent more commonly impacted by drought stress. Approximately 27 million ha of rainfed rice area got affected by drought. Due to diminished soil moisture and extreme drought in 2002, more than 65 % of South Asia experienced loss of about 400kg ha^{-1} in rice yield [10].

Drought is a main challenge to production and a leading root of instability in yield in rainfed lowland rice systems. It stands as primary hurdle to rice production in rainy areas, impacting approximately 10 million hectares upland rice and more than 13 million hectares rainfed lowland rice throughout Asian continent [15].

Intense and frequent dry period mainly affect Eastern India region, Thailand, Myanmar and Laos. In India, lowland rice fed by rain is cultivated over about 20 million hectares, accounting for 32.4% country's total rice-growing region. Of this, about 7.3 million hectares of lowland rice are facing drought. Across Asia, about 50% of water resources used for irrigation are allocated to production of rice [19].

Approximately 55% of the rice-growing area of globe is irrigated, giving to 75% of the world's total rice production. By 2025, it is believed that 15–20 million hectares of irrigated land of rice will face some level of water shortage. Tuong and Bouman assumed that by 2025, 2 million hectares of irrigated rice of dry season and 13 million hectares of irrigated rice of wet-season could face "actual water scarcity".

Additionally, the remaining 22 million hectares of irrigated rice of dry-season in South and Southeast Asia may be impacted by "water scarcity economically" Growing rice in flooded fields requires approximately 2,500–3,000 m^3 of water to produce 1 ton of grain, compared to about 1,000 m^3 needed for 1 ton wheat. Seasonal water use in lowland flooded rice systems can range from 660-5,280 mm,

depending on texture of soil and climate conditions [20].

Even modest reductions in water usage through adjustments to current practices can significantly decrease the overall consumption of freshwater in rice farming. Many rainfed areas are already susceptible to drought under present climatic conditions and are anticipated to experience more frequent and intense drought events in the future. The timing of drought whether it occurs during the early, mid, or terminal stages of the crop growth cycle, plays a critical role in determining the extent of yield loss [21]. Due to its semi aquatic basis and the wide range of ecosystems and conditions of growth in which it thrives, rice production systems are heavily dependent on abundant water supplies. This dependence makes rice cultivation more susceptible to water shortage compared to other systems of cropping [22].

2.6.2 Impacts of Submergence (Flood) on Rice

Plant growth and productivity is badly affected by flooding. Due to floods in lowland and deep-water rice areas at about 16 million ha, $600 USD million economic loss per year is observed. Flash floods that occur unpredictably at any stage of rice crop also affects it badly. Flooding causes loss in quality and amount of paddy specifically at reproductive and adult stage. Due to submergence flowering and maturity is delayed, grain yield reduced, biomass of shoot as well as yield reduced overall. Due to flooding, less grain production occurs due to decreased grain count, less grain filling and less grain weight [10]. Rainfed lowland areas and flood affected rice areas in Asia account for approximately 47 million hectares, representing 35% of the rice growing area globally. This figure is assumed to rise significantly in the next years due to rising sea levels and the increased occurrence and flood intensity due to harsh weather events [23].

2.6.3 Salt Stress Effect on Rice

Salt stress is another important factor affecting rice crop badly. About 25–30% of Plants affected by salt stress show late germination of seeds, delayed seed set,

reduced area of leaf, and decreased pollen viability. Less water potential, less nutrient uptake and increased uptake of sodium (Na^+) and chlorine (Cl^-) is observed in salt stressed rice plants. Other phenomenon like peroxidase activity, proline and Anthomycin contents, concentration of chlorophyll, H_2O_2, Calcium, potassium and sodium ions are also affected by salt stress [10].

Salt stress is most important limitation to production of cereal all over the world. 21.5 million hectares are affected only in Asia with 12 million hectares being saline and 9.5 million hectares being alkaline or sodic. Rice is a salt sensitive plant, yet it is the one of the cereals regarded as a desalinization crop because of it can grow under flood conditions also. The water that is standing in fields of rice helps in leaching of salts from the topsoil to lower level that favors following crops to grow. Despite its high sensitivity to salinity, significant different tolerance to salt stress is observed in rice [24].

Stress due to salt is a significant challenge to production of rice, as advanced varieties of rice are much sensitive to salinity. It is problematic in coastal areas of tropics due to effect of sea water and its concerning in few inland areas because of secondary salinization, often due to uncontrolled water and fertilizers use in irrigated lands. Salt-affected lands are extending all over the world substantially. A rough calculation tells that worldwide, 37% irrigated land is affected from waterlogging and salinity, with 20% of this land being badly affected. Additionally, it is estimated that each year, 2–3 million hectares of potential productive agricultural land is lost because of salinization. The extent to which this land is reclaimed and put back into cultivation is unclear. However, there is significant difference in the figures given by many institutions. In It is estimated that in India, salt-affected land range from 7 to 26 million hectares, in which 17% and 60% of the irrigated land is included [15].

Rice is comparatively resistant of salt stress when it is in germination, tillering stage, and in maturity, but during early seedling and reproductive stages it becomes more sensitive. The mechanisms involved in salt tolerance in early seedling stage are known well. Main features include high seedling strength, salt emission at the root level, compartmentalization of ions in older tissues, high tolerance of

tissues, active stomata and antioxidant systems, especially glutathione pathway to oxidative stress tolerance. During development of reproductive tissues, salt resistant genotypes exclude out salts from the flag leaves [25].

Despite these characteristics are normally independent, salt-tolerant landraces are not known that combine with them, and there is much variation in the expression of special traits in cultivars. This suggests the potential for identifying more better donor varieties and useful alleles of genes. Salinity tolerance during the seedling and reproductive stages are not strongly correlated, meaning that combining the contributing traits from both stages is necessary to develop more resilient, salt-tolerant cultivars [26].

2.6.4 High Temperature Effect on Rice

Heat stress greatly affects morphology, physiology and molecular changes in rice. In morphological changes are genotypes which maintain low temperature of spikelet's for increasing fertility. Some plants prefer to flower at start of the day so that effects of high temperature can be avoided [10].

Rice, like other cultivated crops, has different temperature ranges in all its growing season. Deviations from the required temperature for each stage can change physiological activities or cause changed pathways of development. The response of rice to more temperature's changes regarding stage of development, as high temperature tolerance at one stage and low tolerance during other stages.

In the same way tolerance to cold at the first stage has no correlation with tolerance in the flowering stage in highly productive rice varieties. Therefore, the impact of high temperatures at various developmental stages needs to be evaluated in separate in order to assess, identify, and characterize the genetic mechanisms involved in tolerance [27].

The crop growth cycle of rice can be broadly divided into three stages: the vegetative, reproductive, and grain filling or ripening phases. Of these developmental stages, the reproductive stage is the most sensitive to high temperatures [15].

2.6.4.1 Vegetative Stage

Rice can tolerate comparatively high temperatures of about 35°C in Day and 25°C in night However, temperatures above this limit affect plant height, tillers number, and dry matter accumulation. A study conducted in temperature gradient chamber, rice given temperatures 3.6°C and 7.0°C showed 11.2 to 35.6% reduction in photosynthesis. It lead to changes in structures , organization of thylakoid , defective stacking of granum in chloroplast or loss in swelling [27]. Rice plants exposed to heat stress in Free Air Temperature Increment (FATI) chambers and Temperature Gradient Tunnels (TGT) showed significant reductions in growth duration, leaf area index, biomass, yield, and harvest index, along with a substantial increase in spikelet sterility and stability indices [28].

2.6.4.2 Reproductive Stage

It is more sensitive to heat than vegetative stage. Seed set in rice is highly vulnerable even to short periods of high temperatures during anthesis, which are likely to become more frequent with future climate changes. Temperatures exceeding 35°C at anthesis for one hour or more can cause high spikelet sterility in rice. Anthesis/flowering is the most sensitive process during the reproductive stage to high temperatures, followed by micro gametogenesis. As temperature increases, the diameter and length of the pollen tube decrease, resulting in poor pollen grain germination. Low land rice cultivars are more sensitive to heat stress than upland varieties. Therefore, the male reproductive organ plays a key role in spikelet sterility under high temperatures and has been targeted for efforts to increase tolerance to warmer climates. Heat stress during anthesis has an irreversible effect, reducing panicle dry weight even if environmental conditions improve afterward [15].

2.6.4.3 Ripening Stage

High temperatures in the stage of grain filling not only lessons yield of grain but affect the rice grains quality. This is because of bad impact of high temperatures

on cellular as well as developmental processes in the ripening stage. Common effects of heat stress during the grain filling stage include grain weight reduction, decreased grain filling, more of white chalky and milky white rice percentage, and a reduced grain size and amylase content. Additionally, after anthesis temperature stress decreases test weight, accumulation of dry matter, and grain filling, resulting yield reduction [29].

2.6.4.4 High Temperature at Night

Peng et al. analysed weather data from the IRRI farm between 1979 and 2003 to examine temperature trends and their relationship with rice yields. They found that grain yield declined by 10% for every 1°C increase in minimum temperature during the dry season, while the effect of maximum temperature was insignificant. Similarly, Pathak et al. estimated that the rate of change in the potential yield trend of rice from 1985 to 2000 ranged from -0.12 to 0.05 Mg ha-1yr-1. The decline in rice yield was attributed to decreased radiation and increased minimum temperatures. Although high temperatures during the day and night shortened the grain growth duration, the growth rate was slower during the early or middle stages of grain filling. High night temperatures (22/34°C) also reduced cell size in the central and surface areas of the endosperm compared to high day temperatures (34/22°C). However, the effects of high night temperatures are not yet fully understood and should be prioritized for research, especially given the predicted increase in mean night temperatures [30].

2.6.5 CO_2 Impact on Rice

In pre industrial era levels of CO_2 reached up to 400 ppm. It is estimated that, atmospheric CO_2 levels will reach up to 550 ppm in 2050.This might have effects on biochemistry, physiology and morphology of rice. For studying the effects of eCO_2 on rice, a meta-analysis of 125 studies revealed that hybrid cultivars respond more with much biomass and yield than indica and japonica types. eCO_2 favors more root biomass than shoot biomass. An experiment of 3 years in a free-air

CO_2 enrichment (FACE) facility showed decreased amount of milled, head and brown rice at 200ppm of eCO_2 compared to atmospheric CO2. Incresed CO2 levels also impacts quality as well as nutritional value of rice [10].

Increase in concentration of atmospheric CO_2 boost production of rice biomass, but it may negatively affect grain yield if accompanied by higher air temperatures, as projected under climate change. An Increase of 75-ppm in atmospheric CO_2 is assumed to enhance rice yield by 0.5 tons ha-1On the other hand, increasing average air temperature 1°C in the growing season is estimated to lessen rice yield by 0.6 tons ha-1. As rice is a C3 crop, experiencing less photosynthetic efficiency because photorespiration occurs in hot temperatures [31].

Higher CO_2 levels can also reduce transpiration cooling and increase maintenance respiration when night air temperatures exceed 21°C. The rice response to increased CO_2 levels is affected by availability of Nitrogen; More CO_2 levels with less nitrogen and a decrease of sinks for extra carbon can stop capacity of photosynthesis and growth.

Additionally, crop canopy increases and more biomass due to more CO_2 concentrations can create a larger host size for pathogen populations. This increased biomass can also lead to greater amounts of crop remains, which can favour survival of pathogens and more inoculum levels for other crops and nearby fields [32].

Changes in the host physiology and anatomy is related to the impact of higher concentrations of atmospheric CO_2 on plant diseases. Alterations in leaf wax and in epidermal and mesophyll cells, higher plant content fiber, increased carbohydrate concentration in leaves and reduced concentrations of nutrients are the changes included for contribution of disease. Reduced silicon content in affected rice varieties is related to increased severity of sheath blight and leaf blast under elevated CO_2 levels [33].

Changes in rice leaf such as epidermal thickness, increased leaf wax increase the susceptibility to pathogens, while influencing pathogen virulence changes, pathogen fecundity, abundance, distribution and activity which is enhanced. Rice

blast produces great number of spores in high humidity environments, that disperse through wind which serve as inoculum for newly infections. Disease diversity can be reduced by decreased humidity and lack of rainfalls. Rice blast produces great number of spores in high humidity environments, that disperse through wind which serve as inoculum for newly infections. Disease diversity can be reduced by decreased humidity and lack of rainfalls. Openings to pathogens for penetration are caused by rice injury which are the result of strong winds that blow particle. Transpiration is stimulated by strong winds which promote silicification of leaf tissues and host's resistance reactions are strengthened [34].

2.6.6 Impact of Soil Nutrient Deficit on Rice

Most of world depends on rice as a major source of nutrition. Nitrogen has positive effects on nutritional quality of rice. As more Nitrogen application increase content of proteins but many qualities are negatively impacted by it like low quality of grinding, amylose content, floor viscosity, cooking and eating quality etc. Nutrient deficiency specifically affects the rice crop at time of seedling emergence, start of panicles, harvesting, preparing and maturity [10].

2.6.7 Climate Change Impact on Rice Production

2004 was declared as international year of rice by UN General Assembly on 16 December 2002. The key goal of the United Nations is to reduce the poverty and hunger. But the rate of rice yield improvement has slowed over time. During the first decade of the current century rice yield growth has declined 1.0% and 2.3% per year during the 1970s and it dropped to 1.5% in the 1990s [35]. However, quantitative analyses of change in climate effects on pathogens are low in field studies, research in labs, and modelling assessments. Therefore, controlling the impact of biotic stresses on rice is necessary for increasing and providing stability to rice yields. Fungal diseases alone are assumed to reduce global rice production by 14% each year. The increased frequency and intensity of weather phenomena, along with rising air temperatures and CO_2 atmospheric concs, are projected to

spread rice diseases into new area.

Rice is cultivated in diverse agro-climatic settings globally, and its yield is impacted by biotic stresses. Climate change can worsen these stresses, which may compromise resistance of varieties to rice blast. Climate changes could impact the distribution of pathogens, rates of development and tolerance, metabolism and growth. Climatic factors as precipitation, temperature, and dew drops influence every stage of the cycle of blast disease of rice, from germination of spore to lesion developing. These factors probably may affect distribution of pathogens due to difference in preventive measures. Climate change will have important impact on strategies to manage that depend on host resistance [36].

It is essential to adopt strategies that can reduce the bad effects of climate change on rice yield. Grasping climate change effects on precipitation, air temperature, and adjustments in sea level can aid in modifying crop management practices to improve rice yields. The impacts of chemical control measures can be majorly influenced by variations in the time, intensity level , and occurrence of rainfall. Rainfall after fungicide application can enhance coverage on foliage, but if the rainfall is excessive or intense, it can wash away the fungicide and diminish its effectiveness. Extended rainy and overcast conditions reduce rice growth and its defenses against rice blast. Extended rainy periods, lack of sunlight, and dew create conditions conducive to conidia release, thereby promoting rice blast epidemics. Conidia dispersion is most affected by rainfall at the onset of the rainy season and during intense rain events [24].

2.6.8 Warm Air Temperature Effects on Rice

According to IPCC, the average global annual temperature of air is estimated to increase by 1.8 to 5.8 °C from 1990 to 2100, which would be a significant threat to productivity of rice and food security. The maximum daily optimum air temperature for rice grain yield during the growing season is between 23 and 26 °C. Temperatures more than 33 °C impact anther dehiscence, viability of pollens, fertility of spikelets, and accumulation of dry matter in grains negatively. A 2 °C

increase in average temperature of air during the grain-filling phase could reduce yield of rice by 15% to 17%. Additionally, increased air temperatures due to change in climate are expected to enhance the growth and rice blast pathogen sporulation. Rice cultivation temperature plays a crucial role in determining its susception to blast disease [37].

In subtropical cold regions, air temperature increase is believed to intensify the intensity of rice blast due to a higher infection risk. Leaf dampness for long time, high humidity, and temperatures ranges between 17 and 28 °C promote rice blast development. Conversely, less humidity or dew enhances the infection process. When night temperatures exceed 20 °C, spore liberation is reduced, and infection is less likely. However, rapid lesion growth is favoured by changing daily temperatures Less air temperatures ranging from 16 to 24 °C can sustain sporulation capacity in lesions. Nevertheless, the higher temperatures projected by climate change may decrease the rice blast incidence in most rice-growing areas. Further modelling in detail and monitoring of climate, considering additional factors that affect rice blast, would aid in more effective disease management [34].

2.7 Symptoms of BLB

The symptoms on BLB include small lesions of yellow color on margins of leaves, progressing over hydathodes and stomata. In start the lesions have pale green, changing to yellowish white, at last whole leaf is covered. When tillering stage is reached, these yellow lesions cause necrosis and death of leaf. Mainly i t affects vascular tissues and may affect at any stage either vegetative phase, seedling or reproductive stage. The wholeentire leaf becomes yellow and dry out at the end. Above 70% of humidity and 25 to 4degree temperature is most favorable for BLB development. At affected parts pale amber color dots appear. Kresek phase is most destroying one in tropical rice crops. It causes infection from nursery stage or in seed. If it infects at tillering stage, 20 to 40 % yield is lost and yield loss is 50% if it occurs at initial stage [38].

FIGURE 2.5: Symptoms of BLB in Rice [39].

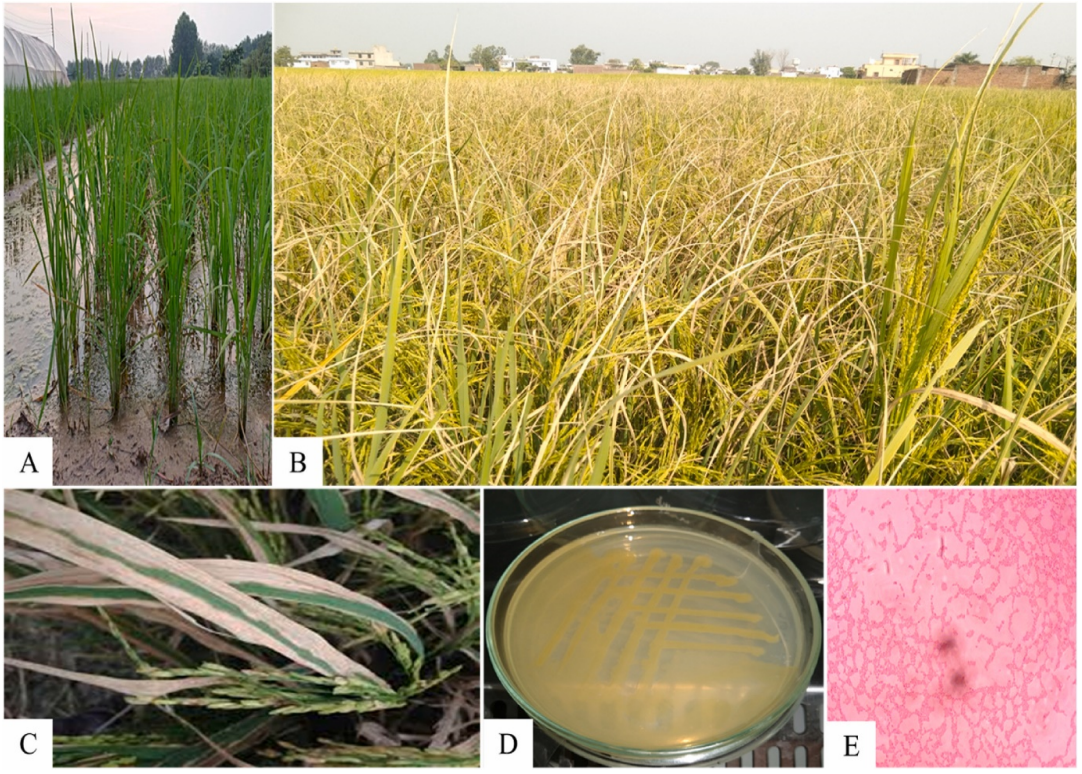

FIGURE 2.6: (A) Healthy Crop of rice (B), Infected Crop with BLB (C), Symptoms of BLB, (D) Culture of *Xanthomonas oryzae pv. oryzae*, (E) *Xanthomonas oryzae pv. oryzae*'s microscopic view [40].

Hydathodes or wounds are entry points for *Xoo*, then it moves to intercellular spaces and finally reach to xylem vessels. The pathogen crosses the veins and propagate further. Spots filled with water are seen on tips of leaves. Chlorosis and necrosis occur in leaf veins [41].

An important staple food crop is rice. Rice paddy had a global production of 496 million tons and 746.9 million tons. Under different environmental conditions the crop is widely spread all over the world. Rice is considered a strategic crop by food

and agriculture organization in the world. Especially in different areas of Asia it is used as staple food. Rice is the second crop after wheat which is consumed exported and cultivated. Super Basmati rice variety among various cultivated varieties is the most wanted variety because of its aroma and grain high quality it is popular among exporters, traders and farmers. But the major problem is susceptibility to different diseases of this variety. Among this bacterial leaf blight is more common which is caused by *Xoo*, this causes severe crop damages [42].

This disease is broadly predominant amongst various rice diversities globally. Traditionally, BLB was reported in Japan and then stated in other rice growing countries. Rice crop was badly affected by this disease in tropical areas and Asia with heavy rainfall in monsoon. Majid et al reported the occurrence of BLB in Pakistan initially. Basmati varieties of rice were later indicated with alarming levels in BLB occurrence in country growing areas. Different growth stages of rice are reported with BLB which is manifested later by acute wilting of young plants known as leaf blight. *Xoo* invades plants via water pores or wounds [42].

Water pores are found at the tips of leaf. *Xoo* enters the plant in water pores or wounds. Since the water pores are found near the edges of the higher portions of the leaf, lesions with wavy margins begin at the leaf tip. The plant eventually dies as a result of these water- soaked lesions growing larger and turning yellow. Many disease management techniques have been used in the past to lower output losses and prevent disease outbreaks, but the use of chemicals has not proven effective since different pathogenic races have varying levels of susceptibility to applied chemicals. An important cereal crop that is grown in many nations worldwide is rice (*Oryza sativa* L.). According to Anon (2010), it produced 89.3MT in 2009–10 and occupies 44.62m hectares in India. Eleven of the 70 recognized crop diseases are caused by bacteria. Seedling, foliar, leaf sheath, grain, culm, and root diseases are the categories into which these illnesses fall. When environmental and cultural factors encourage the development of bacterial infections, the most significant ones, including BLB and BLS, can completely destroy a crop [42].

One of the most destructive rice diseases in the world, bacterial blight can be found in both tropical and temperate climates. In 1884, it was first noted in Japan. With

the introduction of high-yielding cultivars like TNI and IR8, which were sensitive to the disease, bacterial blight spread to other rice-growing regions of Asia in the 1960s. The illness is found in the United States, Australia, Africa, Latin America, and the Caribbean in addition to Asia. Economically, it has affected West Africa, especially Niger, where irrigated rice was severely destroyed in 1982, and Asia, where multiple epidemics have occurred in the last three decades. Under ideal circumstances, vulnerable cultivars may experience crop losses of almost 70% [42].

Economic Significance In some nations, the illness is to blame for a 20–30% loss. Depending on the stage and intensity of the infection as well as the kind of cultivars, yield losses in India ranged from 6 to 60% in various states. In Kerala Palghat area, the illness spread like wildfire and caused a significant crop fatality rate. Reddy et al. (1979) discovered a linear correlation between grain yield and disease severity and created a critical point model to forecast disease-related crop losses [42].

The three primary signs of bacterial blight are yellow or pale-yellow leaves, wilt or Kresek, and leaf blight. The most prevalent condition, leaf blight, typically manifests itself starting at the maximum tillering stage. On the leaf blades, it first appears as water-soaked stripes. The stripes get wider and longer, turn yellow, then turn white, and sometimes they merge together to cover the whole leaf blade. On immature lesions, drops of bacterial exudates may be seen. Saprophytic fungal development may cause older diseased leaves to appear gray. Severe infections can also cause small, round lesions with water-soaked edges to develop on the glumes. Grain from infected plants is of low quality, lighter, and produced in smaller quantities. The most damaging is the Kresek or wilt syndromes [43].

The wilt syndrome, known as 'Kresek,' is the most destructive form of the disease and is typically observed within temperatures ranging from 28°C to 34°C. This condition is prevalent in tropical regions and affects rice plants from the seedling stage to early tillering. Infected plants exhibit wilted leaves that curl up and turn greyish-green. As the disease progresses, the leaves turn yellow or straw-coloured and wither, often leading to the death of the entire plant. Surviving plants are usually stunted and have a yellowish appearance. Kresek can result in total crop

failure in some cases. In tropical areas, the yellow leaf or pale-yellow syndrome is commonly linked to bacterial blight. In this condition, the youngest leaf of the plant becomes uniformly pale yellow or develops a broad yellow stripe. While the bacteria are not present in the leaf itself, they can be found in the internodes and crowns of the affected stems [43].

2.8 Development of BLB

In pathogenic races improvement in mutation is a first-rate problem in growing persistent control. furthermore, because of poisonous remains, antibiotics use and chemical substances to rice BLB, has barriers. The usage of resistance genes to host appears practicle, single gene (*Xa4*) primarily based breeding for BLB management has been proven to be not much effective due to evolution of new populations that conquer those resistance genes.

As end result, organic control seems to be a value effective and environmentally friendly manner to control this extreme risk. Maximum of the Rhizospheric antagonistic microorganism which includes *Pseudomonas* can indirectly boom plant resistance through enhancing the plant increase. Responses of the host plant are because of root colonization of a plant by means of opposed rhizobacteria that play an important position in sickness suppression [44].

Lysobacter antibiotics had been documented as biocontrol sellers in opposition to *Xoo* because of their speedy growth, easy utility and powerful leaf colonization. Plant boom promoting Bacillus spp. have been located to suppress BLB in rice underneath greenhouse situations. Li et al (2011) pronounced *Streptomyces globisporus* for the suppression of rice blast caused by *Magnaporthe oryzae*. The prevalence of sheath blight was decreased through a few biofilms forming and surfactant producing lines of *Bacillus subtilis* [44].

Streptomyces phliantid and a industrial components of *B. subtilis* have been located to be biologically energetic in opposition to rice sheath blight whilst incorporated with chemical fungicide. Hydrogen cyanide (HCN) producing *Pseudomonas*

chlororaphis substantially inhibited the boom of *M. oryzae*, displaying its biocontrol residences in opposition to the causal agent of rice blight. those antagonistic microorganisms can at once suppress plant pathogens by using generating antibiotics, enzymes like chitinases, glucanases, proteases, and siderophores or indirect mechanisms wherein the adverse microorganism compete with the pathogen for a gap or nutrient websites. those bacteria have been said to reduce the sickness prevalence drastically beneath managed in addition to below herbal discipline situations [44].

There are many approaches including chemical, cultural and biological used for BLB control. These are based on disinfection of seeds or using antibiotics. In aqueous solution of Ceresin and Agaricine, seeds are soaked for 12 hours, then soaked in hot water for 30 minutes at 53°C.But these methods are not good for environment. Most effective way to control BLB is identifying disease resistant varieties. It can help to reduce yield losses due to BLB in rice [45].

BLB is found in Asia, Africa, USA and Australia affecting rice. *Xoo* enters the circulatory system via wounds present in leaves and roots. Usually, it spreads in irrigation water or in flood. Sometimes it may be in rain or in wind. This bacteria mostly get in to plant via open wounds or small pores and its proteins get inside. These proteins are transcription activator like effectors. They start leaf blight phase producing lesions. As the bacteria becomes successful to metastasize in leaves, saprophytic growth is exhibited by lesions, hence grain sterility and quality is affected. Other symptoms of Kresek phase like drooping, deterioration and appearance of grey color in leaves may occur. Plants natural system response to this by producing phenolic compounds [46].

The entry points for *Xoo* in rice plant are water pores or wounds. At edges of leaves water pores are found. Lesions appear on margins of leaves at top upper side. Appearing in watery form then changing to yellow whitish color, they expand in form of square and create circular lesions. These lesions are mixed with unaffected areas of leaves giving the disease specific sign of wavy margins clearly visible on blade of leaf. Mostly one or two margins show the lesions which become visible newly affected veins under humidity. In developing the disease and symptoms

environment has more significant role. There are two phases of disease called leaf blight phase and Kresek phase [1].

2.8.1 Leaf Blight Phase

At very start of the disease this phase develops usually in temperate regions of rice growing countries. It starts at lower parts of plants, then slowly moves to the upper parts. When tillering is at its maximum, symptoms are seen on leaf blades. The vulnerable genotypes display more severe symptoms when extreme nitrogen fertilization environment is provided. When attack is more severe, lines of yellow white color changing to pale yellow and necrotic are visible inside of margins of blades [1].

2.8.2 Kresek Phase

Kresek word is taken from java term which means "dead leaves sound". It was first seen in Indonesia assumed to be a different rice disease due to its symptoms. After many studies the agent causing disease was identified. Pervasive infection is main characteristics of Kresek phase. When plants transplanted in nursery, it takes 1to 2 weeks for symptoms to appear. Rarely it may be seen in mature plants. The leaves colour changes from greyish green to white and withering of leaves occur. Usually, symptoms are seen on leaves but sometimes stem rotting may occur [1].

2.9 Resistance Genes for BLB in Rice

Different genes for *Xoo* resistance are being identified and arranged in a series from *Xa1* to *X45*.Nine of them are recessive. Eleven genes encode different proteins involved in different resistance mechanisms. A few of them are entered in to commercial rice varieties in Asia. *Xa21, Xa7* which are dominant as well as *Xa5, Xa13* being recessive have broad spectrum resistance against many *Xoo* races. So majorly used in breeding programs in Asia [47].

As every race of *Xoo* have changed virulence and a virulence factors, so are the genes resistant to special *Xoo* race. For determining the interaction of *Xoo* with plants pathogenicity genes and hypersensitive response is used. For identifying genes against pathogenesis of *Xoo* and determining their role in disease causing many attempts are made. The knowledge of genes important in pathogenesis have become clearer via genome sequence of *Xoo* strains like PXO99A, KACC10331and MAFF311018 [38].

There is a single locus in plants that gives resistance to complimentary a virulence. Plant pathogen interactions genetic analysis have shown it. BLB resistance inheritance in rice has been studied in many countries like Sri Lanka, Japan, IRRI, India and China. It has been too difficult to tell the characteristics as well as the differentiation of resistance genes because there is too much diversity in *Xoo* strains in different countries. In rice, at present 42 genes for BLB resistance are reported in rice. Mutant populations, wild rice and cultivated rice species are used to identify genes giving resistance to *Xoo* strains [38].

There are 14 recessive genes from 42 genes and 9 are being cloned and characterized. Every gene coding different type of protein has a unique mechanism for *Xoo* resistance. Most of these genes have race specific resistance to pathogens. Each gene has been introduced either as single or in combination with others. Evolution is obvious in bacterial races because of different types of selection of these genes. So, it is necessary to discover and identify new genes which can provide resistance and protect against evolving races of *Xoo* [38].

For plants disease control and losses reduction, gene conferred host plant resistance is one of the best, economical and environment friendly way. In last 20 years, a lot of studies done for more resistance to BLB. The genes for resistance is 40 to different strains of *Xoo*. 11 are being classified as *Xa1, Xa3, Xa26, Xa4, Xa5, Xa10, Xa13, Xa21, Xa23, Xa25, Xa27* and *Xa41*. Resistance genes have been classified in to four groups named as (SWEET) genes (*Xa13, Xa25, and Xa41*), (RLK) genes (*Xa21*, *Xa3/Xa26* and *Xa4*), executor genes (*Xa10*, *Xa23* and *Xa27*) and other types of genes (*Xa1* and *Xa5*). To control BLB in Asia, *Xa3*, *Xa26* and *Xa4* have been considered most important genes for resistance [41].

Due to broad spectrum resistance in rice to many *Xoo* strains *Xa21* which has locus on chromosomes 11 with origin of *Oryza longi staminate* has attracted most of the breeders. Being dominant, it gives more stable and long-lasting resistance to *Xoo* in Vietnam and India. In India it is considered most important gene for resistance to 88% zoo strains. *Xa21* gene gives resistance not only at adult stage but also at juvenile stage. *Xa21* has resistance for 6 *Xoo* races in China. It is most extensively used gene in breeding programs. So, it can be said that *Xa21* is the most effective gene [47].

Xa5 is a recessive gene which is present on chromosome 5. It was first observed in DZ192. It is formed by a natural mutation in a sensitive allele. There is a difference of a single amino acid changing valine to glutamine. This occurs due to two nucleotide substitution. Actually, it is a helpful change giving resistance to multiple *Xoo* strains. It is one of those genes that are used extensively in Asia in breeding programs. It offers resistance to many races of as well [47].

Due to global warming, at present, although a lot of genes identified but they do not confer permanent resistance. Among the factors responsible for reduced tolerance in rice varieties to *Xoo*, high temperature is most important one. It is mainly important in those varieties which have *Xa4* gene. Those lines which have *Xa7* gene show more resistance at high temperatures like IRBB7 line. It means that *Xa7* gene has more durable resistance and broad-spectrum response to various *Xoo* strains. It will be helpful to use this gene in rice crop for improving rice production [47].

Among most of resistance genes reported, *Xa13* confers resistance against most pathogenic races of *Xoo* so it is more commonly used in Asian rice programs. By introducing these types of genes in rice will help to get rid of vascular diseases. Furthermore, if more resistant genes of this kind are identified, more cultivars of rice which are resistant to *Xoo* can be developed [47]. Arif et al conducted a molecular survey for identification of *Xa4* a BLB resistant gene in rice lines. They used PCR with specific primers for *Xa4* gene. From 100 germplasm lines, 49 lines showed *Xa4* gene. Out of nineteen basmati breeding lines named as (KSK1, KSK4, KSK6, KSK7, KSK8, KSK12 and KSK16), taken from Rice Research Station

Kala Shah Kaku (KSK RRI), 7 lines have showed *Xa4* gene They also surveyed Pakistani released Basmati varieties. Among eight varieties in Basmati breeding lines of Pakistan, Basmati 385, Basmati 198, Shaheen Basmati and Basmati 2000, *Xa4* gene was found to be present [48]. Another survey was being conducted by Muhammad et al for identification of *Xa13* in some varieties and advanced lines. Specific primers were used with PCR for presence of *Xa13* resistance genes for screening 6 varieties and 52 rice lines. Out of all these, only four varieties and 23 advanced lines contained *Xa13* gene [49].

Allah Ditta et al conducted research by transferring three BLB resistance genes *Xa4, Xa5, Xa21* from a coarse yet BLB resistant variety, IRBB57, Super Basmati, through marker-assisted breeding. Several super basmati lines were developed with different BLB resistance gene combinations. Further studies conducted for degree of tolerance to BLB at two different locations: Pakistan and Philippines. Several Super Basmati Introgressed (SBIL) lines with combination of *Xa5+ Xa21* and *Xa4 + Xa5+ Xa21* conferred broad-scale resistance against both the highly virulent Pakistani and Philippines *Xoo* strains. Therefore, in addition to sustaining Basmati rice yield against BLB, the SBILs developed in this study may represent a useful resource for transferring resistance to BLB-susceptible rice varieties [50].

Arif et al introduced three BLB resistant genes *Xa5*, *Xa13* and *Xa21* in to populations. These genes were very important against BLB in Bangladesh. A variety of rice named as IRRI 154 was crossed with IRBB66 which is resistant to BLB to create such populations. For BLB infection fifteen virulent bacterial isolates were being used. The resulting combinations from F5 and BC2F4 generations were resistant. By using gene specific primers on F5 and BC2F4 ,60 recombinant lines were found having BLB resistance genes. RILs showed broad spectrum tolerance to BLB [51]. Abbasi et al identified *Xa5*, BLB resistance gene by using molecular and conventional approaches. PCR along with specific primers to *Xa5* gene were being used in this research. There were total 60 rice lines used. Out of these 60 lines,31 had *Xa5* gene and other 29 did not have *Xa5* gene.

Along with this a survey was conducted on ten Pakistani Basmati varieties. *Xa5* gene was found present in Basmati-622, Basmati Pak, Kashmir Basmati and

Shahley Basmati. The others including Basmati-385, Basmati-2000, Basmati-370, Basmati-198, Super Basmati and Dokri Basmati had no *Xa5* gene. The narrow genetic base in cultivated rice is reason of vulnerability to BLB as the pathotypes with more virulence evolve with greater frequency and affect rice. The only way to have rice varieties with greater resistance is either discovering new genes which have broad spectrum resistance or pyramiding already known genes [52].

Chimene et al conducted a study for identifying Beninese *Xoo* strains which cause BLB and find resistance genes in Benin rice by screening these. For this purpose leaves of diseased plants having specific symptoms of BLB were collected from different rice fields in three areas of Benin. For sequence specific identification of *Xoo* PCR was used. Seventy-five rice samples were taken for finding resistance genes including *Xa5, Xa7, Xa13 and Xa21.* Results showed that *Xanthomonas oryzae* was found in two fields of Banikouara and one was in Melanville. Out of seventy five rice accession, forty seven showed *Xoo* resistance genes, .4% showed *Xa5* and 40% showed *Xa21* [53].

A set of *Xa21* gene derived lines against BLB pathotypes was generated and evaluated in Bangladesh for understanding resistance mechanisms. It was found that Line Stage Testing population which carried *Xa21* gene showed significant increased resistance to many BLB strains. So it is important potential gene for enhanced resistance and genetic manipulation in rice varieties. Based on this finding Bangladesh Rice Research Institute (BRRI) produced BLB resistant variety BRRI dhan101. Those lines having *Xa21* gene gave higher yield performance to even aggressive BLB strains. It would be helpful for deploying disease resistance genes in hybrid rice varieties for sustaining production of rice even in presence of bacterial disease threat [54].

A study by Shahzad et al was done for *Xa5* gene presence in Pakistani rice including Basmati varities. As *Xa5* is very significant recessive gene against BLB. The whole procedure involved collection of seeds from various institutes and sowing of seeds in pots at NIBGE. Then DNA extraction and use of DNA markers.45 lines including MB 2, MB 33 MB 57 and MB 66, out of 88 germplasm lines had *Xa5* gene. Shahzad et al also surveyed 10 Basmati varieties in Pakistan to find *Xa5.*

Xa5 gene was absent in all these Basmati varieties [54].

Wang et al genotyped five resistant genes named as *Xa4*, *Xa7*, *Xa21*, *Xa23*, and *Xa27* with specific markers in seventy cultivars in Guangdong Province of South China. They found that *Xa4* gene was present in 61 varieties, three varieties had *Xa27* gene while no variety showed *Xa7, Xa21* or *Xa23*. 33 varieties showed resistance in *Xoo* strains. They suggested that *Xa4* was not much suitable in rice breeding. Similarly, *Xa23* was considered more effective against pathotype 9 strains. They breeded two novel varieties of rice with two thermos and photoperiod sensitive genic male sterility lines along with *Xa23* resistance gene using markers and phenotypic selection. All resulting lines had increased resistance to BLB with much yield [55].

Another research was being carried in China by Chen et al for identifying and mapping *Xoo* resistant gene in mutant line H120 as it was resistant to all Chinese *Xoo* races. They mapped *Xa46*(t) in between RM26984 and RM26984 on chromosome 11. The results indicated *Xa46*(t) gene is not identical to *Xa23* [56]. Lu et al isolated a new bacterial blight resistance gene *Xa47* from G252 rice line. It was located in cytoplasm and nucleus. They tested 180 rice materials, certain contained *Xa47* gene. The knockout mutants have more susceptibility to *Xoo* in contrast to wild-type G252.Resistance to BLB was increased in JG30 due to overexpression of *Xa47*. They suggested that *Xa47* is involved in regulating *Xoo* stress response. It may play important role in improving plant disease resistance [57].

Chapter 3

Research Methodology

The methodology opted for this reasearch work can be noted in Fig 3.1.

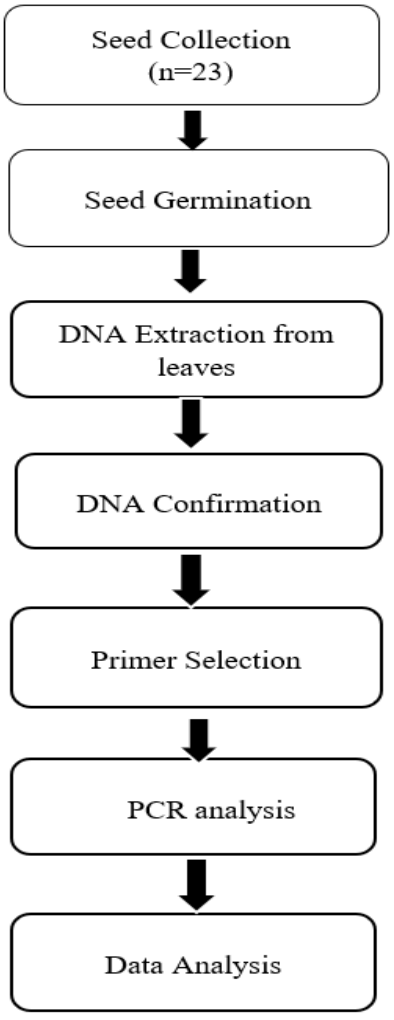

FIGURE 3.1: Methodology flow sheet.

3.1 Seed Collection

23 local rice genotypes were collected from different growing areas of Pakistan including Gujranwala (Punjab), Okara (Punjab), Upper Dir (KPK), Lower Dir (KPK), Swat (KPK), and Mansehra (KPK) (Table 3.1).

TABLE 3.1: Detail of local rice germplasm used (n=23)

Sr. No.	**Acc. No.**	**Source**
1	GW-260	Gujranwala, Punjab
2	GW-262	Gujranwala, Punjab
3	OK-265	Okara, Punjab
4	OK-270	Okara, Punjab
5	UD-205	Upper Dir, KPK
6	LD-167	Lower Dir, KPK
7	LD-173	Lower Dir, KPK
8	LD-175	Lower Dir, KPK
9	SW-225	Swat, KPK
10	SW-228	Swat, KPK
11	SW-231	Swat, KPK
12	SW-234	Swat, KPK
13	SW-240	Swat, KPK
14	SW-243	Swat, KPK
15	SW-244	Swat, KPK
16	MS-247	Mansehra, KPK
17	MS-249	Mansehra, KPK
18	MS-250	Mansehra, KPK
19	LD-153	Lower Dir, KPK
20	LD-155	Lower Dir, KPK
21	LD-180	Lower Dir, KPK
22	UD-201	Upper Dir, KPK
23	UD-207	Upper Dir, KPK

3.2 Plant Germination

The fresh and mature seeds of all rice genotypes were sown on disposable glass having rice nutrients of soil. The plants were checked regularly and watered. After 1 to 2 weeks seed germination was noted.

3.3 Genomic DNA Extraction

Genomic DNA was extracted from young fresh leaves by using the standard Doyle and Doyle (1987) method of DNA isolation [58] with minor modification. 100mg of leaves sample was ground by using mortar and pistil by the addition of 700μl CTAB solution along with Mercaptoethanol. The sample was kept at 65°C in water bath for 40 minutes and inverted 4 times after every 10 minutes. In next step 600 μl of Chloroform/Isoamyl Alcohol (24:1) was added followed by centrifugation at at 12000 rpm for 10 minutes. After centrifugation the upper supernatant was separated and added to fresh append of tube. The sample then was treated with 300 μl Ice chilled 2 Propenol. It was centrifuged again at 12000rpm for 10 minutes. After centrifugation a sample was kept in refrigerator at -4 °C for 45 minutes. Pellet was checked if not formed. Then it was washed about 200ml of 70% Ethanol and centrifuged, followed by drying, ethanol removal and addition of TE buffer.

3.4 DNA Confirmation

1% agarose gel was prepared by adding 1g Agarose powder in 100 µl of 1X TAE Buffer in a flask. After mixing well, the flask was put in microwave and heated for 30 to 60 seconds. Then cooled on room temperature.1 µl of Ethidium Bromide dye was added to cooled solution and shaked well. Then prepared agarose solution was added in to gel tray and allowed to solidify for about 20-30 minutes. DNA samples were run on 1% agarose gel and visualized via UV based gel documented system.

3.5 Primer Selection

Two primers (specific to BLB resistant gene) named as *Xa5* and *Xa7* were selected from previous literature and used for identification of resistant genes (BLB). These primers are chosen because of their availability. The sequence of the primers is given below in table 3.2.

TABLE 3.2: Detailed information of primer sequences for each studied gene.

Target Gene	**Primer**	**Primer Sequence**
Xa5	*Xa5* (F+R)	F: TGTTCTTTTCTCAGGGCCAC R: AGTTTGGAATCACAGGCCAC
Xa7	*Xa7* (F+R)	F: CTGGATACGGAACCTTCTAAC R: AGAGAACCTTCTCCTTCAGTG

3.6 PCR Analysis

PCR profile for denaturation, annealing and extension was optimized for each primer. PCR was performed to identify resistance genes among the selected germplasm. For detection of bacterial blight resistance gene two markers each of 100 bp tightly linked to *Xa5* and *Xa7* genes were used. PCR reaction mixture was formulated using 2 μl PCR Master Mix, 1 μl of each Forward and Reverse Primer, 2 μl dNTPs, 0.2 µl Taq DNA,12.3 μl PCR water and 1 microliter of Genomic DNA solution.

The PCR program involved: 4-minute initial denaturation step at 94°C, 35 cycles of denaturation at 94°C for 30s, annealing at 50 to 58°C for 30s and elongation at 72°C for 1-minute followed by elongation phase at 72°C for 7 minutes.

PCR products of *Xa5* and *Xa7* were resolved in 1% Agarose gel using 1X TBE Buffer at 80V-120 min. The gel was stained with Ethidium Bromide dye and DNA bands visualized under UV Gel Doc System.

3.7 Data Analysis

Bi variate data (1,0) 1 for presence of bands and 0 for absence of bands, was added to MS excel sheet. The data was analyzed by using NTSYs PC 2.1 software.

Chapter 4

Results

In the present study, 23 rice genotypes were collected from the different areas of Pakistan. The details of genotypes and their sources are given in Table 3.1. After DNA extraction, all samples run on 1% agarose gel for presence of DNA (Fig 4.1) Then they were further screened for the presence and absence of *Xa5* and *Xa7* genes using PCR-based markers linked to these genes.

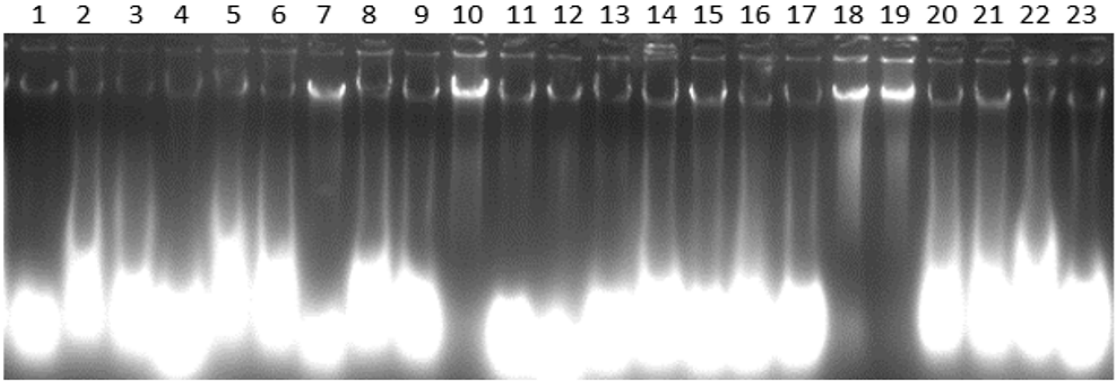

FIGURE 4.1: Showing DNA bands of all 23 samples run on 1% agarose gel.

4.1 Molecular Analysis

Molecular analysis was performed using gene-specific primers. The amplification products appearing as bands in the electrophoresis gel are shown in Fig 4.2 and

Fig 4.3. Banding patterns indicating the presence and absence of *Xa5* and *Xa7* genes in rice germplasm, amplified 141 bp and 294 bp, respectively.

4.1.1 Gel Electrophoretic Patterns for Gene *Xa5*

Gel electrophoretic bands of 23 rice genotypes (table 3.1) generated for BLB resistance gene *Xa5* are shown in Fig 4.2. Out of 23 genotypes, the *Xa5* gene fragment of size 141 bp was present in 22 genotypes and absent in 1 genotype. The genotypes in which the *Xa5* gene was present are GW-260, GW-262, OK-265, OK-270, LD-167, LD-173, LD-175, SW-225, SW-228, SW-231, SW-234, SW-240, SW-243, SW-244, MS-247, MS-249, MS-250, LD-153, LD-155, LD-180, UD-201 and UD-207. *Xa5* was absent in the UD-205 genotype.

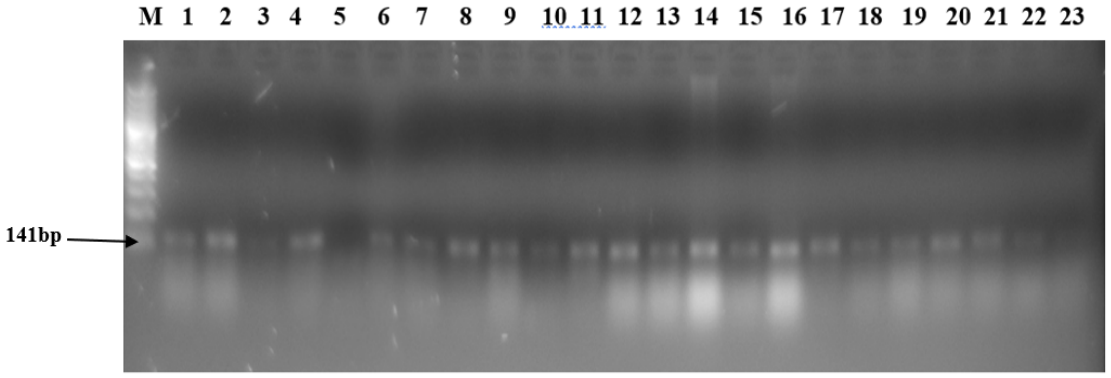

FIGURE 4.2: The *Xa5* marker bands of 23 rice genotypes via gel electrophoresis. M = Marker(100bp), band 1 = GW-260, band 2 = GW-262, band 3 = OK-265, band 4 = OK-270, band 5 = UD-205, band 6 = LD-167, band 7 = LD-173, band 8 = LD-175, band 9 = SW-225, band 10 = SW-228, band 11 = SW-231, band 12 = SW-234, band 13 = SW-240, band 14 = SW-243, band 15 = SW-244, band 16 = MS-247 , band 17 = MS-249, band 18 = MS-250, band 19 = LD-153 , band 20 = LD-155, band 21 = LD-180 , band 22 = UD-201 , band 23 = UD-207

4.1.2 Gel electrophoretic Patterns for Gene *Xa7*

Gel electrophoretic bands of 23 rice genotypes (Table 3.1) generated by using the marker for BLB resistance gene *Xa7* of size 294bp is shown in Fig 4.3. The findings

indicate that the *Xa7* gene was present in 22 genotypes: GW-260, GW-262, OK-265, OK-270, UD-205, LD-167, LD-173, LD-175, SW-225, SW-228, SW-231, SW-234, SW-240, SW-243, SW-244, MS-247, MS-249, MS-250, LD-153, LD-155, LD-180 and UD-207. The *Xa7* gene was absent in the UD201 genotype.

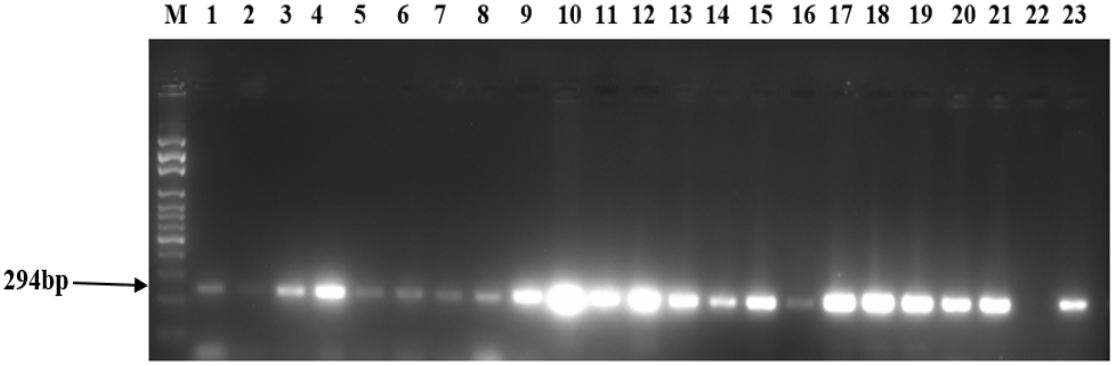

FIGURE 4.3: DNA bands of 23 rice accessions for identifiying BLB *Xa7* gene. M = Marker(100bp), band 1 = GW-260, band 2 = GW-262, band 3 = OK-265, band 4 = OK-270, band 5 = UD-205, band 6 = LD-167, band 7 = LD-173, band 8 = LD-175, band 9 = SW-225, band 10 = SW-228, band 11 = SW-231, band 12 = SW-234, band 13 = SW-240, band 14 = SW-243, band 15 = SW-244, band 16 = MS-247 , band 17 = MS-249, band 18 = MS-250, band 19 = LD-153 , band 20 = LD-155, band 21 = LD-180 , band 22 = UD-201 , band 23 = UD-207.

4.1.3 Genetic Similarity Between Rice Genotypes

Among the selected genotypes, the maximum genotypes showed similarity with one another, as shown in Table 4.1. The *Xa5* gene was present (+) in 22 genotypes and absent (-) in one genotype, as shown in Table 4.1. The genotypes in which the *Xa5* gene is present are GW-260, GW-262, OK-265, OK-270, LD-167, LD-173, LD-175, SW-225, SW-228, SW-231, SW-234, SW-240, SW-243, SW-244, MS-247, MS-249, MS-250, LD-153, LD-155, LD-180, UD-201 and UD-207. In genotype UD-205, the *Xa5* gene is absent (-).

Out of 23 genotypes, the *Xa7* gene was present (+) in 22 genotypes and absent (-) in 1 genotype, as shown in Table 4.1. *Xa7* gene was present (+) in GW-260, GW-262, OK-265, OK-270, UD-205, LD-167, LD-173, LD-175, SW-225, SW-228,

SW-231, SW-234, SW-240, SW-243, SW-244, MS-247, MS-249, MS-250, LD-153, LD-155, LD-180 and UD-207 genotypes. The *Xa7* gene was absent (-) in the UD-201 genotype.

TABLE 4.1: Rice genotypes used in the present study to show the presence (+) and absence (-) of *Xa5* and *Xa7* genes.

Sr. No	**Genotype**	***Xa5***	***Xa7***
1	GW-260	++	++
2	GW-262	++	++
3	OK-265	++	++
4	OK-270	++	++
5	UD-205	- -	++
6	LD-167	++	++
7	LD-173	++	++
8	LD-175	++	++
9	SW-225	++	++
10	SW-228	++	++
11	SW-231	++	++
12	SW-234	++	++
13	SW-240	++	++
14	SW-243	++	++
15	SW-244	++	++
16	MS-247	++	++
17	MS-249	++	++
18	MS-250	++	++
19	LD-153	++	++
20	LD-155	++	++
21	LD-180	++	++
22	UD-201	++	- -
23	UD-207	++	++

4.2 Similarity Matrix Analysis

Among the 23 rice genotypes, similarity indices were computed for every pair that could exist. A scale from 0 to 1 was used to quantify similarity; a value of 1 indicates that different genotypes are similar, whereas values less than 1 indicate varying degrees of similarity. The range of genetic similarity was 0.00 to 1.00. The greatest similarity matrix of 1.00 was displayed by numerous combinations. Some combinations had the lowest similarity matrix, 0.00. GW-260, GW-262, OK-265, and OK-270 were among the genotypes that showed strong resistance ratings (all scored 1) in multiple comparisons. This implies that significant resistance genes against BLB may be present in these genotypes. In many comparisons, genotypes such as UD-205 and LD-167 demonstrated moderate resistance with scores of 0.67. These genotypes might be useful in breeding initiatives meant to increase resistance to BLB. The similarity matrix among rice genotypes is shown in table 4.2.

TABLE 4.2: Similarity matrix table showing similarity among 23 rice genotypes. Greatest similarity = 1.00, Lowest similarity = 0.00.

Acc. No.	**GW-260**	**GW-262**	**OK-265**	**OK-270**	**UD-205**	**LD-167**	**LD-173**	**LD-175**	**SW-225**	**SW-228**
GW-260	1.00									
GW-262	1.00	1.00								
OK-265	1.00	1.00	1.00							
OK-270	1.00	1.00	1.00	1.00						
UD-205	0.67	0.67	0.67	0.67	1.00					
LD-167	1.00	1.00	1.00	1.00	0.67	1.00				
LD-173	1.00	1.00	1.00	1.00	0.67	1.00	1.00			
LD-175	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00		
SW-225	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	
SW-228	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
SW-231	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
SW-234	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00

Table 4.2: (Continued).

Acc. No.	**GW-260**	**GW-262**	**OK-265**	**OK-270**	**UD-205**	**LD-167**	**LD-173**	**LD-175**	**SW-225**	**SW-228**
SW-240	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
SW-243	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
SW-244	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
MS-247	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
MS-249	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
MS-250	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
LD-153	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
LD-155	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
LD-180	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00
UD-201	0.67	0.67	0.67	0.67	0.00	0.67	0.67	0.67	0.67	0.67
UD-207	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00

Acc. No.	**SW-231**	**SW-234**	**SW-240**	**SW-243**	**SW-244**	**MS-247**	**MS-249**	**MS-250**	**LD-153**	**LD-155**	**LD-180**	**UD-201**	**UD-207**
SW-231	1.00												
SW-234	1.00	1.00											
SW-240	1.00	1.00	1.00										
SW-243	1.00	1.00	1.00	1.00									
SW-244	1.00	1.00	1.00	1.00	1.00								
MS-247	1.00	1.00	1.00	1.00	1.00	1.00							
MS-249	1.00	1.00	1.00	1.00	1.00	1.00	1.00						
MS-250	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00					
LD-153	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00				
LD-155	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00			
LD-180	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
UD-201	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	
UD-207	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Acc. No.	UD-201	UD-207
UD-201	1.00	
UD-207	0.67	1.00

4.3 Cluster Analysis of Rice Genotypes Against BLB

Genotypes were further analyzed with NTSYS-PC software version 2.1. The dendrogram was generated using the (UPGMA) Unweighted Pair Group Method with Arithmetic Mean, which divided the 23 genotypes into three groups Fig 4.4.

The genetic distance between rice varieties is calculated based on a statistical index from 0 to 1. The dendrogram arranges the germplasm hierarchically according to the coefficients.

It shows the degree of similarity or difference between the samples. The dendrogram divides genotypes into three main groups based on genetic similarity. Rice germplasms with similar disease resistance are grouped together.

4.3.1 Cluster A (Highly Resistant)

This cluster consists of genotypes with similar patterns with coefficients 1.00. Clusters with high similarity scores indicate that these genotypes are very similar to one another. This group consists of 21 genotypes.

The genotypes were GW-260, GW-262, OK-265, OK-270, UD-207, LD-180, LD-155, LD-153, MS-250, MS-249, MS-247,SW-244,SW-243,SW-240,SW-234,SW-231,SW-228,SW-225,LD-175,LD-173 and LD-167.

These germplasms may share both the same BLB resistance genes. This makes them a strong candidate for breeding programs focused on BLB resistance Fig 4.4.

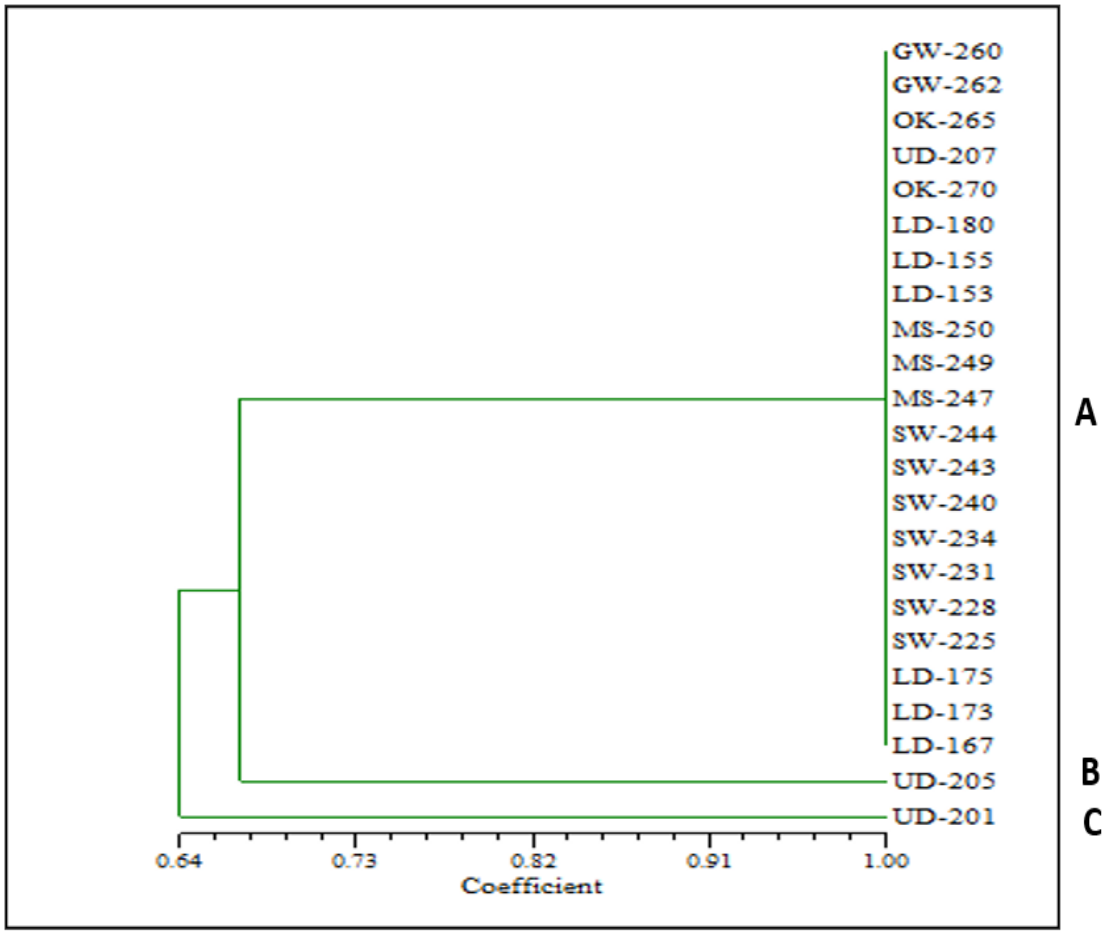

FIGURE 4.4: A dendrogram based on scoring revealed patterns of resistance to bacterial blight in Pakistani rice varieties.

4.3.2 Cluster B (Moderately Resistant)

This cluster represents the moderate resistance among genotypes. Genotypes in this group form a unique group with similar coefficients near 0.67 including only one genotype UD-205. Genotype in this cluster is moderately resistant having only one resistance gene *Xa5* Fig 4.4.

4.3.3 Cluster C (Moderately Resistant)

UD-201 make up this cluster. Its exhibit higher genetic variety, as seen by its lower similarity coefficients of almost 0.64.This genotype is moderately resistant.This cluster's varied genotypes could contain new resistance genes or characteristics, which would make them intriguing subjects for additional research Fig 4.4.

Clustering is used to identify genetic relationships between rice germplasm. Different patterns of resistance to bacterial leaf blight have been identified in rice varieties. Genotypes with high similarity values indicate low genetic distance between genotypes. Less genetic similarity indicates higher divergence between pairs.

4.4 Principal Coordinate Analysis or PCA Biplot and 3D Plot

The two-dimensional scatter plot from the Principal Coordinate Analysis (PCA) provides additional evidence for this clustering trend. Fig 4.5. A visual representation of the genetic links among the rice germplasm examined for bacterial leaf blight (BLB) resistance was produced using the similarity or dissimilarity matrices. An intuitive comprehension of the relationships between various germplasms based on how they react to BLB is made possible by the PCA plot. In the plot, germplasms that are closer together show more similarity, while those that are farther apart show more different genetic characteristics.

The wide distribution of data points between Dim-1 and Dim-2 suggests differences in the studied germplasm. Genotypes UD-201 and UD-205, are far from each other. This indicates significant differences in BLB resistance genes, meaning they may have opposite genes. This 2D plot shows the 21 rice genotypes (GW-260, GW-262, OK-265, OK-270, LD-167, LD-173, LD-175, SW-225, SW-228, SW-231, SW-234, SW-240, SW-243, SW-244, MS-247, MS-249, MS-250, LD-153, LD-155, LD-180 and UD-207), grouped into a single cluster, showing maximum similarity with one another Fig 4.5. We performed a 3-dimensional analysis for further confirmation of molecular and genetic analysis. The 3D graph showed how germplasm arranged themselves across the variations in the collection in three dimensions. This 3D graph illustrates how 23 rice samples (table 3.1) relate genetically with one another. Similar resistance genes are more likely to be shared by varieties that cluster together. This analysis explained the genetic variation based on the three groups named as group 1, group 2 and group 3, Fig 4.6.

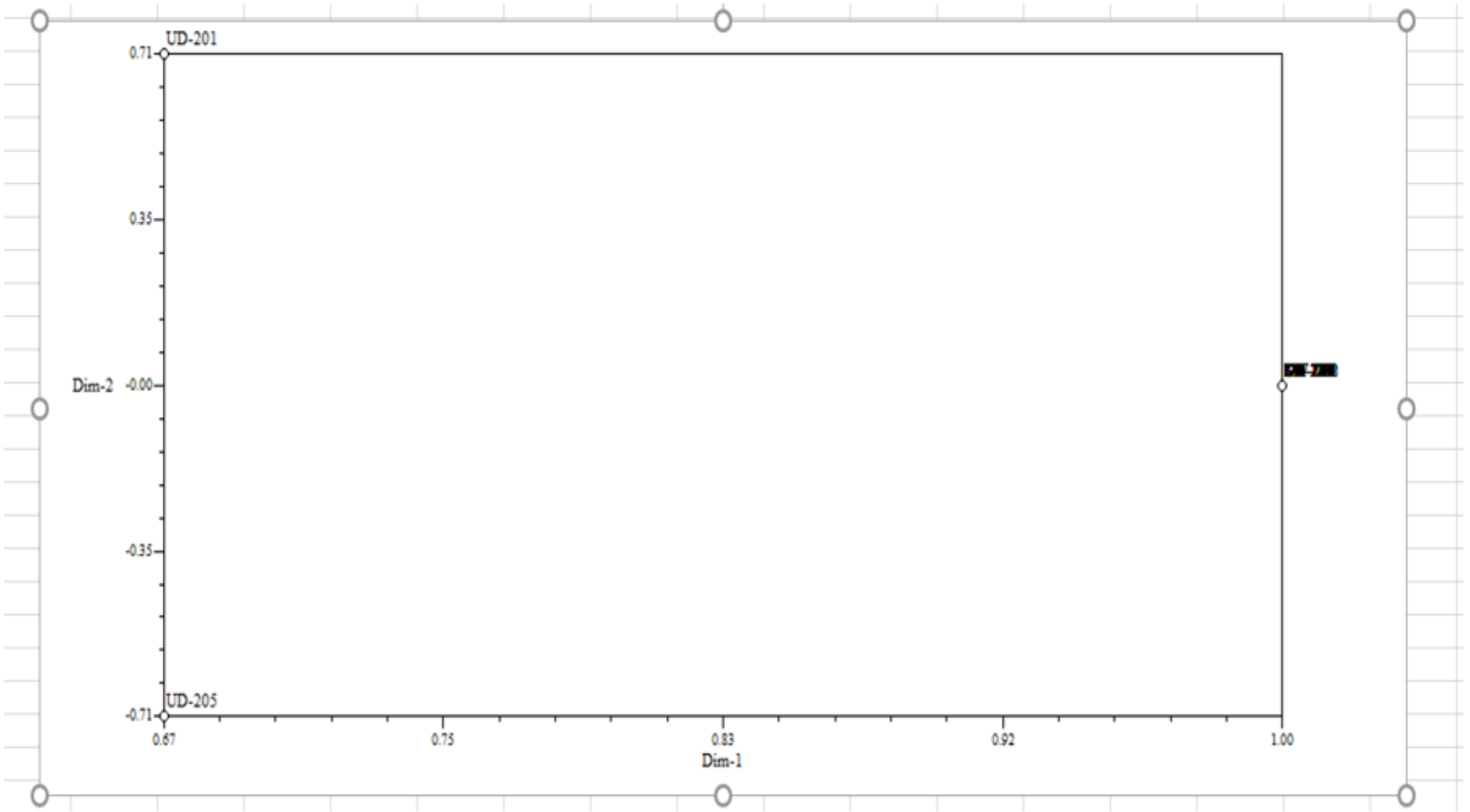

FIGURE 4.5: Two-dimensional scatter plot constructed to show the similarity and dissimilarity among 23 rice genotypes in two dimensions.

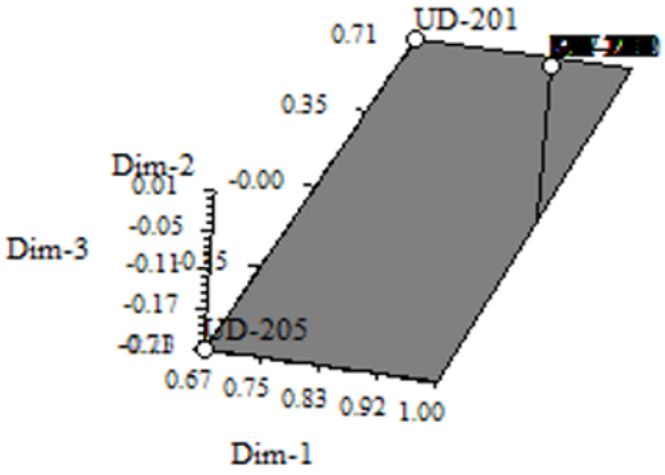

FIGURE 4.6: MDS plot Analysis demonstrating similarity and dissimilarity among 23 rice genotypes in three dimensions.

Group 1 consisted of 21 genotypes forming a cluster. Genotypes are GW-260, GW-262, OK-265, OK-270 , LD-167, LD-173, LD-175, SW-225, SW-228, SW-231, SW-234, SW-240, SW-243, SW-244, MS-247, MS-249, MS-250, LD-153, LD-155, LD-180 and UD-207. Group 2 consisted of only one genotype, which is UD-201. This genotype appears near the highest scores of 1.0 and 0.71, which demonstrate the close genetic match with other genotypes. Group 3 also consisted of only one genotype, UD-205. UD-205 is more genetically dissimilar as compared to other genotypes.

Chapter 5

Discussion

One of the most destructive diseases of rice is bacterial blight, which is brought on by *Xoo*. In severe situations, the severity of BLB disease spreads like wildfire and results in large yield losses. Using resistant cultivars is the most efficient way to fight BLB [59].

This study aimed to identify the presence of *Xa5* and *Xa7* resistance genes in 23 local rice genotypes in Pakistan, using molecular markers and genetic analysis. The *Xa5* and *Xa7* genes were detected in 22 of the 23 rice genotypes in this study, respectively. This suggests that these resistance genes are highly prevalent in the local germplasm. Many genotypes, including GW-260, GW-262, OK-265, and OK-270, showed great genetic similarity with genetic similarity indices ranging from 0.67 to 1.00. On the other hand, UD-205 and UD-201 were shown to have lower levels of resistance and genetic similarities. Similar studies have identified and differentiated between rice varieties who are sensitive to *Xoo* and those who are resistant to *Xoo*.

Using PCR amplification and gel electrophoresis, the molecular investigation verified the existence of the *Xa5* (141 bp) and *Xa7* (294 bp) genes in studied genotypes. These genes' role in BLB resistance is confirmed by the consistent banding patterns seen for them across the majority of genotypes. The lack of *Xa5* in UD-205 and *Xa7* in UD-201 draws attention to the genetic diversity present in the germplasm and marks these genotypes as special situations that require additional

research.

Many studies have documented the use of gene-linked molecular markers to identify the genotype of resistance genes in rice. For example, *Xa4, Xa5*, and *Xa21* resistance genes in Pakistani rice were found using gene-linked markers in a research by Muhammad *et al* [60] and Xia *et al* [61] resistance genes for *Xa5, Xa13, Xa21*, and *Xa27* in wild rice species. Resistance to Xoo is conferred by a number of resistance genes. Interestingly, it has been observed that *Xa21, Xa13, and Xa5* provide high levels of resistance against common *Xoo* strains in India [62]. In a research by Zhao *et al* [63], Molecular markers can efficiently identify the presence of BB resistance genes in rice and significantly aid in the development of new disease-resistant materials. Javed *et al* [64] analyzed genomic data of 38 rice cultivars from Malaysia were examined for allelic variation using one STS marker and thirteen SSR markers. found that whilst *Xa13* was absent from 25 cultivars, *Xa2* was present. Using a dendrogram, cultivars were grouped into seven clusters according to their genetic closeness.

Gautam *et al* [65] study used marker-assisted backcross breeding (MABB) to introduce many resistance genes (*Xa4, Xa5, Xa13, and Xa21*) into salt-tolerant rice variants. The study showed that resistance gene combinations offer long-lasting defense against BLB in a variety of settings. According to a related study by [8], their cluster analysis of 48 rice varieties showed that 75% of all examined varieties belonged to the same group, suggesting limited genetic diversity at the protein level [66].

Pyramiding numerous resistance genes can greatly increase disease resistance without sacrificing yield, as the study [67] showed using a set of 265 RILs with different combinations of resistance genes. This strategy is consistent with our research, which emphasizes how crucial genetic variety is to successful breeding. Several resistance genes (*Xa4, Xa5, and Xa21*) were found in the investigation done by Biswas *et al* [68] using linked molecular markers. Their results confirm that molecular markers are a reliable way to select for BLB resistance. Similar research by Javed *et al* [69] was carried out on 38 rice varieties and found the *Xa2* gene in 25 varieties, and the *Xa13* genes were absent in all tested germplasm.

The results showed that multigenic resistance to bacterial blight is present in Malaysian rice cultivars. In order to assess genetic variety and pinpoint characteristics that contribute to resistance against BLB, the study of Singh et al [70] used Principal Coordinate Analysis (PCA) to ascertain the variability among 184 rice genotypes for yield-related parameters and BLB resistance.

The results demonstrate how local rice germplasm might be a useful resource for creating BLB-resistant genotypes. Because of their high levels of resistance and genetic homogeneity, genotypes like GW-260, GW-262, OK-265, and OK-270 can be used as parental lines in breeding programs. Conversely, genotypes that are moderate resistant, such as LD-167, can help expand the genetic basis of resistant germplasm. Because of their distinct genetic profiles, UD-201 and UD-205 are interesting candidates for additional study in order to find new resistance genes or mechanisms that can enhance current BLB management techniques.

Future scientific research needs to identify and describe new resistance loci which will improve resistance durability. The recent genomic technology tool CRISPR/-Cas9 described by Zafar *et al* [71] enables precise modifications to introduce defense genes that make rice cultivars more resistant to BLB.

Chapter 6

Conclusion and Future Prospects

6.1 Conclusion

Bacterial leaf blight (BLB) disease, which is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), remains a significant threat to rice production worldwide, resulting yield losses. One of the effective and sustainable way to control BLB is development of rice cultivars with more resistance containing resistance genes.

Present study aimed to identify and analyse the BLB resistance genes *Xa5* and *Xa7* in 23 rice genotypes collected from local regions of Pakistan including KPK and Punjab. Molecular screening and genetic analysis gave valuable insights into the genetic similarity, genetic diversity, and potential breeding applications of these germplasms.

Our findings showed that 96% of the rice accessions contained both *Xa5 and Xa7* resistance genes, while 4% had either *Xa7 or Xa5*. No accession was noted to lack of both genes, which showed a strong prevalence of BLB resistance traits in the studied germplasm.

The presence of *Xa5* in 22 out of 23 accessions indicates its important role in resistance mechanisms. Accession UD-205, which lacks *Xa5* but possesses *Xa7*, had partial resistance, suggesting that *Xa7* alone may contribute to BLB resistance, though its effectiveness with *Xa5* needs further study. Genetic similarity analysis

categorized the rice accessions into two primary clusters. Cluster A consisted of highly resistant accessions such as GW-260, SW-244, MS-250, and LD-167, which demonstrated complete genetic similarity. Cluster B included accessions such as UD-205 and UD-201, which exhibited moderate genetic variation, making them valuable candidates for future breeding programs aimed at enhancing genetic diversity in resistant rice varieties. The identification of genetically distinct accessions within the genotypes highlights the potential for integrating novel resistance factors to combat evolving BLB pathogen strains.

The results along with previous studies suggesting that pyramiding multiple resistance genes, such as *Xa5 and Xa7*, enhances BLB resistance. Our findings also support the notion that genetic diversity plays a crucial role in the long-term sustainability of resistance breeding. The identification of accessions like UD-205 and UD-201, which show genetic distinctiveness, opens ways for further genomic studies, including whole-genome sequencing, to discover additional resistance loci and determine the molecular mechanisms involved in BLB resistance.

In short, this study gives a comprehensive understanding of BLB resistance gene presence in local rice germplasm, emphasizing the importance of genetic diversity in resistance breeding. The identified genotypes with both *Xa5 and Xa7* genes serve as valuable resources for breeding programs for enhancing rice resistance to BLB. Applying molecular tools and genomic advancements techniques will be valuable in developing resistant rice cultivars that will play role in food security and sustainable agricultural practices.

6.2 Future Prospects

Future research on BLB resistance in rice should focus on several key areas to improve the effectiveness of breeding programs. First, the potential of pyramiding *Xa5* and *Xa7* genes should be further explored, with a focus on testing their stability and effectiveness under diverse environmental conditions. In addition to these genes, there is a need to identify and incorporate additional resistance genes

to enhance resistance across a broader range of BLB strains and climatic zones. The unique resistance observed in UD-205, which lacks *Xa5* but possesses *Xa7*, suggests the presence of novel resistance mechanisms, requiring further investigation into these mechanisms and their potential use for breeding programs.

Expanding the screening of rice genotypes to include a larger and more diverse set of varieties, both local and exotic, would also give valuable findings into genetic diversity and the overall effectiveness of these resistance genes. Modern molecular breeding techniques, such as CRISPR/Cas9 gene editing, gives an opportunity to enhance the development of rice varieties with increased resistance, allowing for precise modification of resistance genes.

Using genomic selection and marker-assisted breeding (MAS) into future breeding programs could help in the development of BLB-resistant rice cultivars by the precision of trait selection. Finally, an environmental impact assessment should be conducted to evaluate the long-term sustainability of BLB-resistant varieties, considering factors such as ecological interactions and the potential for pest resistance. If these recommendations are given importance, will help to increase the development of BLB-resistant rice varieties, resulting to more sustainable and climate resilient rice farming systems. It will also improve the livelihood of local farmers as well.

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