Elucidation of multicomponent coordinated defense strategies in rice plant against *Drechslera oryzae*

Thesis submitted to the

University of North Bengal

For the award of Doctor of Philosophy

in

BOTANY

By

SWEATA KHATI

Supervisor

Professor B.N.Chakraborty

Immuno-Phytopathology Laboratory
Department of Botany
University of North Bengal
June 2017

DECLARATION

I declare that the thesis entitled "**Elucidation of multicomponent coordinated defense strategies in rice plant against *Drechslera oryzae***" has been prepared by me under the supervision of Professor B.N. Chakraborty, Immuno Phytopathology Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

(Sweata Khati)

Department of Botany
University of North Bengal
Raja Rmmohanpur, P.O-NBU
Dist- Darjeeling, Pin- 734103

Date:

UNIVERSITY OF NORTH BENGAL
Department of Botany

Professor B. N. Chakraborty
Ph.D, FPSI, FNRS, FISMPP, FISPC, FMSI, FIMS
FAScT, FRSC (Lond.), FNAAS

ENLIGHTENMENT TO PERFECTION

IMMUNO-PHYTOPATHOLOGY LABORATORY
PO. N.B.U 734013, Siliguri, West Bengal, India
Phone : 0353-2776337 (O), 09800266298 (M)
Fax : 0353-2699001
E-mail : bncnbu@gmail.com
Website: www.iplnbu.ac.in

CERTIFICATE

This is to certify that Ms. Sweata Khati has prepared the thesis entitled "**Elucidation of multicomponent coordinated defense strategies in rice plant against *Drechslera oryzae***", for the award of Ph.D. degree of the University of North Bengal under my guidance. She has carried out the work at the Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

(Professor B.N. Chakraborty)

(Professor B. N. Chakraborty)
Immuno-Phytopathology Laboratory
Department of Botany
University of North Bengal

ABSTRACT

It is a well-known fact that a plant respond to a pathogen by mobilizing a complex network of active defense mechanisms. The success of the plant in warding off the pathogenic attack depends upon the coordination among the different defense strategies and rapidity of the response. It is generally believed that the plants defend themselves against pathogenic fungi by producing fungi toxic substances such as phytoalexins, pathogenesis related (PR)-proteins, oxidised phenols and several other component. But in most cases the role of a single defence component has been reported at a time while working on disease resistance of a host–pathogen system. Hence, the present study was undertaken to determine whether a multicomponent coordinated mechanism is operative in disease resistance of rice with special reference to brown spot.

Initially, screening of resistance of fifteen rice cultivars against the brown spot disease was carried out. Growth rate, the amount of production/ accumulation of several bio chemical components, Percent Disease Index and defense enzyme activities of Phenylalanine ammonia lyase (PAL), Peroxidase (POX), Chitinase (CHT) and β-1,3-Glucanase (GLU) of healthy and infected samples was measured. Depending upon the results two ethnic cultivars (Brimful and Champasari) and one local cultivar (Black nuniya) was selected for further experimental purpose based upon their poor performance against the disease.

The causal organism was isolated and its morphological characteristics was studied to identification and understanding. Polyclonal antibodies against these pathogen was raised in male white rabbits and immunological assays were optimized for easy and early detection of these pathogens in rice leaf tissues. Detection of pathogen in infected leaf was carried out using Plate trapped antigen coated Enzyme Linked Immunosorbent Assay (PTA-ELISA) and Dot-immunobinding assay. Early detection of infection in artificially inoculated leaves was studied using PTA-ELISA and Dot-blot technique. It was noted that using these immunotechniques the fungal diseases could be detected as early as 24hrs after inoculation whereas the disease symptoms appeared only after 90-120hrs after inoculation.

Molecular detection of the fungal pathogen, *Drechslera oryzae* (RI.DO.01) was carried out using 18S rDNA sequencing of their conserved region using ITS1/ITS4 primer pair. The BLAST query of the 18S rDNA sequence of the isolates against GenBank database confirmed the identity of the isolate RI.DO.01 as *Drechslera oryzae*. The sequences have been deposited in NCBI GenBank database, under the accession no KT768092.

The present study examined the diversity of Arbuscular Mycorrhizal Fungi (AMF), colonisation nature and histopathological study in rice cultivars. Dominant arbuscular mycorrhizal fungi (*Rhizophagus fasciculatus*) isolated from the rhizosphere of rice plants were mass multiplied and used for application as bioinoculant for root colonization in rice plants. *In vitro* antagonistic effect of ten selective Plant growth promoting rhizobacteria (PGPR) and six selective Plant growth promoting fungus gave positive result against both the pathogens. These PGPR (*Bacillus altitudinus*, NAIMCC-BO1485) and PGPF (*Trichoderma harzianum*, NAIMCC-F-03288) were mass multiplied and applied to the rice plants to evaluate their effects on growth promotion and biochemical changes.

The main objectives of the present study was to determine the efficacy of these bioformulations using AMF, PGPF and PGPR singly and in combinations on plant growth promotion and bio control of brown spot disease of rice caused by *Drechslera oryzae* along with determination of cell defense responses in rice plants associated with induction of resistance toward *D. oryzae* by microbial formulation.

Growth enhancement was evaluated in terms of height. Results revealed that growth promotion occurred in all cultivars of rice plants following application of bioinoculants, singly or jointly and in different combinations. Activities of defense enzymes (peroxidase, phenylalanine ammonia lyase, chitinase and β-1,3glucanase) following treatment were analysed. HPLC profile of phytoalexin viz. Phytocassanes and phenolic acids were also determined. Enhanced increase in activities of these defense enzymes were noted in leaf of rice cultivars that were grown in field condition after joint application of bioinoculants. Disease incidence was found to be decreased in treated plants in comparison with untreated control plants. Radial growth bioassay of the antifungal compound obtained from the control and treated inoculated samples were also conducted to observe their activity towards the pathogen *in vitro*.

Apart from enzymatic assessment of defense enzymes, an attempt was also made to conduct fluorescent immunological studies to locate the sites of chitinase and glucanase enzyme expression within the leaf of rice tissues. Immunolocalization of chitinase and glucanase in *T. harzianum+ B. altitudinus + R. fasciculatus* treated and *D. oryzae* inoculated as well as control rice leaves were observed by immunofluorescence study.

ACKNOWLEDGEMENTS

Having unflinching faith on to "omnipotent creator" for his never ending blessing, it's a great pleasure to express my profound gratitude to my respected parents, who digested much hardship inspiring for prosecuting my studies, thereby receiving proper education.

I feel proud to convey my earnest respect, sincere appreciation and enormous indebtedness to my supervisor Professor B.N. Chakraborty, Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal, for his continuous direction, constructive criticism and valuable suggestions in carrying out the research work and preparation of this thesis. I express my gratitude and best regards to Professor Usha Chakraborty of the Department of Botany for her helpful commentary, supportive attitude and unvarying inspiration throughout the research work.

I am highly grateful to the Head of this department for all administrative support during the entire course of my research work and humble acknowledgements to all my honourable teachers of the Department of Botany, University of North Bengal for their valuable teachings, direct and indirect advice and encouragement and co-operation during the whole study period. I also wish to extend my thanks to all the non-teaching staffs of the Department.

I am also indebted to the Honourable Vice Chancellors, both past and present of the University of North Bengal for letting me to carry out my work in this University. My sincere thanks are also due to The Registrar, NBU for his positive response during my registration and submission of Ph.D. Thesis.

Without the financial support from the National Bureau of Agriculturally Important Microorganism, my work would start and end in nothingness and hence I sincerely thank the NBAIM, for providing me resource to study. I also owe special thanks to SciGenom Labs Pvt. Ltd., Kerala, for fungal identification.

I would also like to express my warm personal gratitude to all my friends of the Immuno- Phytopathology Lab especially Dr. Pannalal Dey, Dr. Kiran Sunar, Dr. Arka Pratim Chakraborty, Dr. Utanka Dey, Dr. Sanjita Allay, Dr. Amrita

Acharya, Dr. Somnath Roy, Mr. Shibu Barman, Ms. Priyanka Bhattacharjee, Ms. Puja Sashankar and all research scholars of Plant Biochemistry Lab for all their ideas that we argued about and constant support and assistance in completion of this thesis. Also my sincere thanks to my friend Mr. Praveen Jirwan for his support and inspiration. I wish to thank fieldsman Sambhu Banik and Dolly Mahato for maintaining plants in good health.

I found no words to thank my parents Mr. Krishna Khati and Mrs. Benuka Subba (Khati), my sister Ms. Sabiya Khati and my brother Mr. Bikesh Khati for their unquantifiable love and continuous support, their sacrifice never ending affection, immense strength and untiring efforts for bringing her dreams to proper shape. They were constant source of inspiration and enthusiasm in the critical moment of her studies.

Immuno-Phytopathology Lab
Department of Botany
University of North Bengal
Date:

Sweata Khati

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure 19: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Brimful). (A) *Glomus* sp.; (B) *Glomus* sp.;(C) *Glomus* sp.;(D) *Glomus fasciculatum*; (E) *Glomus mosseae* (juvenile); (F) *Scutellospora* sp.;(G) *Glomus* sp.;(H) *Glomus mosseae* (mature).

Figure 20: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Tulai panji).(A) *Glomus* sp. ;(B) *Glomus* sp.;(C) *Glomus* sp.;(D) ruptured spore of *Glomus* sp.;(E) *Scutellospora* sp.;(F) mature *Glomus* sp.;(G) *Glomus constrictum*

Figure 21: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-5).(A) *Acaulospora* sp.;(B) ruptured *Gigaspora* with conspicuous hyphal attachment;(C) *Scutellospora* sp.;(D) *Glomus* sp.;(E)*Scutellospora* sp.;(F) *Scutellospora* with hyphal attachment (magnified)

Figure 22: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-4).(A)*Scutellospora* sp.;(B) *Gigaspora* sp.;(C) *Glomus* sp.;(D)*Glomus* sp.;(E) Hyphal attachment of *Gigaspora* sp.;(F) *Scutellospora* sp.

Figure 23: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Black Nuniya).(A) *Glomus* sp.;(B) *Glomus* sp.;(C) *Scutellospora* sp.;(D*) Glomus* sp.;(E) *Glomus* sp.; (F) *Acaulospora* sp.; (G) *Glomus* sp.;(H) *Glomus* sp.; (I) *Glomus* sp.

Figure 24: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Champasari).(A) *Gigaspora* sp.;(B) *Glomus* sp.;(C) Ruptured spores of *Scutellospora* sp.;(D) *Scutellospora* sp.(matured)

Figure 25: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Adde).(A) *Glomus constrictum*;(B) *Glomus constrictum*; (C) *Glomus mosseae*; (D) *Acaulospora* sp.; (E) *Acaulospora* sp.;(F) *Acaulospora* sp.;(G,H&I) *Glomus mosseae* in different angles

Figure 26: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-1).(A) *Gigaspora* sp.;(B) *Glomus* sp.;(C)*Glomus* sp.;(D)*Glomus* sp.

Figure 27: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Swarna masuri).(A) *Scutellospora* sp.; (B) Ruptured *Glomus* sp.;(C) *Glomus* sp. with hyphae

Figure 28: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Maiti). (A) *Scutellospora* sp.;(B) *Scutellospora* sp.;(C) *Gigaspora margarita*

Figure 29: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Attheu).(A) *Glomus* sp.;(B) *Scutellospora* sp.;(C) *Glomus* sp;(D) *Acaulospora* sp.;(E) *Glomus constrictum*;(F)Ruptured AMF.

Figure 30: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Sanomasuri).(A) *Scutellospora* sp.;(B) *Scutellospora* sp.;(C) *Glomus* sp.;(D) *Gigasporarosea*.

Figure 31: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Loknath 505).(A) *Glomus* sp. juvenile;(B) *Entrophospora* sp.;(C)*Glomus* sp.;(D) *Scutellospora* sp. with hyphal attachment

Figure 32: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Kaberi 9090).(A) *Glomus* sp.;(B) *Glomus* sp.;(C) *Glomusbadium* ;(D) *Glomus badium*

Figure 33: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Gouraknath 509).(A) Glomus fasciculatum;(B) *Glomus multicaule*;(C) *Glomus* sp.;(*Glomus* sp.)

Figure 34: Root colonization of rice cultivars. (A-C) Brimful; (D-F) Black nuniya; (G-I) Champasari and (J-L) Attheu. (Ih- Intracellular hyphae, Vs- Vesicle, Ar- Arbuscule).

Figure 35: Root colonization of rice cultivars. (A-C) Maiti; (D-F) Sano masuri; (G-I) Tualai panji and (J-L) Swarnamasuri. (Ih- Intracellular hyphae, Irs- Intra radicle spore, Vs- Vesicle, Ar- Arbuscule).

Figure 36: Root colonization of rice cultivars. (A-C) UBKV-1; (D-F) UBKV-4; (G-I) UBKV-5 and (J-L) Adde. (Ih- Intracellular hyphae, Vs- Vesicle, Ar- Arbuscule)

Figure 37: Root colonization of rice cultivars. (A-C) Kaberi 9090; (D-F) Loknath 505; (G-I) Gouraknath 509. (Ih- Intracellular hyphae, Ca- Coiled arbuscule, Vs- Vesicle, Ar- Arbuscule)

Figure 38: Mass multiplication of AMF spores in maize plant (A-C) Pots; (D) Experimental field

Figure 39: Histopathological study of AMF colonized maize roots.(A)Vesicles and intracellular hyphae, (B) Spores bearing hyphae,(C) Vesicles,(D) Vesicle and Intracellular hyphae.(Vs- Vesicle, Ih-Intracellular hyphae, Sp-Spore)

Figure 40: *In vitro* antifungal activities of foliar fungal pathogen *Drechslera oryzae* against selected PGPR isolates. Inhibition of *Drechslera oryzae* in dual plate culture assay by NAIMCC-B01485 (B), NAIMCC-B01483 (C), NAIMCC-B01486 (D), NAIMCC-B01488 (E), NAIMCC-B01489 (F) and Control (A)

Figure 41: *In vitro* antagonistic test of foliar fungal pathogen (*D. oryzae*) against different PGPF.(B) *T. harzianum* (NAIMCC-F-03289), (C) *T.harzianum* (NAIMCC-F-03288), (D) *T. harzianum* (NAIMCC-F-03290), (E) *T. asperellum* (NAIMCC-F-03291), (F) *T. asperellum* (NAIMCC-F-03292), (G) *T. asperellum* (NAIMCC-F-03293) and (A) Control(*D. Oryzae*)

Figure 42: Growth promotion of rice plants following treatment with different PGPR.C-Untreated control (UI),T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01484), T3-*Bacillus altitudinis* (NAIMCC-B01485), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (NAIMCC-B01487), T6-*Bacillus pumilus* (NAIMCC-B01488),T7- *Burkholderia symbiont* (NAIMCC-B01489), T8-*Bacillus aerophilus* (NAIMCC-B01490), T9- *Paenibacillus polymyxa* (NAIMCC-B01491), T10-*Bacillus methylotrophicus* (NAIMCC-B01492).

Figure 43: Activity of defense enzymes in rice leaf samples (A) Peroxidase and (B) Chitinase. C-Untreated control,T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01485), T3-*Bacillus altitudinis* (NAIMCC-B01484), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (NAIMCC-B01487), T6-*Bacillus pumilus* (NAIMCC-B01488),T7-*Burkholderia symbiont* (NAIMCC-B01489), T8-*Bacillus aerophilus* (NAIMCC-B01490), T9- *Paenibacillus polymyxa* (NAIMCC-B01491), T10-*Bacillus methylotrophicus* (NAIMCC-B01492).

Figure 44: Activity of defense enzymes in rice leaf samples (A) PAL (Phenyl alenine ammonia lyase) and (B)β-1,3-Glucanase. C-Untreated control,T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01484), T3-*Bacillus altitudinis* (NAIMCC-B01485), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (NAIMCC-B01487), T6-*Bacillus pumilus* (NAIMCC-B01488),T7- *Burkholderia symbiont* (NAIMCC-B01489), T8-*Bacillus aerophilus* (NAIMCC-B01490), T9- *Paenibacillus polymyxa* (NAIMCC-B01491), T10-*Bacillus methylotrophicus* (NAIMCC-B01492).

Figure 45: HPLC profile of Phytocassanes obtained from leaf extracts of rice plant (cv. Black nuniya) showing different peaks of Phytocassanes following treatment with *Bacillus altitudinus* (NAIMCC-B01485) and pathogen challenge. (UH= Untreated Healthy, UI= Untreated Inoculated, TH= Treated Healthy and TI= Treated Inoculated)

Figure 46: Growth promotion of rice cultivars following treatment with different PGPF. (C= Untreated Control, T1= *T. harzianum* NAIMCC-F-03288, T2= *T. harzianum* NAIMCC-F-03289, T3=*T.harzianum* NAIMCC-F-03290, T4= *T.asperellum* NAIMCC-F-03291, T5=*T. asperellum* NAIMCC-F-03292 and T6= *T. asperellum* NAIMCC-F-03293)

Figure 47: Defense enzyme activity (A) Phenylalanine ammonia lyase and (B) Peroxidase of rice cultivars following treatment with PGPF and inoculated

LIST OF APPENDICES

Chapter 1
INTRODUCTION

Rice (*Oryza sativa* L.) is the seed of the monocotyledonous plant, belonging to the grass family (Poaceae). As a cereal grain, rice is the most important staple food for a greater part of the world's human population especially in Asia. India is the world's second largest producer only after China. Rice being the chief staple food crop of India, is grown in most of the states and occupies more than thirty per cent of the total cultivated area as reported by (Adhikari *et al*., 2012; Chakravorty *et al*., 2013). According to (Chakravorty and Ghosh, 2013), its cultivation is mostly concentrated in the river valleys, low-lying coastal areas and deltas of north eastern and southern India, especially in the states of Andhra Pradesh, Assam, Bihar, Chhattisgarh, Karnataka, Kerala, Odisha, Tamil Nadu, Uttar Pradesh and West Bengal, which together they count about ninety seven per cent of the country's rice production. West Bengal is called as 'bowl of rice' with over 450 rice landraces according to (Deb, 2005; Chatterjee *et al*., 2008). Rice is cultivated in West Bengal on over 65 per cent area under agricultural crops as reported by (Adhikari *et al*., 2012) in three different seasons i.e., Aus (autumn rice), Aman (winter rice) and Boro (summer rice).

Since ages India is home to a wide range of varieties of rice cultivars, landraces and many lesser known types that have been under cultivation by indigenous farmers as well as local entrepreneurs according to (Vinita *et al*., 2013). Also as according to (Roy *et al*., 1985; Paroda and Malik, 1990; Khush, 1997) more than 75,000 local cultivars/landraces of rice are present in here. Moving towards the north-eastern region of India where also rice occupies the most important position of staple food with estimated 10,000 indigenous cultivars of rice landraces grown in this region. Since rice is the most important crop and also of the known fact that it earns a lot of money for the country and also for the state, the rate of production of rice need to remain constant. So to meet up to that level of production a lot experiments are done on rice plant to improve its disease resistance and life span.

Among the most important limiting factors that affect rice production is diseases and fungal disease infection is one of the most serious rice diseases in the rice sector. It results in poor production, poor quality, poor milling returns and reduced income. Rice

brown spot is a chronic disease that affects millions of hectares of rice every growing season, grown by some of the most resource-poor farmers. Still much needs to be understood about brown spot disease despite of its widespread occurrence and impact. It causes an annual yield loss conservatively estimated at 5%. Brown Spot is conventionally perceived as a secondary problem that reflects rice crops that experience physiological stresses as for example drought and poor soil fertility, rather than a true infectious disease especially occurring in environments where water supply is low.

Brown spot has been associated with two major epidemics in India, the first in the Krishna Godavari delta in 1918 to 1919, and the second, during 1942 in today's India and Bangladesh according to (Chakrabarti, 2001). The latter of which has been associated with the Great Bengal Famine as according to (Chakrabarti, 2001; Padmanabhan, 1973). However one needs to remember that the relationship between plant disease epidemics and famines probably is never simple, as because so many other factors lead to major social consequences as reported by (Chakrabarti, 2001; Zadoks, 2008). According to (Reddy *et al*., 2010), rice brown spot is still widely reported across India and more often in the South and South-East Asian countries as per (Savary *et al*., 2000a). Also according to (Savary *et al*., 2000b, 2006) it causes a huge loss in the yield that, on an average, are in the range of 10 % of the attainable yield wherever it occurs in the lowlands of tropical and subtropical Asia. Therefore, Rice Brown Spot is by far one the strongest yield reducers amongst rice diseases today. Further, there is indication that BS (Brown Spot) is becoming more frequent and severe as drought is becoming more frequent as per (Savary *et al*., 2005), perhaps due to increased variability in rainfall. Also (Bedi and Gill, 1960) reported that the range of reported yield losses to brown spot, often articulated in absolute terms, is variable from 4 to29 %, and about 12 %, as per (Aluko, 1975), from 8 to 23 % as per (Fomba and Singh, 1990) from 26 to 52 % according to (Chakrabarti, 2001). The latter figures represent a broader and extreme range as because it is responsible for losses that have been caused by grain infection. Heavily infected grains are not suitable for consumption of humans, which partially explain the effect of BS in the Great Bengal Famine.

Brown spot symptoms initially show a small oval to circular spots on the first seedling leaves. Leaf spots are seen all throughout the growing season and may differ in shape, size and colour as per the environmental conditions, age of the spots, and the degree of susceptibility of the rice cultivars. Small brown spots are dark brown to

reddish brown while large brown spots are light, reddish-brown or gray in the middle surrounded by a dark to reddish-brown in the margin .Older spots may have a bright yellow halo surrounding the lesion. Disease development is also favoured by optimum temperature between 16 and 36°C and high relative humidity (86-100%). Leaves must be wet for at least 8-24 hours for infection to occur. Decrease in yield of rice due to brown spot epidemic in Bengal in 1942 was attributed to constant temperature of 20-30°C for two months, abnormally cloudy weather, and higher-than-normal temperature and rainfall at the time of flowering and grain-filling stages.

Originally the causal pathogen of brown spot in rice was classified in the *Helminthosporium* group, but was subsequently transferred to *Bipolaris*, which is characterized by bipolar germinating conidia. However (Della and Madonna, 2005) reported that it might be caused by *Drechslera* due to observance of intercalary germination in some of the isolates. *Drechslera* and *Bipolaris* species are important plant pathogens and are associated with symptoms of dark spots on leaves, and also root rot of seedlings. These fungi contain dark pigmentation which is due to the presence of a substance called melanin in their cell walls, which is measured as an important factor of virulence. The agent responsible for brown spot in rice (*Oryza sativa* L.) is *Bipolaris oryzae* (Breda de Haan) according to Shoemaker, 1959 [syns. *Drechslera oryzae* (Breda de Haan) as reported by Subramanian and Jain, 1966 and *Helminthosporium oryzae* (Breda de Haan) as reported by Miybabe & Hori, 1901]. According to (Ito & Kurib, 1942) the teleomorph of this species is *Cochliobolus miyabeanus* The generic name *Helminthosporium* is deeply entrenched in the literature of phytopathology, and separation of the graminicolous species into *Drechslera, Bipolaris*, and *Exserohilum* has not been accepted universally according to (Alcorn, 1988). It has been suggested that knowledge of the systematics of the two genera, *Drechslera*, and *Bipolaris*, is insufficient as per (Hawksworth, 1986). The classification of the causal agents of "*Helminthosporium*" diseases is based mainly upon morphological and, to a lesser degree, on biochemical and physiological characteristics according to (Matsumura *et al*., 1998). Some authors have also used molecular markers to study the relationship among these species according to (Bakonyi *et al*., 1995; Abadi *et al*., 1996) for more understandings.

Natural enemies that are used to control disease are called biological control. Biological control is an innovative, cost effective and eco friendly approach. Biological

control is also an alternative to the use of harmful chemical pesticides. Biological fungicides may also be used to repress the population of the pathogenic organisms through competition with pathogenic organisms and also stimulate plant growth, which allows plants to quickly drive away any pathogen effects, or damage the pathogen by means of toxins produced according to (Cook, 2000; Gilreath, 2002). Bio control agents come from natural materials such as animals, plants, bacteria, fungi and certain minerals also. Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. As agricultural production intensives over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations. However, according to De Weger *et al*., 1995 and Gerhardson, 2002, increasing use of chemical fertilizers causes several negative effects such as, development of pathogen resistance to the applied agents and their non target environmental impacts. Furthermore, the growing cost of pesticides, particularly in less- affluent regions of the world, and consumer demand for pesticide-free food has made it necessary to a search for substitutes for these products. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent as per (Gerhardson, 2002). Biological control is therefore being considered as an alternative way of reducing the use of chemicals in agriculture as reported by (De Weger *et al*., 1995, Gerhardson, 2002, Postma *et al*., 2003 and Welbaum *et al*., 2004).

There have been only a few reports on the improvement of Brown Spot control involving biological control agents. However, the use of antagonistic microbes for plant health management has emerged as a viable technology in the recent past. According to (Singh *et al*. 2005) commercially available antagonistic microbes, mostly belonging to the genera *Pseudomonas* and *Trichoderma*, can reduce the damage by direct effects on the pathogens (mycoparasitism, antibiosis, competition for iron) or by improving plant immunity (induced resistance, IR). Direct antagonism has been the key factor in suppression of many soil-borne pathogens, while IR is active against diverse foliar pathogens including both bacteria and fungi as reported by (Shoresh *et al*., 2010). Seed treatments with *Trichoderma viride* or *T. harzianum* have yielded up to 70 % disease reduction according to (Biswas *et al*., 2010b). Over 70 % disease reduction has been

achieved from the use of selected *Pseudomonas* sp. Isolates according to (Joshi *et al*., 2007; Ludwig *et al*., 2009).

Not only fungi but research on Plant Growth-Promoting Rhizobacteria (PGPR) with non-legumes such as rice have shown beneficial effects through biological nitrogen fixation according to (Malik *et al*., 1997), increased root growth as per (Mia *et al*., 2012) with enhanced nutrient uptake as per (Yanni *et al*., 1997), phytohormone production according to (Chabot *et al*., 1996), plant growth enhancement stimulation by other beneficial bacteria and fungi according to (Saharan and Nehra, 2011) and disease control as per (Ramamoorthy *et al*., 2001). Peng *et al*. (2002), however, reported that rhizobial inoculation known for their symbiotic relationship with legumes, could also increase rice grain yield, but little is known about the mechanisms involved. The beneficial effects of the selected rhizobial isolates could be due to their plant growth-promoting abilities namely biological Nitrogen fixation, phosphate solubilization and plant growth regulator or phytohormone similar to the known valuable effects of PGPR according to (Boddey *et al*. in 1997; Verma *et al* in 2001; Araujo *et al*.in 2013 and Kloepper *et al*. in 1980). Also Yanni *et al.* (1997) have shown beneficial interactions of rhizobial isolates on growth of rice, which was believed to be due to increased root efficiency in water and nutrient uptake.

The role of soil organisms that are present below the ground and which interact with plant roots has gained increased attention in recent years according to (Reynolds *et al.,* 2003;van der Putten, 2003; Callaway *et al.,* 2004), and the interactions between beneficial and harmful pathogenic organisms have been known and identified as being particularly relevant due to their important implications for plant fitness according to (Schippers *et al.,*1987; Fitter and Garbaye, 1994;Bever, 2003).Penetration and establishment of fungus in the roots of host plant involves a complicated series of events and intracellular modifications according to (Bonfante-Fasolo and Perotto,1992).Symbiosis between plants and beneficial soil microorganisms like Arbuscular Mycorrhizal Fungi (AMF)are known to support plant growth and help plants to manage with biotic and abiotic stresses. Intense physiological changes take place in the host plant when roots are colonized by AMF affecting the interactions with different organisms below- and above-ground. Defensive effects of the symbiosis against pathogens, pests, and many parasitic plants have been reported for many plant species that includes agriculturally important crops. Other than those mechanisms such

as improved plant nutrition, pathogenic competition and experimental proof supports a major role of plant defences in the observed defence mechanism. During establishment of mycorrhiza, accent of plant defence responses occurs thus attaining a functional symbiosis. As a result of this modulation, a gentle, but effective establishment of the plant immune responses are seen to occur, not only in local defence but also systemically. This activation leads to a prepared state of the plant that allows a more competent activation of defence mechanism in reaction to attack by potential enemies. Maiti *et al*. in 1995 earlier reported colonization by native AMF in rice plant. Earlier workers like Saha *et al*. in 1999 also reported incomplete dependency of upland rice on native AMF for phosphorus acquirement. Dubey *et al*. in 2008 also studied the occurrence of VAM fungi at altering stages of growth of rice plants.

Generally a plant responds to a pathogen by mobilizing a composite network of active defence mechanisms. The success of the plant in driving off the pathogenic attack depends upon the organization among the different defence strategies and the quickness of the response. It is usually believed that plants defend themselves against pathogenic fungi by producing certain fungitoxic substances such as phytoalexins, pathogenesis related (PR)-proteins, oxidized phenols and several other components. Also in most cases the role of a single defence component has been reported at a time while working on disease resistance of a host–pathogen system according to (Bera and Purkayastha**,** 1999).Plants usually display an antibiotic reaction (hypersensitive reaction) when they come across with a pathogenic fungi, and are known to produce phytoalexins which shows antifungal activity against the pathogenic fungus in the tissue around the reaction spot. Examples of phytoalexins include momilactones A and B, oryzalexins A, B, C, D, E, F, and S, sakuranetin, oryzalic acids A and B, oryzalides A and B, and phytocassanes A, B, C, and D (Japanese Patent Application No. 7-43520/1995) revealed by the present inventors.

The present investigation was undertaken taking into consideration many factors but the focus was mainly on study of defence response of rice plants with bioinoculants. The reason being that the on field observation as related to the symptomology on preliminary assessment indicated that the degree severity was more due to fungal pathogen causing brown spot causing crop loss. The goal of this investigation as apparent was to initiate an understanding of the dynamics of micro-flora dwelling in the rhizosphere of the rice plants and their state of interaction with prevailing pathogens

affecting the rice plants. The present study was designed with an aims to explore the possibility of using beneficial bio inoculant, plant growth promoting fungi (PGPF) and arbuscular mycorrhizal fungi (AMF) isolated from rhizosphere of rice plants (*Oryza sativa* L.), specially cultivars grown in West Bengal and Sikkim hills for management of brown spot disease caused by fungal pathogen (*Drechslera oryzae*).

To evaluate and corroborate the goal certain objectives were outlined:

- Screening of resistance of rice cultivars towards *Drechslera oryzae*.
- Raising polyclonal antibody against *Drechslera oryzae* and their immunological detection and identification using immunoassays.
- Determination of level of phenolics and ascertaining their antifungal activity associated with rice cultivars to infection against *Drechslera oryzae*.
- Induction of resistance in rice plants against *Drechslera oryzae* using using bioinoculants (AMF, PGPF and PGPR).
- Evaluation of defence enzymes [Phenylalanine ammonia lyase (PAL), Chitinase (CHT), β-1,3 Glucanase (β GLU) and Peroxidase (POX)] in rice cultivars following induction of resistance.
- Cellular localization of chitinase and glucanase in rice leaf following induction of resistance using bioinoculants.
- Extraction of phenolics and antifungal compound and their evaluation in rice cultivar following induction of resistance against *D. oryzae* using bioinoculants.

Chapter 2
REVIEW OF LITERATURE

Rice being one of the most important cereal crops for all over the world is the seed of the grass species *Oryza sativa* (Asian rice) or *Oryza glabarrima* (African rice). Almost half of the population of the world feeds on rice and it also adds for more than 50% of the regular calorie intake (Maclean *et al*., 2002). It is the staple food for more than three billion peoples all around the world. Rice is grown in almost 114 countries all over the world and more than 50 countries have an annual production of 100,000 tons or more. In comparison to South America and Africa where rice is consumed in equal quantity to wheat and maize but here in Asia rice is the most highly consumed staple food in their daily life. In worldwide production on agricultural commodities rice stands out to be in the third highest position just after sugarcane and maize according to FAO (2012). Grains such as maize and wheat serves for other various purposes other than human food but rice serves as a valuable grain that adds up to the nutritional value and input of calorie accounting for almost one fifth of the calorie taken by humans worldwide.

In the pass thirty years due to advancement in the cultivation techniques of rice farming and availability of much improved high yielding rice varieties the rate of production has been almost doubled but unfortunately it is still being difficult to meet up to the needs of the worldwide rapidly increasing population (Fischer *et al*., 2000; Sasaki and Burr, 2000).

In India almost all the state produces rice but the cultivation is mainly carried out in low lying coastal areas, deltas and river valleys. Seven major rice producing states are: West Bengal, Uttar Pradesh, Andhra Pradesh, Punjab, Tamil Nadu, Odissa and Bihar. These states produce more than half of the total rice produced in the whole country. West Bengal is the leading producer of rice in India and accounts for 14 % of total rice produced in the country. The major rice producing districts in the state are Bardhaman, Medinipur, North and South 24 Parganas. East and West Midnapur, Jalpaiguri, Bardhaman, Bankura, Birbhum, North and South Dinajapur etc. Around the world there are nearly 40,000 different varieties of rice under the species name *Oryza sativa* with a total of about 74 varieties are from India. Most of the varieties in the

hands of the farmers have not yet been studied which still the potential to compete with the now has advanced varieties with organic mode only.

Table1 . Nutrient content of rice per 100 gm portion (Nutrient data laboratory).

Nutrient components	**Value per 100 gm**
Water (gm)	12
Energy (KJ)	1528
Protein (g)	7.1
Fat (g)	0.66
Carbohydrate (g)	**80**
Fibre (g)	1.3
Sugar (g)	0.12
Calcium (mg)	28
Iron (mg)	0.8
Magnesium (mg)	25
Phosphorus (mg)	115
Potassium (mg)	115
Sodium (mg)	5
Zinc (mg)	1.09
Copper (mg)	0.22
Manganese (mg)	1.09
Vitamin (mg)	0.1

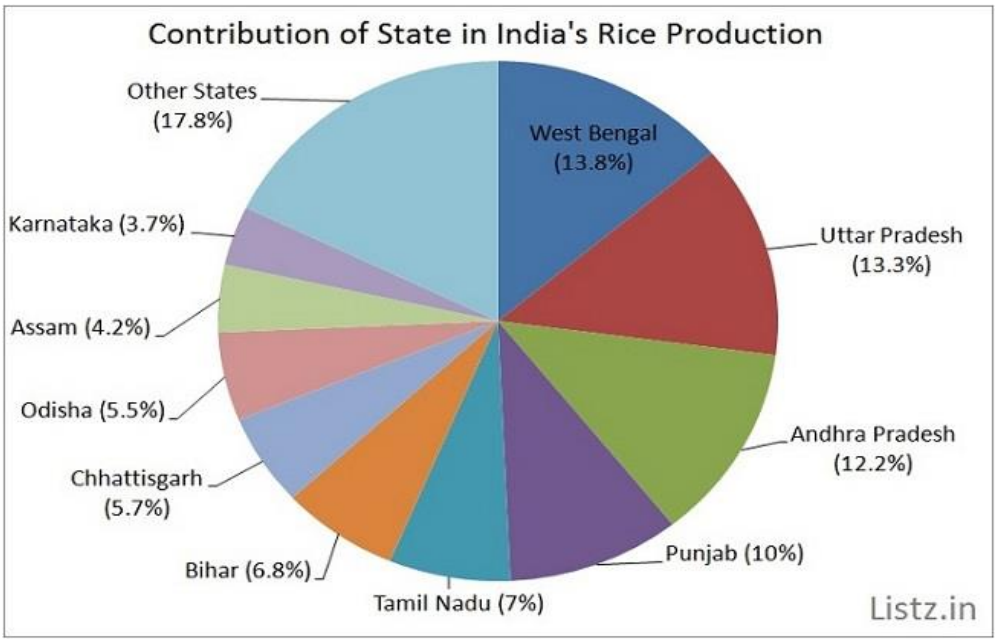


Figure 1. Contribution of State in India's Rice Production. Data collected from: www.wikipedia.com

Diseases

The geographical distribution and the seasonal development of any plant diseases is mostly influenced by the climatic condition, weather and the soil factors like humidity, moisture, temperature, pollutant, pH of the soil, light etc.(Jones, 1924).Severe damage can be caused to the rice plant by the pathogens that often may result in the reduced productivity of food grain. Some of the rice diseases commonly found in nurseries and in the field are given.

Table 2. Some common rice disease

Nursery diseases	**Main Field Diseases**
Blast – *Pyricularia grisea*	Brown Spot – *Helminthosporium oryzae*
Bacterial Leaf Blight – *Xanthomonas oryzae*	Sheath Rot – *Sarocladium oryzae*
Rice Tungro disease – *Rice tungro virus*	Sheath Blight – *Rhizoctonia solani*
	False Smut – *Ustilagonoidea virens*
	Grain discolouration – Fungal complex
	Leaf Streak – *Xanthomonas oryzae pv. oryzicola*

Data collected from:Crop Protection, agritech.tnau.ac.in.

Rice Brown Spot

Rice brown spot is the most aggressive and important rice disease in the world affecting millions of hectares of land every year (Chakrabarti, 2001; Padmanabhan, 1973; Savary *et al.*, 2000a; Zanao Junior *et al*., 2009). Brown spot prevails in almost every place where rice is grown especially in China (Singh, 2005). It can reach upto such a severe state in cool summer and soil deficiency of nitrogen that it may cause a loss in the yield and reduction in the weight of the kernel and the total number of grains the panicle bears (Mew and Gonzales, 2002).This conditions are usually associated with poor farmers with much less resources (Ou, 1985; Zadoks,1974).High humidity level (> 92.5%),leaf wetness and favourable temperature (24-30^{O} C) are favourable condition for disease development (Picco and Rodolfi, 2002).Spores can be transported to other parts of the same plant or other plants by wind and rain. Brown lesions formed in the leaf can reduce nutrient uptake and areas of photosynthesis which ultimately results in the decrease of tillering nodes. Brown spot of rice may be caused by a combined effect of physiological disorders, processes involved in the disease development and mechanisms for disease resistance which brings about a genuine questions regarding the involvement of different metabolic pathways their bases of genetics and interacting genes and cluster of genes (Igawa *et al*., 2005; Timmusk and Wagner, 1999).

Importance of Brown Spot

Two major epidemics has been associated with Brown spot in India: First in Krishna-Godawari delta 1918-1919 and second in today's India and Bangladesh in 1942 (Chakrabarti, 2001). The latter case is associated with Great Bengal Famine (Chakrabarti, 2001; Padmanabhan, 1973). It is a point to note that the disease epidemics and the famines caused are never simple as because the whole process is a combined effects of many consequences (Chakrabarti, 2001; Zadoks, 2008).

Rice brown spot is still reported widely from all across India (Reddy *et al*., 2010) and also more frequently in South and South- East Asian countries (Savery *et al*., 2000a) .It also causes losses in the crop yield upto a limit of 10% in whichever area it occurs (Savery *et al*., 2000b, 2006) especially in the lowlands of tropical and Sub-tropical Asia. This result proves Brown spot to be the strongest disease resulting in the decrease of yield of rice so far. Also as because nowadays the drought conditions all over the world is becoming very serious problems the indication of increase of severity

of brown spot disease has also been found(Savary *et al*., 2005) which is due to the variations in rainfall.

A rapid decrease in the grain yield due to brown spot has been reported which may vary from 4 to 29% (Bedi and Gill, 1960) 12 % (Aluko, 1975) about 8 to 23% (Fomba and Singh, 1990) and about 26 to 52% as reported by (Chakrabarti, 2001).The increase in the range of the later figures shows the effects in the yield losses due to the infection. Grains that are intensely infected are not at all ready for the human intake which almost explains the effect of brown spot on the Great Bengal Famine. Rice brown spot caused by *Drechslera oryzae* (Breda de Haan), Subram. And Jain has two distinct types of symptoms, first is leaf spot and the other one grain discolouration (Drechsler, 1934).

Host plant Resistance

In nearly all natural habitats, plants are surrounded by an massive number of potential enemies (biotic) and a variety of abiotic environmental stress. Roughly all ecosystems contain a wide variety of bacteria, viruses, fungi, nematodes, mites, insects, mammals and other herbivorous animals, significantly responsible for heavy diminution in crop yield. In natural systems, plants face a surplus of antagonists and thus posses a innumerable defence and have evolved multiple defence mechanisms by which they are able to deal with various kinds of biotic and abiotic stress according to (Ballhorn *et al*., 2009). Moreover antimicrobial nature, some of which are performed and some of which induced by infection. There are various other modes of defence including the building up of polymeric barriers to pathogen penetration and the synthesis of enzymes that degrade pathogen cell wall according to (Hammond *et al*., 1996). A very powerful and cost effective method to deal with Brown spot is development of disease resistance in plants. Despite of the severity of brown spot and its importance not much consciousness has been given towards this disease rather than leaf blast and bacterial blight (Savery *et al.*, 2011).

Various Source of resistance

It has been a very long period of continuous efforts regarding the search for different sources of resistance towards Brown spot (Chakrabarti, 2001; Nagai and Hara, 1930). Indentified fifteen different Oryza sativa entries out of one hundred and twenty four were classified as resistant (> 5% severity) according to Satija *et al.* (2005). Also

Hossain *et al*. (2004) identified one resistant variety out of 29 entries. After screening of upland rice germplasm which are exotic and indigenous to Eastern India has proved that several field genotypes has expressed partial and complete resistance to the brown spot pathogen (Shukla *et al.*, 1995). It appears that the sources of disease resistance is very few in case of *Oryza sativa* entries and as per recent studies such as Goel *et al.* (2006) search for other pools is recently being practised specially *Oryza nivara*.

Biological control of Brown spot

A very few research works has been done for the advancement of the use of bio control agents in suppression of brown spot. Moreover, there has been a rapid emergence of viable technology regarding the application of antagonistic microbes for the management of plant health in recent years. *Pseudomonas* and *Trichoderma* are the most commercially used antagonistic microbes that has the power for the reduction of the disease by direct effects on the pathogen such as antibiosis, mycoparasitism ,competition for iron or by the improvement of plant immunity system such as induced resistance (IR) (Singh *et al*., 2005).Many soil borne pathogens are suppressed by direct antagonism whereas Induced resistance is effective against broad range of foliar pathogens which includes both bacteria and fungi (Soresh *et al.*, 2010).

The use of fungicides to control the disease causes several unpleasant effects i.e. development of resistance in the pathogen, residual toxicity, pollution in the environment, high cost etc. Therefore, it has become essential to adopt eco friendly approaches for better crop health and for yield. According to (Mason and Mathew, 1996; Singh, 1994), the practical use of natural compounds as control agents is receiving increased attention and this is partly due to their non-toxicity to humans, their systemicity and biodegradability. Investigations on mechanisms of disease suppression by plant products have suggested that the active principles present in them may either act on the pathogen directly as per (Amadioha, 2000) or induce systemic resistance in host plants resulting in reduction of disease development according to (Narwal *et al*., 2000; Olivieri *et al*., 1996; Paul and Sharma, 2002; Schneider and Ullrich, 1994). Also (Hammerschmidt and Kuc, 1995) reported that, induction of plant's defence genes by prior application of inducing agents is called induced resistance When plants are treated with non-pathogenic or some less harmful pathogens [eg. rhizobacteria-induced systemic resistance (RISR)], it then go onto triggers the production of defence-related gene products according to (Harman *et al.*, 2004). The defense gene products include

peroxidise (PO), polyphenol oxidase (PPO) that catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexins and phenolics synthesis according to (Ramamoorthy *et al*., 2002) . So, these are the enzymes whose activity needs to be increased to prevent fungal diseases like brown spot and this can be done by application of various bioinoculants.

Plant growth promoting rhizobacteria (PGPR)

According to (Glick, 1995; Hallman *et al*., 1997; Rovira, 1965; Sturz *et al*., 2000; Welbaum *et al*., 2004), biocontrol agents are liable to interact with other disease management elements, especially host plant resistance, as well as plant growth related or abiotic stress related, genes: research leading to an perceptive of such interactions at the molecular level could in itself provide light on the physiology of environmental stresses, of disease and their interactions. There has been a huge amount of literature describing potential uses of plant associated bacteria as agents stimulating plant growth and managing soil and plant health .Plant growth-promoting bacteria (PGPB) according to (Saha *et al*., 1999) are associated with many plant species and are commonly present in many environments. The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) as reported by (Kloepper and Schroth, 1978) colonizing the root surfaces and the closely adhering soil boundary, the rhizosphere as per (Kloepper and Schroth, 1978; Kloepper *et al*., 1999). As reported by Kloepper *et al*. (1999) or, more recently, by Gray and Smith (Gray and Smith, 2005), some of these PGPR can also enter root interior and establish endophytic populations. Many of them are able to go beyond the endodermis barrier, crossing from the root cortex to the vascular system, and subsequently flourish as endophytes in stem, leaves, tubers, and other organs according to (Bell *et al*., 1995; Compant *et al*., 2005; Gray and Smith, 2005; Hallman *et al*., 1997).

According to (Gray and Smith, 2005; Hallman *et al*., 1997), the degree of endophytic colonization of host plant organs and tissues shows the ability of bacteria to selectively adapt to these specific ecological niches. Accordingly, close associations between bacteria and host plants can be formed as reported by (Compant *et al*., 2005; Hallman *et al*., 1997, Kloepper *et al*., 1999) without harming the plant as per (Hallman et al.,1997, Kloepper *et al*.,1992; Kloepper *et al*.,1999; Lodewyckx *et al*., 2002; Whipps, 2001). Even though, it is generally believed that several bacterial endophyte communities are the result of a colonizing process initiated in the root zone according to

(McInroy and Klopper, 1995; Sturz *et al*., 2000; Van Peer *et al*., 1990; Welbaum, 2004), they may also instigate from other source than the rhizosphere, such as the phyllosphere, the anthosphere, or the spermosphere according to (Hallman *et al*., 1997). Regardless of their different ecological niches, free-living rhizobacteria and endophytic bacteria use some of the same mechanisms to promote plant growth and control phytopathogens as reported by (Bloemberg *et al*., 2001; Dobbelaere *et al*., 2003; Glick, 1995; Hallman *et al*., 1997, Lodewyckx *et al*., 2002; Sturz *et al*., 2000).

The broadly recognized mechanisms of bio control mediated by PGPB are competition for an ecological niche or a substrate, production of inhibitory allele chemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens according to (Glick, 1995; Haas *et al*., 2000; Haas *et al*., 2002; Lugtenberg, 2001; Ryu *et al*., 2004a) and/or abiotic stresses (Mayak, 2004; Nowak and Shulaev, 2003). According to (Thomashow, 1996) despite their potential as low-input practical agents of plant protection, relevance of PGPB has been hampered by incompatible performance in field tests; this is usually attributed to their poor rhizosphere proficiency as reported by (Schroth, 1981 and Weller, 1988). Rhizosphere competence of biocontrol agents comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora according to (Lugtenberg and Dekkers, 1999; Parke, 1991; Weller, 1988; Whipps, 1997). Given the importance of rhizosphere competence as a precondition of effective biological control, understanding root-microbe communication as per (Bais *et al*., 2004), as affected by genetic according to (Kilic-Ekici and Yuen, 2004; Okubara *et al*., 2004) and environmental as per (Pettersson and Baath, 2004) determinants in spatial as per (Bais *et al*., 2004) and temporal as per (Ping and Boland, 2004) contexts, will significantly contribute to improve the efficacy of these biocontrol agents.

According to (Kerry, 2000; Ping and Boland, 2004; Ramamoorthy *et al*., 2001; Ryu *et al*., 2004b; Thomashow, 1996) biopriming plants with some PGPB can also endow with systemic resistance against a broad spectrum of plant pathogens. Diseases of fungal, bacterial, and viral origin and in some instances even damage caused by insects and nematodes can be reduced after application of PGPB. Certain bacteria trigger a phenomenon known as ISR phenotypically similar to systemic acquired resistance (SAR). SAR develops when plants successfully activate their defence

mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue as reported by (Van Loon, 1998). As SAR, ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPB does not cause evident symptoms on the host plant according to (Van Loon, 1999).

Plant growth promoting fungi (PGPF)

Free-living fungi such as *Trichoderma* spp. are common in soil and root ecosystems. Recent discoveries show that they are opportunistic, avirulent plant symbionts, as well as being parasites of other fungi. At least some strains establish robust and long-lasting colonizations of root surfaces and penetrate into the epidermis and a few cells below this level. They generate or liberate a diversity of compounds that induce localized or systemic resistance responses, and this explains their lack of pathogenicity to plants. These root–microorganism associations cause substantial changes to the plant proteome and metabolism. Plants are protected from numerous classes of plant pathogen by responses that are similar to systemic acquired resistance and rhizobacteria-induced systemic resistance. Root colonization by *Trichoderma* spp. also frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients.

The induction of resistance in plants by *Trichoderma* spp. has been inadequately studied compared with the responses that are induced by rhizobacteria, possibly because the *Trichoderma* research community has focused on factors that are connected with direct effects on other fungi, especially mycoparasitism and antibiosis. That was probably the first clear expression of induced resistance by *Trichoderma* (Bigirimana *et al.,* 1997*)* .They showed that treating soil with *Trichoderma harzianum* strain T-39 made leaves of bean plants resistant to diseases that are caused by the fungal 41 pathogens *B. cinerea* and *Colletotrichum lindemuthianum*, even though T-39 was present only on the roots and not on the foliage. The same group extended their findings from *B. cinerea* to other dicotyledonous plants reported by (De Meyer *et al*., 1998). Analogous studies have now been carried out with a wide range of plants, including both monocotyledons and dicotyledons, and with different *Trichoderma* species and strains. The ability of *T. harzianum* strain T-22 to induce systemic resistance is particularly notable to pathogens in maize as there are, so far no similar reports of

resistance being induced in this crop by any other root associated commensal or symbiotic microorganism. As a result, the capacity to induce resistance to a range of diseases — which are caused by diverse classes of plant pathogen (including fungi, bacteria and viruses) — in a variety of plants seems to be widespread in this fungal genus.

Constant, improper and non-discriminative use of chemicals is known to cause unwanted effects such as residual toxicity, development of pathogen resistance to fungicides, environmental pollution, health problems to humans and animals and increased expenditure for plant protection. As an alternative, plant pathologists have focused their attention to developing environmentally safe, long-lasting and effective biocontrol methods for the management of plant diseases. Among various fungal and bacterial biocontrol agents, *Trichoderma* spp. was most frequently used against various plant diseases. Research during the previous two decades has led to the possibility of biological control as an increasingly realistic option for rice disease management according to (Tsahouridou and Thanassoulopoulosh, 2002). This organism has been shown to be efficient for the control of brown spot disease and the increase of plant growth on rice according to (Harish *et al.*, 2007). Rice plants sprayed with spore suspension of *T. harzianum* obtained a significant reduction in the sternness of disease under greenhouse conditions as reported by (Abdel-Fataah *et al*., 2007). Also, these species are able to colonize the root surface and rhizosphere from the treated seeds, protecting them from fungal diseases and stimulate plant growth and productivity according to (Baker, 1988).

As reported by (Abdel-Fattah *et al*., 2007) direct foliar application of *T. harzianum* was also found to reduce the disease severity and appreciably improve grain yield, total grain carbohydrate and protein, in addition to a significant improvement in the total photosynthetic pigments in rice leaves .The use of *Trichoderma* spp., well-known mycoparasites, can help by improving nutrients uptake and mobilization, enhancing nitrogen use efficiency, promoting root growth and plant biomass, and improving tolerance to various physiological stresses, including soil salinity and drought through the reduction of oxidative damage that stresses cause according to (Harman, 2011; Shoresh *et al*., 2010). Use of these microbes could suppress disease through direct antagonism against the pathogen because imbalanced plant nutrition and drought stresses are predisposing factors for Brown Spot development. This effect

would be combined with improved plant nutrient supply and delayed onset of water shortage in plant tissues according to (Bae *et al*., 2009) varying plant physiology to the disadvantage of the pathogen.

Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizas are mutualistic associations which are formed in between the roots of 80 percent of terrestrial plant species and fungi belonging to the small phylum Glomeromycota (Schubler *et al.*, 2001). The word symbiosis is named after the Greek word "mycos" and "rhiza" which means "fungus-root" and it is probably the oldest and most widespread plant symbiosis on Earth. However, through the fossil records and phylogenetic evidence it shows that their existence is more than 459 million years as in accordance to (Smith and Read, 2008), indicating a selection advantage for both the partners. The fungi forming the arbuscular mycorrhiza (AMF) are obliging biotrophs requiring the host plant for the completion of their life cycle. The fungus colonizes the root cortex and forms intracellular structures called arbuscules (from the Latin "arbusculum", meaning bush or little tree) where the exchange of nutrients between the partners takes place. The extracellular hyphal network spreads widely into the surrounding soil, thereby reaching out of the nutrient depletion zone and improving the supply of inorganic nutrients, especially phosphate and nitrate (Smith *et al*., 2011). In return, receiving the photosynthates from the heterotrophic fungal partners or the host plant (Smith and Smith, 2011).

According to (Mohadevan *et al*., 1988) vesicular arbuscular mycorrhizal fungi forms symbiotic association with roots of most terrestrial plants including many agricultural crops. These are known to occur worldwide in a broad range of unlike environments from artic to tropic and occupy a wide range of ecological niches as per (Shrivastava *et al*., 1996). The role of VAM fungi in the improvement of crop plant is well recognized as reported by (Krishna and Bagyaraj, 1982; Katiyar *et al*., 1994; Rao *et al*., 1995). According to (Bagyaraj and Varma, 1995), VAM fungi are known to improve the nutrient status of the plants, increase growth and development protects plant against pathogen and gives fight to drought and salinity.Colonization by native AMF in rice plant has been reported earlier by (Maiti *et al*., 1995). Partial dependency of upland rice on native AMF for phosphorus acquisition has also been reported by earlier worker such as (Saha *et al*., 1999). The occurrence of VAM fungi at altering stages of growth of rice plants has been studied by (Dubey *et al*., 2008). In recent years,

the application of artificially produced inoculum of VAM fungi has increased its significance in the field of agriculture, horticulture and forestry. Application of mycorrhizal inoculum increased the soil nutrients and root colonization in rice plants as reported by earlier worker such as (Yeasmin *et al*., 2008).

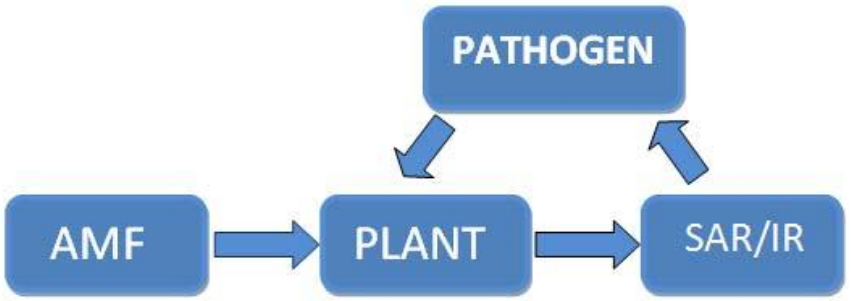
. The ancient mutualist and biotroph of plants, Arbuscular mycorrhizal fungi (AMF), has been found to improve the supply of water and nutrients, such as phosphate and nitrogen to its host plant. In its exchange, it takes a part of photosynthate sugar to complete its life cycle. Although having its own immune system, the plant upon pathogen attack gets weaken and needs reinforcement to fight back and become stabilize in the battle ground. AMF fulfils the need of host plant and provides with support in many ways by induction of attenuated defence signaling for fighting against phytopathogen. This increase not only makes plant more tolerant towards the attack of phytopathogen but also, enhances the genetic, biochemical and signaling factors responsible for its defence purpose.

According to (Kachroo and Kachroo, 2009), it is well known that the plants fights with phytopathogens by means of their own immune system (IS). These comprise physical and chemical barriers and several active mechanisms performed. IS is further divided into primar and secondary immune system. The primary immune system involves interaction of strain- specific avirulent (AVR) protein from the pathogen with a associated plant resistance (R) protein according to (Dangl and Jones, 2001). This in turn initiates systemic acquired resistance (SAR) in systemic tissues to provide with immunity against the secondary infections by related and unrelated pathogens according to (Durrant and Dong, 2004; Klessig *et al*., 2009). A further mode of secondary immunity, termed induced systemic resistance (ISR), is activated upon colonization of plant root by non-pathogenic rhizosphere microbes according to (Van Loon *et al*., 1998). Very often it has been observed that despite having their own defence system, plants require support to fight against phytopathogens. This may be due to slow response or low induction level of defence related factors. According to (Schubler *et al*., 2001), AMF have long been known as a dominant among symbionts of plants. They belong to the phylum, Glomeromycota and colonize 70–90% of land plant species according to (Smith and Read, 2008).

Although the colonization specificity does matter but not much, depending upon many factors including the genotype of the host plant as reported by (Koide and

Schreiner, 1992; Meghvansi *et al*., 2008). There are several benefits of AMF colonization in plants, mainly the increase in nutrient uptake according to (Smith and Read, 2008). In spite of this, still there is a doubt that the AMF has any direct involvement in the host's defence signaling against phytopathogens. Although, there are some indirect functions which donate to strengthen the plant defence responses including rise of plant nutrition as reported by (Smith and Read, 2008) and damage compensation. Moreover, it includes anatomical alterations in the root system according to (Wehner *et al*., 2010), microbial changes in the rhizosphere and enhancing the attenuated plant defence responses by altering the host's signaling pathways according to (Pozo and Azcon-Aguilar, 2007). Defense strategies by VAM are carried primarily through modulation in Jasmonic acid (JA) and salicylic acid (SA) dependent pathways (Pozo and Azcon-Aguilar, 2007). AMF is also reported to play a significant role in inducing the hydrolytic enzymes in defense response (Pozo *et al*., 1999), increased levels of pathogenesis- related (PR) proteins, various phytoalexins (Harrison and Dixon, 1993; Morandi, 1996; Larose *et al*., 2002), accumulation of callose (Cordier *et al*., 1998) and generation of reactive oxygen species (Salzer *et al*., 1999). Therefore, several records have been discovered which potrays the ability of AMF in controling and reducing the severity and incidence of phytopathogens for a longer duration. Also it is a well known fact that the knowledge reagarding the mechanism behind it is very little.

Interaction of AMF induced plant defence responses (SAR/IR) with the pathogen



Colonization of the root system by arbuscular mycorrhizal fungi (AMF) can improve plant resistance as well as tolerance to biotic stresses. Moreover this bioprotection has been adequately described in different plant systems, but the mechanisms still remains mainly unknown. Moreover, experimental evidences on mechanisms such as improved plant nutrition and competition, supports the association

of plant defence mechanisms in the observed protection. During the establishment of the mycorrhiza, inflection of plant defence responses occurs in response to the detection of the AM Fungi in order to attain a functional symbiosis. As a result of this inflection, a mild but effective establishment of the response of plant immunity might take place not only locally but systemically also. This establishment provides a protective state of the plant that allows a more effective establishment in response to the danger by the harmful enemies.

The basis of the success behind the interaction that has taken place during the course of evolution is the mutual benefits, which is however ensured through a strictly bidirectional control of the mutualism (Kiers *et al*., 2011). With regards to the plant, this guideline shows vital alteration in both the primary and the secondary metabolism in result regulating the defense mechanism in the plants (Harrison, 1999; Hause and Fester, 2005). However these alterations have n immense impact on the physiology of the plant that alternates the capacity of the plant to handle the stresses. Earlier works on mycorrhizas have shown the increase in the growth as well as the yield of the mycorrhizal plants, which might be considered mostly due to the increased levels of nutrition of the plant (Linderman, 1994). Also, in the later times many other researchers showed an extremely better tolerance of the mycorrhizal plants to abiotic stresses, such as salinity, presence of the heavy metals, or drought (Miransari, 2010; Smith *et al*., 2010). It has also been proved that the mycorrhizal plants provide a better resistance to the soil borne fungal as well as the bacterial pathogens, nematodes, or root- chewing insects (Azcón-Aguilar and Barea, 1997; Whipps, 2004).In the last few years it has

also been recorded that the mycorrhizal plants can develop induced resistance in response to the shoot pathogens(Pozo and Azcón-Aguilar, 2007; Koricheva *et al*., 2009; Campos Soriano *et al*., 2012).

The persistence of mycorrhizas during the course of evolution can be considered as one of the important phenomenon for the need of assistance in overcoming stressfull conditions, even in the plant systems where the symbiosis dosenot provide any growth benefits(Newsham *et al*., 1995).As because of the properties such as of biofertilizer and its bioprotectivity mycorrhizal symbiosis has proved to become a subject of main focus for researchers as an alternative to the chemical fertilizers and also pesticides with regard to sustainable agriculture(Harrier and Watson, 2004; Mukerji and Ciancio, 2007; Fester and Sawers, 2011).

Despite the obvious benefits of an improved nutritional status for stress tolerance/ resistance, mineral supply experiments have shown that the protective effect observed in mycorrhizal plants cannot be attributed to improved nutritional status alone (Fritz *et al*., 2006; Liu *et al*., 2007). AM associations bring about significant changes in the host plant and its environment: at the rhizosphere level, they influence soil structure, carbon deposition in soil, and microbial diversity, in part through changes in root exudation. These shifts in the microbial communities of the rhizosphere may indirectly influence the out-come of plant interactions with other organisms, including pathogens and beneficial microbes (Berta *et al*., 2002; Barea *et al*., 2005; Artursson *et al*., 2006; Lendzemo *et al*., 2007; Cipollini *et al*., 2012; Effmert *et al*., 2012). Apart from the changes in the rhizosphere, multiple modifications also occur within the host plant.

In the roots, changes in architecture, alterations of the metabolic profile, and accumulation of certain defence compounds may occur (García Garrido and Ocampo, 2002; Strack *et al*., 2003; Hause *et al*., 2007; Schliemann *et al*., 2008; Péret *et al*., 2009; López Ráez *et al*., 2010 a, b). For example, the accumulation of apocarotenoids (cyclohexenone and mycorradicin derivatives) can be observed in mycorrhizal roots, which are the main component of the yellow pigment found in many plant species upon colonization by AMF and have been proposed to play a role in control of the degree of colonization and mycorrhizal functionality (Strack *et al*., 2003; Strack and Fester, 2006; Flob *et al*., 2008; Schliemann *et al*., 2008).Qualitative and quantitative changes in flavonoid contents have been observed, the changes depending on the host plant, AMF, and developmental stage of the symbiosis (Vierheilig and Piché, 2002; Akiyama *et al*., 2002). Changes in phenolic compounds, defence-related phytohormones, and reactive oxygen species also have been reported (Fester and Hause, 2005; López-Ráez *et al*., 2010a, b). Noteworthy, the symbiosis also has a considerable impact on the aerial parts of mycorrhizal plants, some of the reported changes being related to defense or stress tolerance (Liu *et al*., 2007; Kaschuk *et al*., 2009; Fiorilli *et al*., 2009; Pozo *et al*., 2009; Fester *et al*., 2011; Aloui *et al*., 2011).

As for the higher resistance to pests and pathogens of AMF-colonized plants, observations of systemic protection against pathogens in non-colonized root fragments from mycorrhizal plants and enhanced resistance of the aerial parts to certain attackers have pointed out the involvement of plant defense mechanisms (Cordier *et al*., 1998; Pozo *et al*., 2002; Pozo and Azcón-Aguilar, 2007). Defence mechanisms are

coordinated by the plant immune system, strikingly similar in some aspects to the innate immune system in animals (Ausubel, 2005). This system allows the plant to distinguish non-self-alien organisms by recognizing structurally conserved microbe-associated molecules, such as flagellin, lipopolysaccharides, or peptidoglycans, which are collectively, termed microbe-associated molecular patterns (MAMPs, or PAMPs in the case of pathogens). PAMPs are recognized by transmembrane pattern recognition receptors (PRRs), which leads to the induction of the appropriate responses in the host and to PAMP-triggered immunity (PTI) (Ausubel, 2005; Jones and Dangl, 2006; Boller and He, 2009; Thomma *et al*., 2011).

In an evolutionary “arms race," microbes have evolved effector proteins that are secreted into the host and suppress PTI, thus allowing successful host colonization by the pathogen, thus causing effector-triggered susceptibility of the plant to the disease. In some cases, intracellular proteins of the plant recognize pathogen effectors or their modified target proteins and activate immune responses that are quicker, more prolonged, and more robust than those in PTI, resulting in effector-triggered immunity (ETI) (Jones and Dangl, 2006; Boller and He, 2009; Thomma *et al*., 2011). Plant defense responses are coordinated by small molecules that act as signal transducers and tailor the coordinated expression of genes that code for defence-related proteins and compounds (Ausubel, 2005; Jones and Dangl, 2006). Among these molecules, the phytohormones jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and ethylene (ET) play key roles (Pieterse *et al*., 2009). According to the challenger lifestyle, one signalling pathway will prevail over the others.

It is generally assumed that the SA-dependent pathway regulates responses such as programmed cell death, effective against biotrophic organisms, and the JA-dependent pathway regulates responses to necrotrophs and chewing insects (Glazebrook, 2005). However, these hormone signalling pathways do not act independently, but influence each other through a complex network of regulatory interactions, JA and SA pathways in general being mutually antagonistic (Pieterse *et al*., 2009). As biotrophs, mycorrhizal fungi share some similarities with biotrophic pathogens, and are able to trigger plant defence responses at initial stages (Paszkowski, 2006).

Thus, for a successful colonization, the fungus has to cope with these reactions and actively modulate plant responses. We have proposed that this modulation may result in pre-conditioning of the tissues for efficient activation of plant defences upon a

challenger attack, a phenomenon that is called priming (Pozo and Azcón-Aguilar, 2007). Priming sets the plant in an "alert" state in which defences are not actively expressed but in which the response to an attack occurs faster and/ or stronger compared to plants not previously exposed to the priming stimulus, efficiently increasing plant resistance. Thus, priming confers important fitness benefits (Conrath *et al*., 2006; Van Hulten *et al*., 2006; Walters and Heil, 2007).

In the past decade, many priming causing agents have been identified. It has been observed that some chemicals that induce stress responses in plants also induce priming when applied at lower doses, and several fungicides have been shown to prime defenses in treated plants in addition to their primary antifungal activity (Conrath *et al*., 2006; Beckers and Conrath, 2007). Other well-studied examples of priming by chemicals include increased resistance to downy mildew in Arabidopsis thaliana after treatment with the non-protein amino acid Baminobutyric acid (BABA), as well as primed defence responses in tomato and Arabidopsis pre-treated with hexanoic acid and subsequently infected with grey mold (Ton *et al*., 2005; Vicedo *et al*., 2009; Kravchuk *et al*.,2011). Remarkably, priming events occur as a result of inter individual or even inter-species communication. For example, green leaf volatiles released by wounded or infested plants are also able to induce a more efficient activation of defences in neighbouring plants upon subsequent attacks (Kessler *et al*., 2006; Ton *et al*., 2007; Yi *et al*., 2009).

In Arabidopsis seedlings exposed to volatile blends from two Bacillus species, the disease severity caused by a bacterial pathogen was significantly reduced (Ryu *et al*., 2004). Moreover, priming seems to be the mechanism underlying the Induced Systemic Resistance (ISR) observed in plants interacting with beneficial microorganisms (Conrath *et al*., 2006; Goellner and Conrath, 2008; Van Wees *et al*., 2008). Interestingly, priming of the plant immune responses by beneficial microbes is often dependent on a functional JA signaling pathway, as has been described for rhizobacteria and AMF (Verhagen *et al*., 2004; Pozo *et al*. 2004, 2010; van der Ent *et al*., 2009). The molecular mechanisms behind priming of plant defences and its biological relevance in plant resistance are now being uncovered (Pastor *et al*., 2012), and evidence for trans-generational effects of priming have been a major advance in plant research (Luna *et al*., 2012; Rasmann *et al*., 2012; Slaughter *et al*., 2012). Here, we give a summary of the impact of the arbuscular mycorrhizal symbiosis on plant

interactions with other organisms. We give special emphasis to the spectrum of protection against deleterious organisms (Mycorrhiza-Induced Resistance, MIR) and provide an overview of the underlying mechanisms, focusing on the priming of plant defences associated with mycorrhization.

Systemic protection by a mycorrhizal association can even be observed in the aerial parts of a colonized plant, but in contrast to below-ground interactions, reports on AM effects on pests and pathogens attacking shoots are less studied, and the outcome of the interaction is more variable. Early studies described a higher susceptibility of AM plants to viruses, and biotrophic pathogens appear to thrive better on mycorrhizal plants, although an increased tolerance has been observed in terms of plant mass and yield (Gernns *et al*., 2001; Whipps, 2004). Concerning hemibiotrophs, the impact of the symbiosis varies from no effect to reduction of the disease, for example, against *Colletotrichum orbiculare* in cucumber (Lee *et al*., 2005; Chandanie *et al*., 2006). However, pathogens with a necrotrophic lifestyle are hampered in their proliferation, and symptom development is less severe on mycorrhizal plants. Examples are the fungi *Alternaria solani* in tomato (Fritz *et al*., 2006; dela Noval *et al*., 2007), *Magnaporthe grisea* in rice (Campos-Soriano *et al*., 2012), and *Botrytis cinerea* in roses and tomato (Moller *et al*., 2009; Pozo *et al*., 2010).

A functional mycorrhizal association requires a high degree of coordination between both partners. The fungus has to deal with the plant's immune system, contend with the defence mechanisms and overcome them for successful colonization of the host (Kloppholz *et al*., 2011; Zamioudis and Pieterse, 2012). Once established, the plant has to regulate the level of fungal proliferation within the roots to prevent excessive colonization and carbon drainage, thus maintaining the interaction at mutualistic levels. For example, under conditions of high exogenous phosphate supply, the plant actively inhibits proliferation of the fungus within the roots (Breuillin *et al*., 2010). Similarly, plants possess a feedback system that prevents excessive colonization over a critical threshold, a phenomenon termed autoregulation of the symbiosis, described initially in the rhizobium-legume symbioses (Vierheilig, 2004; Vierheilig *et al*., 2008). Mechanistic similarities between the autoregulation of mycorrhization and nodulation and the induction of systemic resistance by beneficial microbes have been pointed out (Vierheilig *et al*., 2008; Zamioudis and Pieterse, 2012). In summary, from presymbiotic stages and throughout a well-established AM association, plant defence mechanisms are

tightly regulated to control the symbiosis. As a side effect, this regulation may directly impact root pathogens.

During the early stages of the interaction, the plant reacts to the presence of AM fungi by activating some defense-related responses that are subsequently suppressed (García-Garrido and Ocampo, 2002; Liu *et al*., 2003). Before penetration of the roots, the fungus seems to trigger the plant's immune system as a biotrophic pathogen would (Guimil *et al*., 2005; Paszkowski, 2006). In response to colonization by AMF, a quick but transient increase of endogenous salicylic acid (SA) occurs in the roots with a concurrent accumulation of defensive compounds, such as reactive oxygen species, specific isoforms of hydrolytic enzymes, and the activation of the phenylpropanoid pathway (Pozo *et al*., 1998; Blilou *et al*., 1999; Dumas-Gaudot 2000; Fester and Hause, 2005; de Román *et al*., 2011). These reactions are temporally and spatially limited compared to the reaction during plant-pathogen interactions, suggesting a role in the establishment or control of the symbiosis (Dumas-Gaudot *et al*., 1996; García-Garrido and Ocampo, 2002). Indeed, SA signaling seems to have a negative effect on AM colonization (de Román *et al*., 2011; Herrera-Medinaetal., 2003), and AM establishment requires inhibition of certain SA-regulated responses (Dumas-Gaudot, 2000) as described for other mutualistic symbiosis (Soto *et al*., 2009).

Defence strategies of plant

Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR)

Almost all the plant when attacked either by a herbivores or by any sort of pathogens locally would react with the production of compounds that would either reduce or inhibit further attack or activity of the pathogens. Local response generally occurs in the plant organ where it is originally attacked and also in the organs at far distance and unaffected ones generally known as systemic resistance. One of these type of resistance used by the plants as defence strategies against the pathogens is Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR).(Hunt *et al*., 1996; Schneider *et al*., 1996; Sticher *et al*.,1997; Mauch-Mani and Metraux, 1998; Hammerschmidt, 1999a).

SAR is an induced defence mechanism providing a long-lasting protection against a huge range of microorganisms. The signalling molecule Salicylic Acid is required in association with pathogenesis related protein which also helps in the

contribution of resistance in plants. In accordance to Vallad and Goodman (2004) who had worked both on positive and negative responses and also in future improvement in the utility of both chemical and biological elicitors of induced resistance in agricultural use along with the incorporation of the potentiality to use genetic variation within the crop species population for the better utilization of SAR and ISR in the field.

Later it was discovered that the isochorismate pathway is the important sourse of Salicylic acid during SAR which was done using the *Arabidopsis* as plant model. The positive regulator protein NPR1, in response to the SA, moves towards the nucleus where it interacts with TGA transcription factors as a result inducing the expression of defence genes ultimately activating SAR. It has been suggested by Durrant and Dong (2004) that the mobile signal for SAR might be a lipid molecule. The search for the mobile signal that travels through the phloem from the site of infection for the establishment of systemic immunity has been going on for a long time. In the past few years several candidate signalling molecules have been emerged which includes the methylated derivatives of well known defence hormone (methyl salicylate), jasmonic acid, glycerolipid derived factor and a group of peptides which is involved in cell to cell basal defence signalling. Signal amplification of systemic SAR appears increasing on parallel salicylic acid dependent defence responses in fine tuning with auxin (Volt *et al.,* 2008).

A very important role is played by Salicylic Acid (SA) (Raskin, 1992) in the signalling pathway that leads to ISR (Mauch-Mani and Metraux, 1998; Cameron, 2000; Metraux, 2001). The endogenous levels of SA in the phloem increase both locally and systemically after the infection before the ISR occurs (Malamy *et al*.,1990; Metraux *et al*., 1990; Rasmussen *et al*., 1991).SA is seen to be produced in response to the infection both locally and systemically therefore *de novo* production of SA in plant parts not infected might however contribute in the systemic expression of ISR (Meuwly *et al.,* 1995).The SA levels is positively correlated with the level of resistance of plants that exhibit constitutive expression of SA which has been proved to be true for natural cultivars of rice (Silvermann *et al.*,1995), and for within plant differences in SA levels in potato (Coquoz *et al*.,1995), and also for arabidopsis plant that can express a noble hybrid enzyme salicylate synthase (SAS) activity so have a increase in the level of SA (Mauch *et al*., 2001).One important experiments showing the role of SA in one or the other forms of the ISR have bought into utilization transgenic plants that express the

bacterial *nahG* gene encoding for naphthalene hydroxylase G. Therefore such types of plants fails to accumulate SA and a blockage occurs in their response of their ISR(Delaney *et al*., 1994; Gaffney *et al*., 1994).

Several experiments done using the combination of nahG and the wild type of shoots grafted onto the nahG and also the wild type plants revealed the elicitation of ISR in the tissue of wild types even when the nahG transformed parts of the plant received the inducing infection, which ultimately suggests that the signal that emanates from the inducing tissue is not SA (Vernooij *et al*., 1994).These nahG plants might suffer defects which is yet to be discovered (Cameron, 2000).According to Rasmussen *et al*.(1991) time duration in the process of induction and the accumulation of SA within the phloem in combination with experiments involving removal of leaf were not l all consistent with SA being the first systemic signal in cucumber. All these evidence throws light upon the understanding that the SA and other systemic signals are involved in the signalling of ISR (Sticher *et al*., 1997).

Moreover local responses in the cell surrounding may include changes in the composition of the cell wall that can ultimately result in the inhibition of entry of the harmful pathogens, and *de novo* synthesis of antimicrobial compounds such as pathogenesis related (PR) proteins and phytoalexins (Kuc, 1995; Hammerschmidt, 1999b). Phytoalexins are mainly associated to the local response whereas the occurrence of the PR proteins is mainly associated with both local and systemic response.

Phytoalexins

Phytoalexins were first conceived as antifungal substances produced by cells of potato tubers as they underwent hypersensitivity to penetration by infection hyphae of incompatible races of *Phytophthora infestans* (Mont.) de Bary (Muller and Borger, 1941). The hypothetical phytoalexins were suggested to prevent further growth of the hyphae in these cells and also to confer cross-protection against infection by compatible races inoculated at the same time or some hours later. Although no chemical entities were isolated from potato at this time, Muller & Borger suggested that phytoalexins were non-specific in their effect on fungi, and that a major difference between hypersensitive and susceptible varieties of plant was the speed of formation of phytoalexins in response to infection. Thus it was considered that the speed of this

reaction was dependent upon the sensitivity of the host cell to the fungus attempting infection. The precise meaning attached to this word 'speed' was not clear in these writings, but it is of current interest as revealed by studies on phaseollin accumulation in beans to be discussed later.

Muller (1958) thought that phytoalexins as envisaged in his earlier work with potato might be of general occurrence in plants. He attempted to demonstrate that such substances formed in bean (*Phaseolus vulgaris* L.) as it underwent hyper- sensitivity in response to attempted infection by the fruit pathogen *Sclerotinia fructicola* (Wint.) Rehm. Droplets of spore suspension were placed in the cavities of opened bean pods from which the seeds had been removed. The spores were observed to germinate, and to cause brown flecks in the underlying tissue within 24 h. Host damage was indicated by uptake of the stain rhodamine B and also by a decrease of pH in affected cells. Infection droplets were collected, combined, made spore-free by centrifugation and tested for their effects on other spores in vitro. They became increasingly antifungal after incubation in seed cavities for 14 h and completely fungistatic after 24 h. Control droplets of water became highly stimulatory to test fungi after incubation in pods. The antifungal principle in the fungi static infection droplets was removed by partition with petroleum spirit leaving a highly stimulatory water-phase. The properties of the anti-fungal fraction suggested that a distinct chemical entity had been extracted as a phytoalexin.

Plants are constantly attacked by many potential pathogens and respond by the activation of defence genes, the formation of reactive oxygen species (ROS), and the synthesis of pathogenesis-related (PR) proteins, localized cell-wall reinforcement and the production of antimicrobial compounds. Low molecular mass secondary metabolites with antimicrobial activity that are induced by stress are collectively named phytoalexin, and are an important part of the plant defence repertoire (Hammerschmidt, 1999b; Pedras *et al*., 2011). Phytoalexins are a heterogeneous group of compounds (Shinbo *et al*., 2006; Schmelz *et al*., 2011; Huffaker *et al*.,2011) that show biological activity towards a variety of pathogens and are considered as molecular markers of disease resistance. The concept of phytoalexins was introduced over 75 years ago (Muller and Borger, 1940) based on the finding that potato (*Solanum tuberosum*) tuber tissue that had previously been infected with an incompatible race of *Phytophthora infestans* induced resistance to a compatible race of *P. infestans*. It was hypothesized

that the tuber tissue, in response to the incompatible interaction, produced substances (phytoalexins) that inhibited the pathogen and protected the tissue against later infection by other compatible races of the pathogen (Coleman *et al*., 2011). Since then, the field has evolved extensively, not only with respect to studying the roles of phytoalexins in defence against pathogens and pests, but also with respect to their health-promoting effects (Boue *et al*., 2009; Ng *et al*., 2011; Smoliga *et al*. ,2011; Holland, *et al*.,2010; Jahangir *et al*.,2009; Yang *et al*. 2009). For example, indole phytoalexins contribute to the antioxidant, anticarcinogenic and cardiovascular protective activities of Brassica vegetables (Jahangir *et al*. 2009). Peanut (*Arachis hypogea*) phytoalexins have antidiabetic, anticancer and vasodilator effects .The biological activities of glyceollin, a soybean (*Glycine max*) phytoalexin, include antiproliferative and antitumor actions (Ng *et al*., 2011). The sorghum (*Sorghum bicolor*) phytoalexins, 3-deoxyanthocyanins, might be useful in helping to reduceincidence of gastrointestinal cancer (Yang *et al*., 2009). The phytoalexin resveratrol from grapevine (*Vitis vinifera*) has anti-aging, anticarcinogenic, anti-inflammatory and antioxidant propertiesthat might be relevant to chronic diseases and/or longevity in humans (Smoliga *et al*., 2011).Phytoalexins accumulate at infection sites and they inhibit the growth of fungi and bacteria in vitro therefore, it is logical to consider them as possible plant-defence compounds against diseases caused by fungi and bacteria. There is a evidence of phytoalexin being taken role in plant defence. Concerning the accumulation of pisatin in pea and phaseollin in green bean, it was apparent that the phytoalexins accumulated to fungitoxic concentrations not only in inoculum droplets placed on opened pea or bean pods but also in the tissues immediately below the inoculum droplets (Cruickshank and Perrin, 1968) . These data supported a role for phytoalexins in plant disease resistance, but there were and still are exceptions. There are also examples that phytoalexins accumulated during compatible plant-pathogen interactions. These include the induction of pisatin by the virulent Oomycete *Aphanomyces eutiches (*Pueppke and Van Etten, 1976) and by the pathogenic strains of the fungus *Nectria hematococca* and induction of spirobrassinin by virulent races of *Leptosphaeria maculans* (Pedras and Seguin-Swartz, 1992). Similarly Glazebrook and Ausubel (1994) reported that the virulent pathogen *Pseudomonas syringae pv. maculicola* elicits the synthesis of high levels of camalexin in *Arabidopsis thaliana*. Mert-Türk *et al*. (1998) also showed that camalexin accumulated during both compatible and incompatible interaction in *A. thaliana* when challenged with an Oomycete, *Peronospora parasitica*. If the results

exemplified above are interpreted, in incompatible interactions, phytoalexin accumulation limits or stops pathogen growth, thereby conferring resistance to the plant. In compatible interactions, the pathogen apparently, tolerates the accumulated phytoalexins, detoxifies them, suppresses phytoalexin accumulation, and/or avoids eliciting phytoalexin production (Mansfield, 1982). To develop disease protection strategies, plant pathogen research in the field of phytoalexins has also focused on interpreting their biosynthesis pathways and regulation in different crop plants by using different cultivars, transgenic plants and mutants, and by applying -omics, molecular biology and biochemical approaches.

Plant defence reactions against pathogens, including fungi, bacteria, and viruses, involve induced synthesis of low molecular weight compounds called phytoalexins. Biotic elicitors that are derived from the cell surface of pathogenic microbes as well as host plants trigger the defence response. lt is considered that an elicitor molecule combines with a plant membrane receptor (Cosio *et al*., 1992; Shibuya *et al*., 1993) and that the complex activates a series of specific genes, resulting in the synthesis of phytoalexins (Brooks and Watson, 1991). It has been suggested that JA could be an integral part of a general signal transduction system regulating inducible defence genes in plants (Farmer and Ryan, 1992; Gundlach *et al*., 1992). In suspension-cultured rice (Oryza *sativa* L.) cells, treatment with an elicitor (N-acetylchitoheptaose) induces production of phytoalexins (Yamada *et al*., 1993). In this report, we present evidence that JA is a key signal transducer between recognition of N-acetylchitoheptaose and the production of a phytoalexin, momilactone A, in the rice cells.

Hypersensitive reaction or response (HR) is the programmed cell death of infected plant cells to restrict pathogen invasion to the initially infected regions and is included in the resistance (*R*) gene–mediated resistance to pathogens. Upon HR, the biosynthesis of low molecular-weight defence signal compounds, such as salicylic acid, jasmonic acid, and ethylene (De Laat *et al*., 1983; Iwai *et al*., 2006; Malamy *et al*., 1990; Seo *et al*., 2001), is induced for the signal transductions of disease resistance. As well as these signal compounds, antifungal molecules called phytoalexins (PA),which are also low molecular-weight compounds produced in host plants in response to parasite infection (Muller and Borger ,1940) or stress (Kuc, 1995), are induced in many plant-microbe interactions. Although the roles for PA in disease resistance were proposed (Kuc, 1995), structures of the PA were found to be diverse, including

flavonoid, isofla- vonoid, diterpenoid, sesquiterpenoid, and indole (Grayer and Kokubun, 2001), and precise studies to reveal the biological significance of each PA for resistance in individual experimental system shave been required.

Pathogen-induced defence mechanisms in higher plants may involve de novo synthesis of antifungal compounds, known as phytoalexins, which play an important role in the disease resistance of various plant species. Plants that are attacked by pathogenic microorganisms respond with a variety of defence reactions. One such reaction is the production of secondary metabolites that serve as plant antibiotics, known as phytoalexins, which are generated through the perception of signal molecules called elicitors, which are mostly derived from pathogens. More than 300 structures of phytoalexins were isolated from approximately 900 plant species, including the rice phytoalexins momilactones A and B,oryzalexins A through F4–and S, phytocassanes A through E (diterpenes), and sakuranetine (a flavanone).Umemura *et al*.(2003) reported that phytocassanes play an important role in disease responses of rice plants.

Elicitors have the property of inducing the production of phytoalexins in rice plants, as well as to agents for controlling rice diseases. More particularly, the present investigation relates to evaluation of phytoalexins characterized in rice plants which are cultivated for tests. Phytoalexins synthesized in the rice plant bodies are extracted so as to screen the property of elicitors of inducing the production of phytoalexins such as phytocassanes and rnornilactones and the like in rice plants. Because such phytoalexins have potent antimicrobial activity against causal organisms of rice plant diseases such as rice blast fungus (*Magnaporthe grisea*, previously designated as *Pyricularia oryzae*), rice sheath blight fungus (*Rhizoctonia solani*), and rice brown spot fungus (*Drechslera oryzae*) elicitors having the property of inducing the production of phytoalexins in rice plants Would be useful as active ingredients in agents for controlling rice diseases.

Fifteen phytoalexin compounds have been identified in suspension-cultured rice cells treated with biotic elicitors such as a chitin oligosaccharide or a cerebroside (Kuc,1995 and Harborne, 1999) and/or from rice leaves that were either infected with the rice leaf blast pathogen Magnaporthe grisea or exposed to UV irradiation (Cartwright *et al*.,1977; Akatsuka *et al*.,1983; Kono *et al*.,1984; Sekido *et al*.,1986; Kato *et al*.,1993; Kodama *et al*.,1992; Koga *et al*.,1995; Koga *et al*.,1997). With the exception of the flavonoid sakuranetin, all of these rice phytoalexins are diterpenoids. These compounds have been classified into four structurally distinct types of polycyclic

diterpenoid phytoalexins based on the structures of their diterpene hydrocarbon precursors: phytocassanes A to E, oryzalexins A to F, momilactones A and B, and oryzalexin S (Kodama *et al*., 1992). The common precursor geranylgeranyl diphosphate is cyclized to ent-copalyl diphosphate (ent-CDP) and then to ent-cassa-12, 15-diene and ent sandaracopimaradiene, leading to phytocassanes A to E and oryzalexins A to F, respectively. Geranylgeranyl diphosphate is also cyclized to syn-CDP and then to 9 _H-pimara-7,15-diene and stemar-13ene, leading to momilactones A and B and oryzalexin S, respectively. Phytocassane A to D are produced upon elicitation by fungal pathogen *Magnaporthe grisea* and isolated from rice stems infected with *Rhizoctonia solani* (Koga *et al*., 1995). Phytocassane E, which is induced by the potato pathogen *Phytophthora infestans* also shows antifungal property against *Magnaporthe grisea* (Koga *et al*., 1997). These studies indicated that the antifungal activities of phytocassane B, C and E (EC50 values, 4–7 μg/mL) are about four-folds stronger than the activities of phytocassane A and D. This higher antifungal activity was attributed to the β-hydroxyl group in C-1 position of phytocassane B, C and E, which can form intra molecular hydrogen bond with the carbonyl group in position C-11 (Koga *et al*., 1997).

PR- proteins

A mechanism of plants defence from pathogen attack is induced resistance (Metraux *et al*., 2002). During induced resistance, a biological or a chemical stimulus is recognised by the plant setting in motion a signal transduction pathway that activates resistance responses, such as programmed cell death and defence gene expression (Edreva, 2004). Induced Systemic Resistance (ISR) is a type of induced resistance which is produced in roots by nonpathogens, like the very important soil-borne plant growth promoting rhizobacteria (PGPR) and beneficial soil-borne plant growth-promoting fungi (PGPF), and is transmitted through the signal molecules of ethylene (ET) and jasmonic acid (JA) (Vallad and Goodman, 2004). In monocotyledonous plants, transcripts related to ET and/or JA signalling pathways have been induced or primed after treatment with beneficial fungi, bacteria band insects associated with ISR (Kanno *et al*., 2005; Muyanga *et al*., 2005; Djonovic *et al*., 2007).

According to van Loon (1999) the PR proteins were originally suggested to have been defined and also detected its accumulation in huge amounts only after infection and absent in healthy plants (van Loon and van Kammen, 1970).They have now been discovered in more than 40 species belonging to 13 families. Two distinct groups of PR

proteins have been recorded. Prevalent in the intercellular spaces are the acidic PR proteins and basic PR proteins that are more or less functionally similar but have different molecular weights as well as the amino acid sequences located mainly in the intracellular vacuoles (Legrand *et al.,* 1987; Niki *et al.,* 1998; van Loon 1999).

Some of the PR proteins have also been reported to have Chitinase (Legrand *et al.,* 1987) or β 1, 3-glucanase activity. Chitinase are basically structurally and functionally different group of enzymes having the capacity to hydrolyse the chitin and many of them are believed to play a significant role in the defence of plant against several fungal pathogens (Sahai and Manocha, 1993; Jackson and Taylor, 1996). Chitinase also shows effective antifungal activities (Schlumbaum *et.al*., 1986) and over-expression of chitinase in the plants shows decrease in the susceptibility towards the infection by the fungus with chitin- containing cell wall (Broglie *et.al*., 1991; Datta and Datta, 1999). In contrary to this the function of many other PR protein is still not known (van Loon and van Strein, 1999) and many PR proteins can be functionally active only when it is combined. Specifically the basic PR proteins are also expressed in specific tissue and in a controlled manner throughout the development as for example during leaf senescence (van Loon, 1999).Expression of specific defence related genes such as *PR-1* and *β-glucanase* 2 often used as ISR markers can be uncoupled from phenotypic pathogen resistance(Greenberg *et.al*., 2000) which ultimately indicates that these compounds are not necessary for an effective resistant phenotype.PR proteins are normally used as ISR markers but yet it has not until been reported any antiviral or antibacterial activity.

Chapter 3
MATERIALS AND METHODS

3.1. Plant material

3.1.1. Collection of seeds

Rice seeds were collected from different regions of West Bengal and Sikkim. Brimful, Champasari and Black Nuniya from Bijanbari (27° 02'N 88° 07'E/ 27.04° N 88). Kaberi 9090, Loknath 505 and Gouraknath 509 from Indo-Japan hybrid seed centre, Siliguri (26.7100° N, 88.4300° E). Sano masuri and Adde from Sikkim (26.7100° N, 88.4300° E). Attheu and Maiti from Kalimpong (N 27° 12'457'' E 88° 14' .574'') .Swarnamasuri and Tulai panji from Malda(25.0000° N,88.1500° E) and finally UBKV-1,UBKV-4 and UBKV-5 from Uttar Bangha Krishi Vishwavidyalaya (UBKV), Coochbehar (26° 24'15" N, 89° 23'5"E) respectively for initial morphological screening and comparative analysis of brown spot disease(Table 3)

Table3. Rice cultivars and its GPS location.

Sl. No	Rice Cultivars	Cultivar type	Origin	GPS Location
1.	Brimful	Ethnic	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
2.	Champasari	Ethnic	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
3.	Black Nuniya	Local	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
4.	Kaberi 9090	Commercial	Siliguri	26.7100° N,88.4300° E
5.	Loknath 505	Commercial	Siliguri	26.7100° N,88.4300° E
6.	Gouraknath 507	Commercial	Siliguri	26.7100° N,88.4300° E
7.	Sano Musuri	Ethnic	Sikkim	27.3300° N,88.6200° E
8.	Adde	Ethnic	Sikkim	27.3300° N,88.6200° E
9.	Attheu	Ethnic	Kalimpong	27° 04'N 88° 28'E/27.06,88.47°
10.	Maiti	Ethnic	Kalimpong	27° 04'N 88° 28'E/27.06,88.47°
11.	Swarnamasuri	Local	Malda	25.0000° N,88.1500° E
12.	Tulaipanji	Local	Malda	25.0000° N,88.1500° E
13.	UBKV-1	Research	UBKV	26° 24'15" N, 89° 23'5"E
14.	UBKV-4	Research	UBKV	26° 24'15" N, 89° 23'5"E
15.	UBKV-5	Research	UBKV	26° 24'15" N, 89° 23'5"E

3.1.2. Viability test

To check the viability of the seeds, they were initially surface sterilized with 0.1% (w/v) $HgCl_2$ for 3-4 minutes, and then washed with sterile distilled water and then

transferred to sterile Petri plates under aseptic conditions. The seeds were allowed to germinate for one week and percentage of germination was observed for each rice cultivars (Figure 2) .The seeds were stored in air tight bags at -4˚C for further use.

Figure 2: Rice cultivars grown in petriplates for viability test

3.1.3. Maintenance of rice seedlings in glass house

Fifteen different rice cultivars were given for germination and maintained in glass house of Immuno- phytopathology Laboratory, Department of Botany, University of North Bengal prior to its transfer to the experimental field. They were initially given for germination in earthen plates of 7 cm height and 16 cm diameter containing sandy

loam soil and farmyard manure in the proportion of 2:1 by weight. Rice nursery was used for transplanting within 25-30 days after the date of sowing (Figure 3).

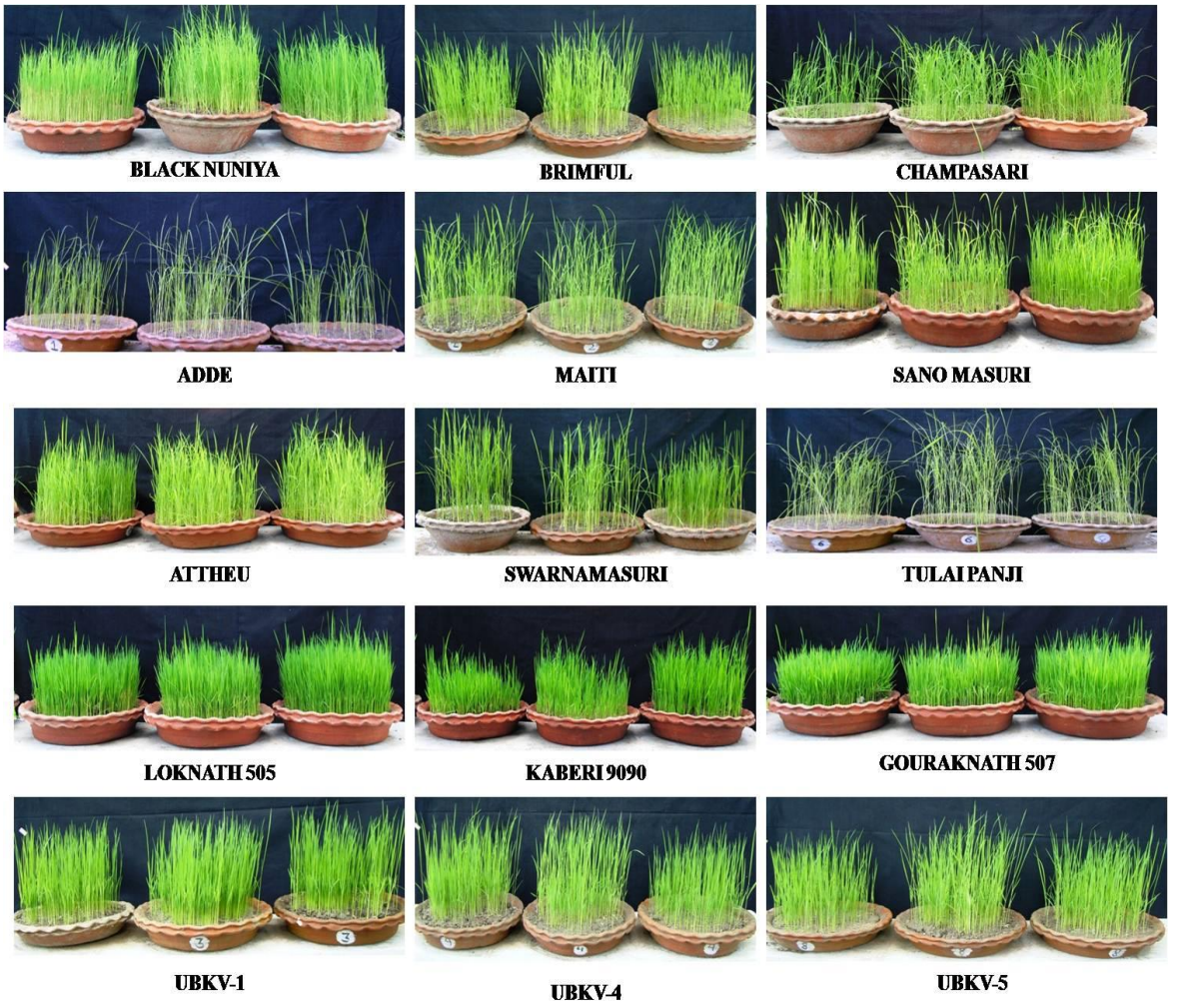


Fig 3: Maintenance of rice seedlings in glass house.

3.1.4. Maintenance in field condition

After germination the rice seedlings were then transferred to experimental fields of Immuno - Phytopathologyl Laboratory, Department of Botany, University of North Bengal. Eight inch row to row and plant to plant distance was maintained for each cultivar. Each block consisted of 10 lines of each cultivar with 10 transplanted rice plants. Healthy samples were maintained in glass house condition. The plants were grown during June to December. Plants were watered regularly twice in a day and weeding was done once a week (Figure 4).

Figure 4: Maintenance of rice cultivars in the field

3.2. Fungal culture

3.2.1. Isolation and maintenance

Fungal pathogen was isolated from samples of diseased leaves of rice (*Oryza sativa*) plants grown in experimental fields of Immuno- phytopathology Laboratory, Department of Botany, University of North Bengal by culturing pieces of internal tissues. Infected leaves tissues were thoroughly washed in sterile water, treated with

0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to potato dextrose agar (PDA) slants and incubated at 28ºC for one week. The fungal mycelium grown was transferred to PDA slants and kept for further identification.

3.2.2. Morphological and microscopic observation

The isolated fungus was allowed to grow in petriplates (7cm) containing sterile PDA medium for 7 days, then nature of mycelia growth, growth rate were observed. For identification, spore suspension was prepared. Drops of spore suspension was placed in a clean , grease free glass slides mounted with lacto phenol- cotton blue, covered with cover slip and sealed with wax. The slides were then observed under microscope following which spore characteristics were determined and size of spores measured.

3.2.3. Completion of Koch's postulate

Healthy leaves of rice (*Oryza sativa*) plants (1 month old) were further inoculated with this isolated organism and incubated for a period of 4 weeks for completion of Koch's postulate .Subsequently, after the development of the disease the infected leaves were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to PDA slants and incubated at 28ºC. At the end of two weeks, the re isolated organism was examined, compared with the original stock culture and its identity was confirmed.

3.2.4. Assessment of mycelia growth

Mycelial growth of the fungal cultures was assessed in both solid media and liquid media to study and evaluate their cultural characteristics.

3.2.4.1. Solid media

To assess the growth of fungal culture in solid media, the fungus was first grown on Petri dishes, each containing 20ml of PDA followed by incubation for 7 days at 28ºC. Agar blocks (6mm diameter) containing the mycelium was cut with sterile cork borer from the actively growing region of mycelial mat and transferred to each Petri dish containing 20ml of different sterilized solid media. The colony diameter was studied at regular interval of time. The media were as follows:

A. ***Potato dextrose agar (PDA):***

Peeled potato - 40.00g, Dextrose – 2.00g, Agar - 2.00g, Distilled water - 100ml

B. ***Richards agar (RA):***

KNO_3 - 1.00g, KH_2PO_4 - 50g, $MgSO_4$. $7H_2$ O - 0.25g, $FeCl_3$ - 0.002g, Sucrose - 3.00g, Agar - 2.00g, Distilled H_20 – 100ml

C. ***Oats meal agar (OA) :***

Oatsmeal-200g (blended in 600ml water,heated to 40-45^0 C), Agar 20.2g(melted in 400ml water).Both were mixed up,filtered and then autoclaved for 90 min and supplemented with 300µg ml /L Oxytetracycline.

3.2.4.2. Liquid media

To assess the mycelial growth in liquid media the fungus was first grown on petriplates, each containing 20ml of PDA and incubated for 5-8 days at 28ºC. The mycelial block (5mm) from the actively growing region of the fungus in the petriplate was cut with sterilized cork borer and transferred to Erlenmeyer flask (250ml) containing 50 ml of sterilized Potato dextrose broth (PDB), and incubated for 7-10 days with constant stirring at room temperature.

3.3. Soluble protein

3.3.1. Extraction and estimation of soluble proteins

3.3.1.1. Fungal Mycelia

Mycelial protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). The fungal mycelia were grown in 250 ml Erlenmeyer flask each containing 50 ml of potato dextrose broth (PDB) and incubated for 10 days at 30+ 1ºC for extraction of antigen, mycelial mats was harvested washed with 0.2% Nacl and rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05m sodium phosphate buffer (PH-7.2) supplemented with 0.85% NaCl, 10mM sodium meta bisulphite, PVPP (Polyvinyl pyrollidine Phosphate) and 0.5mM magnesium chloride in ice bath. The homogenate mixture was kept for 2h or overnight at 4ºC and then centrifuged at 10.000rpm for 30 min, at 4ºC to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4ºC. After this period, the mixture was centrifuged (10,000rpm) for 30 minute at 4º C, the precipitate was dissolved in the same buffer (pH 7.2). The preparation was dialysed for 72h through cellulose tubing (sigma chemical co., USA) against 1L of 0.005 M sodium phosphate buffer (pH 7.2) with six changes. The

dialysate was stored at -20ºC and used as antigen from the preparation of antiserum and other experiment.

3.3.1.2. Leaf

Soluble protein was extracted from rice leaves following the method of Chakraborty *et al.* (1995). Leaf tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM Na2 S2 O5, 0.5 mM MgCl2 and 2mM PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.3.2. Estimation of protein content

Soluble proteins were estimated following the method as described by Lowry *et al*. (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% CuSO4 and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% $Na_2 CO_3$ in 0.1 NaOH) was added. This was incubated for 15 min at room temperature and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for further 15 min following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.4. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

For the preparation of gel the following stock solutions were prepared:

3.4.1. Preparation of stock solution

Following stock solution were prepared

(A) Acrylamide and N' N'- methylene bis acrylamide

A stock solution containing 29 % acrylamide and 1% bisacrylamide was prepared in water. The pH of the solution was kept below 7.0. The stock solution was then filtered through Whatman No. 1 filter paper, kept in brown bottle and stored at 4ºC and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

A 10 % stock solution of SDS was prepared in warm water and stored at room temperature.

(C) Tris Buffer

(i) 1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4ºC for use.

(ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4ºC for use.

(D) Ammonium Persulphate (APS)

Fresh 10 % APS solution was prepared with distilled water each time before use.

(E) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3) and 0.1 % SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10 % SDS in 1 L of distilled water.

(F) SDS gel loading buffer

This buffer contains 50 mM Tris-HCl (pH 6.8), 10mM β-mercaptoethanol, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol. A 1X solution was prepared by dissolving 0.5 ml of 1 M Tris buffer (pH 6.8), 0.5 ml of 14.4 M β-mercaptoethanol, 2 ml of 10 % SDS, 10 mg bromophenol blue, 1 ml glycerol in 6.8 ml of distilled water.

3.4.2. Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb + 1cm).

After pouring the resolving gel solution, it was immediately over layered with isobutanol and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30 - 45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris - Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Name of the compound	**10 % Resolving gel (ml)**	**5% Stacking gel (ml)**
Distilled water	2.85	2.10
30 % acrylamide	2.55	0.50
Tris*	1.95	0.38
10% SDS	0.075	0.030
10% APS	0.075	0.030
TEMED**	0.003	0.003
*For 1.5 M Tris pH 8.8 in resolving gel and for 1 M Tris pH 6.8 in stacking gel ** N,N,N',N'- Tetramethyl ethylene diamine		

3.4.3. Sample preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with 1X SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 4 min to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b- 97,4000; Bovine Serum Albumin- 68,000; Ovalbumin- 43,000; Carbolic Anhydrase- 29,000; Soyabean Trypsin inhibitor- 20,000; Lysozyme- 14,300) was treated as the other sample and loaded in a separate well.

3.4.4. Electrophoresis

Electrophoresis was performed at a constant 15 mA current for a period of three hours until the dye front reached the bottom of the gel.

3.4.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

The gel was removed from the fixer and stained in this stain solution for 4 hours at 37°C with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5: 1) at 40°C with constant shaking until the background became clear.

3.5. Preparation of antigens

3.5.1. Fungal antigen

3.5.1.1. Pathogen

Mycelial protein was prepared following the method as outlined by (Chakarborty and Saha, 1994). Mycelia mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ in ice bath. The homogenate mixture was kept for 2h or overnight at 4 ºC and then centrifuged at 10.000rpm for 30 min at 4ºC to eliminate cell debris. The supernatant was collected and stored in -20 ºC and used as antigen for the preparation of antiserum.

3.5.1.2. Leaf

Leaf protein was extracted from rice leaves following the method of Chakraborty *et al.* (1995). Leaf tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM Na2 S2 O5, 0.5 mM

MgCl2 and 2mM PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as antigen.

3.6. Raising of polyclonal antibodies

3.6.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal antigens in New Zealand white male rabbits of approximately 2kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Antisera reserves for plant pathogens, Immuno-Phytopathology Laboratory, Department of Botany, NBU. They were regularly fed with green grass, soaked gram, green vegetables and carrots etc. twice a day. After each bleeding they were given saline water for three consecutive days and kept in proper hygienic conditions.

3.6.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera, intramuscular injections of 1ml antigen(protein extracted) mixed with 1ml of Freund's complete adjuvant (Genei) were given into each rabbit 7 days after pre- immunization bleeding and repeating the doses at 7 days intervals for consecutive week followed by Freund's incomplete adjuvant (Genei) at 7 days intervals upto 12-14 consecutive weeks as required. Method of (Alba and Devay, 1985 and Chakraborty and Saha, 1994) was followed for immunization.

3.6.3. Bleeding

Bleeding was performed by marginal ear vein puncture, three days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they were placed on their back on a wooden bored fixed at an angle of 60º, and held the rabbits tight so that it could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol. The ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 -10 ml of blood samples were collected in sterile graduated glass tube. The blood samples were incubated at 37ºC for 1hr for clotting. After clotting; the colt was loosened with a sterile needle. Finally, the serum was classified by centrifugation. (2000g for 10 minute at room temperature) and

distributed in 1 ml vials and stored at -20ºC as crude antisera. The serum was used for Dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA).

3.7. Purification of IgG

3.7.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2 ml) was first diluted with two volume of distilled water and an equal volume of 4 M ammonium sulphate. The pH was adjusted to 6.8 and mixture was stirred for 16 h at 20°C. The precipitate thus formed was collected by centrifugation at 12000 rpm at 22°C for 1 h. Then the precipitate was dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.7.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30cm height) and allowed to settle for 2h. After the column material had settled 25ml of buffer (0.02M sodium phosphate, pH 8.0) washing was given to the column material

3.7.3. Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.03 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying the column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column, buffer (2) was mixed into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV-Vis spectrophotometer.

3.8. Immunological assays

3.8.1. Plate trapped antigen coated (PTA) - ELISA

Plate trapped antigen coated (PTA)-ELISA was performed following the method as described by (Chakraborty *et al.* 1995) with modifications. Antigen were

diluted with coating buffer and the antigens were loaded (200µl per well) in ELISA plate (Coaster EIA/RIA, strip plate, USA) arranged in12 rows in a (Cassette) ELISA plate. After loading, the plate was incubated at 25 ºC for 4 h. then the plate was washed four times under running tap water and twice with PBS-Tween and each time shaken well to dry. Subsequently, 200 µl blocking reagent was added to each well for blocking the unbound sites and plate was incubated at 25 ºC for 1h. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200 µl per well) and incubated at 4ºC overnight. After a further washing, antirabbit IgG goat antiserum labeled with alkaline phosphatase diluted 10000 times in PBS, was added to each well (100 µl per well) and incubated at 37 ºC for 2 h. The plate was washed, dried and loaded with 200 µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 1 h. color development was stopped by adding 50 µg per well of 3 N NaOH solution and the absorbance was determined in an Multiscan Ex (Thermo Electron) ELISA Reader(Multiskan, Thermo Labsystems) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.8.2. Dot immunobinding assay

Dot blot was performed following the method suggested by Lange *et al.* (1989) with modifications. Following buffers were used for dot immunobinding assay.

Carbonate –bicarbonate (0.05 M, pH 9.6) coating buffer.

Tris buffer saline (10mM pH 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.

Blocking solutions 10% (w/v) skim milk powder (casein hydrolysate, SLR) in TBST (0.05 M Tris-HCl, 0.5 M NaCl) 5% v/v Tween 20 , pH 10.3.

Alkaline phosphatase buffer (100 mM tris HCl, 100 mM NaCl, 5mM $MgCl_2$

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size 0.45µm, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate-bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Antigen (5µl) was loaded on to NCM and allowed to dry for 30 min at room temperature. Template was removed and blocking of NCM was done with 19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker. Respective polyclonal antibody (IgG 1:500) prepared against that antigen

was added directly in the blocking solution and further incubated at 4 ºC for overnight. The membrane was then washed gently in running tap water for three min, thrice followed by washing in TBST (pH 7.4), (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2h at 37ºC. The membrane was washed as before. 10 ml of NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized with the intensity of dots.

3.8.3. Western blot

Protein samples were electrophoresed on 10% SDS PAGE gel as suggested by Laemmli (1970) and electo transferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of the pathogen (*Drechslera oryzae*) following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) as substrate. Immunoreactivity of proteins was visualized as violet coloured bands on the NCM.

The following buffers were used for Western blotting-

(i) All the stock solutions and buffers used in SDS-Gel preparation for Western blotting were as mentioned earlier in SDS-PAGE protein.

(ii) Tranfer buffer (Towbin buffer): (25mM Tris, 192mM glycine 20% reagent grade Methanol, pH 8.3).

Tris- 3.03g; Glycine- 14.4g; 200 ml Methanol (adjusted to 1lit. with dist. Water).

(iii) Phosphate buffer Saline, PBS, (0.15M, pH 7.2)

Stocks

A. Sodium dihydrogen phosphate- 23.40g in 1000ml dist. water

B. Disodium hydrogen phosphate- 21.294g in 1000ml dist. water

280 ml of stock A was mixed with 720 ml of stock B and the pH was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

(iv) Blocking solution: 5% non fat dried milk + 0.02% sodium azide in PBS with 0.02% Tween 20.

(v) Washing buffers:

(a) Washing buffer-1: PBS

(b) Washing buffer-2: (50mM Tris-HCl, 150 mM NaCl, pH 7.5). Tris- 6.07 g; NaCl- 8.78g; made up to 1lit. with distilled water.

(vi) Alkaline phosphatase buffer:(100mM NaCl, 5mM $MgCl_2$, Tris- HCl, pH 9.5). Tris- 12.14g; NaCl- 5.84g; $MgCl_2$- 1.015g; made up to 1 lit. with double distilled water.

(vii) Substrate

NBT: 5mg NBT in 100µl of 70% N,N-dimethyl formamide

BCIP: 5mg BCIP in 100µl of 70% N,N-dimethyl formamide

Substrate solution was prepared by adding 66µl NBT and 33µl BCIP in 10ml alkaline phosphatase buffer or, 1 tablet of NBT/BCIP (Sigma Chemical, USA) in 10ml of double distilled water.

(viii) Stop solution: (0.5M EDTA solution in PBS, pH 8.0) EDTA sodium salt-0.0372g in 200µl distilled water, added in 50ml of PBS.

Blot transfer process

Following the SDS-PAGE, the gel was transferred in prechilled transfer (Towbin) buffer for 1h. The nitrocellulose membrane (BIO-RAD, 0.45µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves and soaked in Towbin buffer for 15 min. The transfer process was done in Trans- Blot SD Semi-Dry Transfer cell (BIO-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the semi-dry cell. A pipette glass (or glass rod) was rolled over the surface of the filter paper to exclude all air bubbles. The pre wetted membrane was placed on top of the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and air bubbles were rolled out. Finally another presoaked filter paper was placed on the top of gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The

blot unit was run for 45 min at a constant volt (15V). After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1h. and proceeded for immunological probing.

Immunoprobing

Following drying, blocking was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min with gentle shaking on a platform shaker at room temperature. Subsequently, the membrane was incubated with antibody (IgG) solution (blocking solution: PBS [1:1, v/v + IgG, diluted as 1:100 or as per requirement]. The bag was sealed leaving space for few air bubbles and incubated at 4°C overnight. All the processes were done with gentle shaking. Next day the membrane was washed thrice in 250 ml PBS (washing buffer -1). Final washing was done in 200ml washing buffer-2 to remove azide and phosphate from the membrane before enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with antirabbit goat IgG (Sigma Chemicals) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1h. at room temperature. After enzyme reaction, membrane was washed four times in washing buffer-2. Then 10ml substrate was added and the reaction was monitored carefully. When bands were observed up to the desired intensity, the membrane was transferred to tray of 50ml stop solution.

3.8.4. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia, cross- section of infected leaves were done using FITC labeled goat antirabbit IgG following the method of (Chakraborty and Saha, 1994). FITC was done to locate AMF spores in soil and observe root colonization and cellular location of AMF in rice plants which was mass multiplied in maize plants following colonization with AMF.

3.8.4.1. Fungal mycelia

Fungal mycelia were grown in liquid potato dextrose medium as described earlier. After five days of inoculation young mycelia were taken out from flask and taken in Eppendorf tube and washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 h at room temperature. The mycelia was washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma chemicals) diluted 1:40 with PBS (pH

7.2) and incubated in dark for 45 min at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were observed and photograph under both phase contrast and UV fluorescence condition using Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.8.4.2. Cross section of rice leaves

Initially, cross section of healthy and infected rice leaves were cut and immersed in PBS (pH 7.2). These section were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1 hour at room temperature. After incubation, cross sections were washed thrice with PBS- Tween (pH 7.2) for 15 minute and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC).The sections were incubated for 45 minutes in dark. After that sections were washed thrice with PBS- Tween as mentioned above and then mounted on a grease free slide with 10%glycerol. Fluroescence of the root and leaf sections were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.8.4.3. Localization of chitinase and glucanase by immunofluorescence

Indirect fluorescence staining of cross-section of tea leaves was done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994). Initially, cross sections of healthy and infected tea roots were cut and immersed in phosphate buffer saline (PBS), pH 7.2. These sections were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1h. at room temperature. After incubation, sections were washed thrice with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min in dark. After that sections were washed thrice with PBS-Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Photographs were taken by scope photo instrument directly attached to a computer. Fluorescence of the root section was observed using Leica Leitz biomed Microscope with fluorescence optics equipped with UV-filter set-I-3 and photograph was taken.

3.9. Isolation of genomic DNA

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. Liquid nitrogen was used for crushing the cell mass.

3.9.1. Preparation of genomic DNA extraction buffer

The following buffers for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

Lysis Buffer	**Genomic DNA Buffer**	**CTAB Buffer**
50 mM Tris, pH 8.0 100 mM EDTA 100mM NaCl 1% SDS	10 mM Tris, pH 8.0 0.1 mM EDTA	2% CTAB 1.5% PVP K 30 1.4 mM Nacl 20 mM EDTA 100mM Tris HCL pH 8.0 0.1% B-mercaptoethanol

3.9.2. Genomic DNA extraction

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. The mycelia were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60^0C followed by centrifugation at 12,000 rpm for 15 min., whereas genomic DNA was extracted from isolates of bacteria CTAB buffer. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min, washed in 70% ethanol and air dried.

3.9.3. Purification of genomic DNA

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure was followed by RNAase treatment. Genomic DNA was resuspended in 100 µl 1 X TE buffer and incubated at 37^oC for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both

spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

3.9.4. Measure DNA Concentration using Spectrophotometer

The pure sample was (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), used to quantify DNA. For quantitating DNA absorbance at wavelengths of 260 nm and 280 nm were taken. Quantification was done as follows:

1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

Pure preparations of DNA have OD_{260}/OD_{280} value 1.8. If there is contamination with protein or phenol, this ratio will be significantly less than the value given above, and accurate quantitation of the amount of nucleic acid will not be possible.

3.9.5. Agarose gel electrophoresis to check DNA quality

Gel electrophoresis is an important molecular biology tool. Gel electrophoresis enables us to study DNA. It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion, or the presence of a point mutation; it can also be used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample. Preparing an agarose gel involves melting a specified amount (0.8%) of agarose in 1X TBE buffer, cooling the solution, and pouring it into the gel casting tray with ethidium bromide. Gels solidify in 15-20 minutes.

3.9.5.1. Preparation of DNA samples for electrophoresis

Agarose (0.8%) in 1X TBE buffer was melted, cooled and poured into the gel casting tray with ethidium bromide. Gels solidify in 15-20 min.

3.9.5.2. Run gel electrophoresis for DNA fraction

The electrical lead of the gel tank was attached firmly and applied electric supply at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 minutes. The DNA migrated from cathode to anode. Run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then

electric current was turned off and gel was removed from the tank and examined on UV-transilluminator and photographed for analysis.

3.10. ITS PCR analysis

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o} C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72 ^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µ1) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

3.10.1. ITS PCR primers

The following primers were used to amplify ITS regions:

ITS-Primers pairs	**Primer Seq 5'-3'**	**Mer**	**TM**	**% GC**
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%

3.10.2. Amplification conditions

Temperature profile, 94^{o}C for 4 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 60^{o}C for 30 sec and extension at 70^{o}C for 5 min and the final extension at 72^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.10.3. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bi-directionally using the ITS primer pairs by Sci Genome, Kerela.

3.10.4. Sequence analysis

DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4.

3.10.5. Chromatogram of sequence

The chromatogram of the DNA sequence was analyzed by the software Chromus.

3.10.6. Editing and alignment of sequence data

All the DNA sequences edited by using the software BioEdit and aligned with Clustral W algorithms.

3.11. BLAST analysis of Sequence

The DNA sequences were analyzed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast,Altschul *et al.*, 1997) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism was done on the basis of homology of sequence.

3.12. Submission of rDNA gene to NCBI GenBank

The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the ITS sequence after complete annotation and given accession numbers.

3.13. Multiple sequence alignment and phylogenetic analysis

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson *et al.,* 1994). Phylogenetic inference was performed by the Neighbor Joining (NJ) method (Saitou and Nei, 1987). Bootstrap tests with 500 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained (Tamura *et. al*., 2007).

3.14. Assessment of disease caused by fungal pathogens on rice plants

3.14.1. Detached leaf

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded rice leaves detached from plants were placed in trays lined with moist blotting paper.

Wounds were made on adaxial surface of each leaf with 26 G½ needle and inoculated with 20µl droplets of spore suspension (1.2 X 10^6 conidia ml^{-1}) of the fungus (prepared from 14 days old culture in PDA). Spore suspension was placed (2-4 drops $leaf^{-1}$) on the adaxial surface of each leaf with a hypodermis syringe on the wounds. In control sets drops of sterile distilled water were placed on the wounded leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize drying of the drops during incubation.

Percent drops that resulted in lesion production were calculated after 48, 72 and 96 hours of incubation as described by Chakraborty and Saha (1994). Observations were made based on 6 inoculated leaves for each treatment in average of three separate trials.

3.14.2. Whole Plant

Whole plant inoculation was carried out essentially as described by Mathur *et al*. (2000) with minor modifications. The fungus was grown in PDA for 14 days at 30°±2°C and spore suspension was prepared (1.2 X 10^6 conidia ml^{-1}). Tween-20 was added @ 2ml l^{-1} to facilitate adhering of the spores to leaf surface. 2 year old plants were spray-inoculated with an atomizer @ 100ml per plant so as to wet both ventral and dorsal surfaces. The plants were immediately covered with polythene bags so as to maintain high relative humidity and kept overnight. Next day, the polythene bags were removed and transferred to glass house benches and maintained at 30°±2°C.

The disease severity on plant leaves was recorded using the method of Adlakha *et al*. (1984). Results were always computed as the mean of observations of 25 well-established and fully grown 6 months old rice plants in average of three separate experiments.

3.15. *In vivo* testing for antagonism to fungal pathogens

3.15.1. Antifungal test of Plant growth promoting rhizobacteria (PGPR)

Ten previously isolated characterized sequenced PGPR strains were taken for the antagonistic study against the pathogen *D. oryzae*. The bacterial strains with NBAIM Acc. No. and NCBI(Gen Bank) Acc. No. are as follows *Bacillus pumilus* (NAIMCC-B01483) (JF836847), *Bacillus pumilus* (NAIMCC-B01487) (JQ765579), *Bacillus pumilus* (NAIMCC-B01488) (JQ765580), *Burkholderia symbiont* (NAIMCC-B01489) (JQ765578), *Bacillus aerophillus* (NAIMCC-B01490) (KC603894),

Paenibacillus polymyxa (NAIMCC-B01491) (KC703775), *Bacillus methylotrophicus* (NAIMCC-B01492) (JQ765577), *Bacillus altitudinis* (NAIMCC-B01484) (HQ849482), *Bacillus altitudinis* (NAIMCC-B01485) (JF899300), *Enterobacter cloacae*(NAIMCC-B01486) (KC703974) which are coded as (BRHS/C1), (BRHS/T382), (BRHS/T384), (BRHS/P92), (BRHS/ B104), (BRHS/R72), (BRHS/P91), (BRHS/P22), (BRHS/S73), (BRHS/R71) accordingly, was collected from Immuno- Phytopathology Laboratory, Department of Botany, University of North Bengal were evaluated against leaf pathogens- *Drechslera oryzae* in dual culture using PDA medium. The bacteria were streaked on one side of the Petri plate and 4mm fungal pathogen block was placed at the other side of the plate, incubation was undertaken for 5-7 days at 28º±2ºC and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolates. For each test three replicate plates were used. These bacteria were tested for antagonism to *Drechslera oryzae*, and were used for further evaluation.

3.15.2. Antifungal test of Plant growth promoting fungi (PGPF)

The efficacy of BCA (*Trichoderma* sp.) collected from Immuno- Phytopathology Laboratory, Department of Botany, and University of North Bengal was tested *in vitro* for inhibiting growth of the pathogen (*Drechslera oryzae*) in dual culture using PDA. Six previously isolated, characterized and sequenced *Trichoderma* isolates were taken into consideration for this study. Out of these six, three were *T. harzianum*, viz RHS/S559 (NAIMCC-F-03288, HQ334997), RHS/S560 (NAIMCC-F-03289, HQ334995) and RHS/M511 (NAIMCC-F-03290, GQ995194), and the other three were *T. asperellum*, viz RHS/M512 (NAIMCC-F-03291, HQ265418), RHS/M517 (NAIMCC-F-03292, HQ334994) and RHS/M561 (NAIMCC-F-03293, HQ334996). Each fungal isolate was placed at one side of the agar plate about 1cm away from the edge and a 4mm diameter block of the pathogen, taken from growing edge of the fungal culture was inoculated at the other half of the Petri plate. For each test, three replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28ºC and inhibition zone towards the fungus colony in individual plate was quantified. Results were expressed as mean % of inhibition in presence of the fungal isolate.

3.16. Mass multiplication of bioinoculants and pathogen and their application

3.16.1. Arbuscular Mycorrhizal Fungi (AMF)

3.16.1.1. Isolation of AMF spores

Arbuscular mycorrhizal fungal spores were screened from soil samples of fifteen rice varieties rhizosphere by the wet sieving and decanting method (Gerdeman and Nicholson, 1963). Soil samples (100gm each of the root zone) were collected, suspended in water (1lt.) in order to obtain a uniform suspension. Soil clusters are carefully dispersed in the water and is kept for 10 minutes to settle down the heavy particles. Aqueous suspension was passed through a set of sieves of different pore size (200, 170, 150, 80, 50 µm) arranged one below t other. The spores were picked by the help of bristles / brushes and transferred to grooved slides or vials and observed under dissecting microscope. Few spores were stained with Melzar's reagent and studied under sterio-microscope. Healthy spores are separated by fine brush and are stored in autoclaved glass vials either in sterile distilled water or Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in one litre of boiled distilled water) at 4ºC for further study and observation. It is evident from various studies that each plant has multiple AM fungi population.

3.16.1.2. Identification of AMF spores

Spore samples were separated according to their morphology size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachment etc. for the purpose of identification. The spores were identified with the help of standard keys (Walker, 1981; Schneck and Perez, 1987). Spores were critically examined with special reference to variation in vesicles (size, shape, wall thickness, wall layer, position and abundance), hyphal branching patterns, the diameter, structure (especially near entry points) and the staining intensity of hyphae.

3.16.1.3. Spore count

Rhizosphere soil (100gm) was taken and suspended in 250ml water. Wet sieving and decanting method was used for isolation of spores. Total number of spores was then counted and spore percentage of different genera was obtained.

3.16.1.4. Histopathological studies of rice roots

Fungal association of AM fungi within the root tissues was observed according to Philips and Hayman (1970).Young roots from rice plants were dug out manually.

The root sample was washed with tap water gently to free them from soil particles and stored in FAA (formaline aceto alcohol) prior to staining. For staining , root segment of 1cm each was put into the test tube and boiled in 10% KOH solution for 15-20 minutes on a water bath (sometimes even 60 minutes for hard roots), washed in tap water and stained with chlorazol black E (Phillips and Hayman, 1970). For confirmation of infection, the presence of intercellular hyphae, vesicles and arbuscules or both characteristics was taken into consideration. Percent root colonization was determined following the method of Giovanetti and Mosse (1980).

3.16.1.5. Percent Root colonization

Percent root colonization was estimated by using slide method by (Giovannetti and Mosse, 1980). All the infected and uninfected segments of root tissue and the percentage of infection was calculated as follows AMF infection (%) = [infected root segments / total fragments of root taken] X 100.

3.16.1.6. Mass multiplication of AMF

Maize (*Zea mays*), was used for the mass multiplication of AM fungi. Black plastic pots (12inch) were filled with sterilized soil to discard the presence of other fungal propagules. After the plantation adequate water was given. Spores collected from rice rhizosphere were collected by fine tweezers and needles under dissecting microscope and were washed by distilled water several times to remove the adhered debris followed by inoculation in the roots (7-10 days old). After 45 days the presence of spores were verified and inocula were prepared by mixing the chopped roots of maize plants with the potted soil where extra radical spores of required spores were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.16.2. Plant Growth promoting fungi (PGPF)

3.16.2.1. Selection of PGPF

Three isolate of *Trichoderma harzianum* (NAIMCC-F-03288, 03289, 03290) and another three isolate of *Trichoderma asperellum* (NAIMCC-F-03291, 03292, 03293) were selected as plant growth promoting fungi based on their performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al*., 2014).

3.16.2.2. Mass multiplication and application

3.16.1.1. Wheat bran culture

Inoculum of *Trichoderma harzianum* and *Trichoderma asperellum* was prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 ºC for 10 days. To each pot of soil (2000 g), 10 g of the wheat bran colonized by *T. harzianum* was mixed to give a concentration of 105 cfu/g of soil as described by Chakraborty *et al.* (2003).

3.16.1.1. Foliar spray

The selected PGPF was grown in 100ml PDA medium for 7-10 days till sporulation occured. The spores were then scraped off from the surface of the media with the help of inoculating needle and the spores were collected in sterile distilled water. The spore suspension in sterile distilled water at a concentration of 3X 10^4 spores/ml after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the three months old plants after pruning. The spraying was done forth nightly till the new shoots started appearing. The spraying was done for 4 times after every 3 days interval of time.

3.16.3. Plant growth promoting rhizobacteria (PGPR)

3.16.3.1. Selection of PGPR

Ten efficient bacterial isolates [*Bacillus pumilus* (NAIMCC-B01483) (JF836847), *Bacillus pumilus* (NAIMCC-B01487) (JQ765579), *Bacillus pumilus* (NAIMCC-B01488) (JQ765580), *Burkholderia symbiont* (NAIMCC-B01489) (JQ765578), *Bacillus aerophillus* (NAIMCC-B01490) (KC603894), *Paenibacillus polymyxa* (NAIMCC-B01491) (KC703775), *Bacillus methylotrophicus* (NAIMCC-B01492) (JQ765577), *Bacillus altitudinis* (NAIMCC-B01484) (HQ849482), *Bacillus altitudinis* (NAIMCC-B01485) (JF899300), *Enterobacter cloacae* (NAIMCC-01486) (KC703974)] were selected as Plant growth promoting rhizobacteria based on their performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.,* 2013).

3.16.3.2. Mass multiplication and application of PGPR

3.16.3.2.1. Soil drench

The bacteria were grown in NB for 48 hrs at 28^{o}C and centrifused at 12,000rpm for 15 minute. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following

method to obtain a final density of $3X10^6$ cfu ml^{-1}. The bacterial suspension was applied to the pots during transplantation of seedlings . Applications were done @ 0f 100 ml per experimental field at regular interval of one month for three months subsequently.

3.16.3.2.2. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of 3x106cfu ml-1 after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the three months old plants after pruning. The spraying was done for 4 times after every 3 days interval of time.

3.16.4. Application of different bioinoculants under field condition

For field inoculation, initially chopped maize roots colonized with dominant spores of *Rhizophagus fasciculatus* (AMF) were applied in the experimental field before the transfer of the seedlings. One month following the application of AMF, root colonization status was examined. Then after 15 days of treatment various mass multiplied *Trichoderma* sp (PGPF) in wheat bran was applied in the field. Two weeks after application of PGPF soil drench application or foliar spray application of various PGPR was done for 16 days at 3 days interval of time.In case of joint treatment with all three bioinoculants, they were added to the field sequentially but in case of dual treatments (AMF+PGPF, AMF+PGPR and PGPF+PGPR) application was done accordingly. Growth parameters were finally recorder after two months of application of last treatment.

3.16.5. Inoculum preparation of pathogen

The brown spot pathogen, *Drechslera oryzae* was grown in 100ml PDA medium for 7-10 days till sporulation occured. The spores were then scraped off from the surface of the media with the help of inoculating needle and the spores were collected in sterile distilled water. The spore suspension containing $3X\ 10^4$ spores/ml with 0.01% Tween 20 was sprayed on to the treated as well as untreated plants and covered with plastic bags for 48hrs.

3.17. *In vivo* assessment of plant growth promotion

3.17.1. Assessment of plant growth following application of bioinoculants

Plant growth promotion in terms of height was recorded after 20, 40, 60 and 80 days of application of bioinoculants and total biomass of root and shoot in field grown plants .

3.17.2. Assessment of disease sevierity

Establishment of naturally and artificially occurring brown spot disease was observed and disease severity was assessed in terms of lesion number per leaf and infection index calculated as described by Adlakha *et al.* (1984). Ten diseased leaves were randomly selected and incidence observed and finally Percentage Disease Index (PDI) was calculated.

For percent disease index (PDI) calculation, the following formula was used- [(class rating x class frequency)/(total no. of leaves x maximum rating scale)] x 100. Disease severity was recorded using a score chart consisting of nine different scales (0 = no incidence, 1= Less than 1%, 2 = 1-3%, 3 = 4-5%, 4 = 6-10%, 5=11-15%, 6 = 16-25%, 7 = 26-50%, 8 = 51-75% and 9 = 100%) of infection prepared on the basis of percent diseased area of the total leaf area (IRRI, 2002).

3.18. Extraction and asssay of defense enzymes activities after application of bioinoculants

3.18.1. ß-1, 3- glucanase (E.C. 3.2.3.39)

Extraction of ß-1, 3- glucanase (E.C. 3.2.3.39) was done following the method described by Pan *et al*., (1991). Rice leaves samples (1g) was crushed were crushed in liquid nitrogen and extracted using 5ml of chilled 0.05M sodium acetate buffer (pH 5.0) by grinding at 4^{o}C using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4^{o}C and the supernatant was used as crude enzyme extract.

Estimation of β-1, 3-glucanase activity was done by following the laminarin dinitrosalicylate method described by Pan *et al.*, (1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4 %) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µg glucose released min-1 g-1 fresh tissue

3.18.2. Chitinase (E.C.3.2.1.14)

Extraction of Chitinase (E.C.3.2.1.14) was done by following the method described by Boller and Mauch (1988) with modification. Rice leaves samples (1g) were crushed in liquid nitrogen and extracted using 5ml of chilled 0.1 M Sodium Citrate buffer (pH 5). The homogenates were centrifuged at 12,000 rpm for 10 min and the supernatant was used as cenzyme source.

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10µl of 1M Na-acetate buffer (pH4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 r.p.m for 3 min. 0.3 ml supernatant, 30µl of 1M K-PO_4 buffer (pH7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C.The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released /min/ g fresh wt. tissue.

3.18.3. Phenylalanine Ammonia Lyase (PAL) (E.C.4.6.1.5)

Extraction of PAL (E.C.4.6.1.5) was done by the method described by Chakraborty *et al.,* (1993). Leaf samples were crushed in liquid nitrogen and extracted using 5 ml of sodium borate buffer (pH8.8) containing 2 mM β mercaptoethanol in ice followed by centrifugation at 15000 rpm for 20 min at 4°C. The supernatant was collected and after recording its volume, used immediately for assay or stored -20°C.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic- acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3ml of 300µM sodium borate (pH 8.8), 0.3ml of 3µM L-phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation of 1 hour at 40^oC the absorbance at 290nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced in 1 min $g^{-}1$ fresh weight of tissue.

3.18.4. Peroxidase (E.C.1.11.1.7)

For the extraction of peroxidase (E.C.1.11.1.7) the plant tissues were macerated to powder in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) containing 2 mM β mercaptoethanol under ice cold conditions. The homogenate was centrifuged immediately at 15000 rpm for 20 minutes at 4ºC. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20ºC (Chakraborty *et al.*1993).

For determination of peroxidase activity, 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 0.2M sodium phosphate buffer (pH 5.4), 100µl of 4Mm H_2O_2, 100µl O- dianisidine (5 mg ml-1 methanol) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in the presence of H_2O_2 (Chakraborty *et al.*1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/min^{-1}.

3.18.5. Isozyme analysis of peroxidase

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase. Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a mortar and pestle in 2 M sodium phosphate buffer (pH 7.0) in ice cold condition as described by Davis (1964) and used immediately for the isozyme analysis.

3.18.5.1. Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N‘ N‘ methylene bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of N‘ N‘ bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris- HCl (Resolving gel)

36.6 g of Tris base was mixed with distilled water and 0.25 ml of N,N,N,N-tetramethyl ethylene diamine (TEMED) was added. The pH was adjusted to 8.9 with concentrated HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

Solution D: Tris- HCl (Stacking gel)

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g glycine in 1 L of distilled water.

3.18.5.2. Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E: 79 distilled water in the ratio of 1: 1: 4: 1 by pipette leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions B: D: F:

distilled water in the ratio of 2: 1: 1: 4. Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30- 45 minutes in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoretic apparatus. Tris- Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.18.5.3. Sample Preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixture in ice cold condition. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

3.18.5.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3 - 4 h at 4°C until the dye front reached the bottom of the gel.

3.18.5.5. Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method of Reddy and Garber (1971). The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7 % Acetic acid. After the appearance of clear blue coloured bands, analysis of isozyme was done immediately.

3.18.6. Extraction and estimation of total sugar content

Extraction of total sugar was done following the method of Harborne (1973).With minor modifications under normal room temperature and light conditions. One gm of leaf tissue were weighed and crushed with 10ml of 95% ethanol. The alcoholic fraction was evaporated off on a boiling water bath. The aqueous fraction was centrifuged at 10,000 rpm for 15 min and the supernatant was collected and stored at -4^{o}C.

Estimation of total sugar content was determined following the method given by Plummer (1978). 1ml of test solution was reacted with 4ml of Anthrone's reagent (0.2% anthrone in conc. H_2SO_4) and mixed properly. The reaction mixture was incubated for 10mins in a boiling water bath at about 100^oC taking proper precaution followed by cooling it under running tap water. The absorbance was measured at 620nm in a colorimeter and quantified using a standard curve of D- glucose.

3.18.7. Extraction and estimation of total chlorophyll content

Extraction of chlorophyll from leaves was done according to the method of Harbone (1973). 1g of leaf sample was homogenized in 80% acetone and filtered through Whatman No. 1 filter paper in a dark chamber. Addition of 80 % acetone from the homogenized sample was done repeatedly. The filtrate was collected and the total volume was made up to 10 mL using 80% acetone.

Estimation of chlorophyll was done by measuring the OD of the filtrate at 663 nm and 645nm respectively in a UV-VIS spectrophotometer (UV-VIS spectrophotometer 118 systronics) against a blank of 80% acetone and calculated using the formula as given by Arnon (1949).

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg g $^{-1}$fresh weight

Chlorophyll a = $(12.7\ A_{663} - A_{645})$ mg g $^{-1}$fresh weight

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g $^{-1}$fresh weight

3.18.8. Extraction and estimation of Phenol content

3.18.8.1. Extraction of phenol

Phenol was extracted from the fresh young leaves following the method of Mahadevan and Sridhar (1982). One g of leaf tissues were cut into pieces and immediately immersed in 10 ml of boiling alcohol. After 15 minutes of boiling it was cooled and crushed in mortar using pestle thoroughly at room temperature. The extract was filtered through Whatmann No. 1 filter paper. Final volume was adjusted with 80 % ethanol. The whole extraction of phenol was done in dark to prevent light induced degradation of phenol.

3.18.8.2. Estimation of Total phenol

Total phenol content was estimated by Folin Ciocalteau's reagent, following the method of Bray and Thorpe (1954). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin

Ciocalteau's reagent followed by 2 ml of 20 % sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.19. HPLC Analysis of phenols and phytoalexin

3.19.1 Sample preparation

3.19.1.1 Phytoalexin

Extraction procedure of rice phytoalexin Phytocassanes and was followed as described by (Umemura *et al*., 2003). Twenty grams of samples (about 25 leaves) were cut into pieces and then shaken with 200 ml of ethyl acetate and 200ml of 0.1 N Na_2CO_3 (pH 10.5) for 18h. The ethyl acetate fraction was collected and then mixed with 20ml of 0.02 N HCl and centrifuged at 15,000 × 30 min.

3.19.1.2. Phenolic compounds

Phenol extraction and preparation of the sample for HPLC was done by the method described by Pari and Latha (2004) in the dark. Fresh leaf tissue were taken and chopped into small pieces and soaked overnight in absolute methanol at solid material to methanol ratio of 1:3 (w/v) in dark. The suspension was filtered and the filtrate was evaporated using a rotary evaporator at 40^{o}C and lyophilized. It was re dissolved in 1mL of HPLC grade methanol and filtered through Millipore membrane (0.45μm) filter.

3.20. HPLC analysis

3.20.1. Phytoalexin

To measure the amount of phytoalexin especially Phytocassanes induced, the supernatant collected after the extraction procedure was put through High Performance Liquid Chromatography (HPLC) eluted with 45 % acetonitrile. (UV-VIS Detector and Liquid Chromatogram, SHIMADZU). Phytocassanes were monitored at 280 nm (Koga *et al*., 1998).

3.20.2. Phenolics

For the analysis of total phenol in HPLC a method followed by Pari *et al*., (2007) was used. For the HPLC finger print analysis of phenolic compounds present in extracts a Shimadzu system (Shimadzu Corp., Kyoto, Japan) was used, a flow rate of

1mL/ min, and gradient elution of HPLC grade of acetonitrile-water-acetic acid (5:93:2, v/v/v) [Solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [Solution B], a 0-50 min solvent B from 0-100%; and injection volume of 20μL were applied; whereas the separation of compounds was monitored at 280 nm. The identification and quantification of the phenolic compounds were done using the standards such as caffeic acid, ferulic acid, gallic acid, phloroglucinol, pyrogallol, resorcinol, salicylic acid and vannilic acid.

3.21. Bioassay of antifungal compounds

3.21.1. Radial growth bioassay

Radial growth inhibition bioassay was performed for determining antifungal activity of phytoalexin (Phytocassanes) extracted from rice leaves as described by Van Etten (1973).The sample (50µl) were taken in sterile petridishes (2" dia) and allowed to evaporate. Subsequently 10ml sterilized PDA medium was poured in each petridish, thoroughly mixed and allowed to solidify. Agar block (4 mm dia) containing mycelia of *Drechslera oryzae* (14 days old culture) were taken from the advancing zone and transferred to each Petri dish these were incubated at 28±2^{o}C, until inhibition of mycelia growth was observed. Percentage of mycelial inhibition was calculated using the following formula:

$$\text{Percentage of mycelia inhibition} = (C-T)/C \times 100$$

Where, C and T are the growth diameter (mm) in control and treated samples respectively.

Chapter 4
RESULTS

4.1. Initial screening of rice cultivars

4.1.1. Seed Morphology of rice cultivars

Seed morphology of all the cultivar was observed (Fig.5). Quantitative and qualitative traits in landraces of rice mainly for kernel colour, seed coat colour, aroma type presence or absence of awn and length of seed was recorded (Table 4) and it was seen that a total of 9 landraces had white kernel colour while 4 had brown and 2 had greyed-orange. The seed coat colour variation in different landraces ranged from Golden yellow, Yellow, Red and Black. 6 landraces were having aroma whereas 9 had no aroma and lastly 11 landraces were found to have awn and 4 were awnless. UBKV-4 was longest in length with 1.1 cm and Sano masuri being the smallest of 0.4 cm.

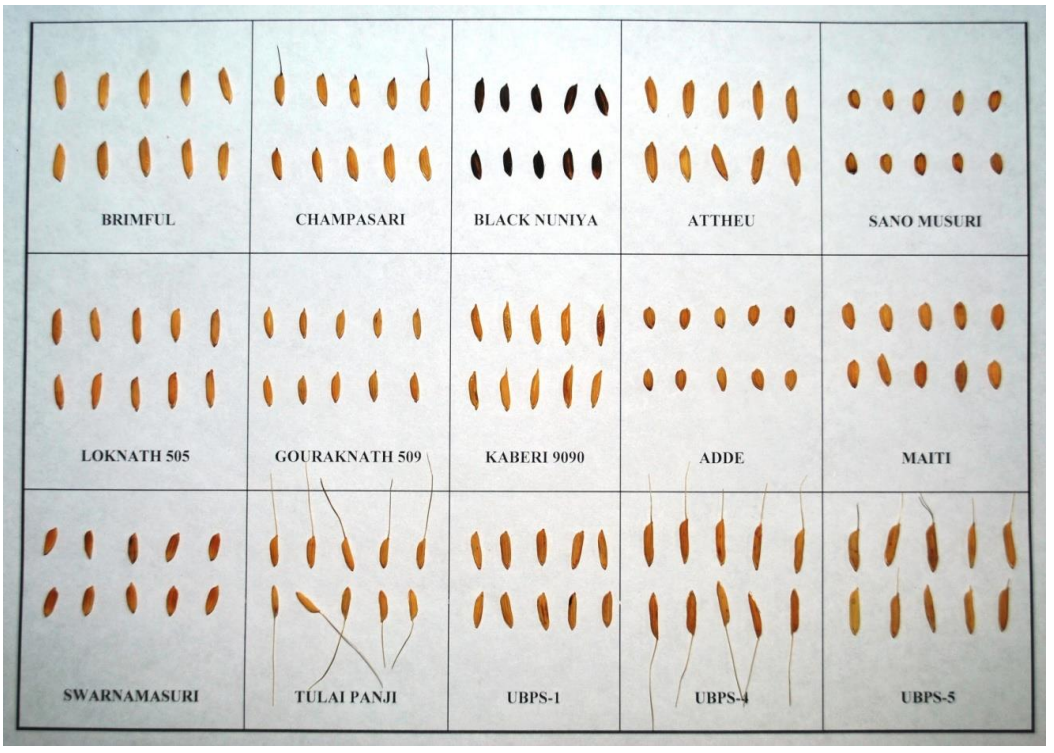


Figure 5: Fifteen different rice cultivars showing morphology

Table 4.Morphological characterization of rice cultivars

Sl. No	Rice cultivar	Area of collection	Kernel colour	Seed coat colour	Aroma	Presence of Awn	Seed Length (cm)
1.	Brimful	Bijanbari	Brown	Red	Present	Absent	0.9±.06
2.	Champasari	Bijanbari	White	Red	Absent	Present	0.8±.03
3.	Black Nuniya	Bijanbari	Brown	Black	Present	Absent	0.7±.04
4.	Attheu	Kalimpong	White	Yellow	Present	Absent	0.9±.05
5.	Sano Masuri	Sikkim	White	Yellow	Absent	Absent	0.4±.0.2
6.	Loknath 505	Siliguri	White	Golden Yellow	Absent	Absent	0.8±0.4
7.	Gouraknath 509	Siliguri	White	Golden Yellow	Present	Absent	0.7±0.4
8.	Kaberi 9090	Siliguri	White	Golden Yellow	Absent	Absent	0.9±0.7
9.	Adde	Sikkim	Brown	Yellow	Present	Absent	0.5±0.9
10.	Maiti	Kalimpong	Brown	Yellow	Absent	Absent	0.6±0.7
11.	Swarnamasuri	Malda	Greyed orange	Red	Absent	Absent	0.7±0.6
12.	Tulaipanji	Malda	Greyed orange	Golden Yellow	Present	Present	0.7±0.5
13.	UBKV-1	UBKV	White	Yellow	Absent	Present	0.9±0.8
14.	UBKV-4	UBKV	White	Red	Absent	Present	1.1±0.6
15.	UBKV-5	UBKV	White	Yellow	Absent	Absent	1.0±0.5

Mean value of ten replicates ± Standard error

4.1.2. Germination ability

Germination percentage was found to be highly variable in all the fifteen cultivars.UBKV-1 cultivar showed highest germination percentage of (99%) while Adde showed lowest germination percentage (22%). Similarly the cultivar Brimful showed 62%, Champasari 56%, Black nuniya 74%, Loknath 505 92%, Gouraknath509

44%, Kaberi 9090 98%, Sano masuri 36%, Maiti 42%, Attheu 30%, Swarnamasuri 34%, Tulaipanji 26%, UBKV-4 90% and UBKV-5 98%.The cultivars showing high germination percentage would be useful in plant breeding programs (Figure 6, A).

4.1.3. Total protein content

Total soluble protein present in each rice leaf samples were recorded after 15 days of growth of the plants. The graph is presented in (Figure 6, B)shows that the soluble protein content of UBKV-4 was found to be maximum (22.76 mg/gm tissue) and that of Tulaipanji was found to be minimum (15.13 mg/gm tissue). Similarly the protein content of rice cultivar Brimful was found to be 17.5, Champasari 18.0, Black nuniya 17.2, Loknath 505 19.5, Gouraknath 509 15.5, Kaberi 9090 20.0, Adde 16.2, Sano masuri 15.5, Maiti 16.0, Attheu 16.5, Swarnamasuri 17.5, UBKV-1 20.0 and UBKV-5 17.5 mg/gm tissue.

4.1.4. Growth rate

Rate of growth in each cultivar was noted after every five days interval in cm. (Figure 6,C) shows that the maximum length (17.5 cm) was recorded in Brimful and minimum length (7.9 cm) was recorded in Sano masurisimilarly the growth of rice cultivar Champasari was recorded to be 11.8, Black nuniya 13.2, Loknath505 10.2, Gouraknath 509 11.3, Kaberi 9090 11.0, Adde 10.2, Maiti 11, Attheu 12.6, Swarnamasuri 11.4, Tulaipanji 13.3, UBKV-1 14.7, UBKV-4 10.1 and UBKV-5 14.7cm after 15 days of the growth of the seedling .

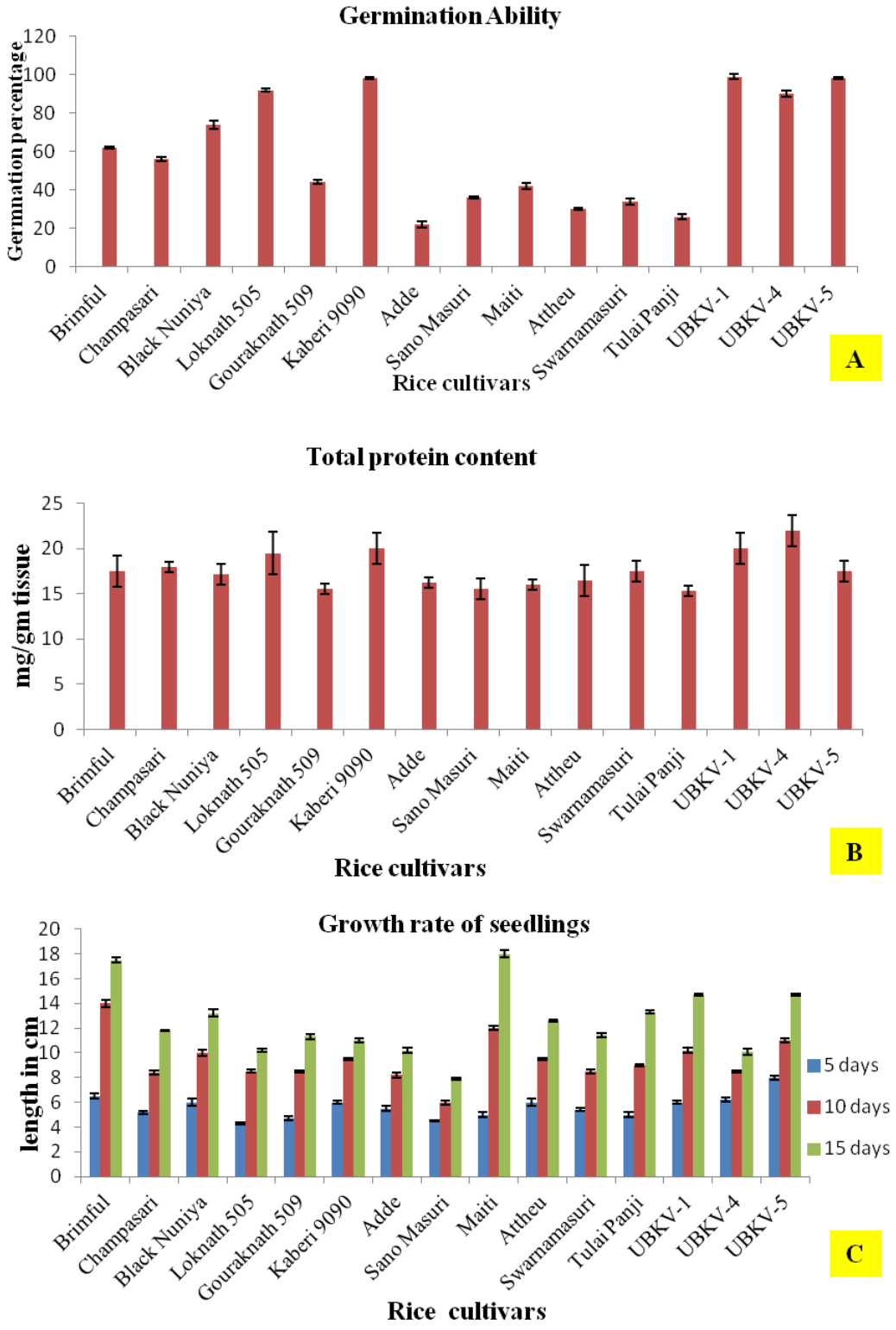


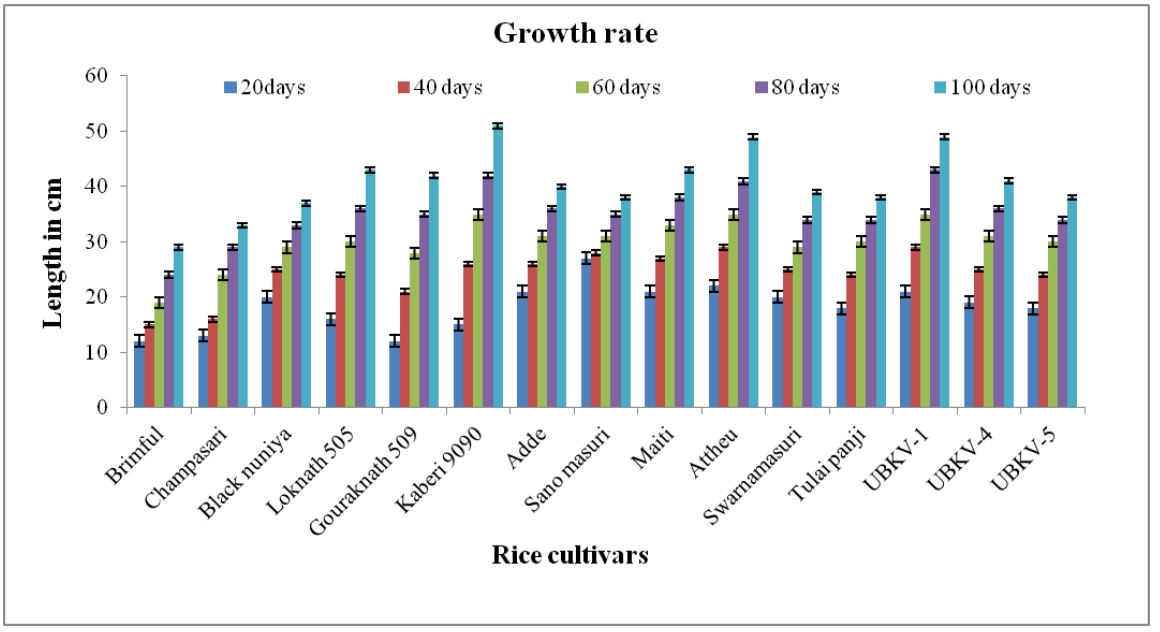
Figure 6: Initial screening of rice cultivars grown in petriplates. (A) Germination ability, (B) Protein content and (C) Growth rate.

4.2. Screening of resistance of rice cultivars towards brown spot pathogen

To conduct different experiments on induction of resistance in Rice plants it became necessary to screen the tolerant and susceptible rice cultivars with distinctive disease reaction for use as test plant material. All the fifteen rice cultivars were tested in plot experiment to screen their various responses to infection with brown spot pathogen.

4.2.1. Growth rate

Rate of growth was observed for all the rice cultivars after every 20, 40, 60, 80 and 100 days keeping a gap of 20 days interval of time (Fig. 7). It was observed that the rice cultivar Kaberi 9090 showed the maximum height of 51 cm followed by UBKV-1 and Attheu 49 cm, Loknath 505 and Maiti 43cm, Gouraknath 509 42cm, UBKV-4 41 cm, Adde 40 cm, Swarnamasuri 39cm, Sano masuri, Tulaipanji and UBKV-5 38 cm, Black nuniya 37 cm, Champasari 33 cm and Brimful showing the minimum growth of 29 cm. It is evident that the rate of growth is quite slow in case of local cultivars such as Black nuniya, Champasari and Brimful.



. Figure 7: Growth rate of fifteen rice cultivars grown in field

4.2.2. Disease development

Under the natural condition the establishment of the brown spot disease was observed after four month growth of the rice plants grown on experimental plots (Figure 8) and Disease index (PDI%) was calculated. DI of rice cultivar was found to

differ significantly from each other in comparison to check the susceptibility towards the infection. The maximum incidence (62.28%) was observed in Black Nuniya followed by Brimful, Champasari, Tulaipanji,Adde, Kaberi 9090, Swarnamasuri, Gouraknath 509 Attheu, Sano masuri, Maiti, UBKV-4, UBKV-5, Loknath 505. The lowest incidence was observed in UBKV-1 (25.62 %). (Table 5) shows that among all the fifteen cultivars three local rice cultivars (Black Nuniya, Brimful and Champasari)are highly susceptible to brown spot pathogen and two rice cultivars (Loknath 505 and UBKV-1) is resistant to the pathogen as evident from the data on percent disease index. The local cultivars Black Nuniya was found to be most susceptible to brown spot.

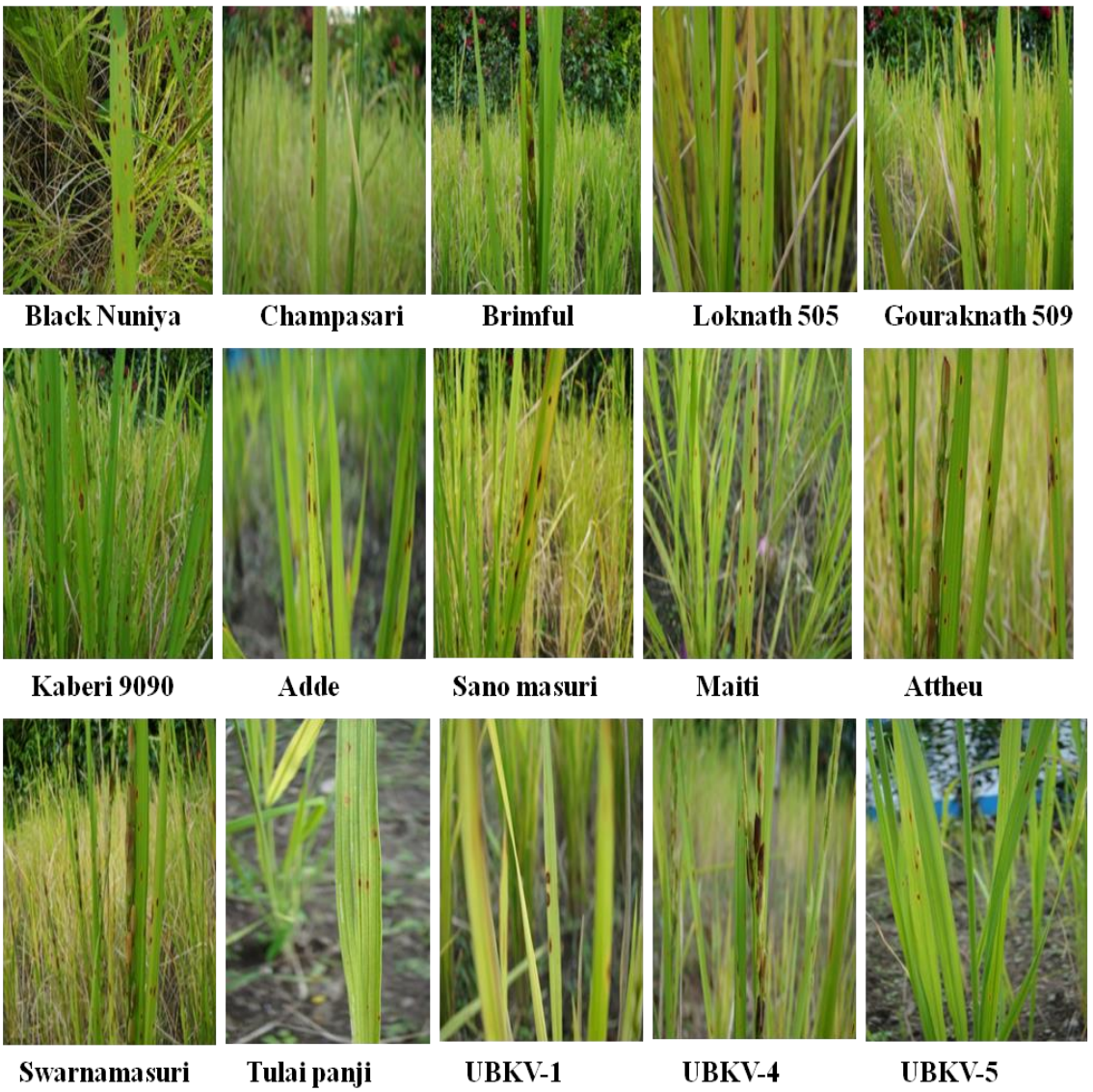


Figure 8: Rice cultivars grown in experimental plot showing natural infection (Brown Spot) caused by *D. oryzae*.

Table 5. Disease index showing the establishment of natural disease.

Sl. No.	Rice cultivars	Disease index (PDI %)
1.	Black Nuniya	**62.28**
2.	Champasari	51.76
3.	Brimful	52.72
4.	Kaberi 9090	48.47
5.	Loknath 505	31.66
6.	Gouraknath 507	40.05
7.	Sano Musuri	48.36
8.	Adde	49.82
9.	Attheu	49.44
10.	Maiti	47.89
11.	Swarnamasuri	43.33
12.	Tulaipanji	50.85
13.	UBKV-1	25.62
14.	UBKV-4	43.78
15.	UBKV-5	43.66

4.2.3. Activity of defense enzymes

4.2.3.1. Peroxidase

Peroxidases are members of a large group of heme-containing glycoproteins that catalyseoxidoreduction between hydrogen peroxide and various reductants. They have an absolute requirement of hydrogen peroxide as electron donor. Peroxidases are implicated to play multiple roles in plant-pathogen interactions. In case of peroxidise activity (quantitative analysis) o-dianisidine was used as substrate and its oxidation was monitored spectrophotometrically. Peroxidase specific activity was assessed in healthy and naturally infected rice leaf tissue for all the fifteen rice cultivars. The results are presented (Fig.9, B) where it can be noted that peroxidise activity has increased in all the infected samples in comparison to the healthy ones. Peroxidase accumulation was found to be maximum in infected samples of rice cultivar UBKV-5 (178.30Δ OD/ gm tissue/min) and minimum in rice cultivar Brimful (70.02Δ OD/ gm tissue/min) in comparison to the healthy samples.

4.2.3.2. Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL) enzyme activity was measured in healthy and infected rice leaves of all fifteen rice cultivars. As shown in (Fig.9; A) PAL activity

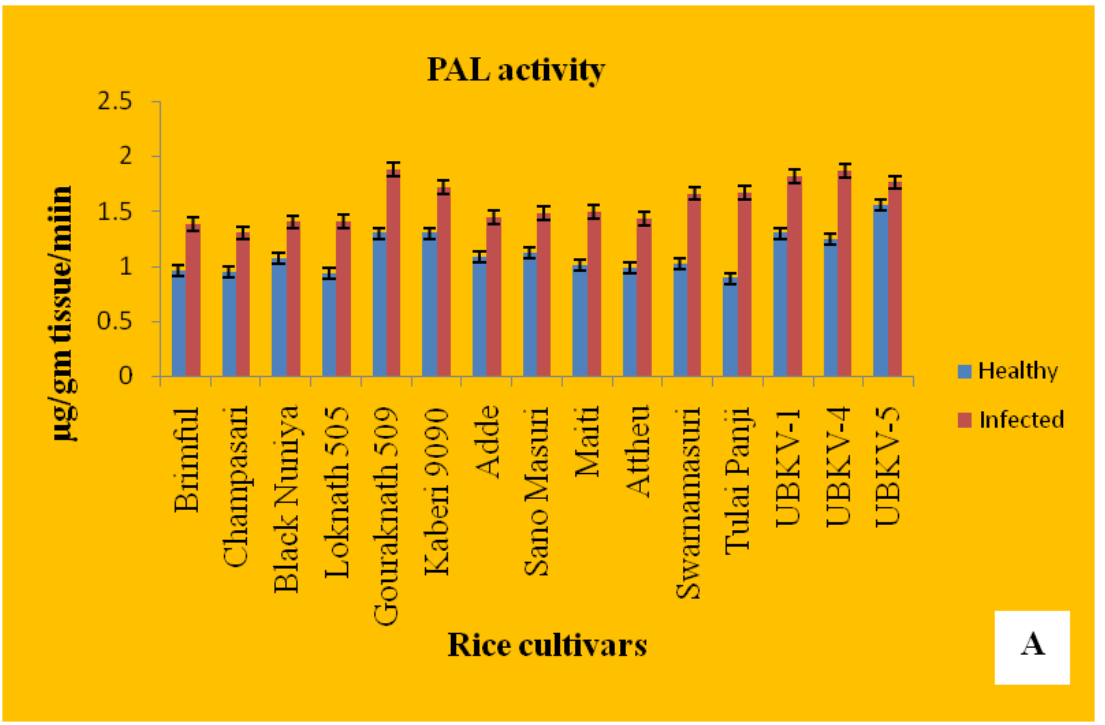
was more in infected samples than in healthy samples. Maximum ammount of PAL accumulation was observed in infected samples of rice cultivar Gouraknath 509(1.88µg/gm tissue/min) and the minimum accumulation was observed in infected samples of rice cultivar Champasari (1.3 µg/gm tissue/min) in comparison to healthy samples. The enzyme activity correlates with disease incidence in all the rice cultivars.

4.2.3.3. Chitinase

Chitinase enzyme is one of the important PR proteins involved in defense mechanism of plants. This enzyme was also analysed for healthy and infected leaves of all the fifteen rice cultivars.Chitinase activity was found to be the maximum in rice cultivar Loknath 505 (0.194 µg/GlcNAC/hr) and minimum in rice cultivar Champasari (0.142 µg/GlcNAC/hr) in comparison to the healthy samples (Fig.10,A).

4.2.3.4. β-1,3 Glucanase

β-1, 3 Glucanase activity was also measured in healthy and infected leaves of all fifteen rice cultivars. In this case also increase of enzyme activity was noted in infected leaves in comparison to the healthy samples in all the rice cultivars. Glucanase activity was found to be maximum accumulated in rice cultivar Kaberi 9090 (535µg glucose/gm tissue/min) and minimum in rice cultivar Black nuniya (445µg glucose/gm tissue/min) in comparison to the healthy samples (Fig.10, B). Hence activity of defense enzymes showed that all the local cultivars are very susceptible to the brown spot pathogen.



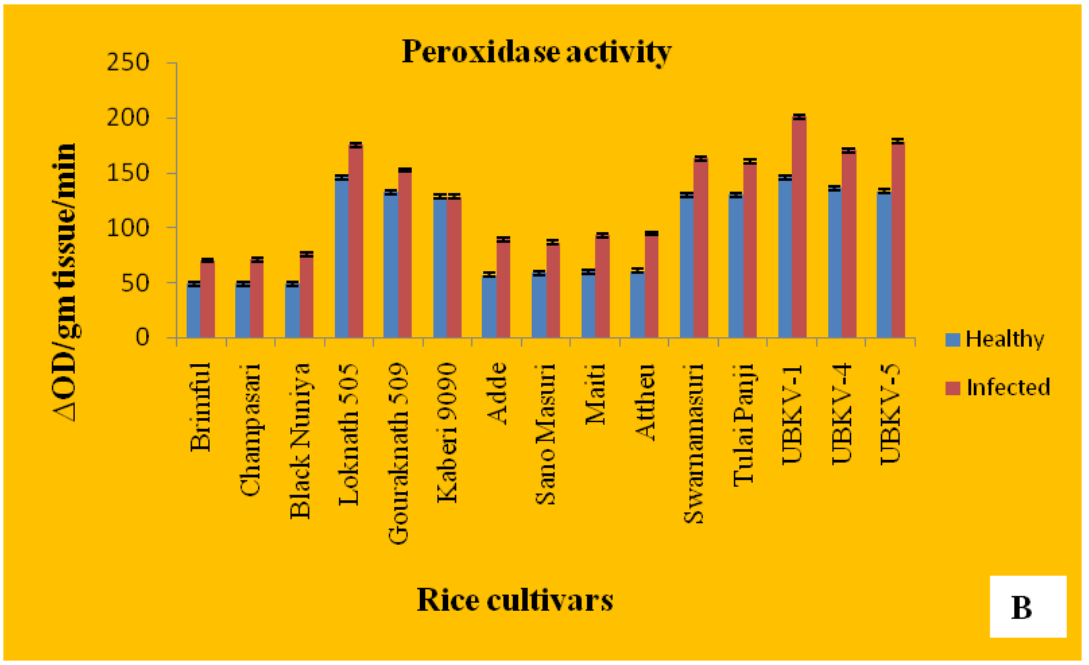
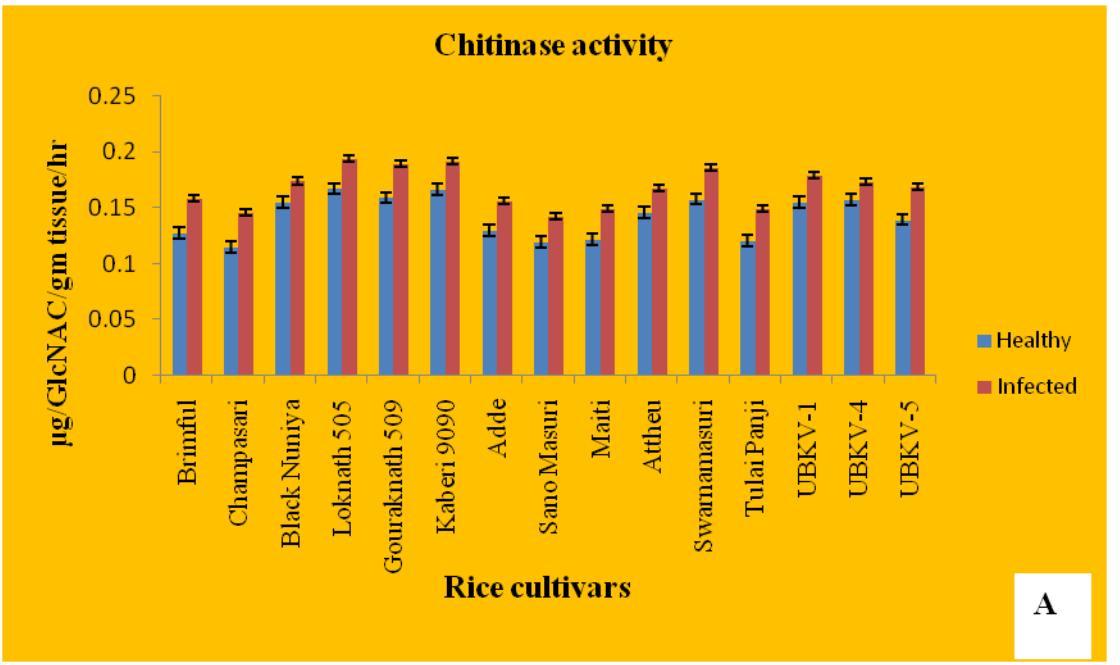


Figure 9: Phenylalanine ammonia lyase (A) and Peroxidase (B) activity in healthy and naturally infected rice leaves.



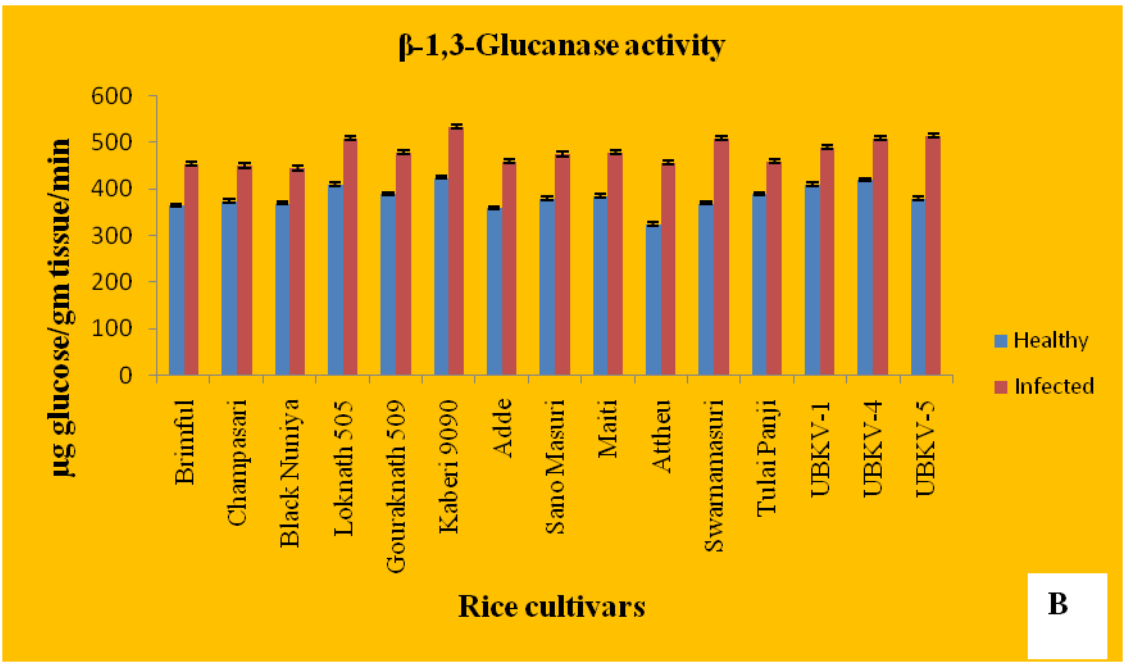


Figure 10: Chitinase (A) and β-1,3-glucanase (B) activity in healthy and naturally infected rice leaf samples

4.2.4. Total phenol content

Total phenol content of healthy and naturally infected rice cultivars were tested. It was found that the accumulation of phenol content in infected rice cultivar of UBKV-4was maximum of 7.22 mg/gm tissue followed by Loknath 505 7.14 mg/gm tissue, UBKV-1 7.1 mg/gm tissue, Kaberi 9090 7.03 mg/gm tissue, UBKV-5

6.62mg/gm tissue, Tulaipanji 6.22 mg/gm tissue, Swarnamasuri 6.24mg/gm tissue, Gouraknath 509 5.82 mg/gm tissue, Maiti 5.52mg/gm tissue, Attheu 5.08mg/gm tissue, Adde 4.83mg/gm tissue, Sanomasuri 4.77mg/gm tissue, Black nuniya 3.46mg,gm tissue, Brimful 3.22mg/gm tissue and Champasari showing the least accumulation of phenol content 3.05 mg/gm tissue (Fig. 11).

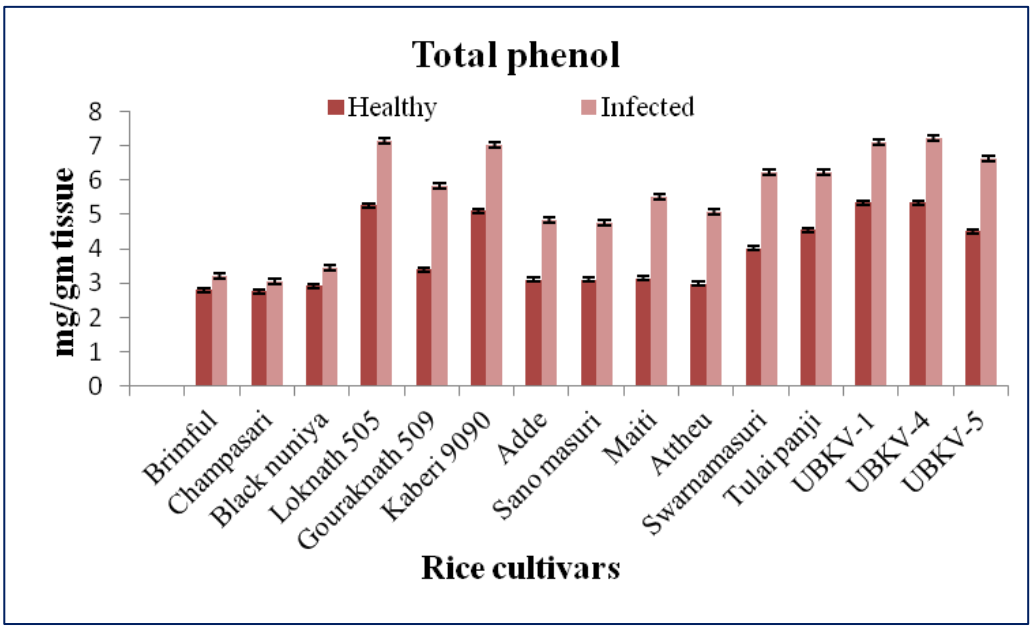


Figure 11: Total phenol content of healthy and naturally infected rice cultivars.

4.3. Morphological and cultural characteristics of the pathogen

4.3.1. Growth in different media

Fungal pathogen isolated from brown spot infected rice leaf was taken for the completion grown in different sterile media *i.e.* Potato dextrose agar (PDA), Richard's agar (RA), Oats meal agar, for 7 -10 days at 28^0 C. Results showed that the maximum growth was recorded in PDA. In culture, the whole area of the Petri plate was readily covered by the mycelium, including aerial hyphae which may cover the lid of the plate. The mycelium initially was observed to have white colour appearance which soon turned light gray after complete growth in petri plate as well as in liquid potato dextrose broth (PDB) media in conical flask giving a fluffy appearance (Figure 12).

4.3.2. Microscopic observation

Microscopic observation under bright field of the isolated pathogen was done. Conidiophore were in single, straight, pale to light brown in colour ranging in length from (357.60µm-296.45µm). Conidia are crescent-shaped ranging in size from

(51.37x273.91µm – 73.15x327.05µm), light brown to brown, widest in the middle or below the middle and tapering towards the rounded ends and germinating from a single pole. On the basis of hyphal structure, mycelia, structure of conidiophore and conidia it was found that the fungus belonged to genera *Drechslera* (Fig.13). The fungus were grown in PDA slants and maintained at 28^0C for further use. Pathogen was also taken for the completion of Koch's postulate for the conformation of disease.

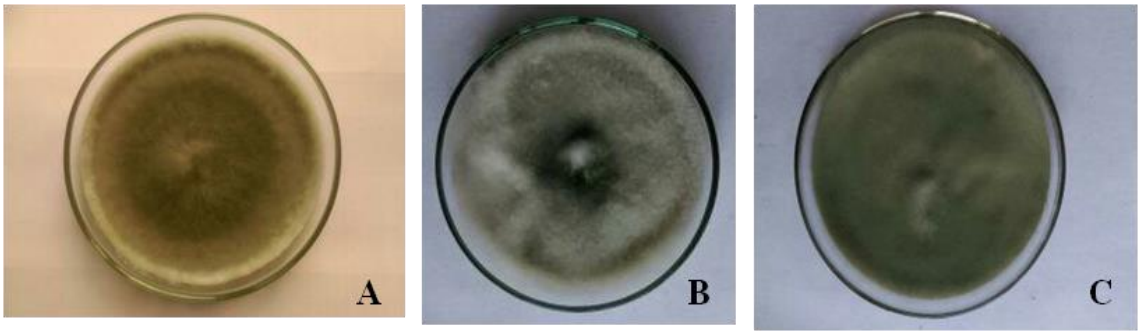


Figure 12: Radial growth pattern of fungi in different media (7-10 days old). (A) Potato dextrose agar, (B) Oats meal agar and (C) Richard's synthetic agar.

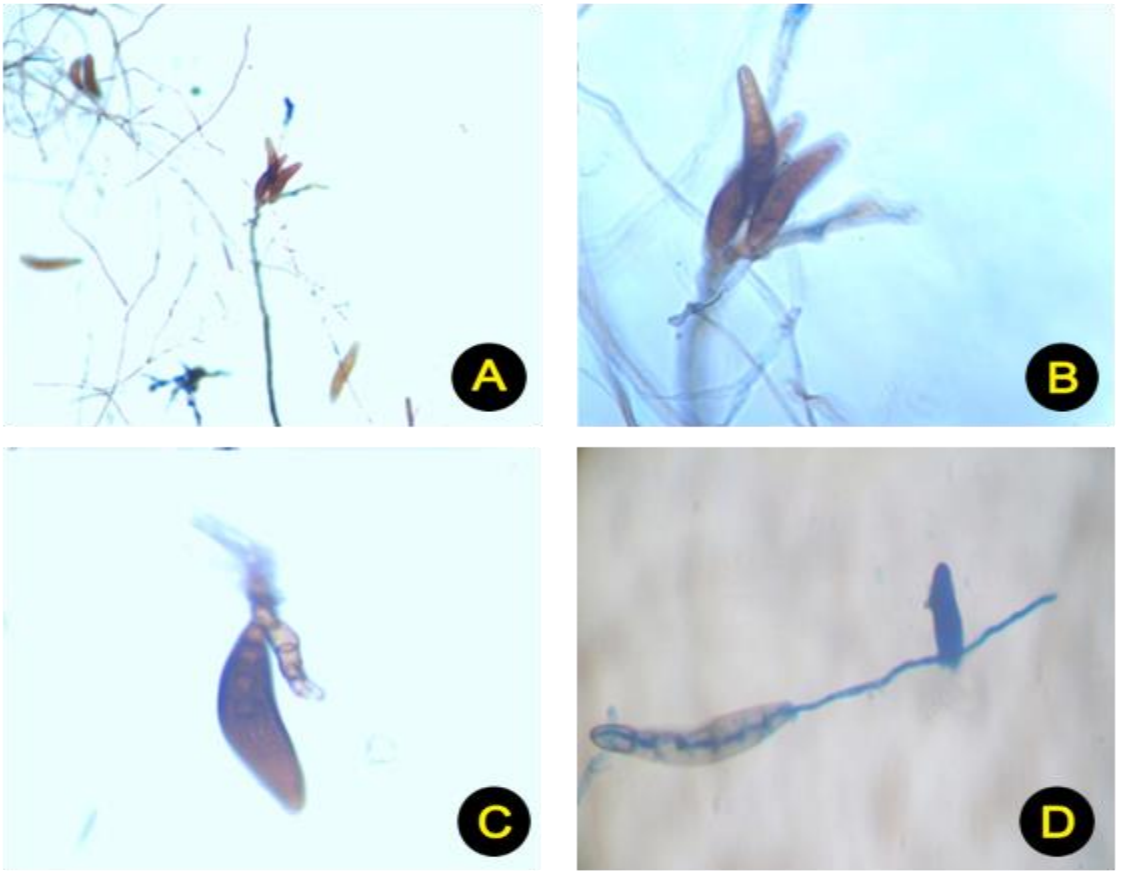


Figure 13: Microscopic view of fungal isolate from infected rice leaves. (A) Conidiophore bearing conidia (10X), (B) Enlarged view of (C 40X), (C) Conidium (40X) and (D) Germinating conidium (40X)

4.4. Bioassay of antifungal compounds

Antifungal activity of the compounds (phytoalexin)collected from the healthy and infected leaves of two resistant cultivars (Loknath 505 and UBKV-1) and three susceptible cultivars (Black Nuniya, Brimful and Champasari)were conducted . The samples were extracted by the process described in Materials and Methods. Prior to use, the extracts were sterilized in disposable Millipore filter (0.22 µm pores) and given in petriplates (50µl) and after evaporation Richard's Agar was given and allowed to solidify. The agar discs of the fungal pathogen were placed and incubated as described in Materials and Methods. All the assays were performed in triplicates. Fungal colony diameter of treated and control sets were measured and percentage of mycelia inhibition was calculated.

4.4.1. Radial growth bioassay of antifungal compound

On the onset, crude extract (ethyl acetate fraction) prepared from healthy and infected rice leaves of all five cultivars were bio assayed following radial growth inhibition assay. Result (Table 6) revealed that mycelia growth of the pathogen was inhibited markedly in the medium supplemented with the extracts of inoculated leaves of resistant cultivars (Loknath 505 and UBKV-1) in comparison to the susceptible cultivars in relation to their respective control.

Table 6. Effects of antifungal compound from rice leaf extracts on radial growth of *D. oryzae*

Cultivars	**Diameter of mycelia (mm)[a]**	
	Healthy	**Infected**
Resistant		
UBKV-1	24.8	9.3
Loknath 505	23.7	11.7
Susceptible		
Black Nuniya	27.9	18.2
Champasari	27.3	18.5
Brimful	28.4	18.8
Control (Richard's Agar)	50	

a= Average of three experimental sets. Diameter was noted after 7 days

4.5. Pathogenicity test of rice cultivars towards *D. oryzae*

4.5.1. Detached leaf

Three most susceptible rice cultivars (Black Nuniya Brimful,and Champasari) were selected for further experiments on the basis of their poor performance among the other rice cultivars against the brown spot disease. Rice leaves inoculated with *D. oryzae* spore suspension, results revealed that disease developed very rapidly in all the three cultivars. Black Nuniya was most susceptible, followed by Brimful and Champasari. After 96h of inoculation (61.03) Percentage of lesion production was obtained in Black Nuniya while in Brimful approximately 59.12 percent lesion production was observed and finally in Champasari approximately 56.22 percent lesion production was observed.(Table 7 ; Fig. 14 C&D).

4.5.2. Whole plant

Three cultivars of well established pot grown rice plants were inoculated with spore suspension of *D. oryzae*. All the three rice cultivars showed the development of disease. The maximum PDI % after 21 days of incubation was observed in case of Black Nuniya 70.05 followed by Brimful 58.22 and Champasari 52.35 as shown in (Table 8; Fig 14A&B).Results obtained from all the three rice cultivars for resistance test performed against *D. oryzae* following detached leaf and whole plant inoculation technique indicated that all the three rice cultivars are susceptible to the fungal pathogen Black Nuniya being the most followed by Brimful and Champasari.

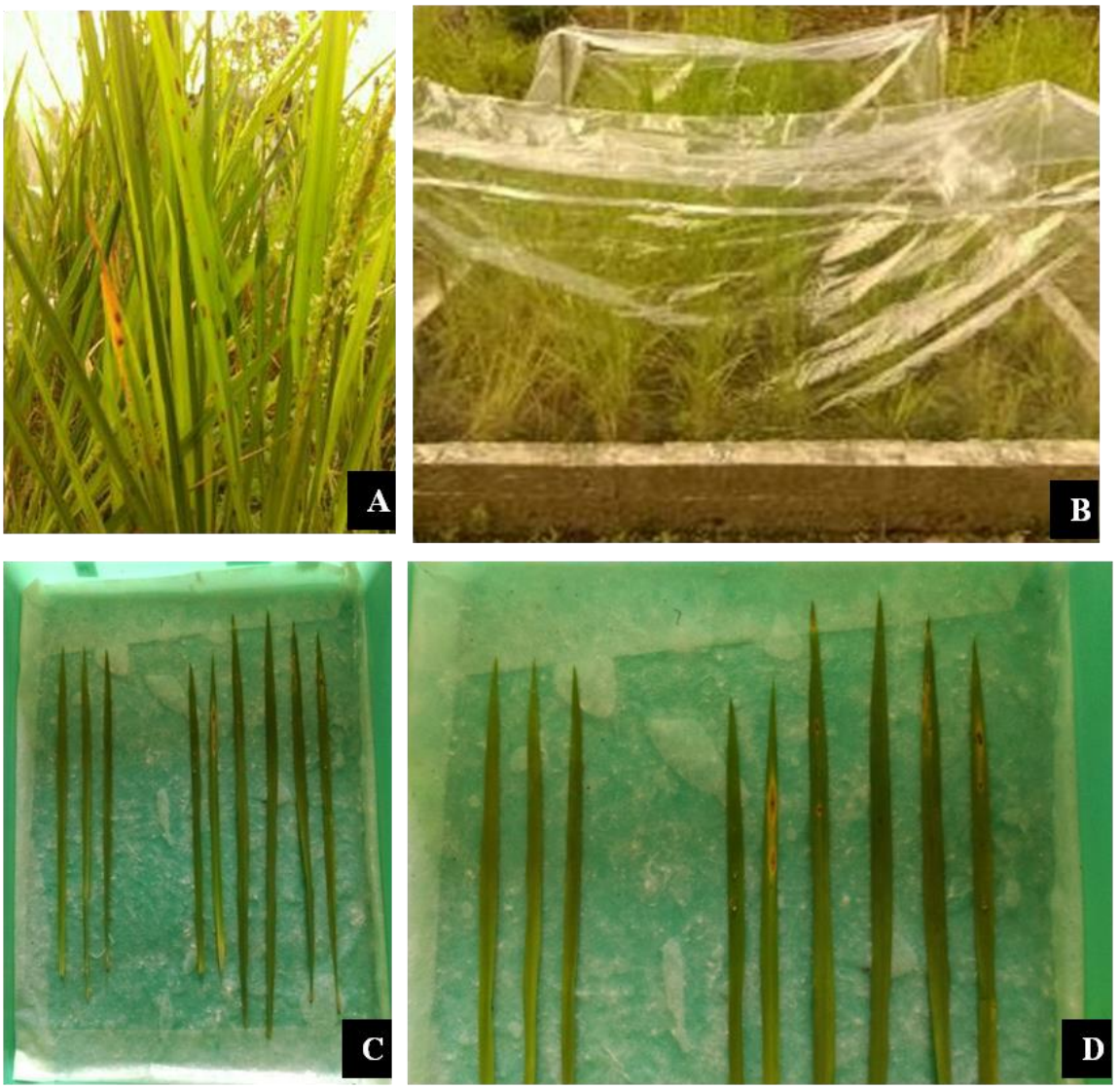


Figure 14: Pathogenecity test of rice cultivars against *D. oryzae*. (A&B) Whole plant (C&D) Detached leaf

Table 7. Pathogenicity test of *D. oryzae* on three rice cultivars following detached leaf inoculation technique

Rice cultivars	**Percent lesion production***		
	Hours after inoculation		
	48	**72**	**96**
Black Nuniya	48.53±1.4	54.23±1.2	61.03±1.2
Brimful	45.02±1.5	50.06±1.8	59.12±1.6
Champasari	40.34±1.9	45.03±1.4	56.22±1.4

*Average of three separate trails, 6 leaves inoculated in each trial ± Standard error

Table 8. Pathogenicity test of *D oryzae* on three cultivars of rice plant following whole plant inoculation technique

Rice cultivars	Percent Disease Index (PDI %)*		
	No. of days after inoculation		
	7	14	21
Black Nuniya	48.14±1.2	58.04±1.5	70.05±1.4
Brimful	46.53±0.5	51.25±1.4	58.22±1.6
Champasari	43.28±0.7	49.34±1.5	52.35±1.2

*Average of three separate trails± Standard error

4.5.3. Immunoenzymatic assays

Pathogenicity tests following detached leaf method as well as whole plant inoculation technique shows that disease symptom development in plants takes almost around 2-3 weeks. However with help of various immunoenzymatic method including PTA-ELISA and Dot immunobinding assays presence of fungal pathogen in plants can be detected as early as 48h of inoculation. These techniques would eventually help in early detection of diseases in plants.

4.5.3.1. PTA-ELISA

After artificial inoculation of rice cultivars with the fungal pathogens it was observed that all the three cultivars were highly sensitive to the organisms. Hence all the three susceptible rice cultivars were selected for further immunological assays. Antigens were extracted at from healthy and artificially inoculated leaves at 24hr interval for 4 days. These antigens (40µg/L) were tested against anti-*D. oryzae* antisera at 1:125 dilution. Infections could be detected from 24hrs onwards in ELISA on the basis of higher absorbance values of infected leaf extracts in comparison to healthy leaf extracts (Table 9).

4.5.3.2. Dot immunobinding assay

For DIBA, total soluble protein extract was prepared from healthy and artificially inoculated leaves of three different rice cultivars. Dot immunobinding assay was performed using these antigen preparations with IgG of *D. oryzae*. Antigens were

spotted carefully on nitrocellulose paper and probed with these IgG. Results have been presented in Table 10 clear and intense colour reactions were observed with homologous antigens. Greater colour intensity was noted in Black Nuniya followed by Brimful and Champasari with the IgG which showed susceptible reaction to the pathogen in pathogenecity tests. The three different cultivars of rice plant showed slight differences in disease reaction with the pathogen infection. The results obtained were similar whether assessed by traditional methods or by immunological techniques, which conclusively proved that all the three rice cultivars are susceptible to the pathogen.

Table 9. ELISA values showing reaction of anti-*D. oryzae* with antigens of healthy and artificially inoculated rice plants after every 24hrs

Rice cultivars	**Anti-*D. oryzae* antisera***	**Time interval (hrs)**			
		24	**48**	**72**	**96**
Black Nuniya	Healthy	0.679±0.003	1.160±0.001	1.255±0.006	1.323±0.008
	Inoculated	0.992±0.010	1.272±0.008	1.329±0.009	1.359±0.012
Brimful	Healthy	0.676±0.002	0.014±0.001	1.135±0.004	1.239±0.002
	Inoculated	0.969±0.010	1.236±0.005	1.262±0.014	1.325±0.014
Champasari	Healthy	0.672±0.003	0.817±0.007	0.931±0.004	0.990±0.009
	Inoculated	0.796±0.006	1.014±0.014	1.202±0.013	1.301±0.010

* Antisera used at 1:125 dilution, Antigen concentration at 40µg/L, absorbance taken at 405nm, ± Standard error

Table 10. Dot immunobinding assay of healthy and artificially inoculated leaf antigen of rice plants using PAb of D. oryzae.

Antigen (40µg/ml)	**PAb of *D. oryzae***	
Rice cultivars	**Healthy**	**Inoculated with *D. oryzae****
Black Nuniya	+++	++++
Brimful	++	+++
Champasari	++	+++
Mycelia: *D. oryzae*	++++	

Colour intensity of dots: + pink; ++ light violet; +++ violet; ++++ deep violet;

NBT/BCIP used as substrate; PAb (1:125); * 48hrs after inoculation.

4.6. Immunological assay for detection of *Drechslera oryzae*

4.6.1. Soluble protein

Mycelia antigen of the pathogen (*D. oryzae*) was initially analysed by SDS PAGE. The molecular weight of protein bands visualized after staining with coomassie blue were determined from the known molecular weight marker. Mycelia protein of *D. oryzae* exhibited 6 bands in SDS PAGE ranging in molecular weight from 97kDa to 14kDa (Fig. 15, C).

4.6.2. Immunological assays

Immunological assays were performed using Polyclonal antibodies (PAb) raised against mycelia protein of *D. oryzae* in rabbit. Effectiveness of antigen in raising antibodies were checked initially using agar gel double diffusion technique followed by dot immunobinding assay and western blot analysis. Optimization of ELISA was done by considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity.

4.6.2.1. Dot immunobinding assay

Dot immunobinding assay using mycelia antigen and PAb of *D. oryzae* was also standardized. Dot immunobinding assays confirm the effectiveness of raising antibodies against *D. oryzae*. Soluble protein obtained from seven-day old mycelia of *D. oryzae* was reacted on nitrocellulose paper with PAb of *D. oryzae.* Result shows development of deep violet colour in homologous reactions indicating a positive reaction suggestive of effectiveness of mycelial antigen in raising PAb against the pathogen. However, faint violet colour was observed in heterologous reactions (Fig 15, A).

4.6.2.2. Indirect immunofluorescence

Indirect immunoflouorescence of young hyphae of *D. oryzae* was conducted with homologous antibody (PAb of *D. oryzae*) and reacted with fluorescein isothiocyanate (FITC) labelled antibodies of goat specific for rabbit globulin. Antibody labelling with fluorescein isothiocyanate is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens shared by host and parasite. Specific detection of cross reactive antigens were confirmed as apple green fluorescence in young mycelia of the pathogen (Fig. 15, B).

4.6.2.3. Western blot analysis

Western blot analysis using PAb of *D. oryzae* was performed to develop strategies for rapid detection of the pathogen. Antibody (3rd bleed) was used to confirm the precipitin reaction done with PAb raised against mycelia protein. For this total soluble protein of 7 days old mycelia was used as antigen source. SDS-PAGE was performed as described previously followed by probing of the localized antigen with alkaline phosphatase conjugate. Sharp bands were produced which was stained blue. The bands on nitrocellulose membrane was compared with corresponding protein bands on the SDS-PAGE. Bands of varying intensities was observed ranging from 50 kDa to 97 kDa (Fig. 15, D).

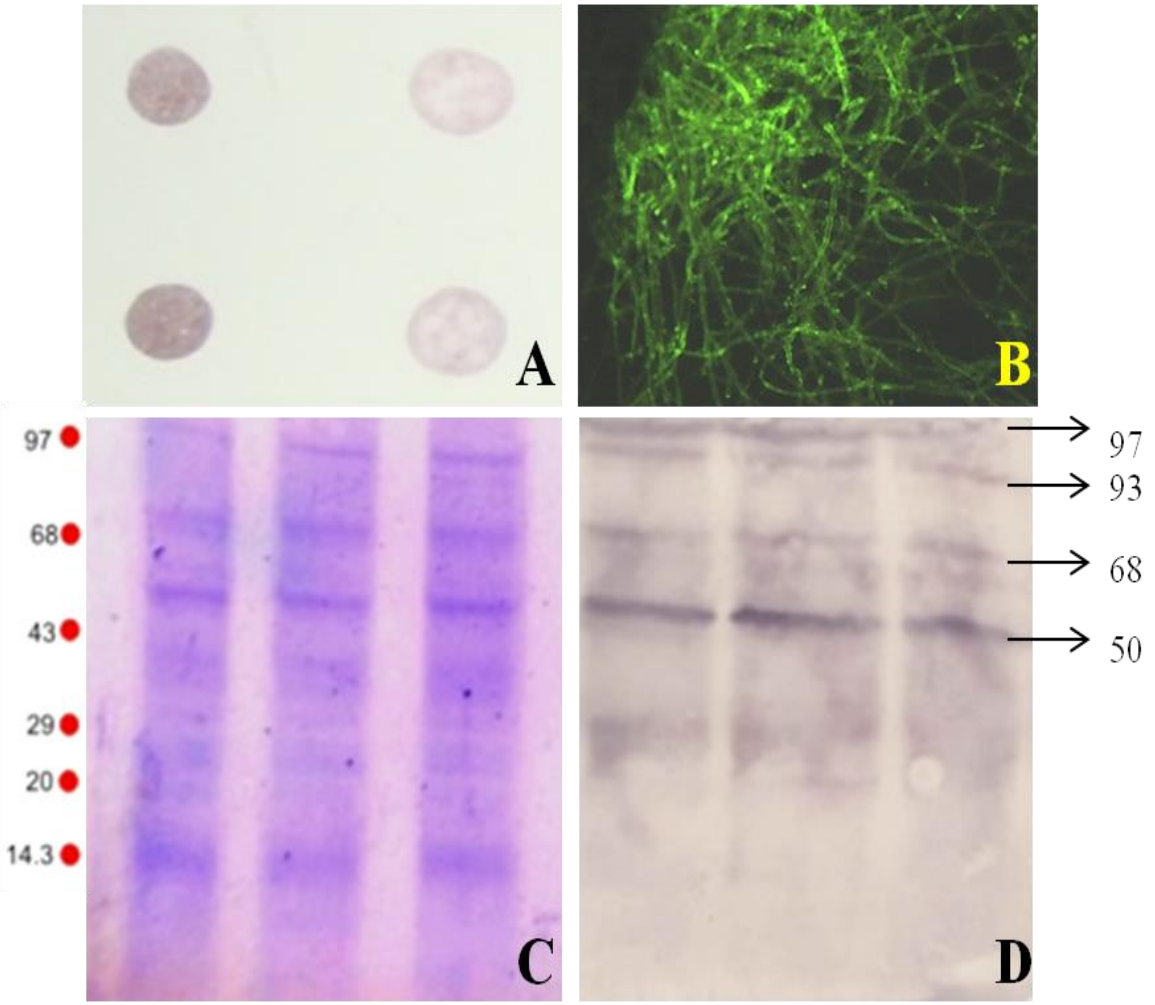


Figure 15: Serological assays of *Drechslera oryzae*; (A) Dot-blot, (B) Immuno-fluorescence of young mycelia treated with PAbs of *D. oryzae* and labelled with FITC, (C) SDS-PAGE and (D) Western blot analysis of *D oryzae*

4.7. Molecular characterization of the pathogen (*D. oryzae*)

4.7.1. 18 S rDNA sequence analysis for identification of pathogens

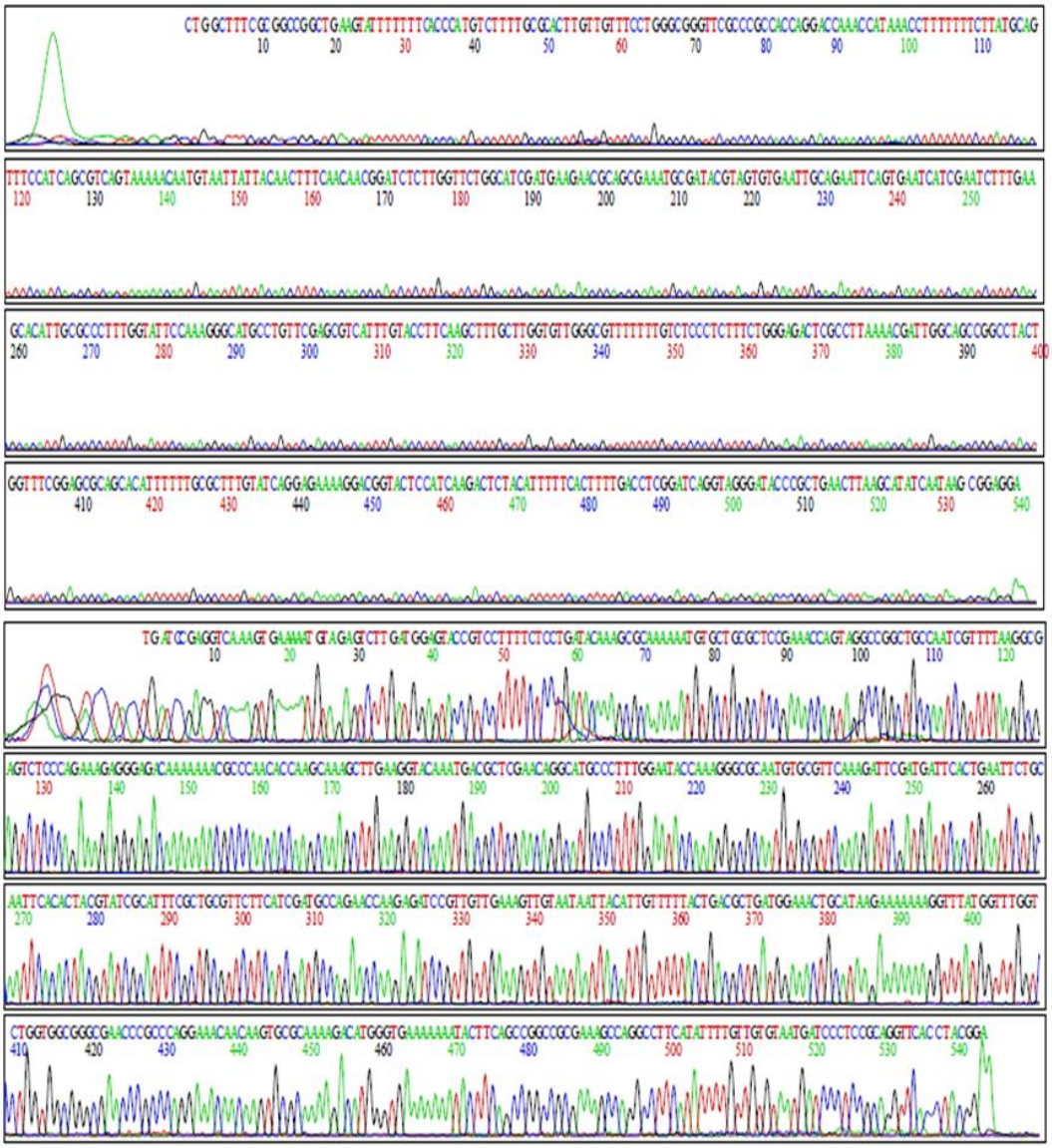
Genomic DNA of *D. oryzae* isolate -R1.DO.01 was isolated and purified and re-suspended in 1X TE buffer until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free. Purity of DNA evaluated in terms of the ratio between absorbance of A_{260} and A_{280} showed that genomic DNA of this pathogen was 1.8. ITS-PCR was performed with the help of ITS specific universal primer pair T/ITS1 and T/ITS4 where a uniform product of 572bp was obtained. The amplicons were sequenced and was further analyzed.

4.7.2. 18S rDNA sequence and BLAST analysis

The BLAST query of the 18S r DNA sequence of the isolate R1.DO.01 against GenBank database confirmed the identity of the isolate as *Drechslera oryzae*. The sequences have been deposited in NCBI, GenBank database under the accession no.**KT768092.** The sequence chromatograms have been represented in Fig. 16.

4.7.3. Multiple sequence alignment

A multiple sequence alignment of ITS gene sequences of *Drechslera oryzae* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1software. The use of CLUSTAL-W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig. 17). Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *Drechslera oryzae* (KT768092) (Table 11).



Partial sequence of 18S ribosomal RNA gene

TAGGTGAACCTGCGGAGGGATCATTACACAACAAAATATGAAGGCCTGGCTTTCG
CGGCCGGCTGAAGTATTTTTTTCACCCATGTCTTTGCGCACTTGTTGTTTCCTGGG
CGGGTTCGCCCGCCACCAGGACCAAACCATAAACCTTTTTTTCTTATGCAGTTTCC
ATCAGCGTCAGTAAAAACAATGTAATTATTACAACTTTCAACAACGGATCTCTTGG
TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAA
TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGG
CATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTTTGCTTGGTGTTGGGCGTTTT
TTTGTCTCCCTCTTTCTGGGAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTG
GTTTCGGAGCGCAGCACATTTTTTGCGCTTTGTATCAGGAGAAAAGGACGGTACTC
CATCAAGACTCTACATTTTTCACTTTTGACCTCGGATCAGGTAGGGATACCCGCTG
AACTTAAGCATATC

Sequence deposited: NCBI
ACCESSION: KT768092
VERSION: KT768092.1
GI: 944552010 DNA linear 572bp

Title: Molecular identification of *Drechslera oryzae* isolated from infected leaf of *Oryza sativa* (L.) (cultivar-Brimful)

Figure 16: Chromatogram and sequence of 18S rDNA region *Drechslera oryzae* strain RI.DO.01 deposited in NCBI Genbank

Figure 17: 18S r DNA sequence alignments of *Drechslera oryzae* (KT768092) with other extypes isolate. The conserved regions of the gene are demonstrated in different colour.

Table 11.Genbank Accession Numbers and Geographic locations of the *Ex-Type* strains of *D. oryzae* that showed homology with the isolate R1.DO.01

Accession No.	Strain/ isolate	rDNA Sequence (bp)	Country
DQ300201	Palawan-Monopolar (PM)	584	Philippines
DQ300204	Cavinti-Monopolar (CM)	584	Philippines
DQ300203	Cavinti-Intercalary (CI)	584	Philippines
DQ300206	San Pablo-Intercalary (SI)	584	Philippines
DQ300207	San Pablo-Monopolar (SM)	584	Philippines
JN093305	PG10	572	India
GU222691	87	569	USA
GU080212	BsDR1	540	Oman
KC916692	TC2-022	448	Canada
KF539843	6g	508	Argentina
GU480916	B54	507	Iran
HM195254	Bs 63	569	India
GU222692	88	572	USA
HM195262	Bs 92	569	India
HM998310	MvNorthCarDukeForU8	578	USA
HM195258	Bs 72	570	India
DQ061108	JTO396b	602	Australia
GU222690	86	515	USA
AY004800	DAOM 126766	552	Canada
FJ746665	ATCC MYA-3300	530	USA
HM998314	MvNorthCarDukeForU13	566	USA
KJ476182	RWB 1035	612	Brasil
KJ476183	RWB 1158	611	Brasil
KJ476184	RWB 1212	616	Brasil
AY004784	DAOM 126772	577	Canada
KT768092	**R1.DO.01**	**572**	**India**

4.7.4. Phylogenetic analysis of *Drechslera oryzae*

Phylogenetic analysis of *D. oryzae* was done (Fig 18).The evolutionary history was inferred using the Neighbor-Joining method (Saitou N &Nei M,1987). The optimal with the sum of branch length = 36.26551106 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein J, 1985). The tree is drawn to scale, with branch length in same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K, Nei M & Kumar S, 2004) and are in the units of the number of base substitutions per site. Codon position included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated

from the dataset (Complete deletion option). There were a total of 435 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura K, Dudley J, Nei M & Kumar S, 2007).

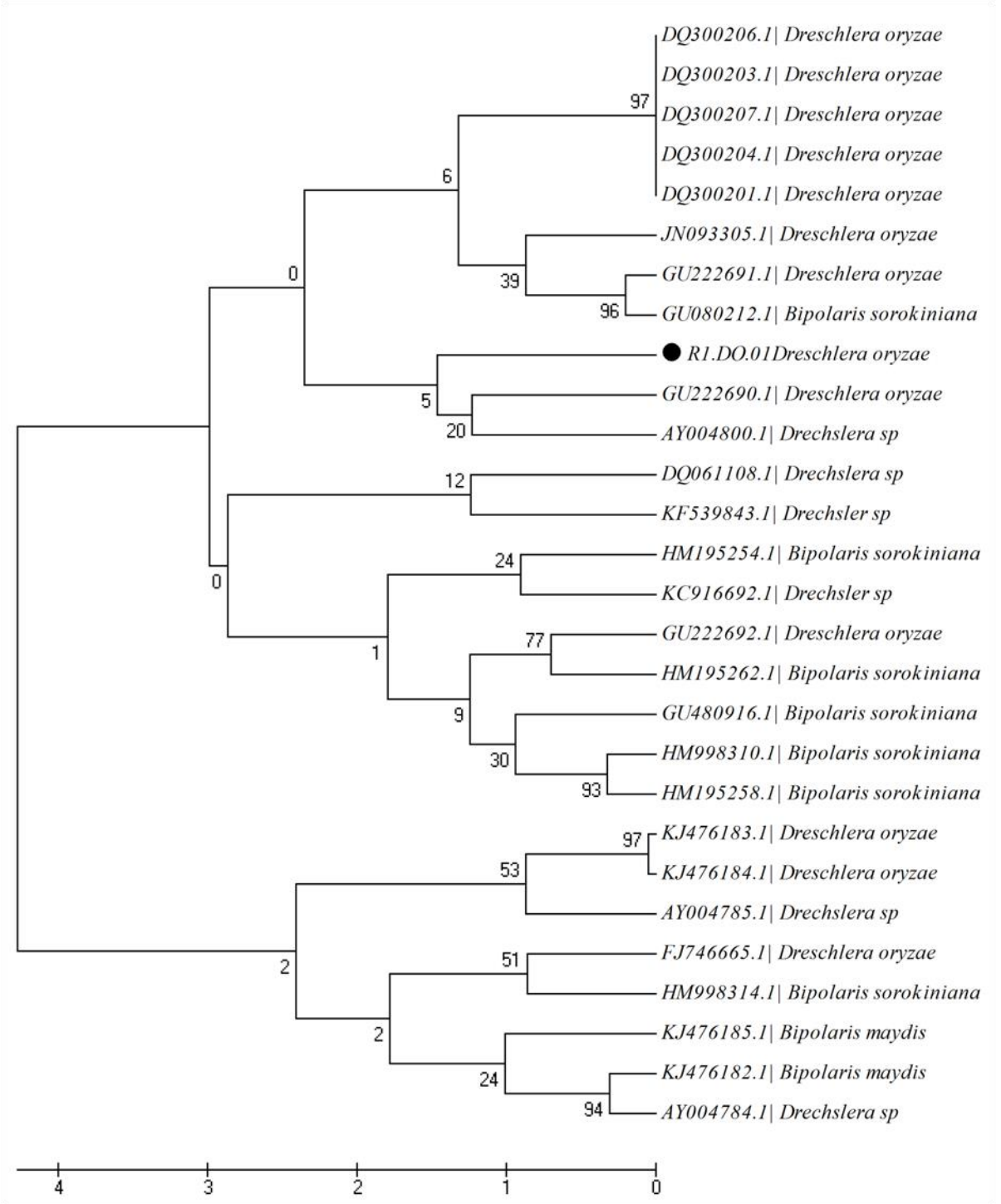


Figure18: Phylogenetic placement of *Drechslera oryzae* KT768092with other ex-type strains obtained from NCBI GenBank Database.

4.8. Association of Arbuscular Mycorrhizal Fungi (AMF) in rice cultivars

Arbuscular mycorrhizal fungal spores from rhizospheric soils of fifteen different rice cultivars grown in experimental plots were isolated and their average spore population and percent root colonization were determined. Morphological features of isolated AMF spores were critically examined with special reference to variation in size, colour, wall thickness, shape, wall layers specially germinal wall, coriaceous wall, amorphous wall and beaded wall layers, hyphal branching patterns, the diameter, structure and the staining intensity of hyphae. Average population of AMF spores obtained from different rice rhizosphere have been presented in Table 12.

Table 12. Population count of AM Fungi in rhizosphere of fifteen different rice cultivars and percentage colonization in root.

Sl. No.	**Rice cultivars**	**Percentage of VAM spores in rhizospheric soil (%)**					**Root colonization (%)**
		Glomus	***Gigaspora***	***Scutellospora***	***Acaulospora***	***Entrophospora***	
1	Loknath 505	78.04	19.59	1.68	00.33	00.33	99 %
2	Gouraknath 509	83.85	15.09	-	01.04	-	91 %
3	Kaberi 9090	67.17	27.30	0.61	04.90	-	93%
4	Champasari	80.00	20.00	-	-	-	90%
5	Brimful	85.52	13.15	1.3	-	-	99%
6	Black Nuniya	83.33	13.88	-	02.77	-	98%
7	Adde	66.66	31.81	-	01.51	-	98%
8	Sano Masuri	65.94	33.74	-	00.30	-	93%
9	Maiti	83.30	13.88	-	02.77	-	99%
10	Attheu	78.02	17.48	1.1	03.36	-	96%
11	Swarnamasuri	69.7	23.25	5.81	01.16	-	99%
12	TulaiPanji	65.19	33.77	01.04	-	-	96%
13	UBKV-1	90.39	5.64	01.12	02.82	-	98%
14	UBKV-4	60.30	36.43	00.75	02.51	-	100%
15	UBKV-5	50.07	41.07	02.52	06.31	-	98%

Among all the genera, the genus *Glomus* was predominant in almost all the rice cultivars. UBKV-5 containing the least amount (50.07%) and that of UBKV-1 containing the highest amount (90.39%) followed by *Gigaspora*, lowest amount found in UBKV-1(5.64%) and highest amount found to be in UBKV-5(41%). *Acaulospora*, found to be lowest amount in Sanomassuri (0.30%) and highest amount in UBKV-5 (6.31%) *Scutellospora*, was found to be in least quantity in Kaberi 9090 (0.61%) and

highest amount reported in Swarnamasuri (5.81%)and lastly *Enthropospora* was found in one of the rice rhizosphere that is Loknath 505 (0.335). Different VAM population found in each of the rice cultivars have been shown in Figure 19-33.

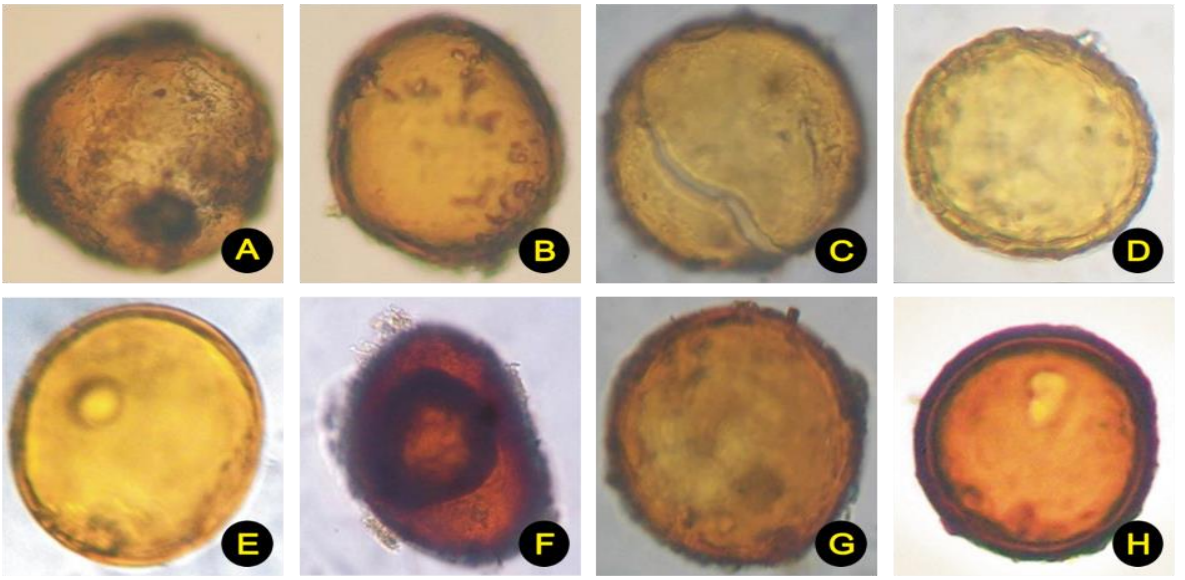


Figure 19:Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Brimful). (A) *Glomus* sp.; (B) *Glomus* sp.;(C) *Glomus* sp.;(D) *Glomus fasciculatum*; (E) *Glomus mosseae* (juvenile);(F) *Scutellospora* sp.;(G) *Glomus* sp.;(H) *Glomus mosseae* (mature).

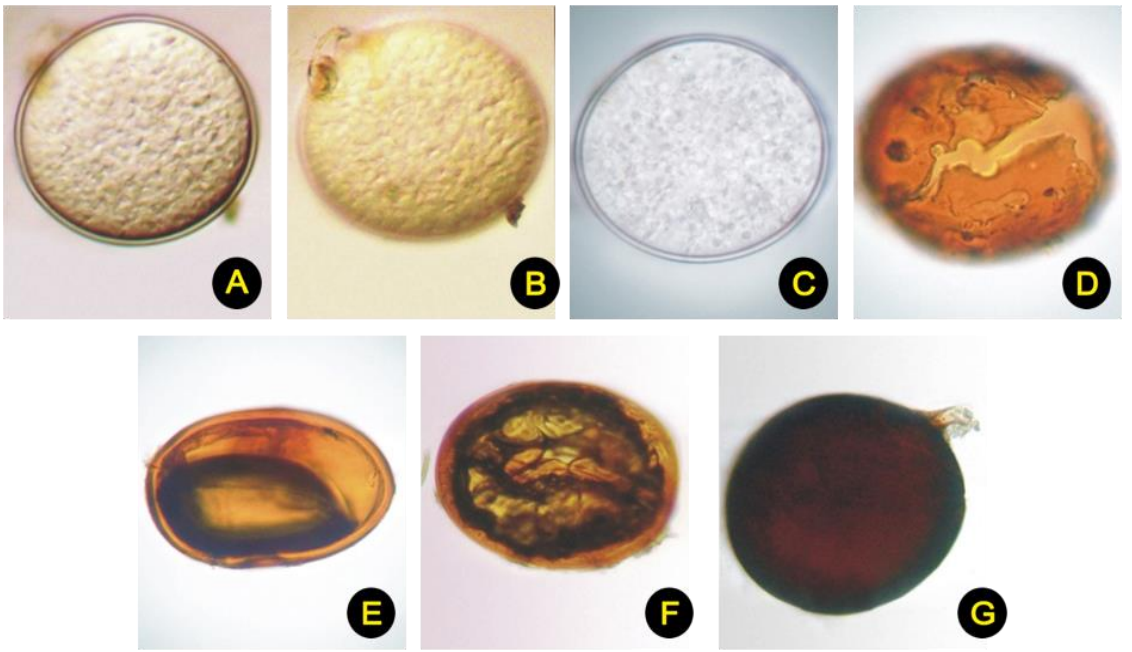


Figure 20: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Tulaipanji).(A) *Glomus* sp. ;(B)*Glomus* sp.;(C) *Glomus* sp.;(D) ruptured spore of *Glomus* sp.;(E) *Scutellospora* sp.;(F) mature *Glomus* sp.;(G) *Glomus constrictum*

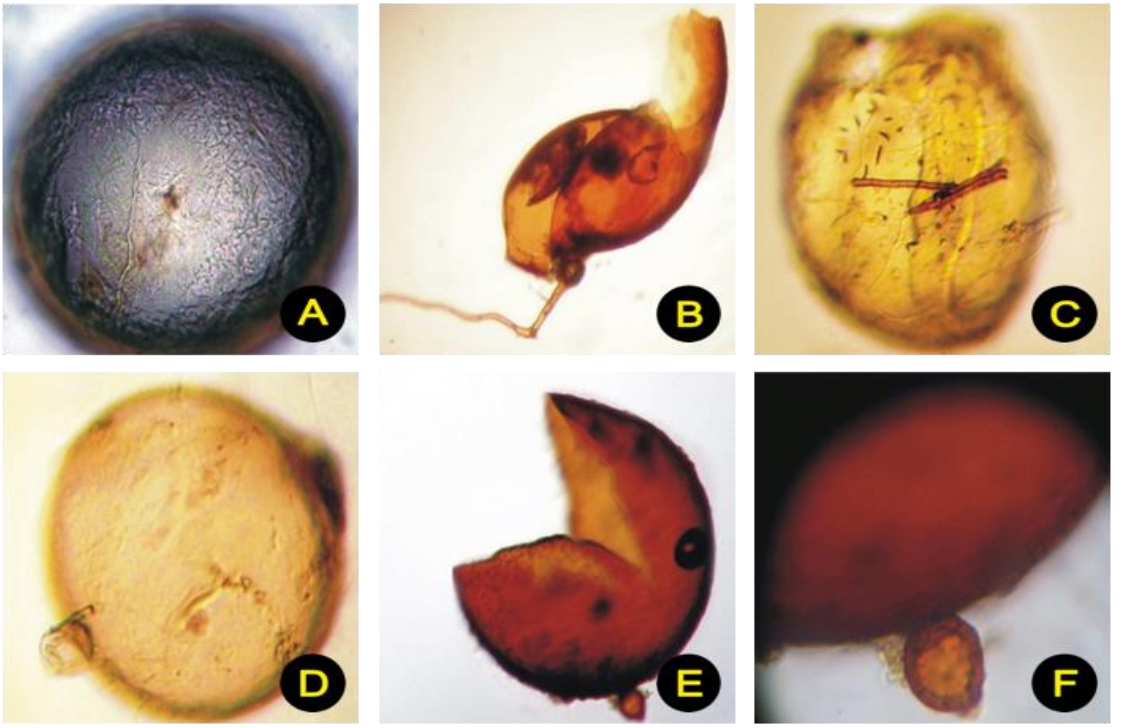


Figure 21: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-5).(A) *Acaulospora* sp.;(B) ruptured *Gigaspora* with conspicuous hyphal attachment;(C) *Scutellospora* sp.;(D) *Glomus* sp.;(E)*Scutellospora* sp.;(F) *Scutellospora* with hyphal attachment (magnified)

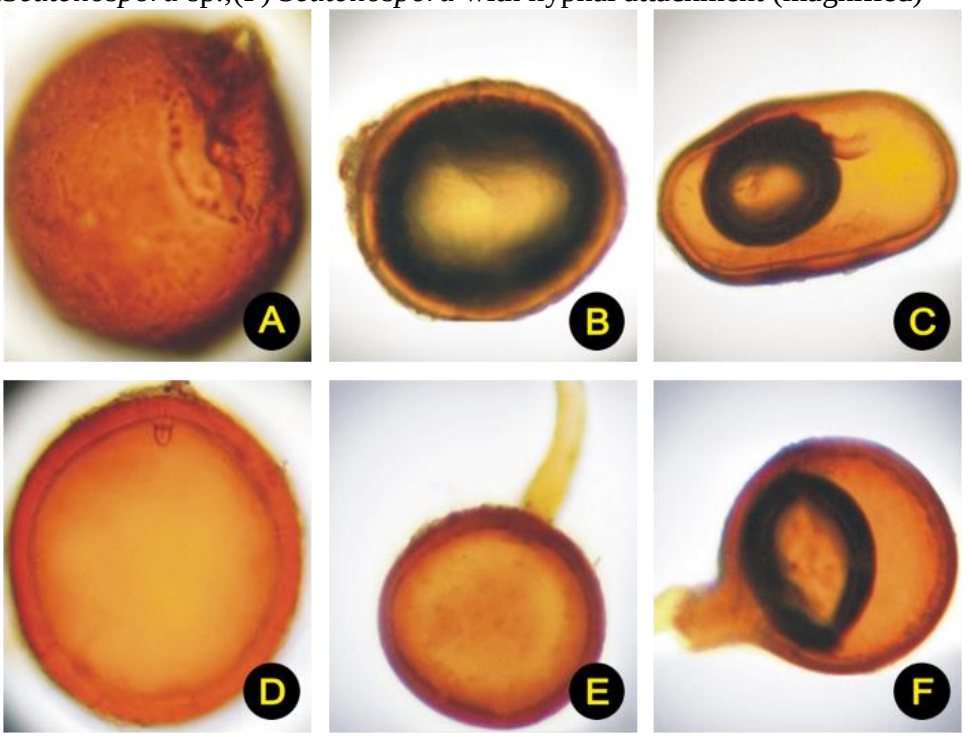


Figure 22: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-4).(A)*Scutellospora* sp.;(B) *Gigaspora* sp.;(C) *Glomus* sp.;(D)*Glomus* sp.;(E) Hyphal attachment of *Gigaspora* sp.;(F) *Scutellospora* sp.

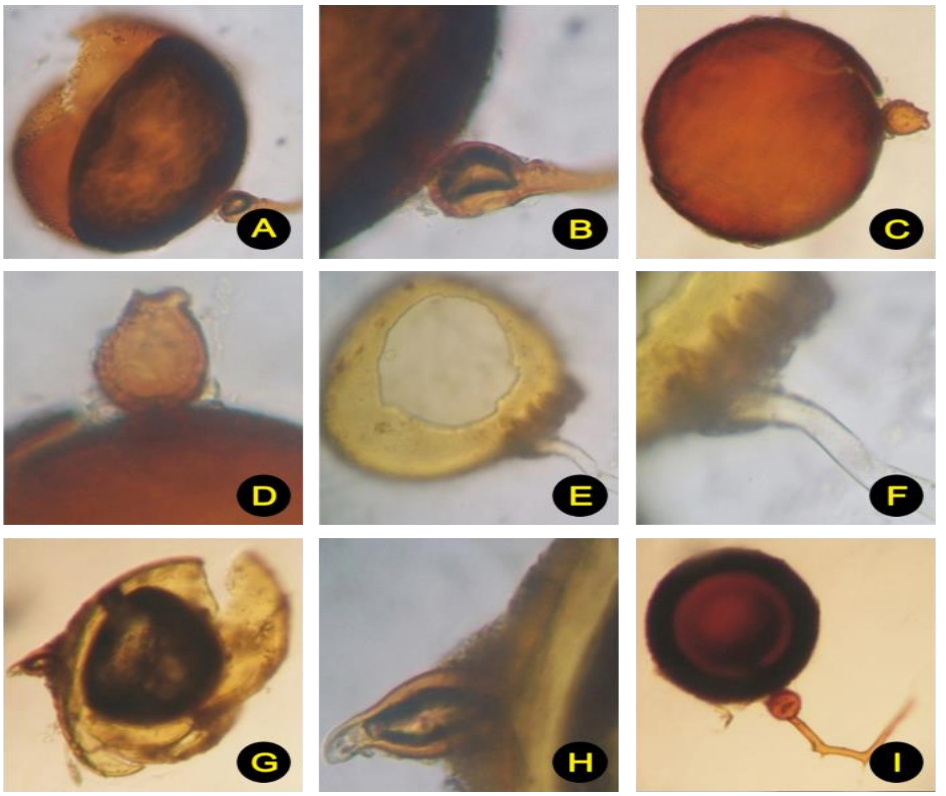


Figure 23: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Black Nuniya).(A) *Glomus* sp.;(B) *Glomus* sp.;(C) *Scutellospora* sp.;(D*) Glomus* sp.;(E) *Glomus* sp.; (F) *Acaulospora* sp.; (G) *Glomus* sp.;(H) *Glomus* sp.; (I) *Glomus* sp.

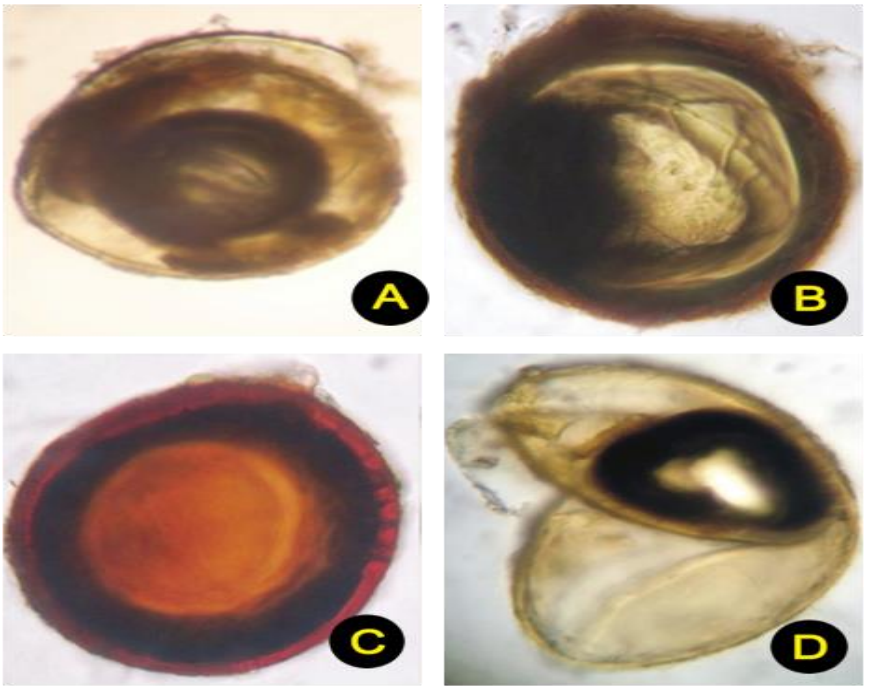


Figure 24: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Champasari).(A) *Gigaspora* sp.;(B) *Glomus* sp.;(C) Ruptured spores of *Scutellospora* sp.;(D) *Scutellospora* sp.(matured)

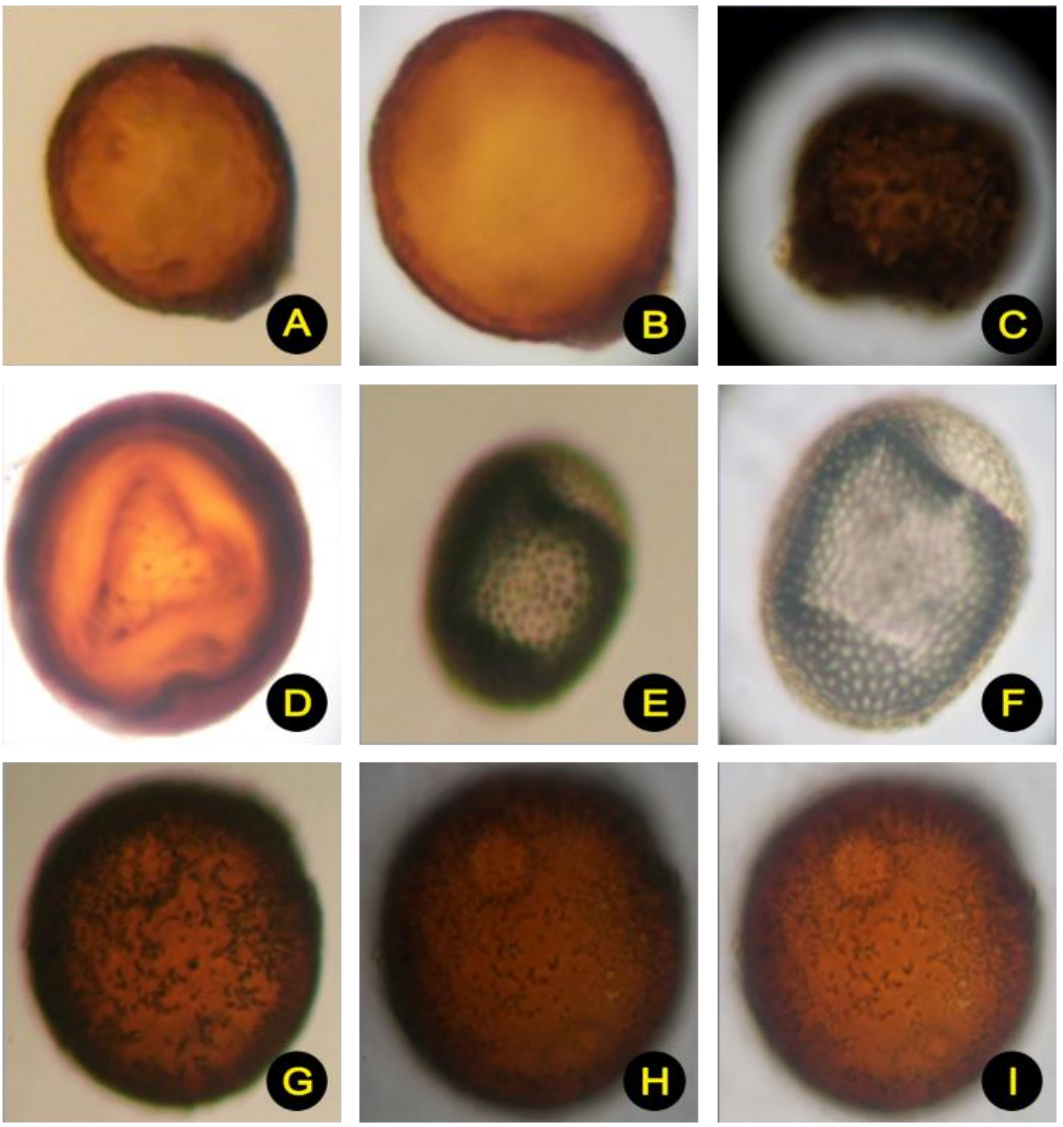


Figure 25: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Adde).(A) *Glomus constrictum*;(B) *Glomus constrictum*;(C) *Glomus mosseae*;(D) *Acaulospora* sp.;(E) *Acaulospora* sp.;(F) *Acaulospora* sp.;(G,H&I) *Glomus mosseae* in different angles

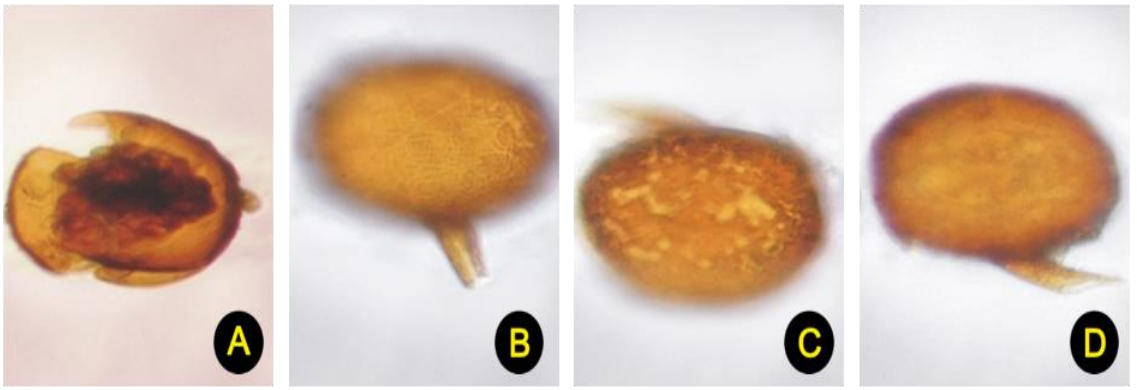


Figure 26: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-1).(A) *Gigaspora* sp.;(B) *Glomus* sp.;(C)*Glomus* sp.;(D)*Glomus* sp.

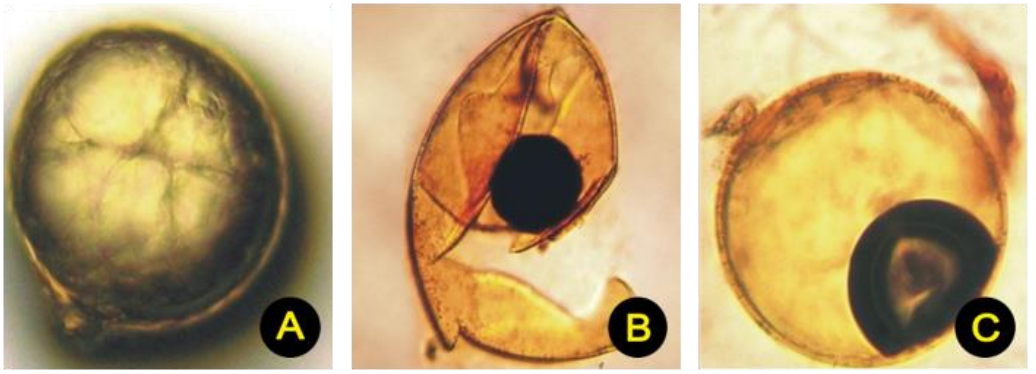


Figure 27: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Swarnamasuri).(A) *Scutellospora* sp.; (B) Ruptured *Glomus* sp.;(C) *Glomus* sp. with hyphae

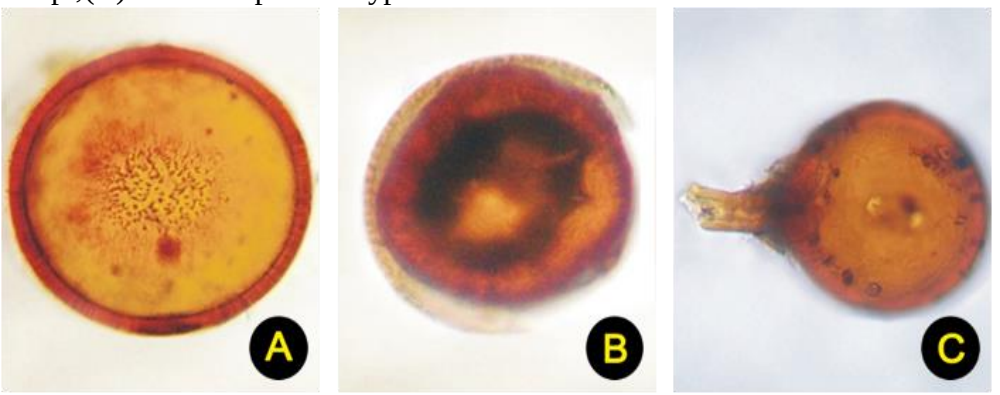


Figure 28: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Maiti). (A) *Scutellospora* sp.;(B) *Scutellospora* sp.;(C) *Gigaspora margarita*

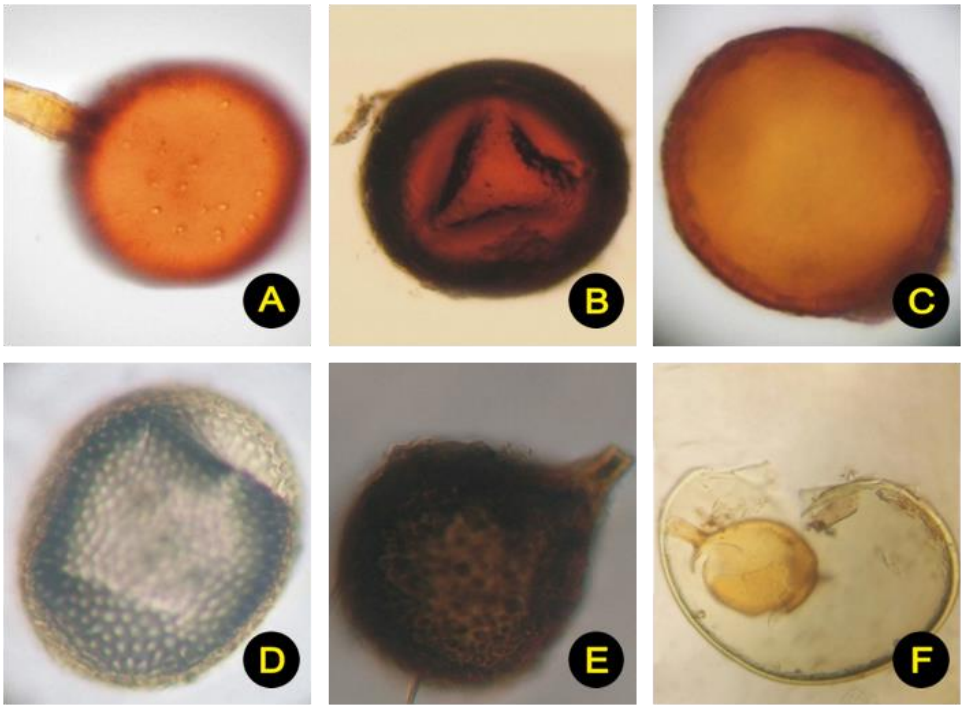


Figure 29: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Attheu).(A) *Glomus* sp.;(B) *Scutellospora* sp.;(C) *Glomus* sp;(D) *Acaulospora* sp.;(E) *Glomus constrictum*;(F)Ruptured AMF.

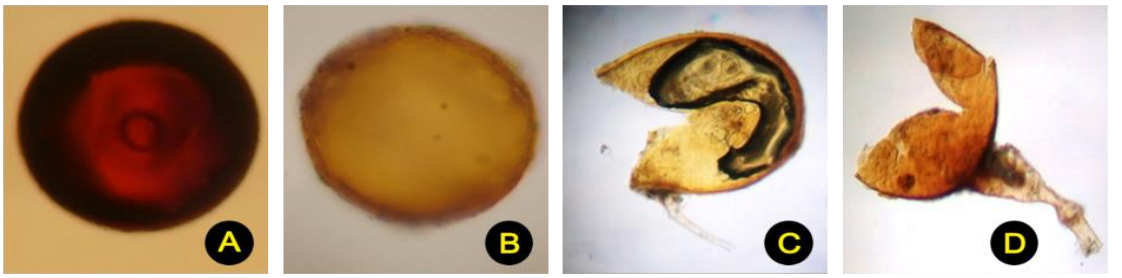


Figure 30: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Sanomasuri).(A) *Scutellospora* sp.;(B) *Scutellospora* sp.;(C) *Glomus* sp.;(D) *Gigaspora rosea*.

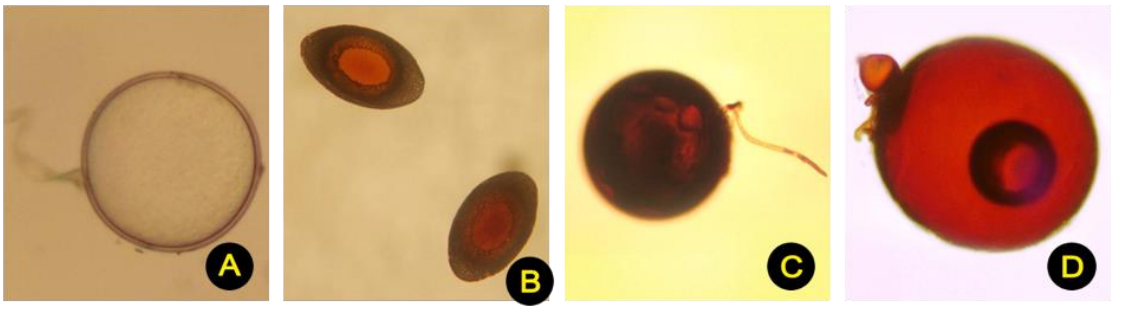


Figure 31: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Loknath 505).(A) *Glomus* sp. juvenile;(B) *Entrophospora* sp.;(C)*Glomus* sp.;(D) *Scutellospora* sp. with hyphal attachment

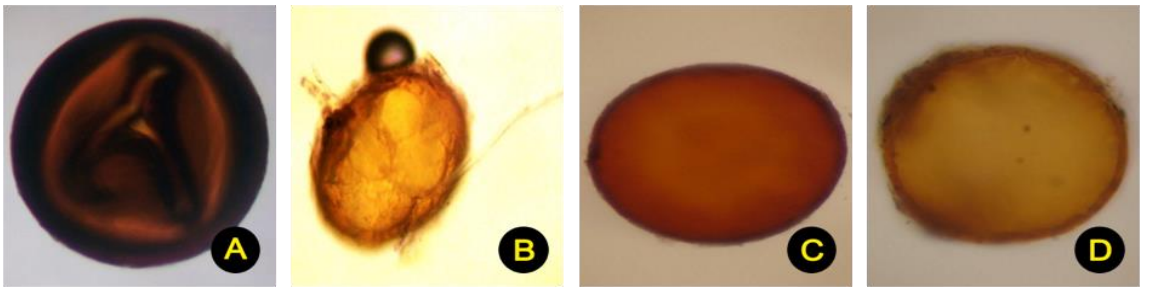


Figure 32: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Kaberi 9090).(A) *Glomus* sp.;(B) *Glomus* sp.;(C) *Glomus badium* ;(D) *Glomus badium*

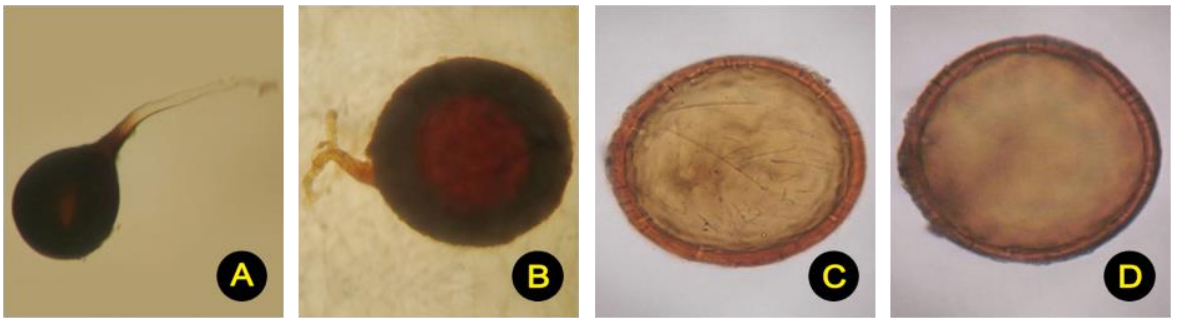


Figure 33: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Gouraknath 509).(A) *Glomus fasciculatum*;(B) *Glomus multicaule*;(C) *Glomus* sp.;(*Glomus* sp.)

4.8.1. Characterization and identification of AMF

AMF spores collected from rhizospheric soil of various rice cultivars were initially characterized on the basis of their morphological features like size, shape, spore wall texture, subtending hyphe, colour, hyphal attachment, spore wall ornamentation as well a microscopical characters like wall layers and wall thickness. The detailed descriptions of the most dominant spores *Rhizophagus fasciculatus* previously named as named as *Glomus fasciculatum* followed by *Funneliformis mosseae* previously named as *Glomus mosseae* , *Glomus badium, Glomus constrictum, Glomus multicaule, Gigaspora margarita* and *Gigaspora rosea* have been presented in Table 13.

Table 13.Morphological and microscopical characters of dominant AMF spores

Rhizophagus fasciculatus Walker and Schubler	Spore colour : Pale yellow to bright brown
	Spore size and shape : Globose to subglobose, size ranges from 70-120µm in diameter.
	Sub cellular structure: Spores produced directly with one or more subtending hyphae attached to it. Spore wall is continuous, consisting of three layers (L1, L2, and L3).
	Subtending hyphae : Single subtending hyphae attached with the spore.
Funneliformis mosseae Walker and Schubler	Spore colour: Brown to orange-brown
	Spore size and shape: Size ranges from 80-180 µm. Globose to sub-globose, sometimes irregular. Sporocarp contains 2-5 spores surrounded in a peridium.
	Subcellular structure: Presence of three hyaline layers with subtending hyphae attached.
	Subtending hyphae: Funnel shaped double layered hyphae.
Glomus badium Oehl, Redecker and Sieverd	Spore colour : brownish orange to reddish brown
	Spore size and shape : Spores occur in dense sporocarps; mainly ovoid to irregular; sometimes globose to subglobose; 250-320 µm diameter
	Subcellular structure : composed of two layer
	Subtending hyphae : from a hyphal plexus and separated by an interspore mycelium and occasionally by cystidium-like

	structures.
Glomus constrictum Trappe	Spore colour : brownish orange to dark brown
	Spore size and shape : globose to subglobose; 160µm diam in average
	Subcellular structure : consists of one wall containing two layers, most juvenile spores with spore wall layer 1 only
	Subtending hyphae : Subtending hyphae brownish orange to dark brown; straight or curved; usually markedly constricted at the spore base, sometimes cylindrical, flared to funnel-shaped
Glomus multicaule Gerdemann and Bakshi	Spore Colour: Brownish orange to dark brown
	Spore size and shape: Size ranges from 149-249 X 124- 162 µm.Ellipsoid,broadlyellipsoid,subglobose or triangular
	Subcelluar structure: Presence of one hyaline layer subtending hyphae attached.
	Subtending hyphae:Subtending hyphae varies from 1-4, thick ornamented spore
Gigaspora margarita W.N. Becker and I.R. Hall	Spore colour : yellowish white to sunflower yellow
	Spore size and shape :globose to subglobose; 357 µm diam; sometimes ovoid; 320 X 370 µm.
	Subcellular structure : Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell that composed of two layers
	Subtending hyphae : single subtending hypha attached with the spore
Gigaspora rosea T.H. Nicolson and N.C. Schenck	Spore colour : Pale cream with a pale pink tint in new healthy spores
	Spore size and shape: Globose to subglobose. Size distribution: 160-280 µm.
	Subcelluar structure : Spore wall consists of three layers (L1, L2, and L3
	Subtending hyphae : single subtending hyphae attached with the spore

4.8.2. Histopathology and root colonization with AMF in rice cultivars

Fifteen rice cultivars grown in the experimental plots (4 months old plant) were studied extensively to explore the diversity and mycorrhization.100% highest root colonization was noticed in UBKV-4 followed by 99% in Swarnamasuri, Maiti, Brimful and Loknath 505, 985 in Black nuniya, Adde, UBKV-1 and UBKV-5, 96% in Tulaipanji and Attheu, 93% in Kaberi 9090, Sano masuri and Gouraknath 509 and finally Champasari was found to have lowest root colonization of 90% (Table 12).Root samples taken from each of the fifteen cultivars were examined under microscope and mycorrhization was documented. The physical nature of arbuscules; vesicles, intraradical hyphae etc were studied extensively to determine the colonization impact of these rice cultivars. Root colonization observed in all the rice cultivars has been shown in Figure 34-37,which clearly shows that the ability of AM colonization is maximum in commercial and research cultivars than the ethnic and local cultivars. The results clearly shows that the roots of ethnic rice cultivar Champasari has the least ability to colonize AM fungi while that of research cultivar UBKV- 4 has the maximum.

After the identification of AMF the fresh cleaned spores of *R. fasciculatus* were given for mass multiplication in maize plant (*Zea mays*).Maize plants were grown in field as well as in pots with sterilized soils (Fig. 38) to discard other fungalpropagules.The plants were regularly watered for 60 days after inoculation.The root colonization behaviour of the AMF spores within the root tissues of the inoculated maize plants were studied. Presence of many vesicles, spores, intracellular hyphae and extraradical hyphae with spores were observed. (Fig.39).

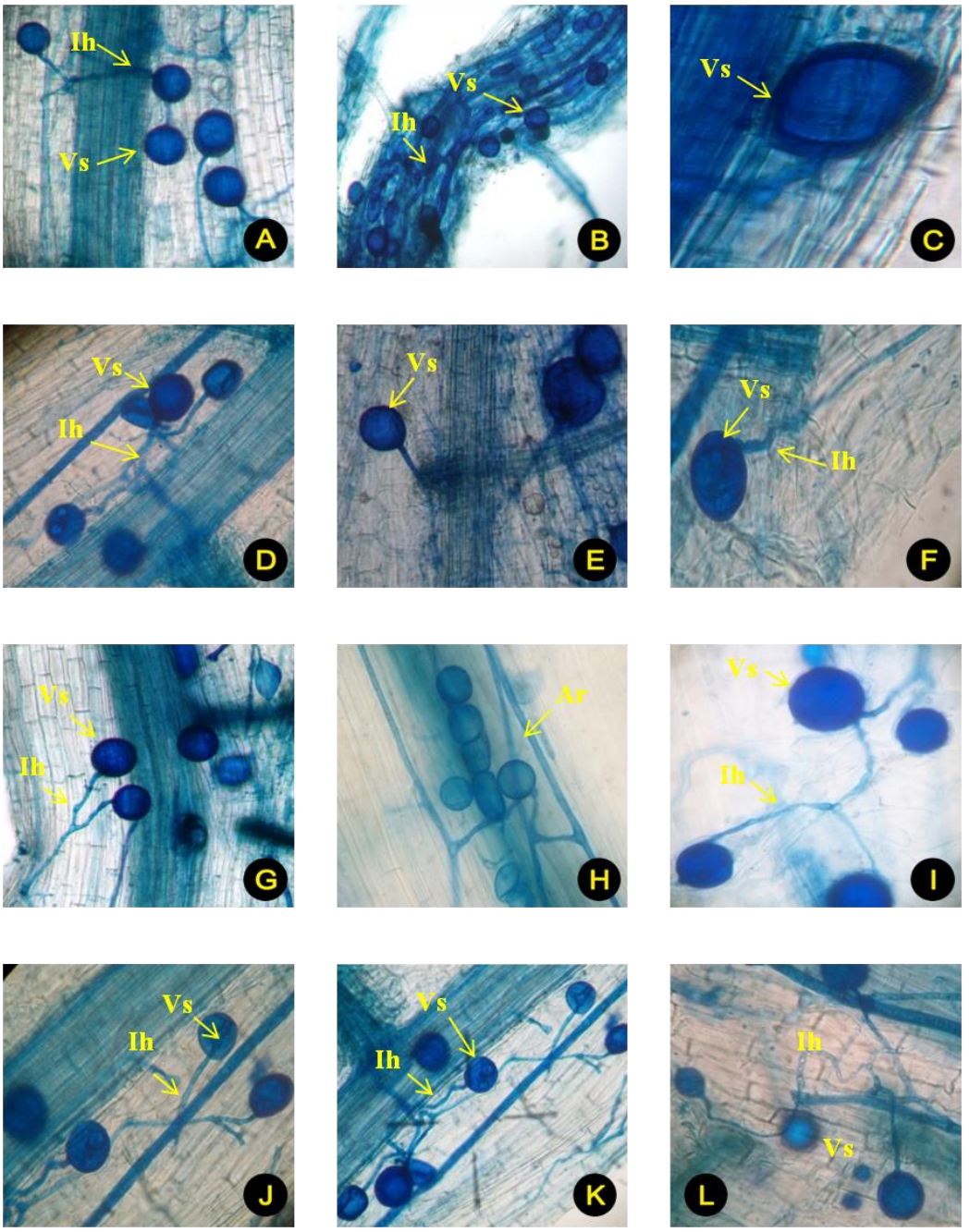


Figure 34: Root colonization of rice cultivars. (A-C) Brimful; (D-F) Black nuniya; (G-I) Champasari and (J-L) Attheu.(Ih- Intracellular hyphae, Vs- Vesicle, Ar- Arbuscule).

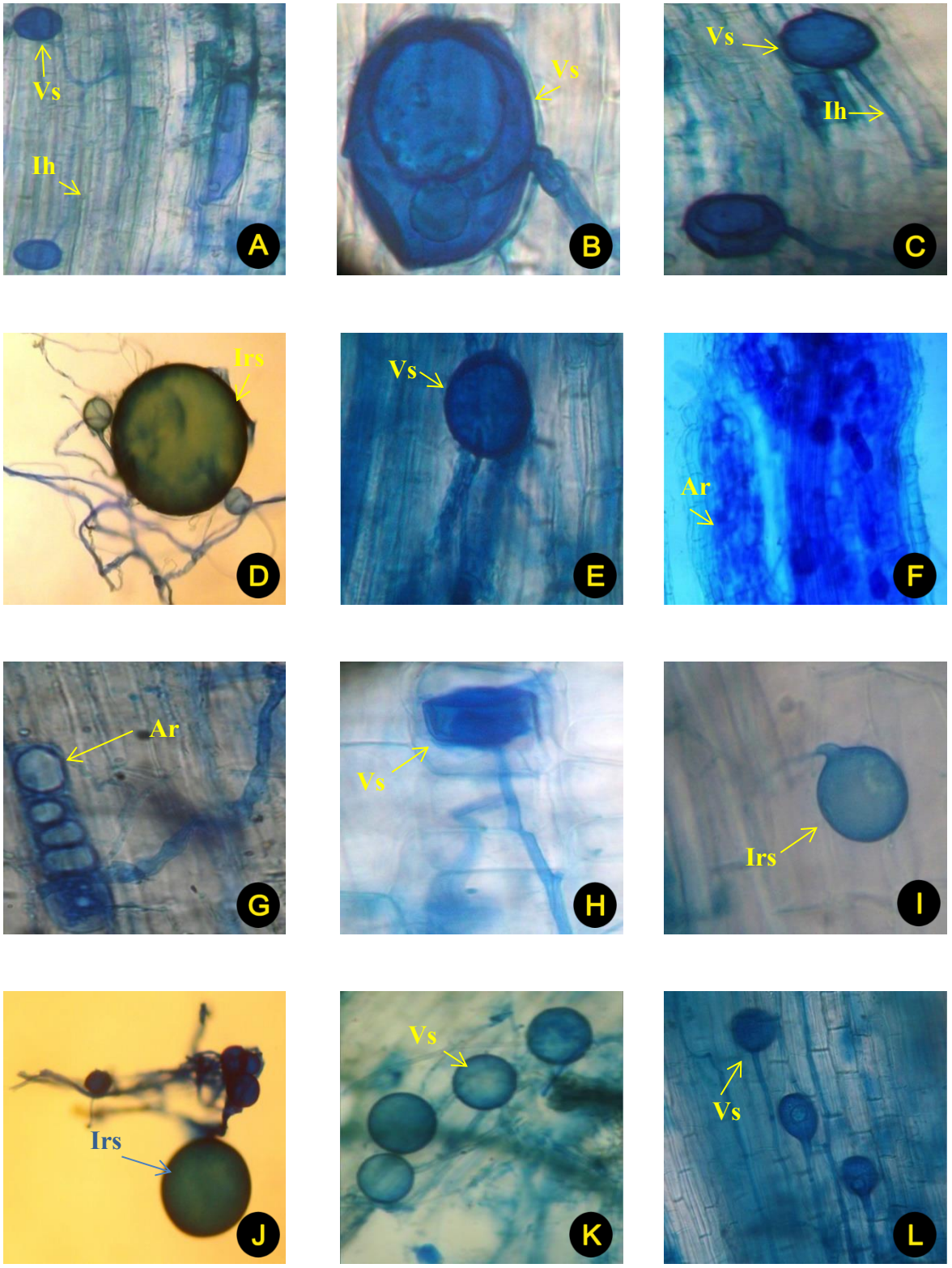


Figure 35: Root colonization of rice cultivars. (A-C)Maiti; (D-F) Sano masuri; (G-I)Tualaipanji and (J-L)Swarnamasuri.(Ih- Intracellular hyphae,Irs- Intra radicle spore, Vs- Vesicle, Ar- Arbuscule).

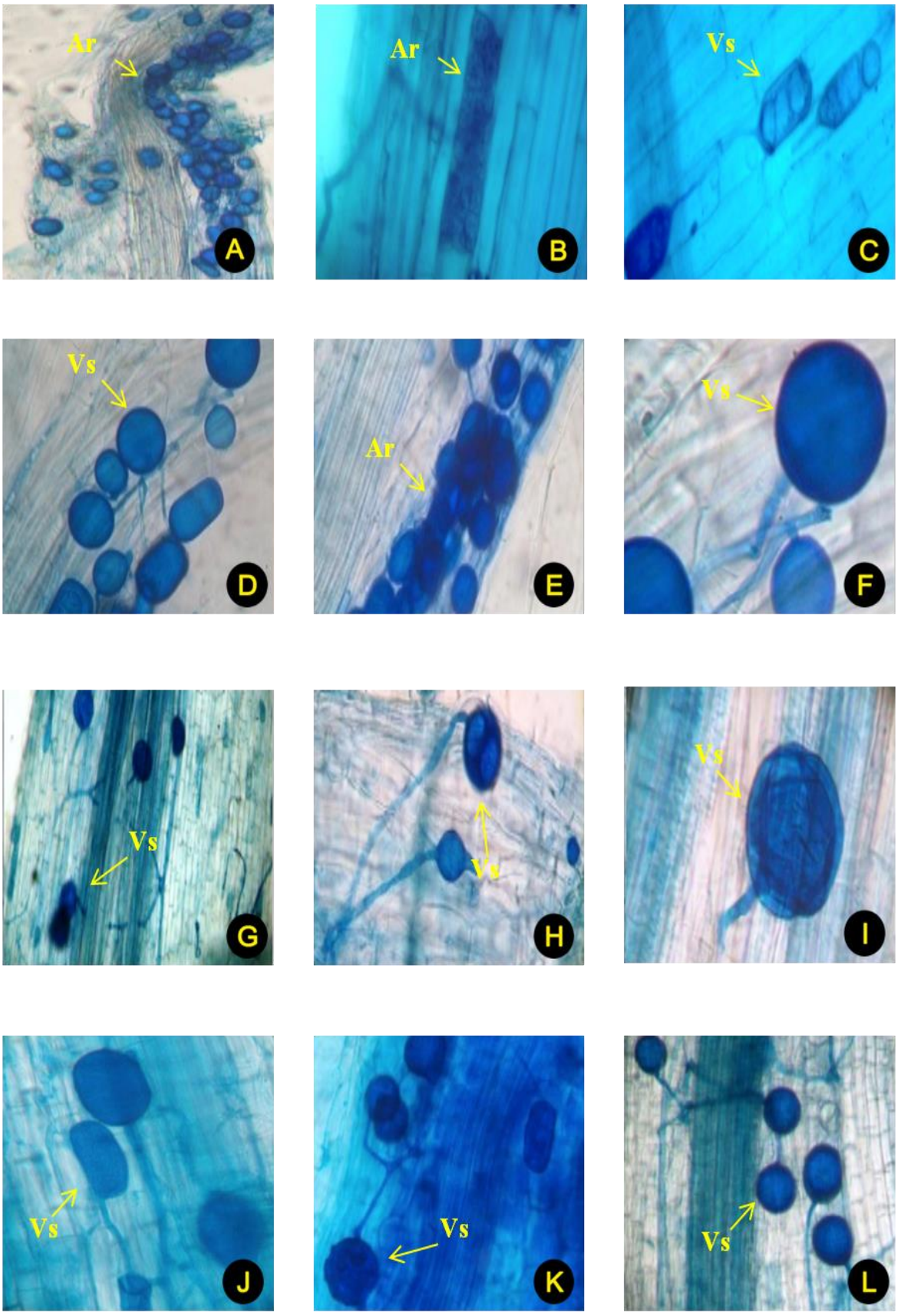


Figure 36: Root colonization of rice cultivars. (A-C)UBKV-1; (D-F) UBKV-4; (G-I) UBKV-5 and (J-L)Adde. (Ih- Intracellular hyphae, Vs- Vesicle, Ar- Arbuscule)

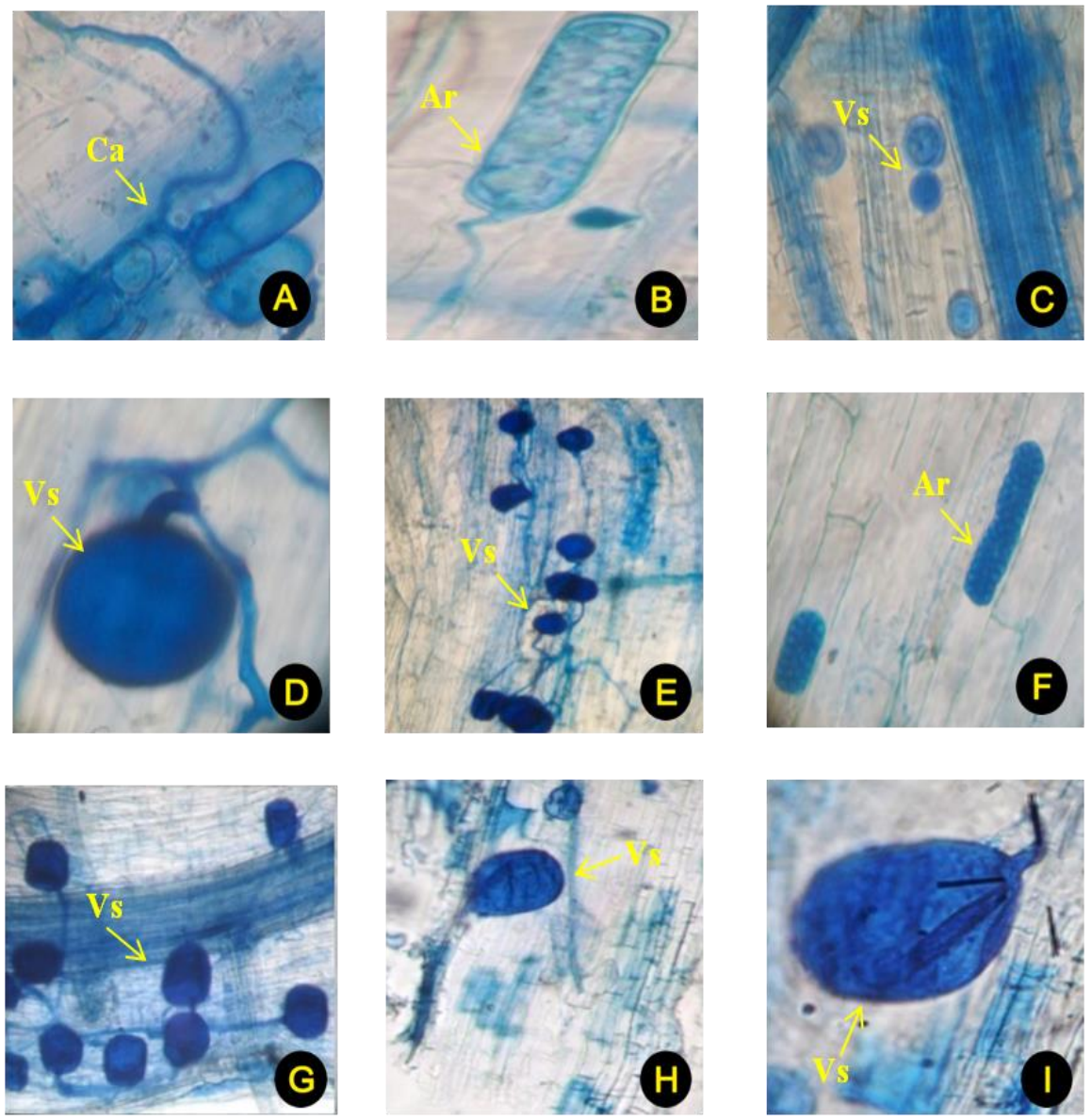


Figure 37: Root colonization of rice cultivars. (A-C)Kaberi 9090; (D-F)Loknath 505; (G-I)Gouraknath 509.(Ih- Intracellular hyphae, Ca- Coiled arbuscule, Vs- Vesicle, Ar- Arbuscule)

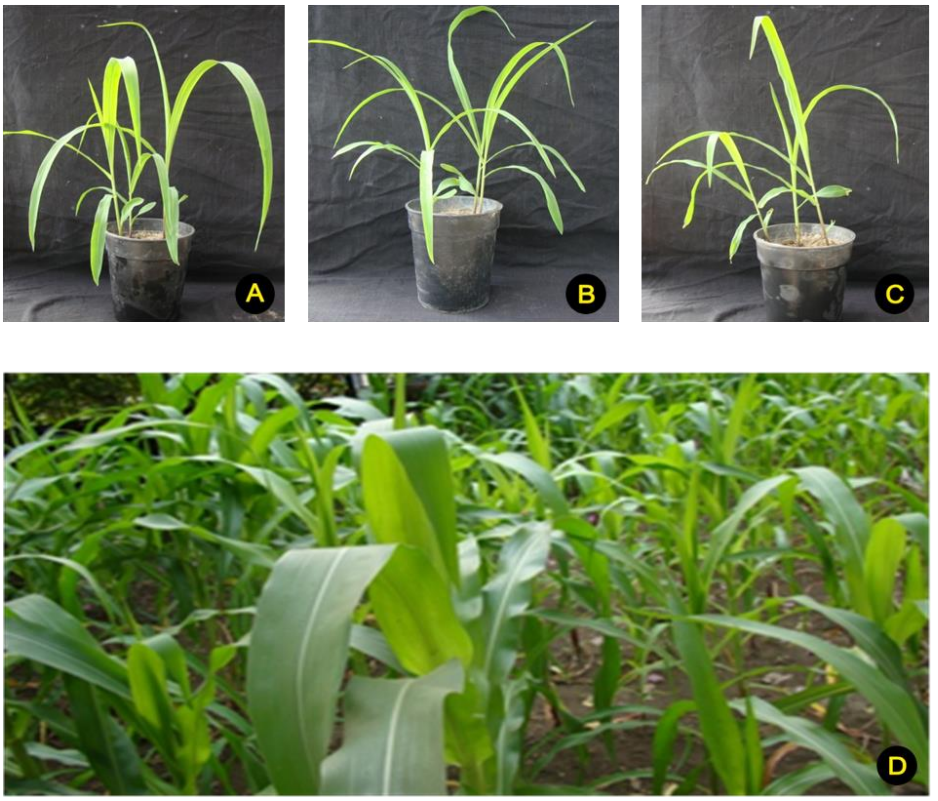


Figure 38: Mass multiplication of AMF spores in maize plant (A-C) Pots; (D) Experimental field

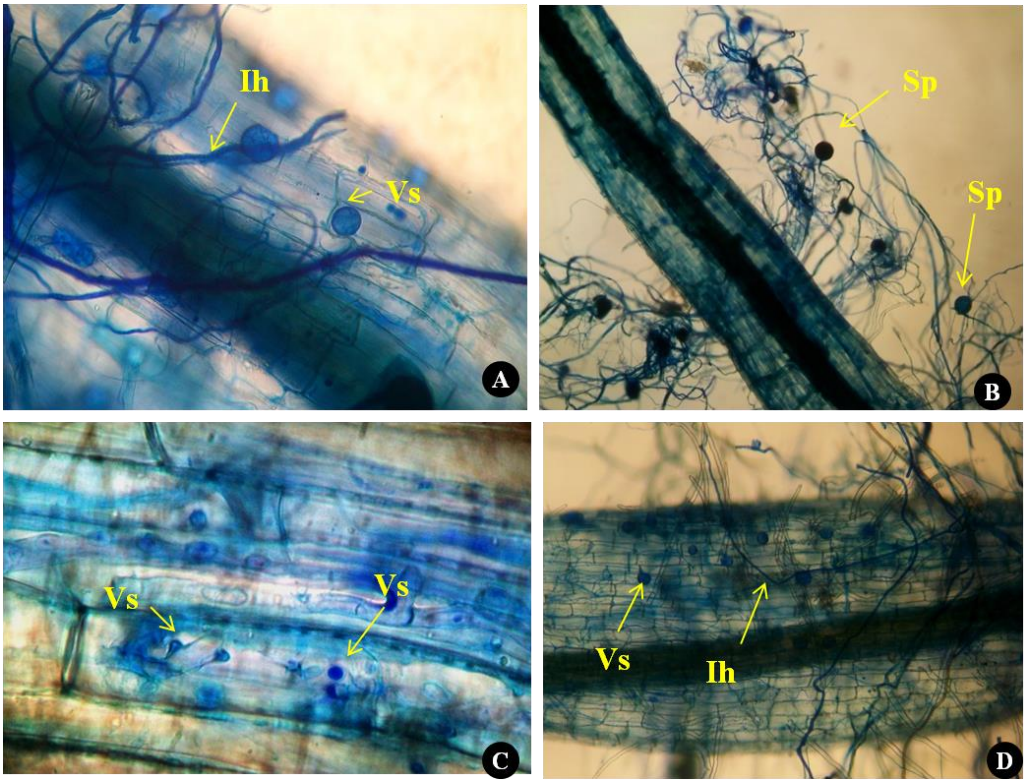


Figure 39: Histopathological study of AMF colonized maize roots. (A)Vesicles and intracellular hyphae, (B) Spores bearing hyphae,(C) Vesicles,(D) Vesicle and Intracellular hyphae.(Vs- Vesicle,Ih-Intracellular hyphae, Sp-Spore)

4.9. *In vitro* Antagonistic activities of bioinoculants against *D. Oryzae*

4.9.1 Antagonistic effect of PGPR isolates

Ten previously isolated characterized sequenced PGPR strains were taken for the antagonistic study against the pathogen *D. oryzae*. The bacterial strains with NBAIM Acc. No. and NCBI (Gen Bank) Acc. No. are as follows *Bacillus pumilus* (NAIMCC-B01483) (JF836847), *Bacillus pumilus* (NAIMCC-B01487) (JQ765579), *Bacillus pumilus* (NAIMCC-B01488) (JQ765580), *Burkholderia symbiont* (NAIMCC-B01489) (JQ765578), *Bacillus aerophillus* (NAIMCC-B01490) (KC603894), *Paenibacillus polymyxa* (NAIMCC-B01491) (KC703775), *Bacillus methylotrophicus* (NAIMCC-B01492) (JQ765577), *Bacillus altitudinis* (NAIMCC-B01484) (HQ849482), *Bacillus altitudinis* (NAIMCC-B01485) (JF899300), *Enterobacter cloacae* (NAIMCC-01486) (KC703974). Almost all the bacterial isolates could inhibit the growth of fungal pathogen markedly; however *Bacillus altitudinis* (NAIMCC-B01485) could inhibit the growth of the pathogen more prominently. The result of the interaction have been presented in Table 14. *In vitro* antifungal activities of foliar fungal pathogen *D.oryzae* against different PGPR have been given in Fig.40.

Table 14. *In vitro* pairing of PGPR isolates with foliar pathogen of rice- *Drechslera oryzae* for evaluation of antifungal activities.

Interacting microorganisms	**Bacterial Strain**	**Diameter of fungal colony (cm)**	**% of inhibition**
Drechslera oryzae		9.50±0.15	-
D. oryzae + Bacillus altitudinis	NAIMCC-B01485	1.50±0.14	**84±1.73**
D. oryzae + Bacillus pumilus	NAIMCC-B01483	1.98±0.21	79±1.63
D. oryzae + Enterobactor cloacae	NAIMCC-B01486	2.10±0.23	77±1.73
D. oryzae + Bacillus pumilus	NAIMCC-B01488	2.21±0.27	76±1.62
D. oryzae + Burkholderia symbiont	NAIMCC-B01489	2.46±0.24	74±1.54
D. oryzae + Bacillus altitudinus	NAIMCC-B01484	2.51±0.22	73±1.52
D. oryzae + Bacillus pumilus	NAIMCC-B01487	2.52± 0.20	72±1.46
D. oryzae + *Bacillus aerophilus*	NAIMCC-B01490	2.53±0.23	72±1.45
D. oryzae + *Paenibacillus polymyxa*	NAIMCC-B01491	2.59± 0.25	71±1.43
D. oryzae + Bacillus methylotrophicus	NAIMCC-B01492	2.60±0.22	70±1.42

Mean value of three replicates; ± Standard error; Diameter of fungal colony after 7 days growth (cm)

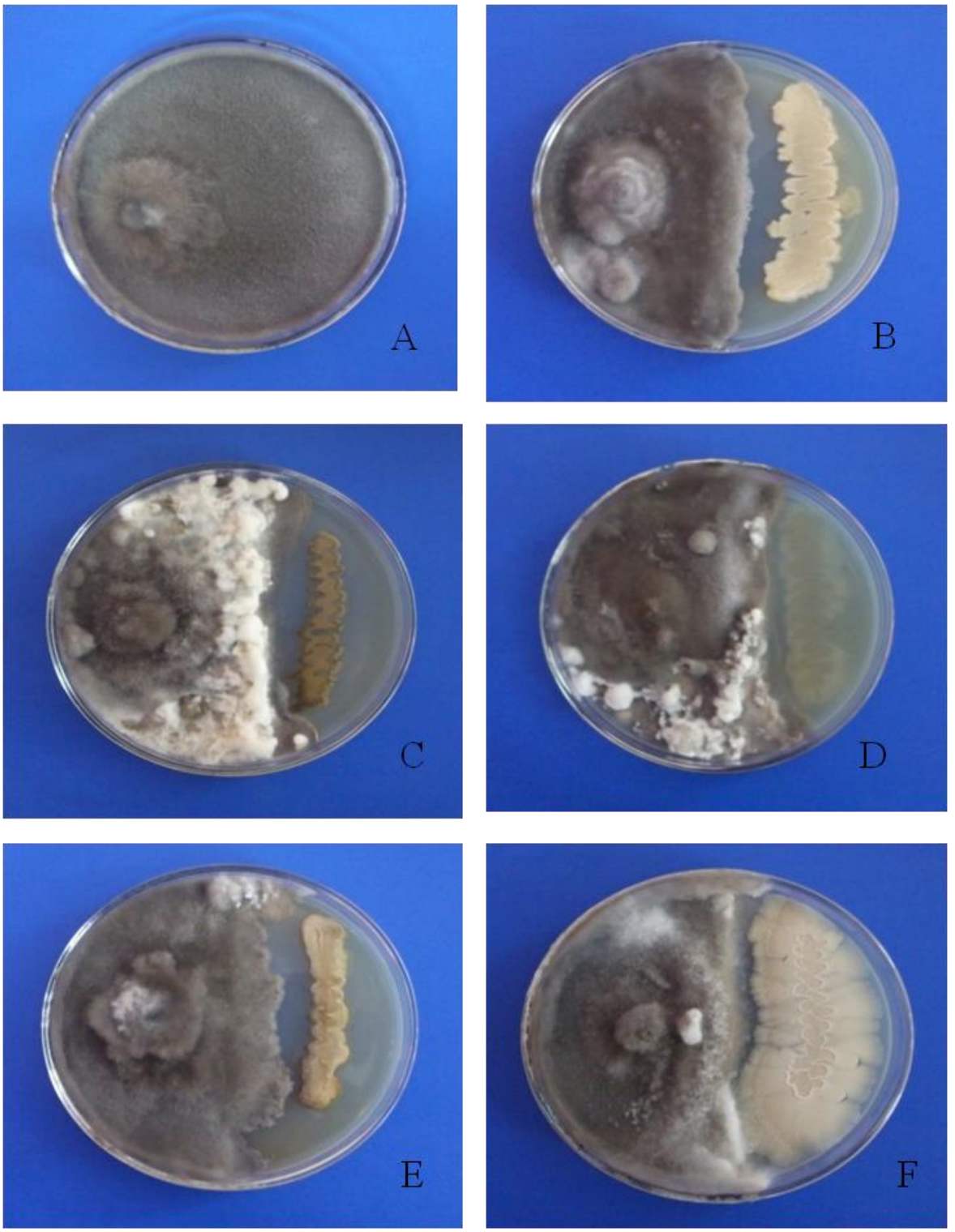


Figure 40: *In vitro* antifungal activities of foliar fungal pathogen *Drechslera oryzae* against selected PGPR isolates. Inhibition of *Drechslera oryzae* in dual plate culture assay by NAIMCC-B01485 (B), NAIMCC-B01483 (C), NAIMCC-B01486 (D), NAIMCC-B01488 (E), NAIMCC-B01489 (F) and Control (A)

4.9.2. Antagonistic effect of PGPF.

Three different isolate of *T. harzianum* (NAIMCC-F-03288), (NAIMCC-F-03289), (NAIMCC-F-03290) and three different isolates of *T. asperellum* (NAIMCC-F-03291), (NAIMCC-F-03292), (NAIMCC-F-03293) was obtained from culture collection of Immuno phytopathology Laboratory, Department of Botany, University of North Bengal. The fungus was initially taken up for its antagonistic effect against the fungal pathogen *D. oryzae.* For the antagonistic test, 5mm disc of fungal isolates were taken from 7 days old culture and placed at the periphery of the Petri plate. Similarly, agar disc of 5mm from pathogen culture was placed in the same Petri plate in the opposite end. The percent inhibition in the radial colony was calculated by the following formula-Percent inhibition =C-T/TX100, Where C= radial growth in control and T= radial growth in treatment. The interaction and inhibition percent was recorded and enlisted in the Table 15. *T. harzianum* isolate NAIMCC-F-03288 showed more profound inhibitory effect (77.94%) against the fungal pathogen and among the *T. asperellum* isolates NAIMCC-F-03292 showed the maximum inhibition 76.47% (Fig. 41).

Table 15.*In vitro* antagonistic tests of selected PGPF isolates against brown spot pathogen *D.oryzae*

Interacting Microorganisms	**Diameter of fungal colony after 7 days of growth (cm)**		**% of inhibition**
	PGPF	*D. oryzae* isolates	
D. oryzae		8.4 ± 0.23	-
T.harzianum (NAIMCC-F-03288)+*D.oryzae*	68.0	15.0±0.11	**77.94±1.65**
T.harzianum(NAIMCC-F-03289)+*D. oryzae*	66.0	22.0±0.23	66.66±1.73
T.harzianum(NAIMCC-F-03290)+*D. oryzae*	65.0	23.0±0.14	64.61±1.42
T.asperellum(NAIMCC-F-03291)+*D.oryzae*	63.0	18.0±0.29	71.42±1.74
T.asperellum (NAIMCC-F-03292) +*D.oryzae*	68.0	16.0±0.08	76.47±1.62
T.asperellum (NAIMCC-F-03293)+*D.oryzae*	64.0	17.0±0.24	73.43±1.68

Values are average of three replicate experiments. ±= Standard Error.

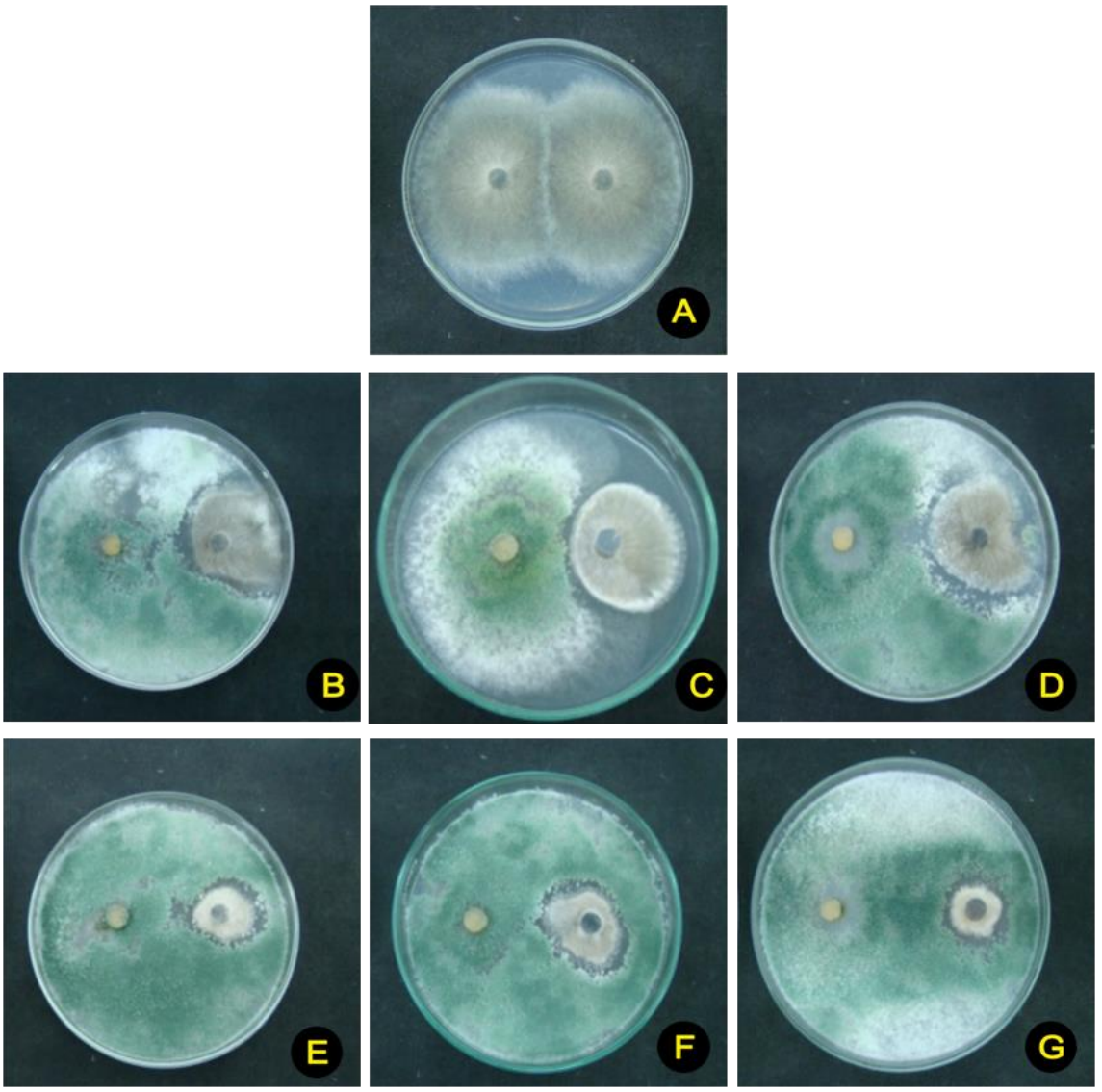


Figure 41: *In vitro* antagonistic test of foliar fungal pathogen (*D. oryzae*) against *Trichoderma* isolates .(B) *T. harzianum* (NAIMCC-F-03289), (C) *T. harzianum* (NAIMCC-F-03288), (D) *T. harzianum* (NAIMCC-F-03290), (E) *T. asperellum* (NAIMCC-F-03291), (F) *T. asperellum* (NAIMCC-F-03292), (G) *T. asperellum* (NAIMCC-F-03293) and (A) Control (*D. Oryzae*)

4.10. Growth promotion and biochemical changes in rice cultivar following application of PGPR.

4.10.1. Screening of PGPR isolates for plant growth promotion

4.10.1.1. Growth enhancement

Three most susceptible rice cultivars (Black Nuniya, Brimful and Champasari) were selected on the basis of their poor performance among the other rice cultivars against the brown spot disease. For the purpose of further experiments these three rice cultivars were selected in order to induce the disease resistance capacity among them and for their better health and development. Growth promotion in three rice cultivars were checked following their treatment with ten most efficient PGPR that was already tested for their antagonistic activity against the pathogen *D. oryzae*. These bioinoculants were added to the soil at different time intervals as mentioned in Materials and Methods. Effects of their application in growth and biochemical changes in rice plants were noted under field conditions.

In the first trial of the experiment effects of different PGPR on the health status of rice cultivars was tested. PGPR were applied by using foliar spray and soil drench as described in materials and methods. Plant growth in terms of height of plant was recorded at 20 days interval from the date of transferring seedlings to the experimental plot. Results revealed that growth was affected by the different bacterial treatments. Maximum growth was observed in plants treated with *Burkholderia symbiont* (NAIMCC-B01489) in cultivar Black nuniya, *Bacillus altitudinis* (NAIMCC-B01485) in cultivar Champasari and in case of cultivar Brimful plants treated with *Bacillus altitudinis* (NAIMCC-B01484) and *Enterobacter cloacae* (NAIMCC-B01486) showed maximum growth (Fig. 42).

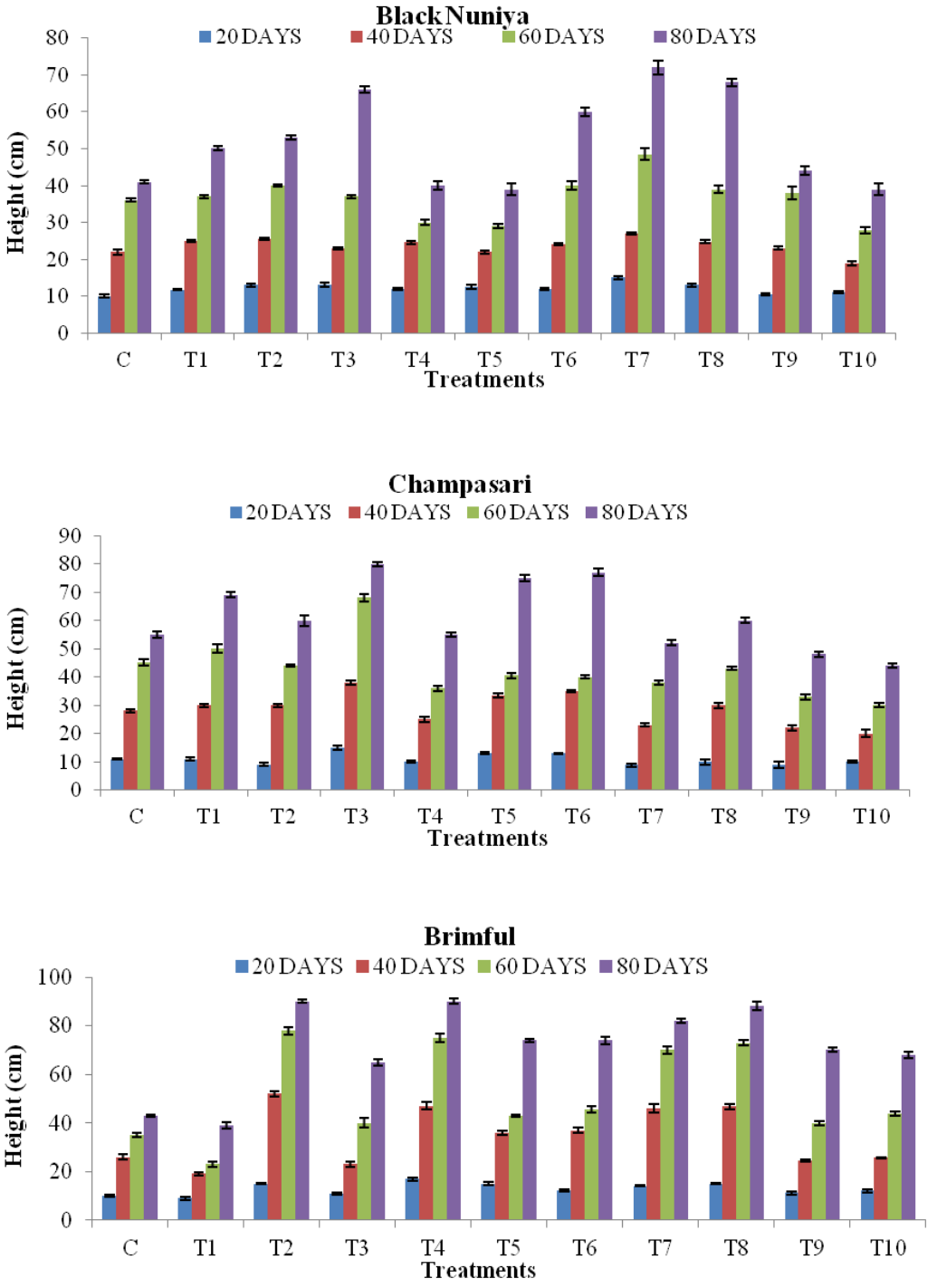


Figure 42: Growth promotion of rice plants following treatment with different PGPR.C-Untreated control (UI),T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01484), T3-*Bacillus altitudinis* (NAIMCC-B01485), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (NAIMCC-B01487), T6-*Bacillus pumilus* (NAIMCC-B01488),T7- *Burkholderia symbiont* (NAIMCC-B01489), T8-*Bacillus aerophilus* (NAIMCC-B01490), T9- *Paenibacillus polymyxa* (NAIMCC-B01491), T10-*Bacillus methylotrophicus* (NAIMCC-B01492).

4.10.1.2. Total sugar content

In case of total sugar, results revealed that here maximum accumulation occurred in treatment with *Bacillus aerophilus* (NAIMCC-B01490) in case of Black nuniya, *Enterobacter cloacae* (NAIMCC-B01486) in case of Champasari and *Bacillus methylotrophicus* (NAIMCC-B01492) in case of Brimful (Table 16)

Table 16.Total sugar content of rice leaves following treatments with PGPR

Treatments	**Total sugar content (mg/gm tissue)**		
	Black Nuniya	**Champasari**	**Brimful**
Untreated Control (UI)	41.33±1.45	27.33±0.40	33.23±0.72
PGPR treated *Bacillus pumilus* (NAIMCC-B01483)	57.70±0.74	51.40±1.05	55.46±1.21
Bacillus altitudinis (NAIMCC-B01484)	46.80±0.55	50.46±0.52	44.77±0.92
Bacillus altitudinis (NAIMCC-B01485)	46.39±0.48	46.83±0.60	40.54±0.89
Enterobacter cloacae (NAIMCC-B01486)	57.65±0.70	59.42±0.67	57.53±0.29
Bacillus pumilus (NAIMCC-B01487)	56.16±0.95	44.36±0.63	48.97±0.48
Bacillus pumilus (NAIMCC-B01488)	34.68±0.15	38.13±0.85	35.80±0.33
Burkholderia symbiont (NAIMCC-B01489)	47.63±0.20	42.20±0.49	41.00±3.01
Bacillus aerophilus (NAIMCC-B01490)	64.48±1.05	47.33±0.48	59.49±0.44
Paenibacillus polymyxa (NAIMCC-B01491)	56.30±0.45	49.20±0.41	51.73±0.93
Bacillus methylotrophicus (NAIMCC-B01492)	59.86±0.75	58.47±0.86	62.87±0.73
CD(p=0.05)	Treatments = 6.78 Cultivars = 3.54		

Mean value of three replicates; ± Standard error

4.10.1. 3. Total chlorophyll content

Total chlorophyll content was also found to increase in treated samples than the untreated control samples. In case of rice cultivar Black nuniya it was found that the maximum accumulation of total chlorophyll content took place in plot treated with *Bacillus aerophilus* and in Champasari with *Burkholderia symbiont* and in Brimful with *Bacillus pumilus* (NAIMCC-B01483) (Table 17).

Table 17. Total chlorophyll content of rice leaves following treatments with PGPR

Treatments	**Total chlorophyll content(µg/ml)**		
	Black Nuniya	**Champasari**	**Brimful**
Untreated Control (UI)	12.17±0.10	11.35±0.06	12.93±0.23
PGPR treated *Bacillus pumilus* (NAIMCC-B01483)	14.67±0.22	14.50±0.15	16.08±0.31
Bacillus altitudinis (NAIMCC-B01484)	12.81±0.21	13.60±0.07	13.50±0.11
Bacillus altitudinis (NAIMCC-B01485)	12.84±0.07	11.98±0.17	12.60±0.18
Enterobacter cloacae (NAIMCC-B01486)	10.50±0.02	11.37±0.06	12.71±0.11
Bacillus pumilus (NAIMCC-B01487)	12.78±0.34	12.44±0.06	13.54±0.18
Bacillus pumilus (NAIMCC-B01488)	14.68±0.04	14.73±0.08	14.82±0.26
Burkholderia symbiont (NAIMCC-B01489)	14.87±0.17	15.64±0.23	15.80±0.10
Bacillus aerophilus (NAIMCC-B01490)	16.11±0.11	15.24±0.14	14.55±0.52
Paenibacillus polymyxa (NAIMCC-B01491)	11.69±0.45	13.07±0.19	12.68±0.43
Bacillus methylotrophicus (NAIMCC-B01492)	12.69±0.09	14.47±0.09	14.62±0.93
CD(p=0.05)	Treatments = 1.00 Cultivars = 0.52		

Mean value of three replicates; ± Standard error

4.10.1. 4. Protein content

Estimation of protein contents in all the rice cultivars following various PGPR treatments revealed enhancement in protein content of which highest accumulation in rice cultivar Black nuniya was obtained in treatment containing *Paenibacillus polymyxa* and in Champasari also it was obtained in treatment containing *Paenibacillus polymyxa* finally in Brimful it was obtained in *Bacillus aerophilus*. (Table 18)

Table 18. Protein content of rice leaves following treatments with PGPR

Treatments	**Protein content (mg/gm tissue)***		
	Black Nuniya	**Champasari**	**Brimful**
Untreated Control (UI)	23.90±0.34	37.25±0.93	31.19±0.67
PGPR treated *Bacillus pumilus* (NAIMCC-B01483)	45.50±0.67	53.86±0.29	50.17±0.54
Bacillus altitudinis (NAIMCC-B01484)	55.25±0.27	50.53±0.54	46.41±0.96
Bacillus altitudinis (NAIMCC-B01485)	94.56±0.35	49.45±0.44	56.72±0.58
Enterobacter cloacae (NAIMCC-B01486)	55.03±0.34	57.72±0.69	55.45±0.72
Bacillus pumilus (NAIMCC-B01487)	66.77±0.56	57.22±0.82	59.42±0.60
Bacillus pumilus (NAIMCC-B01488)	34.93±0.80	30.20±0.68	40.63±0.86
Burkholderia symbiont (NAIMCC-B01489)	45.10±0.70	51.05±1.08	52.00±0.35
Bacillus aerophilus (NAIMCC-B01490)	65.73±2.11	80.03±1.02	87.73±3.00
Paenibacillus polymyxa (NAIMCC-B01491)	49.44±1.70	73.47±2.25	70.84±0.75
Bacillus methylotrophicus (NAIMCC-B01492)	55.44±1.78	39.81±1.33	52.49±1.25
CD(p=0.05)	Treatments = 12.02 Cultivars = 6.28		

*Mean value of three replicates ±Standard error

4.10.1. 5. Total phenol content

Total phenols showed variations according to the treatments. Highest accumulation in rice cultivar Black nuniya was obtained in treatment containing *Bacillus altitudinus*(NAIMCC-B0485) similarly in Champasari was obtained in treatment containing *Bacillus pumilus* (NAIMCC-B01487) and finally in Brimful was observed in treatment with *Bacillus pumilus* (NAIMCC-B01487) (Table 19)

Table 19.Total phenol content of rice leaves following treatments with PGPR

Treatments	Total phenol content(mg/gm tissue)		
	Black Nuniya	**Champasari**	**Brimful**
Untreated Control (UI)	2.71±0.08	3.50±0.20	3.60±0.23
PGPR treated *Bacillus pumilus* (NAIMCC-B01483)	4.23±0.17	4.79±0.15	3.93±0.20
Bacillus altitudinis (NAIMCC-B01484)	4.93±0.20	4.83±0.27	5.06±0.12
Bacillus altitudinis (NAIMCC-B01485)	8.26±0.4	6.22±0.15	5.76±0.08
Enterobacter cloacae (NAIMCC-B01486)	5.58±0.10	6.58±0.16	6.30±0.20
Bacillus pumilus (NAIMCC-B01487)	6.68±0.24	7.13±0.18	7.83±0.17
Bacillus pumilus (NAIMCC-B01488)	5.83±0.23	5.60±0.30	4.93±0.26
Burkholderia symbiont (NAIMCC-B01489)	7.10±0.15	6.80±0.20	6.72±0.13
Bacillus aerophilus (NAIMCC-B01490)	6.76±0.14	6.34±0.17	6.65±0.12
Paenibacillus polymyxa (NAIMCC-B01491)	4.70±0.07	7.06±0.12	7.33±0.21
Bacillus methylotrophicus (NAIMCC-B01492)	5.86±0.26	5.63±0.31	5.96±0.14
CD(p=0.05)	Treatments = 0.78 Cultivars = 0.41		

Mean value of three replicates; ± Standard error

4.11. Activation of defense response of rice cultivars against *Drechslera oryzae* following application of PGPR

4.11.1. Disease suppression

Rice cultivars were under observation from seedling stage to mature stage and data was collected for the establishment of disease caused by *Drechslera oryzae* under artificially inoculated condition and disease index were prepared accordingly which showed higher amount of PDI percentage in control set of plant (76.19%) in comparison with the plants treated with *Bacillus altitudinis* (NAIMCC-B01485) (9.83%) in case of Black nuniya, (18.19%) in comparison to control set with (71.08%) in case of Champasari and (12.67%) in comparison to control set with (69.33%)(Table 20).

Table 20.Evaluation of Disease index for brown spot in rice plants following treatments with PGPR

Treatments	Black Nuniya		Champasari		Brimful	
	PDI (%)	Mean diameter of lesion (mm.)	PDI (%)	Mean diameter of lesion (mm.)	PDI (%)	Mean diameter of lesion (mm.)
Untreated Control (UI)	76.19	2.1	71.08	1.6	69.33	2.0
PGPR Treated *Bacillus pumilus* (NAIMCC-B01483)	26.18	1.7	38.80	0.6	23.33	2.1
Bacillus altitudinis (NAIMCC-B01485)	09.83	2.0	18.19	3.0	12.67	0.3
Bacillus altitudinis (NAIMCC-B01484)	22.54	1.5	41.17	1.9	62.50	1.4
Enterobacter cloacae (NAIMCC-B01486)	16.92	0.6	34.54	2.2	16.54	1.6
Bacillus pumilus (NAIMCC-B01487)	19.73	1.8	36.17	0.9	24.50	1.5
Bacillus pumilus (NAIMCC-B01488)	32.94	0.8	44.14	0.5	47.05	1.0
Burkholderia symbiont (NAIMCC-B01489)	28.40	1.5	44.79	1.5	44.79	1.6
Bacillus aerophilus (NAIMCC-B01490)	54.42	2.0	46.30	1.0	25.8	1.8
Paenibacillus polymyxa (NAIMCC-B01491)	38.45	0.8	52.80	1.5	30.56	0.6
Bacillus methylotrophicus (NAIMCC-B01492)	37.95	0.5	61.12	1.5	14.47	0.4

PDI- Percentage of Disease Index.

4.11.2. Activity of defense enzymes

Defense enzymes activity when tested showed significant variation according to the treatment and higher amount of enzyme activity was found in treated rice plants rather than control set of plants. Significant increase in enzymatic activity were found in plants treated with *Bacillus altitudinis* (NAIMCC-B01485), *Burkholderia symbiont*, *Paenibacillus polymyxa*.(Fig. 43&44).

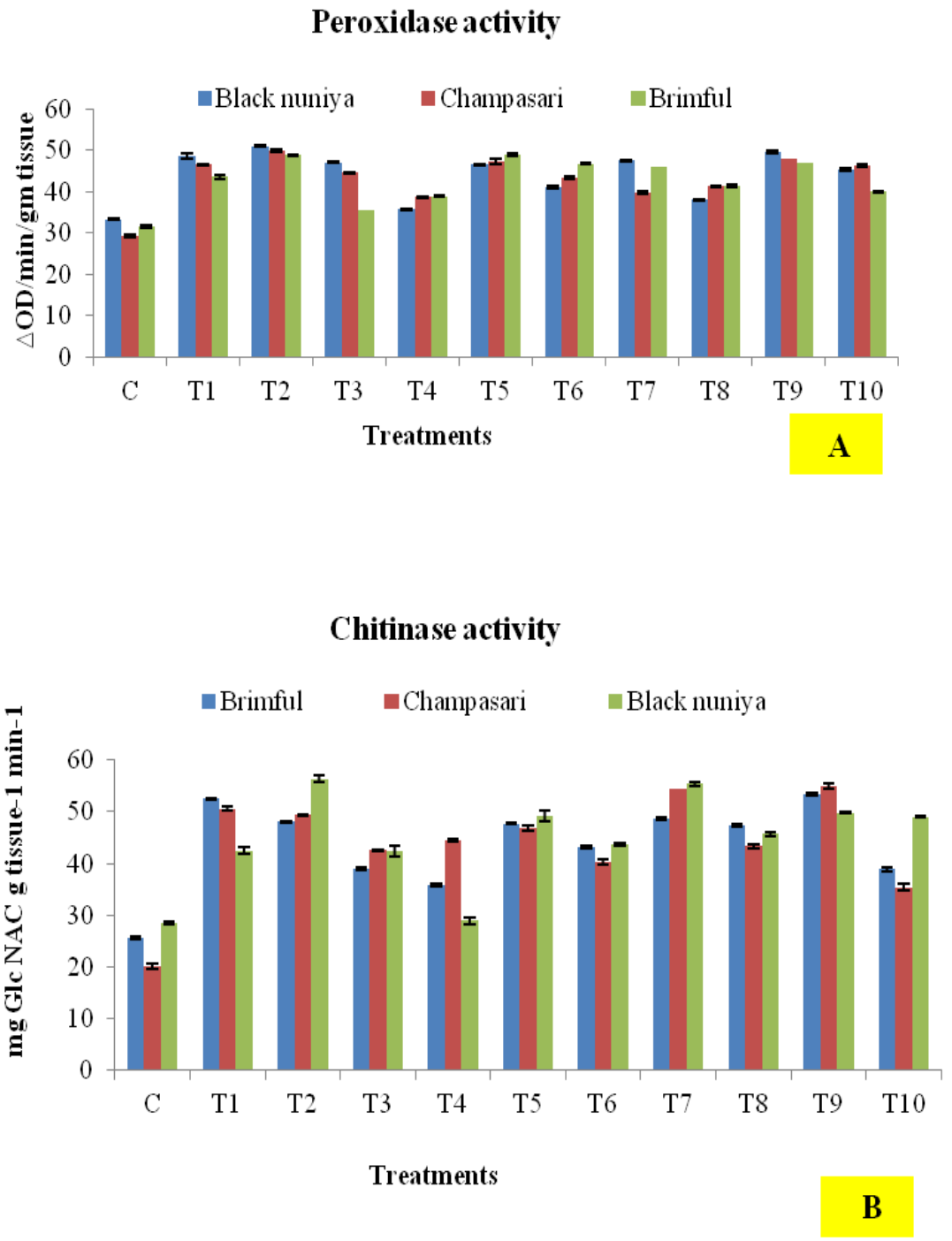


Figure 43: Activity of defense enzymes in rice leaf samples (A) Peroxidase and (B) Chitinase. C-Untreated control,T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01485), T3-*Bacillus altitudinis* (NAIMCC-B01484), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (NAIMCC-B01487), T6-*Bacillus pumilus* (NAIMCC-B01488),T7- *Burkholderia symbiont* (NAIMCC-B01489), T8-*Bacillus aerophilus* (NAIMCC-B01490), T9- *Paenibacillus polymyxa* (NAIMCC-B01491), T10-*Bacillus methylotrophicus* (NAIMCC-B01492).

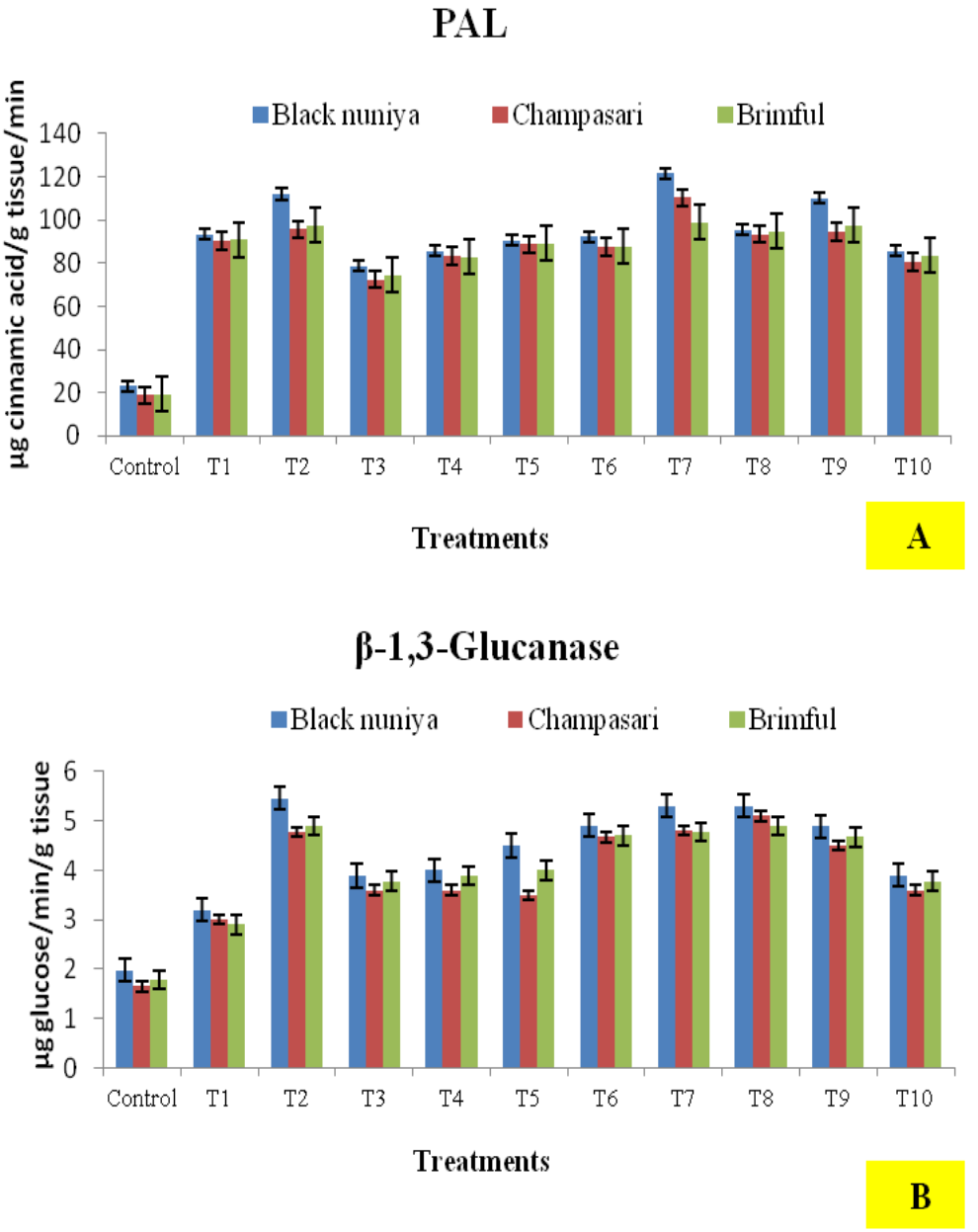


Figure 44: Activity of defense enzymes in rice leaf samples (A) PAL (Phenyl alenine ammonia lyase) and (B)β-1,3-Glucanase. C-Untreated control,T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01485), T3-*Bacillus altitudinis* (NAIMCC-B01484), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (NAIMCC-B01487), T6-*Bacillus pumilus* (NAIMCC-B01488),T7- *Burkholderia symbiont* (NAIMCC-B01489), T8-*Bacillus aerophilus* (NAIMCC-B01490), T9-*Paenibacillus polymyxa* (NAIMCC-B01491), T10-*Bacillus methylotrophicus* (NAIMCC-B01492).

4.11.3. HPLC analysis of phytoalexin

HPLC analysis was done for detecting the phytoalexin namely Phytocassanes from the leaves of rice cultivar Black nuniya in Untreated healthy, Untreated inoculated

and PGPR (*Bacillus altitudinus*, NAIMCC-B01485) treated healthy and treated inoculated plants exhibiting the lowest PDI percentage. A total of 5 peaks were clearly visible in untreated healthy as well as untreated inoculated plants and a total of 7 peaks in treated healthy and treated inoculated with the pathogen. However the compounds increased markedly in treated plants in case of both healthy and inoculated rice plants. Also enhancement of the peaks in case of inoculated samples shows increase in compounds resulting in better defense. (Figure 45; Table 18).

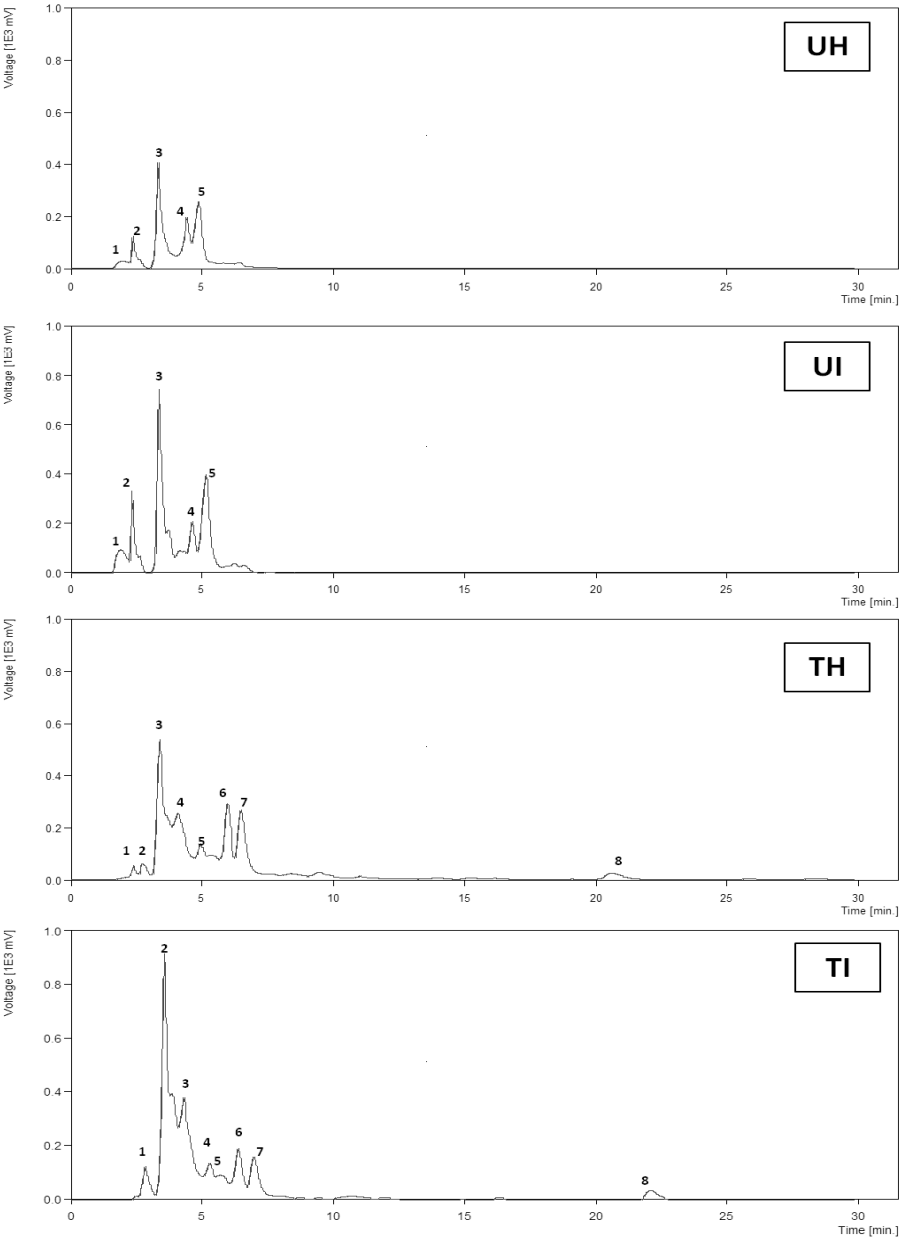


Figure 45: HPLC profile of Phytocassanes obtained from leaf extracts of rice plant (cv. Black nuniya) following treatment with *Bacillus altitudinus* (NAIMCC-B01485) and challenge inoculation with *D. oryzae*. (UH= Untreated Healthy, UI= Untreated Inoculated, TH= Treated Healthy and TI= Treated Inoculated)

Table 21. Peak results of Phytocassanes extracts from leaves of Black nuniya following treatment with *Bacillus altitudinus* (NAIMCC-B01485) and pathogen challenge

Untreated Healthy (UH)		
Peak no	**Retention time (min)**	**Height(mV)**
1	1.980	27.572
2	2.360	125.386
3	3.340	409.069
4	4.440	194.111
5	4.900	255.207
Untreated inoculated (UI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	1.900	96.722
2	2.330	336.235
3	3.370	749.211
4	4.660	210.528
5	5.160	404.511
Treated Healthy (TH)		
Peak no	**Retention time (min)**	**Height(mV)**
1	2.380	56.153
2	2.730	61.288
3	3.410	538.687
4	4.060	257.911
5	4.970	137.982
6	5.970	295.645
7	6.500	269.338
Treated Inoculated (TI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	2.850	123.301
2	3.580	923.959
3	4.300	385.217
4	5.300	137.457
5	5.690	93.042
6	6.410	190.447
7	6.990	161.783

4.12. Growth promotion and biochemical changes in rice cultivar following application of PGPF.

In the second trial experiment growth promotion in all the three rice cultivars viz. Black nuniya Brimful, and Champasari were checked following their treatment with different PGPF (*T. harzianum and T. asperellum*) on the basis of their inhibitory

potentiality *in vitro*. These bio inoculants were added to the soil and leaves at different time intervals as mentioned in Materials and Methods. Effects of their application on growth and biochemical changes in these susceptible rice cultivars were noted under field condition.

4.12.1. Growth enhancement

The effect of treatments *T. harzianum*, viz. NAIMCC-F-03288, NAIMCC-F-03289, NAIMCC-F-03290 and the other three *T. asperellum*, viz.NAIMCC-F-03291, NAIMCC-F-03292 and NAIMCC-F-03293 on the growth of the three rice cultivars were noted after every 20, 40, 60 and 80 days of interval. It was seen that the increase in the height following treatment was variable in all the rice cultivars. Significant increase in the height was observed in all the treatments in comparison to the control after 20, 40, 60 and 80 days after inoculation (Fig.46).Under the field condition, the growth was highest in the plants treated with *T.harzianum* (NAIMCC-F-03288) 75 cm in Black Nuniya followed by 54 cm in Champasari and 69.5 cm in Brimful after 80d treatment compared to the control and other treatments.

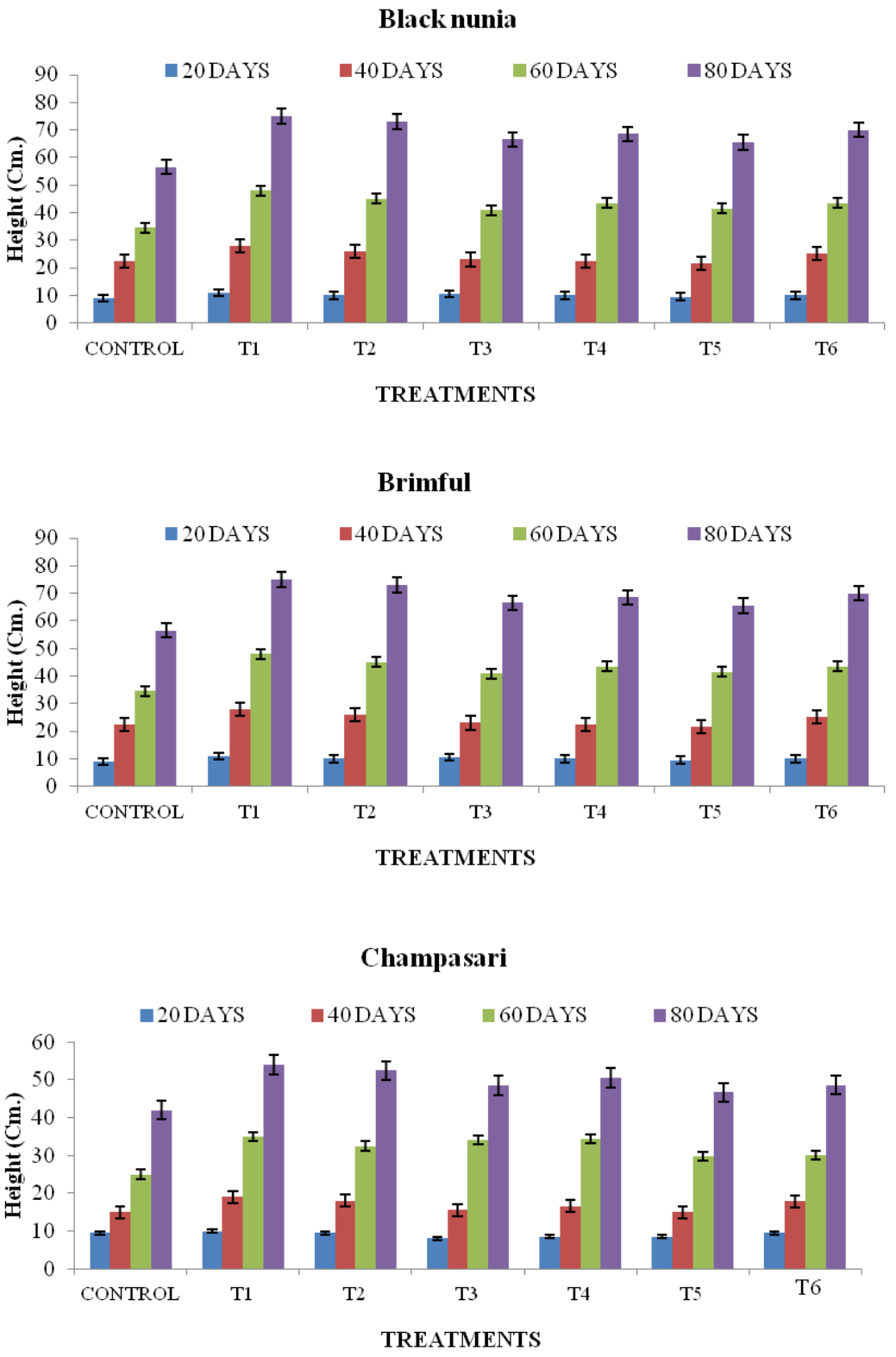


Figure 46: Growth promotion of rice cultivars following treatment with different PGPF. (C= Untreated Control, T1= *T. harzianum* NAIMCC-F-03288, T2= *T. harzianum* NAIMCC-F-03289, T3=*T.harzianum* NAIMCC-F-03290, T4= *T.asperellum* NAIMCC-F-03291, T5=*T. asperellum* NAIMCC-F-03292 and T6= *T. asperellum* NAIMCC-F-03293)

4.12.2. Total sugar content

In case of total sugar, results revealed that here maximum accumulation occurred in treatment with *T. harzianum* (NAIMCC-F-03288) in case of Black nuniya, Champasari and in case of Brimful also. In case *T. asperellum* (NAIMCC-F-03293) showed the maximum sugar content in all the rice cultivars (Table 22).

Table 22.Total sugar content of rice leaves following treatments with PGPF

Treatments	Total sugar content (mg/gm tissue)		
	Black nuniya	**Champasari**	**Brimful**
Unteated Control (UI)	31.50±0.72	31.33±0.48	35.67±0.84
PGPF Treated *T. harzianum* (NAIMCC-F-03288)	65.84±0.84	62.00±0.92	68.87±0.68
T.harzianum (NAIMCC-F-03289)	50.56±1.21	46.57±0.65	55.45±0.95
T.harzianum (NAIMCC-F-03290)	51.73±0.68	49.20±1.21	59.46±0.95
T.asperellum (NAIMCC-03291)	48.90±1.03	47.33±1.21	50.45±1.28
T.asperellum (NAIMCC-F-03292)	48.78±080	45.67±0.87	56.16±1.22
T.asperellum (NAIMCC-F-03293)	59.94±0.94	56.70±0.40	64.67±0.83
CD(p=0.05)	Treatments = 2.79 Cultivars = 1.82		

Mean value of three replicates; ± Standard error

4.12.3. Total protein content

Estimation of protein contents in all the rice cultivars following various PGPF treatments revealed enhancement in protein content of which highest accumulation in rice cultivar Black nuniya was obtained in treatment containing *T. harzianum* (NAIMCC-F-03288) and in Champasari and Brimful also it was obtained in treatment containing *T. harzianum* (NAIMCC-F-03288) showing that the *in vivo* results were in accordance to the *in vitro*. (Table 23)

4.12.4. Total Phenol content.

Total phenols showed same results according to the treatments. Highest accumulation in rice cultivar Black nuniya was obtained in treatment containing *T. harzianum* (NAIMCC-F-03288) similarly in Champasari and Brimful also (Table 24).

Table 23. Protein content of rice leaves following treatments with PGPF

Treatments	**Protein content (mg/gm tissue)**		
	Black nuniya	**Champasari**	**Brimful**
Untreated Control (UI)	26.70±0.66	26.95±1.47	30.50±1.73
PGPF Treated *T.harzianum* (NAIMCC-F-03288)	70.60±1.12	65.50±2.28	73.40±1.53
T.harzianum (NAIMCC-F-03289)	54.60±2.55	50.00±1.62	55.67±1.68
T.harzianum (NAIMCC-F-03290)	57.67±0.67	57.80±1.58	59.87±1.64
T.asperellum (NAIMCC-03291)	45.60±1.03	43.50±1.51	46.50±1.45
T.asperellum (NAIMCC-F-03292)	58.00±2.00	58.60±2.14	60.00±1.47
T.asperellum (NAIMCC-F-03293)	63.40±1.27	64.50±1.84	65.56±1.39
CD(p=0.05)	Treatments = 2.69 Cultivars = 1.76		

Mean value of three replicates; ± Standard error

Table 24.Total phenol content of rice leaves following treatments with PGPF

Treatments	**Total phenol content (mg/gm tissue)**		
	Black nuniya	**Champasari**	**Brimful**
Untreated Control (UI)	2.55±0.30	2.89±0.12	3.69±0.17
PGPF Treated *T. harzianum* (NAIMCC-F-03288)	8.50±0.37	8.00±0.30	9.80±0.46
T.harzianum (NAIMCC-F-03289)	5.86±0.19	5.63±0.14	5.96±0.36
T.harzianum (NAIMCC-F-03290)	6.22±0.12	5.58±0.43	7.76±0.35
T.asperellum (NAIMCC-03291)	6.00±0.81	5.58±0.32	6.35±0.49
T.asperellum (NAIMCC-F-03292)	5.85±0.33	5.60±0.11	6.60±0.12
T.asperellum (NAIMCC-F-03293)	6.57±0.67	4.90±0.64	6.80±0.60
CD(p=0.05)	Treatments = 0.78 Cultivars = 0.51		

Mean value of three replicates; ± Standard error

4.13. Activation of defense response in rice plants following application of PGPF against *D.oryzae*

4.13.1. Disease suppression

From the results collected it is revealed that seed coating as well as foliar application of different strain of *Trichoderma* (PGPF) in rice decreases disease severity. In case of untreated infected plants PDI was quite higher than PGPF treated infected plants. In case of Black nuniya application of *T.harzianum* (NAIMCC-F-03288)bought about the least PDI % similarly in Champasari *T.asperellum* (NAIMCC-F-03293)reduced disease index markedly compared to untreated control and in case of Brimful *T.asperellum* (NAIMCC-F-03292)bought about the least PDI % among treated inoculated set of plants (Table 25).

Table 25. Evaluation of Disease index for brown spot in rice plants following treatments with PGPF against pathogen challenge

Treatments		**Black nunia**		**Champasari**		**Brimful**	
		PDI (%)	**Mean diameter of lesion (mm.)**	**PDI (%)**	**Mean diameter of lesion (mm.)**	**PDI (%)**	**Mean diameter of lesion (mm.)**
Untreated Control (UI)		60.45	2.5	84.50	1.8	55.60	2.0
PGPF Treated *T.harzianum* (NAIMCC-F-03288)	TI1	25.78	1.0	54.56	1.2	30.57	1.5
T.harzianum (NAIMCC-F-03289)	TI2	47.89	1.5	64.80	1.2	38.90	1.4
T.harzianum (NAIMCC-F-03290)	TI3	58.90	1.4	68.90	1.4	52.78	1.0
T.asperellum (NAIMCC-03291)	TI4	40.56	1.5	55.60	1.6	30.55	1.2
T.asperellum (NAIMCC-F-03292)	TI5	40.67	0.8	54.47	1.5	25.78	1.2
T.asperellum (NAIMCC-F-03293)	TI6	35.67	2.0	45.50	1.0	35.56	1.6

PDI=Percent Disease Index

4.13.2. Defense enzymes

Defense enzyme activities in leaves were assessed after the completion of four foliar spray of PGPF at an interval of three days. The results indicated that post PGPF applications, activities of defense enzymes were enhanced markedly. In case of Brimful the values for PAL activity ranged from 75.83(T3)-128(T6)µg cinnamic acid/g tissue/min in comparison to the control 23.29.Similarly in Champasari the values ranged from 75(T3)-98.78(T1) in comparison to the control 21128µg cinnamic acid/g tissue/min and finally in case of Black Nuniya the values ranged from 78.4(T3)-132.4(T6) in comparison to the control 23.4128µg cinnamic acid/g tissue/min (Fig.47, A).

For peroxidise the values in case of rice cultivar Black Nuniya ranged from 45.6(T5)-53.46(T6) ΔOD/min /g tissue in comparison to the control 40.8.Similarly in case of Champasari the values ranged from43.4(T5)-50.78(T6) ΔOD/min /g tissue in comparison to the control 42.46 and finally in Brimful the vales ranged from 43.65(T5)-51.8(T6) ΔOD/min /g tissue in comparison to the control 41.5.(Fig. 47, B).

For chitinase the values in case of rice cultivar Black Nuniya ranged from 23.4(T1)-5.23(T6) µg GLC-NAC/hr/g tissue in comparison to the control 20.89.Similarly in case of Champasari the values ranged from 18.7(T1)-46.66(T6) µg GLC-NAC/hr/g tissue in comparison to the control 19.78 and finally in Brimful the vales ranged from 20(T1)-48(T6) µg GLC-NAC/hr/g tissue in comparison to the control 22.(Fig. 48, A).

For glucanase the values in case of rice cultivar Black Nuniya ranged from 3.6(T1)-6.56(T6) µg glucose/min/g tissue in comparison to the control 2.33.Similarly in case of Champasari the values ranged from 3(T1)-4.98(T6) µg glucose/min/g tissue in comparison to the control 1.69 and finally in Brimful the vales ranged from 3(T1)-5.5(T6) µg glucose/min/g tissue in comparison to the control 1.9.(Fig. 48, B).

4.13.3. HPLC analysis of phytoalexin

HPLC analysis was done for detecting the phytoalexin namely Phytocassanes with the leaves of rice cultivar Black Nuniya in Untreated healthy, untreated inoculated, PGPF (*T. harzianum*, NAIMCC-F-03288) treated Healthy and PGPF (*T. harzianum*, NAIMCC-F-03288) treated and inoculated plants. A total of four peaks were visible in Untreated samples whereas in case of Treated samples an extra peak was visible along

with the enhancement in the level of the compound in case of treated inoculated samples which clearly indicates its better resistivity towards the pathogen. (Fig. 49; Table 26).

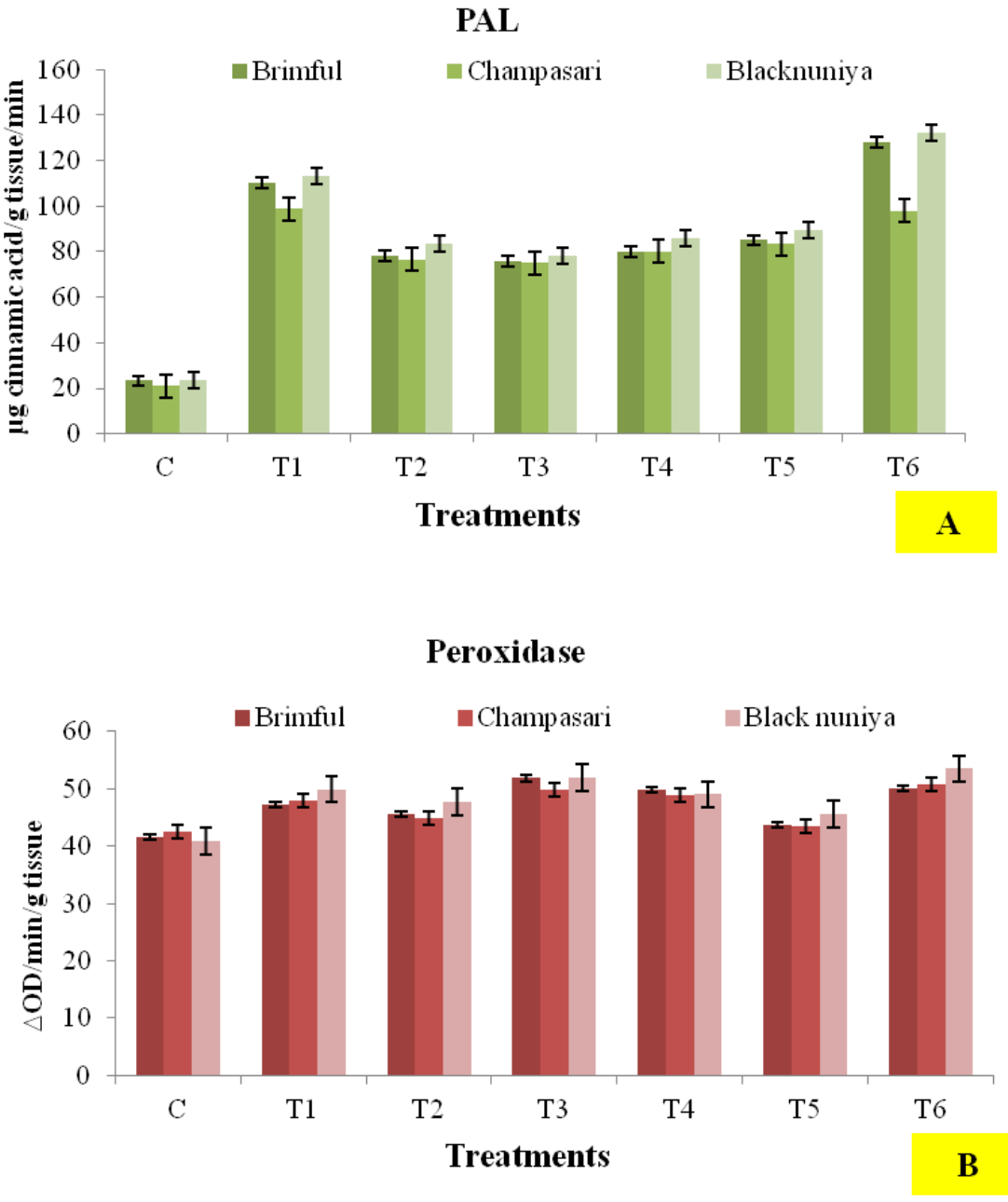


Figure 47: Defense enzyme activity (A) Phenylalanine ammonia lyase and (B) Peroxidase of rice cultivars following treatment with PGPF and inoculated with pathogen (*D oryzae*). C-Untreated Control, T1-T6=Treated Inoculated [T1-*T.asperellum* (NAIMCC-F-03293), T2-*T.harzianum* (NAIMCC-F-03289), T3-*T.harzianum* (NAIMCC-F-03290), T4-*T.asperellum* (NAIMCC-F-03291), T5-*T.asperellum* (NAIMCC-F-03292) and T6= *T.harzianum* (NAIMCC-F-03288)]

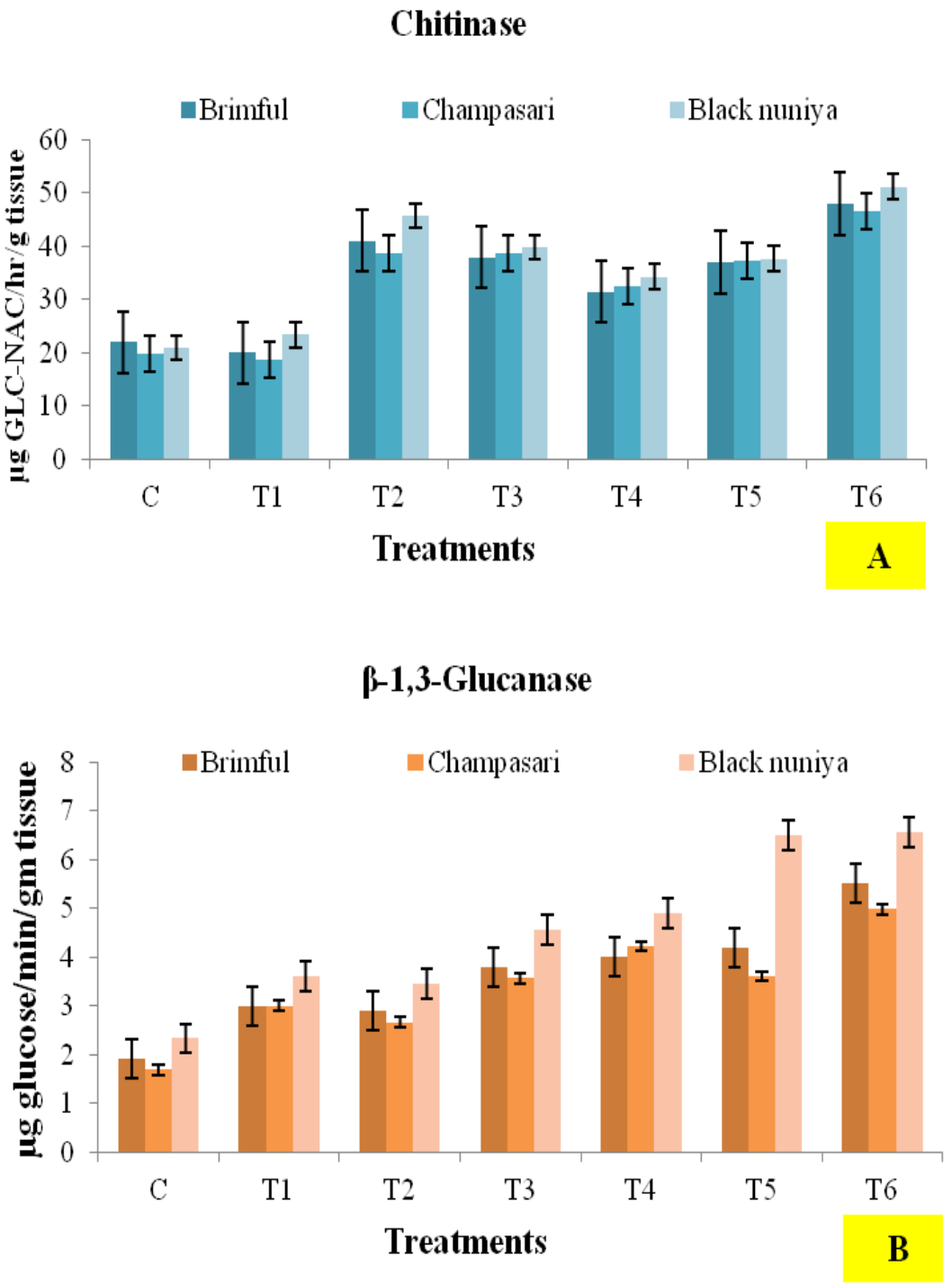


Figure 48: Defense enzyme activity (A) Chitinase (B) β-1, 3-Glucanase of rice cultivars following treatment with PGPF and pathogen challenge. C-Untreated Control,T1-T6=Treated Inoculated [T1-*T.asperellum* (NAIMCC-F-03293), T2-*T.harzianum* (NAIMCC-F-03289), T3-*T.harzianum* (NAIMCC-F-03290), T4-*T.asperellum* (NAIMCC-F-03291), T5-*T.asperellum* (NAIMCC-F-03292) and T6= *T.harzianum* (NAIMCC-F-03288)]

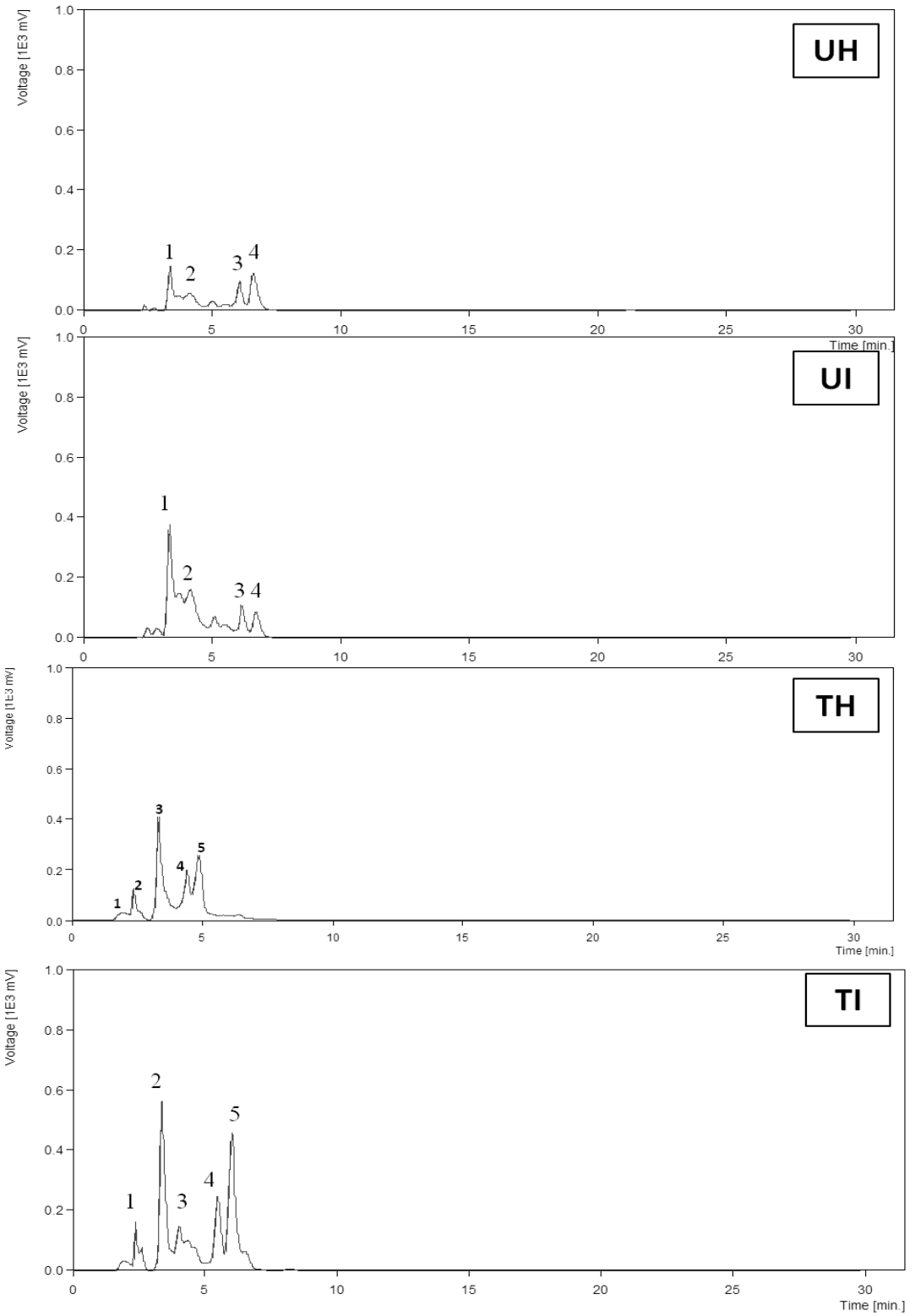


Figure 49: HPLC analysis of Phytocassanes from leaf extracts of rice plant (cv. Black Nuniya) treated with *T. harzianum* (NAIMCC-F-03288) and pathogen challenge. (UH- Untreated Healthy, UI- Untreated Inoculated, TH- Treated Healthy and TI- Treated Inoculated)

Table 26. Peak results of Phytocassanes from leaf extracts of rice plant (cv. Black Nuniya) following treatment with *T. harzianum* (NAIMCC-F-03288) and pathogen challenge

Untreated Healthy (UH)		
Peak no	**Retention time (min)**	**Height(mV)**
1	3.390	152.745
2	4.110	60.635
3	6.080	101.217
4	6.640	129.147
Untreated Inoculated (UI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	3.360	381.265
2	3.740	152.817
3	4.150	163.230
4	6.180	109.617
Treated Healthy (TH)		
Peak no	**Retention time (min)**	**Height(mV)**
1	1.980	27.572
2	2.360	125.386
3	3.340	309.069
4	4.440	194.111
5	4.900	255.207
Treated Inoculated (TI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	3.190	158.840
2	4.300	394.599
3	3.040	163.145
4	6.510	218.813
5	6.890	350.685

4.14. Growth promotion and biochemical changes in rice cultivar following application of AMF (*R. fasciculatus*)and pathogen challenge

4.14.1. Growth promotion

In the third set of trial, AMF fungi *R. fasciculatus* was tested for its effects in inhibiting brown spot of rice plants caused by *D. oryzae* in field condition. For field inoculation, chopped maize roots colonized with dominant spores of *R. fasciculatus* (AMF) were applied in the root rhizosphere following the transplantation of fifteen days old rice seedlings prior to the pathogen challenge. One month following application of

AMF root colonization status was examined and it was confirmed that the rice roots were colonized with the AMF (Fig. 50). The growth of the plants were recorded after every 20 days of interval of time and it was observed that the application of AMF improved the growth of the treated plants in comparison to the control plants (Table 27).

Table 27. Growth promotion of field grown rice plants following application of AMF (*R. fasciculatus*) and pathogen challenge

Rice cultivars	**20 days**		**40 days**		**60 days**		**80 days**	
	Control	**Treated**	**Control**	**Treated**	**Control**	**Treated**	**Control**	**Treated**
Black Nuniya	9±0.02	11±0.44	19±0.43	23±0.08	22±0.46	40±0.92	39±0.93	65±0.01
Brimful	12±0.81	14±0.46	24±0.35	30±0.43	36±0.82	48±0.24	42±0.56	60±0.01
Champasari	11±0.28	13±0.01	22±0.82	29±0.55	31±0.33	42±0.04	40±0.46	58±0.53

± indicates standard error.

4.14.2. Changes in biochemical activity

In field condition total sugar content in all three rice cultivars showed considerable increase in the treated samples in comparison to the control sets. Estimation of total chlorophyll content and total protein content revealed that although there was not much changes in the level of chlorophyll content, protein and phenol content increased in treated plants in relation to the control sets (Table 28).

Table 28.Change in biochemical activity in rice cultivars following application of AMF (*R. fasciculatus*) and pathogen challenge

Biochemical components	Brimful		Black Nuniya		Champasari	
	Control	Treated	Control	Treated	Control	Treated
Total sugar content (mg/g tissue)	34.45±0.54	42.63±0.54	38.16±0.51	45.88±0.54	28.16±0.52	31.06±0.51
Total chlorophyll content (µg/g tissue)	13.62±0.25	14.83±0.02	13.88±0.16	14.01±0.22	12.14±0.32	12.90±0.11
Total protein content (mg/g tissue)	31.06±0.25	42.5±0.22	33.72±0.32	45.81±0.36	28.08±0.33	37.45±0.35
Total phenol content (mg/g tissue)	3.42±0.02	4.85±0.05	3.95±0.07	4.99±0.03	2.70±0.05	4.15±0.04

Mean value of three replicates; ± Standard error.

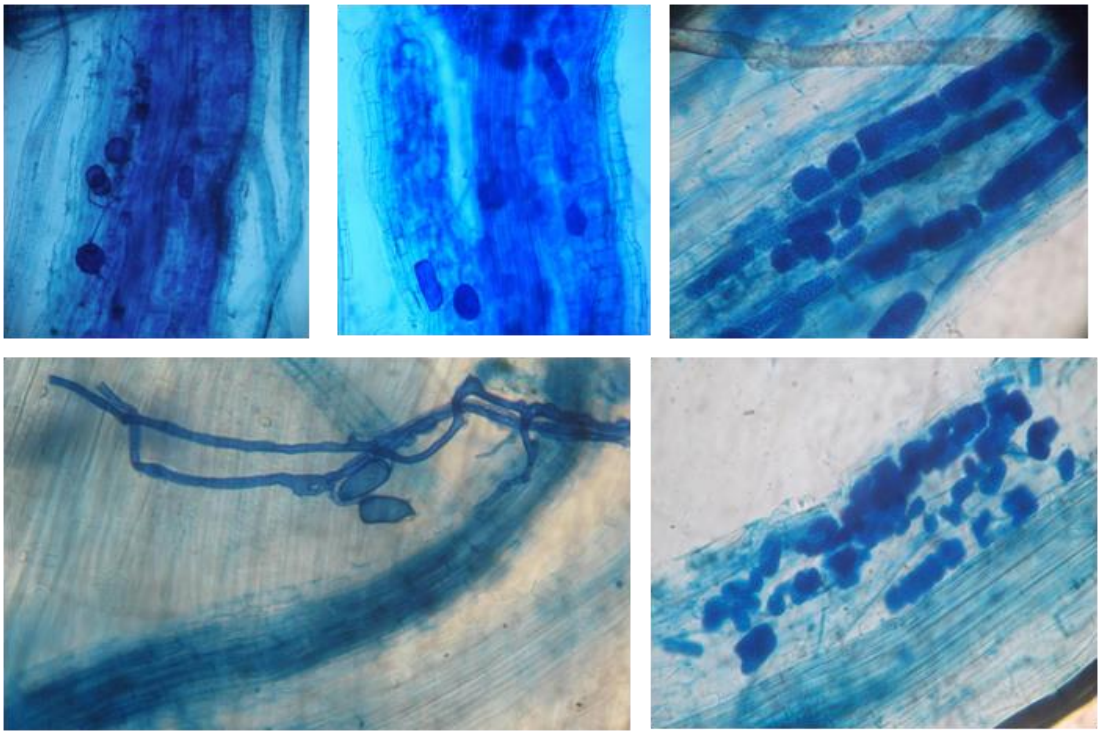


Figure 50 : Root colonization of rice following application of AMF (*R. fasciculatus*).

4.15. Activation of defense response in rice plants following application of AMF (*R. fasciculatus*) against *D.oryzae*

4.15.1. Disease suppression

Percentage disease index for control and treated sets of rice cultivars was recorded after every 7, 14, 21 and 28 days of pathogen challenge. It was evident from the results that the bioinoculant (AMF) could decrease the intensity of infection upto some level (Table 29)

Table 29. Percent Disease Index in rice plants following treatment and pathogen challenge.

Rice cultivars		Time interval (days)			
		7	14	21	28
Brimful	UI	22.8±0.05	33.6±0.05	42.5±0.94	53.6±0.45
	TI	18.5±0.57	25.4±0.01	36.7±0.97	45.4±0.01
Champasari	UI	19.7±0.58	31.2±0.02	40.8±0.02	52.6±0.05
	TI	17.5±0.02	23.6±0.44	34.8±0.01	44.08±0.01
Black Nuniya	UI	20.6±0.11	32.4±0.47	42.8±0.48	54.3±0.05
	TI	17.8±0.08	24.6±0.09	31.8±0.91	41.63±0.05

± indicates standard error. (UI= Untreated Inoculated and TI= Treated Inoculated)

4.15.2. Defense enzyme

Assay of defense enzymes- PAL, POX, CHT and GLU content in the leaves of the inoculated plants was carried out after every 24 hr interval upto96 hrs. Considerable increase in activities of chitinase, β-1,3-glucanase, peroxidise and phenylalanine ammonia lyase in the leaves of rice plants were observed after the application of *R. fasciculatus* and enhanced markedly after challenge inoculation with *D. oryzae* . It was observed that all the enzymes were markedly increased in Black Nuniya followed by Champasari and Brimful. (Fig. 51).

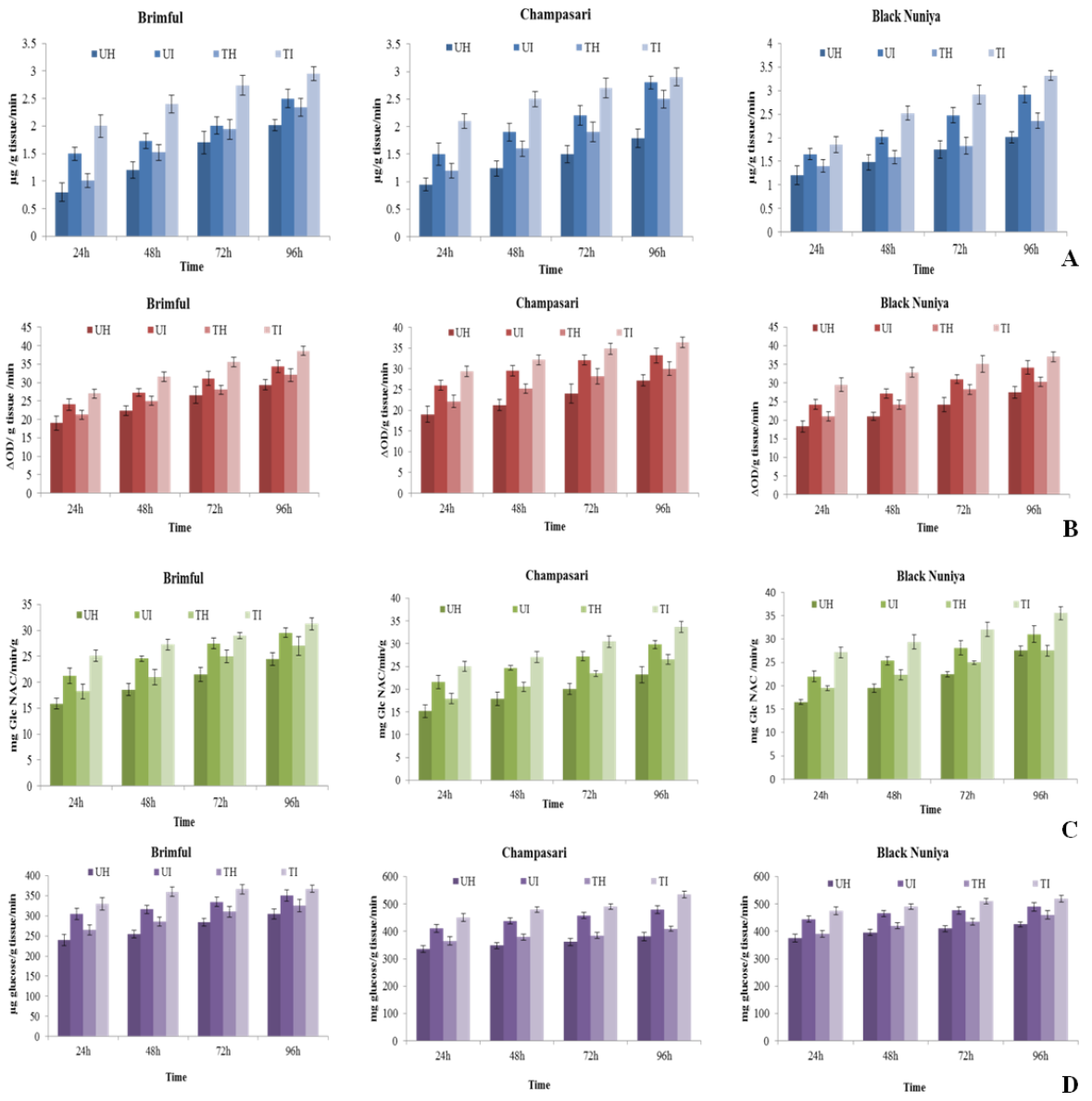


Figure 51: Changes in the level of defense enzyme in rice cultivars treated with *R. fasciculatus* following artificial inoculation with *D. oryzae*.(A) PAL, (B) Peroxidase, (C) Chitinase and (D) β-1,3 Glucanase. (UH- Untreated Healthy, UI- Untreated Inoculated, TH- Treated Healthy and TI- Treated Inoculated)

4.16. Growth promotion and biochemical changes in rice cultivar following dual and combined application of bioinoculants against *D. oryzae*

In the fourth trial experiment all the bioinoculants PGPR (*B. altitudinus*,NAIMCC-B01485), PGPF (*T. harzianum*,NAIMCC-F-03288) and AMF (***R. fasciculatus***) on the basis of their performance in disease suppression PGPR were applied in dual and in combined form for all the three rice cultivars and challenge inoculated with the pathogen *D. oryzae*. These different bioinoculants were added to the

soil at different time interval as mentioned in Materials and Methods. Effects of their application on growth promotion and biochemical changes in rice plants were noted under field condition.

4.16.1. Growth promotion

Growth enhancement in terms of height was measured after 20, 40, 60 and 80 days of final treatment. The results as shown in the Table revealed that growth was significantly improved after each treatment but best growth was obtained in Black nuniya after application of joint treatments with the three bio inoculants. Growth promotion was almost four fold increased following treatment in all the three cultivars (Table 30).

4.16.2. Changes in biochemical activity

Total sugar content and total chlorophyll content was quantified for all three rice cultivars following the treatment and it was observed that the content was in increased amount in comparison to the control. Total soluble protein was quantified in leaves of control and treated rice plants where it was noticed that protein content in leaves increased following treatments. However the contents were more in case of combined application in comparison to the dual application (Table 31).SDS-analysis was conducted for the protein sample of rice cultivar Black Nuniya with combined treatment showing the maximum protein content. Leaf protein exhibited bands in SDS-PAGE ranging in molecular weight (25,29,30,32,34,43,68,69,71,72,97,98KDa) (Fig.55) and bands were of varying intensities. Total phenol content also increased in leaves following treatments and it was recorded to be highest in Black Nuniya as shown in Table 31.

Table 30. Growth promotion in rice cultivars following application of bioinoclants.

Rice cultivars	**Treatment**	**Height (cm) After**			
		20 days	**40 days**	**60 days**	**80 days**
Brimful	Control	9±0.2	18±0.11	23±0.08	40±0.14
	PGPR+AMF	12±0.11	21±0.11	27±0.14	55±0.12
	PGPF+AMF	12±0.06	22±0.15	28±0.11	58±0.11
	PGPR+PGPF	14±0.11	23±0.06	29±0.12	60±0.06
	PGPR+PGPF+AMF	15±0.16	24±0.12	30±0.06	62±0.16
Black Nuniya	Control	12±0.16	23±0.09	34±0.16	54±0.09
	PGPR+AMF	15±0.09	32±0.15	49±0.09	58±0.05
	PGPF+AMF	16±0.03	33±0.08	51±0.12	59±0.12
	PGPR+PGPF	17±0.10	35±0.05	53±0.19	61±0.06
	PGPR+PGPF+AMF	20±0.11	41±0.16	58±0.08	64±0.11
Champasari	Control	11±0.06	21±0.14	32±0.14	38±0.19
	PGPR+AMF	14±0.05	30±0.11	41±0.05	56±0.14
	PGPF+AMF	15±0.14	32±0.05	44±0.11	57±0.12
	PGPR+PGPF	17±0.11	35±0.11	48±0.05	59±0.15
	PGPR+PGPF+AMF	19±0.05	38±0.12	50±0.04	62±0.01
CD(p=0.05)	**Treatments** **Cultivars**	**1.00** **0.77**	**4.66** **3.61**	**6.24** **4.84**	**6.48** **5.02**

±Standard Error, Average of three replicates. (PGPR+AMF- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, PGPF+ AMF- *T. harzianum* (NAIMCC-F-03288)+ *R. fasciculatus*, PGPR+PGPF= *Bacillus altitudinus* (NAIMCC-B01485)+ *T. harzianum* (NAIMCC-F-03288) and PGPR+PGPF+AMF= *Bacillus altitudinus* (NAIMCC-B01485 +*T. harzianum*(NAIMCC-F-03288)+ *R. fasciculatus*)

Table 31. Changes in biochemical components in rice cultivars following treatment with bioinoculants against challenged inoculation with the pathogen

Rice cultivars	Treatment	Biochemical components			
		Total sugar content (mg/g tissue)	Total chlorophyll content (µg/g tissue)	Total protein content (mg/g tissue)	Total phenol content (mg/g tissue)
Brimful	Control	35.02±0.05	13.45±0.52	32.02±0.25	3.68±0.12
	PGPR+AMF	44.23±0.08	15.01±0.50	43.06±0.22	4.91±0.13
	PGPF+AMF	44.68±0.07	15.25±0.57	43.58±0.31	5.02±0.11
	PGPR+PGPF	45.52±0.04	17.45±0.51	44.27±0.26	6.32±0.15
	PGPR+PGPF+AMF	47.28±0.06	17.82±0.52	47.91±0.28	7.02±0.11
Black Nuniya	Control	36.45±0.08	13.68±0.56	32.45±0.32	4.02±0.16
	PGPR+AMF	46.22±0.01	15.82±0.51	45.82±0.21	5.28±0.15
	PGPF+AMF	47.89±0.05	15.91±0.52	46.03±0.28	5.41±0.12
	PGPR+PGPF	51.45±0.04	17.98±0.59	47.08±0.31	6.98±0.11
	PGPR+PGPF+AMF	55.67±0.05	18.05±0.54	51.28±0.26	7.45±0.18
Champasari	Control	29.68±0.06	12.18±0.55	28.45±0.27	3.22±0.16
	PGPR+AMF	33.42±0.04	14.46±0.51	36.80±0.2	4.68±0.11
	PGPF+AMF	36.89±0.06	14.91±0.58	36.92±0.28	4.90±0.16
	PGPR+PGPF	40.02±0.05	15.06±0.54	37.12±0.32	5.81±0.18
	PGPR+PGPF+AMF	45.68±0.06	16.92±0.51	42.28±0.25	6.91±0.11
CD(p=0.05)	Treatments Cultivars	3.85 2.98	0.88 0.68	2.29 1.78	0.27 0.21

±Standard Error,Average of three replicates. (PGPR+AMF- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, PGPF+ AMF- *T. harzianum* (NAIMCC-F-03288)+ *R. fasciculatus*, PGPR+PGPF= *Bacillus altitudinus* (NAIMCC-B01485)+ *T. harzianum* (NAIMCC-F-03288) and PGPR+PGPF+AMF= *Bacillus altitudinus* (NAIMCC-B01485 +*T. harzianum*(NAIMCC-F-03288)+ *R. fasciculatus*)

4.17. Activation of defense response of *Rice* following dual and combined application of bioinoculants against *D. oryzae*

4.17.1. Disease suppression

Upon pathogen spray, the percent disease index was recorded after 7,14,21 and 28 days. It was observed that disease incidence was much less in treated inoculated plants in comparison to untreated inoculated (UI) for all the intervals. It was found that the development of the disease was suppressed maximum in plots with combined application of PGPR (*B. altitudinus*, NAIMCC-B01485), PGPF (*T. harzianum*, NAIMCC-F-03288) and AMF (*G. fasciculatum*) then the plots with dual application for all the three cultivars among which Black nuniya with all the combination showed the highest suppression of only 16.87% of PDI followed by Champasari with 22.46% of PDI and Brimful showing 24.55% PDI respectively (Fig.52).

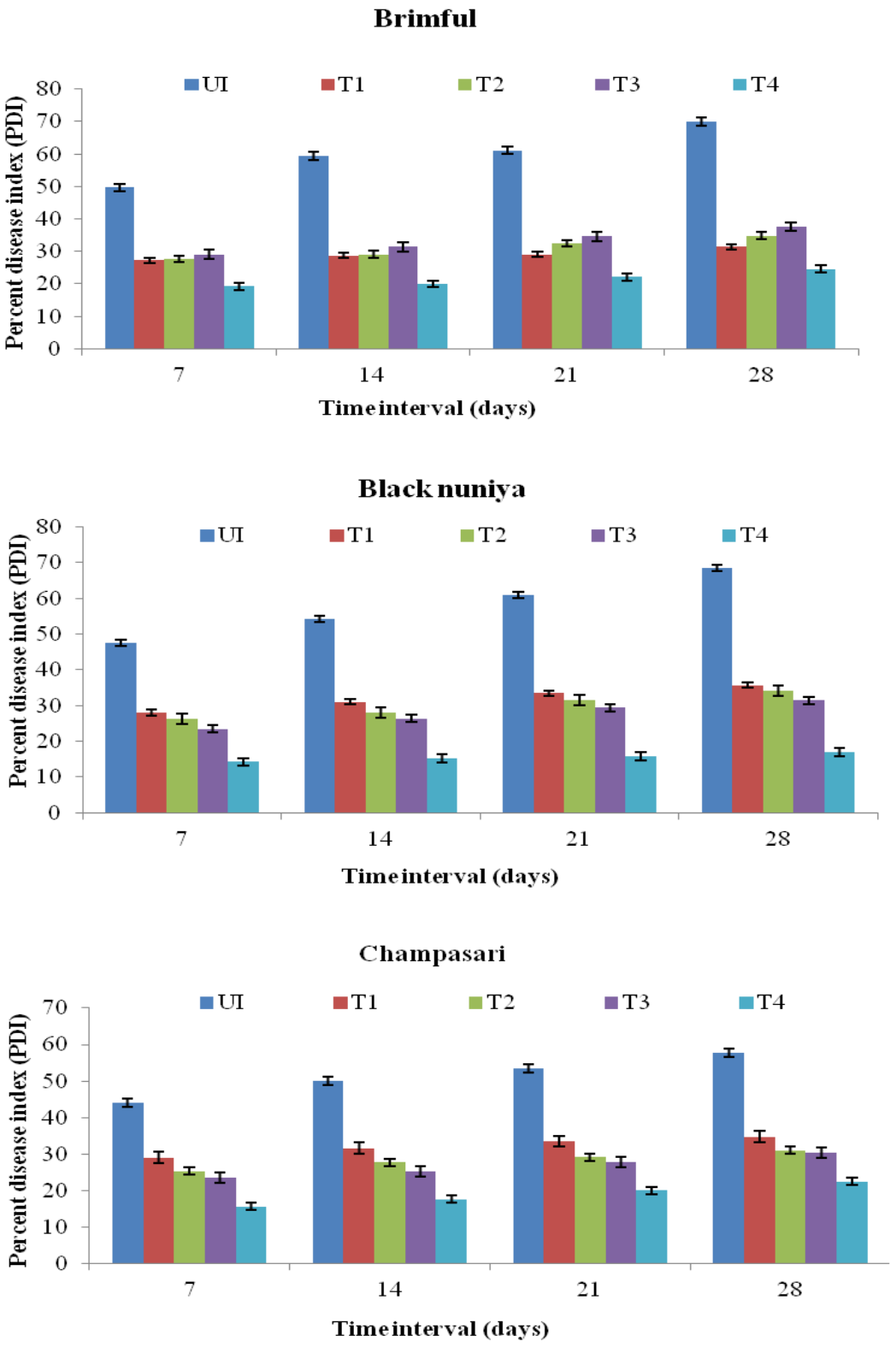


Figure 52: Percent disease index in rice cultivars following treatment and artificial inoculation with *D. oryzae*. (UI- Untreated inoculated, T1- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, T2- *T. harzianum* (NAIMCC-F-03288)+ *R. fasciculatus*, T3= *T. harzianum* (NAIMCC-F-03288)+ *Bacillus altitudinus* (NAIMCC-B01485) and T4= *T. harzianum*(NAIMCC-F-03288)+ *R. fasciculatus* + *Bacillus altitudinus* (NAIMCC-B01485)

4.17.2. Changes in activity of defense enzymes

The changes in the level of four different defense enzymes viz. Phenylalanine ammonia lyase, Peroxidase, Chitinase and β-1,3- Glucanase was analysed after 48 hrs of artificial inoculation of *D. oryzae* spore suspension. The following results as shown in Figure. 53 revealed that levels of defense enzymes were increased in bioinoculant treated PGPR (*B. altitudinus*,NAIMCC-B01485), PGPF (*T. harzianum*,NAIMCC-F-03288) and AMF (*R. fasciculatus*) inoculated plants of all the rice cultivars in comparison to their untreated inoculated sets. The levels of enzymes increased mainly in PGPR+PGPF and PGPR+AMF+PGPF treated plants. This collaborates with the fact the disease incidence was suppressed in these treated plants where the defense enzymes were increased (Fig. 53&54).

Peroxidase variations have been reported to be used as genetic markers at different levels within a taxon. In order to reveal changes in the isozyme patterns on infection, Native PAGE was performed in the rice cultivar Black Nuniya treated with combined application and which showed the least PDI%. Three bands with R_m value 0.27, 0.54 was seen in both Control and treated samples and whereas a presence of a new band of R_m value 0.82 was visible only in treated infected samples(Fig.55).

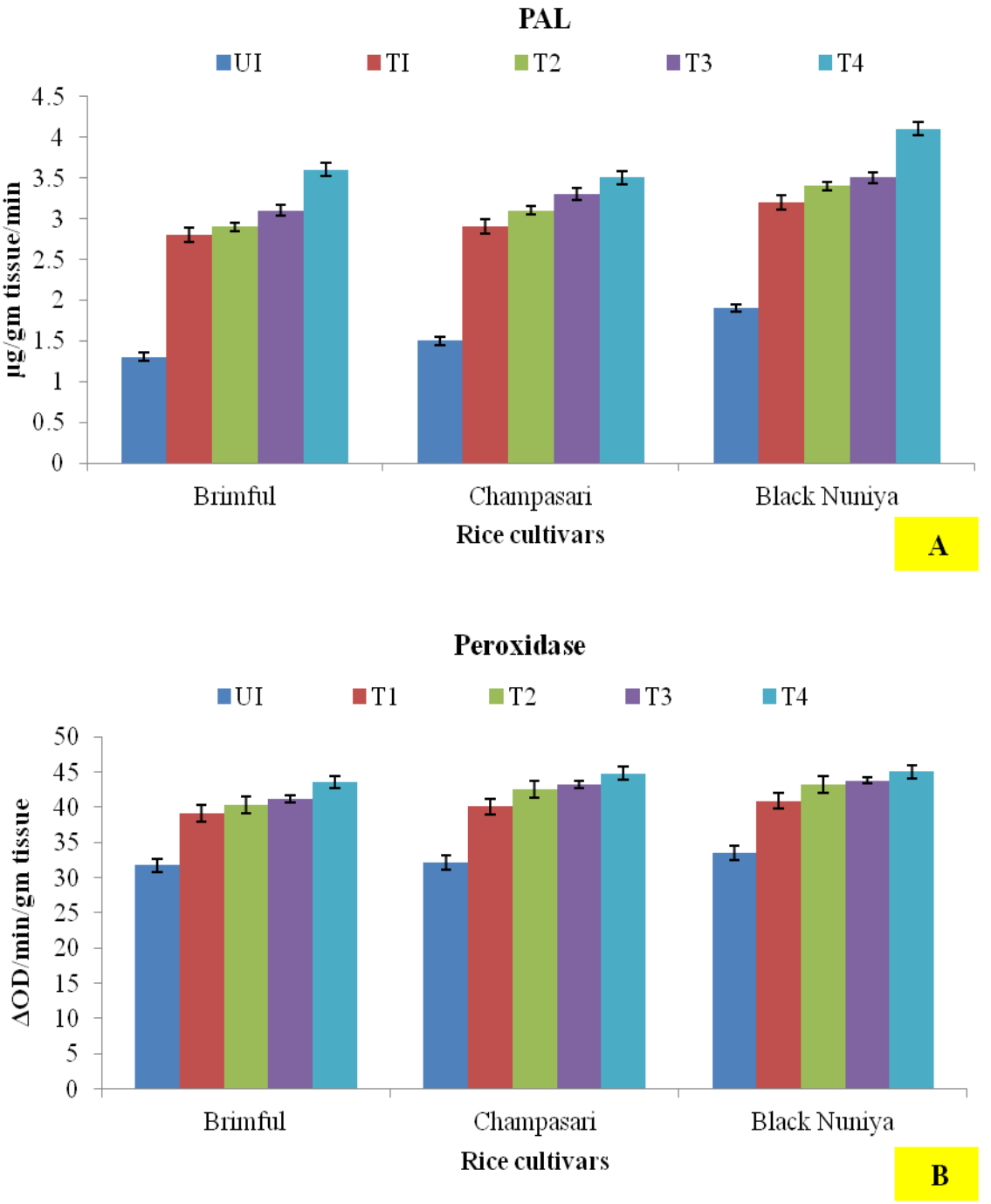


Figure 53: Defense enzyme activity of rice cultivars against *D. oryzae* following dual and combined application of bioinoculants. (A) PAL (Phenylalanine ammonia lyase) and (B) Peroxidase. (UI- Untreated inoculated, T1- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, T2- *T. harzianum* (NAIMCC-F-03288)+ *R. fasciculatus*, T3= *T. harzianum* (NAIMCC-F-03288)+ *Bacillus altitudinus* (NAIMCC-B01485) and T4= *T. harzianum*(NAIMCC-F-03288)+ *R. fasciculatus* + *Bacillus altitudinus* (NAIMCC-B01485)

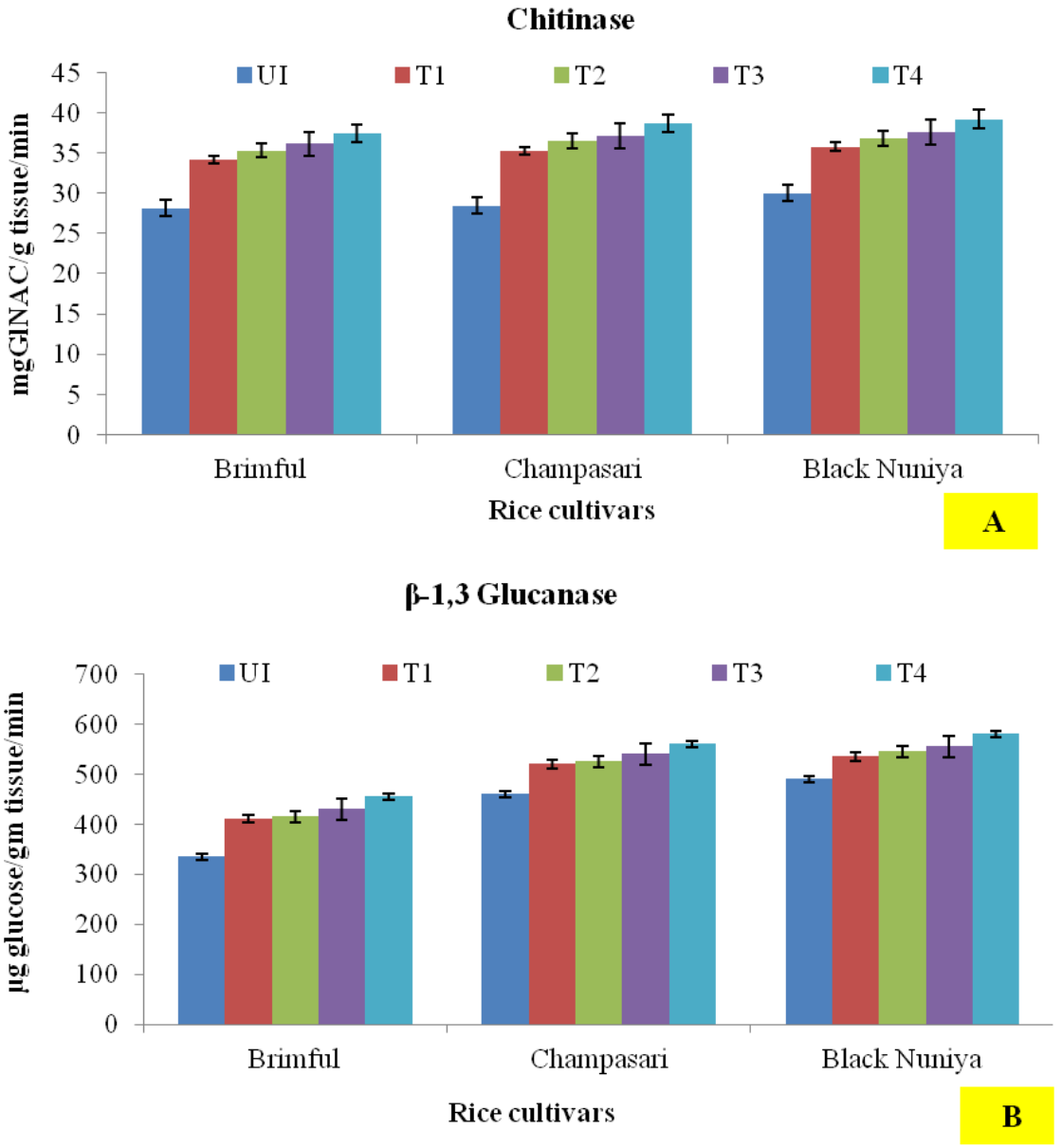


Figure 54: Defense enzyme activity of rice cultivars against *D. oryzae* following dual and combined application of bioinoculants. (A)Chitinase and (B) β-1,3Glucanase. (UI- Untreated inoculated, T1- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, T2- *T. harzianum* (NAIMCC-F-03288)+ *R. fasciculatus*, T3= *T. harzianum* (NAIMCC-F-03288)+ *Bacillus altitudinus* (NAIMCC-B01485) and T4= *T. harzianum*(NAIMCC-F-03288)+ *R. fasciculatus* + *Bacillus altitudinus* (NAIMCC-B01485)

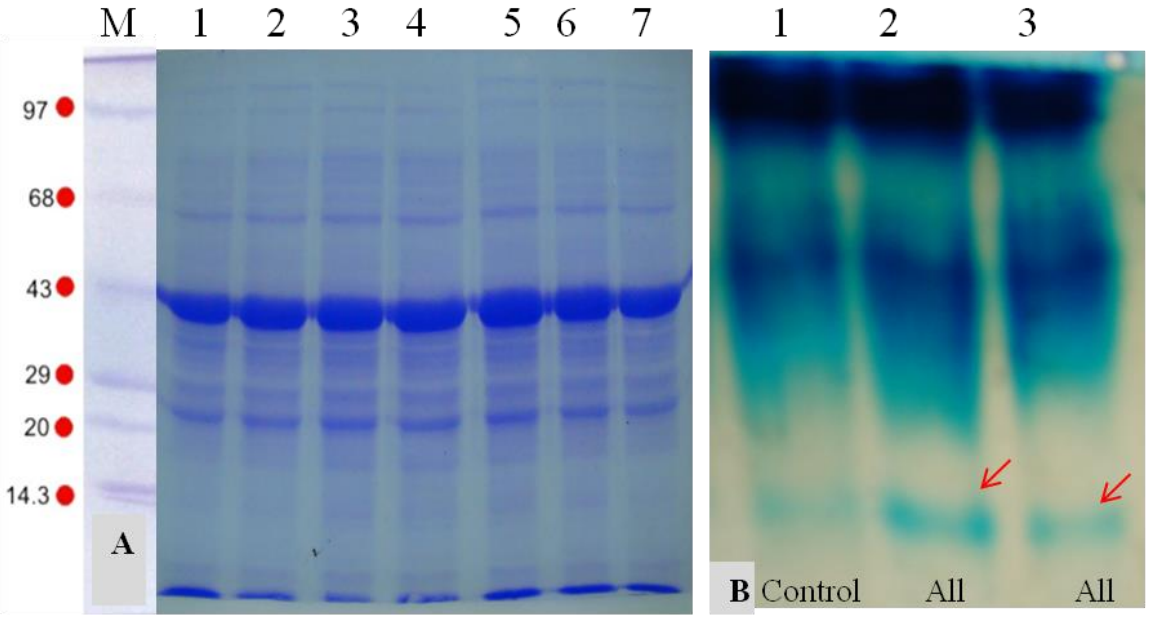


Figure 55: SDS-PAGE (A) and Peroxizyme analysis (B) of leaf proteins of rice cultivar (Black nuniya) following treatment with bioinoculants and challenged inoculation with *D. oryzae*

4.17.3. Radial growth bioassay of antifungal compound (Phytocassanes)

Crude extracts (Ethyl acetate fraction) prepared from Untreated and Treated samples with and without inoculation with pathogen were bio assayed following radial growth inhibition assay as described in Materials and methods .Results (Table.32,Fig. 56) revealed that mycelia growth of *D. oryzae* was inhibited markedly in the medium supplemented with the extracts of treated leaves. Treated inoculated samples showed the maximum inhibition towards the pathogen depicting the induction of antifungal compound following treatment.

Table 32. Effects of antifungal compound (Phytocassanes) from rice leaf extracts of (cv. Black Nuniya) following treatment with bioinoculants [*T. harzianum* (NAIMCC-F-03288), *R. fasciculatus* and *B. altitudinus* (NAIMCC-B01485)] and inoculation with *D. oryzae*

Sample	Diameter of mycelia (mm)[a]
UH	28.6
UI	19.2
TH	10.5
TI	6.3

a= Average of three experimental sets. Diameter was noted after 7 days (UH= Untreated Healthy, UI= Untreated Infected, TH=Treated Healthy and TI= Treated Infected)

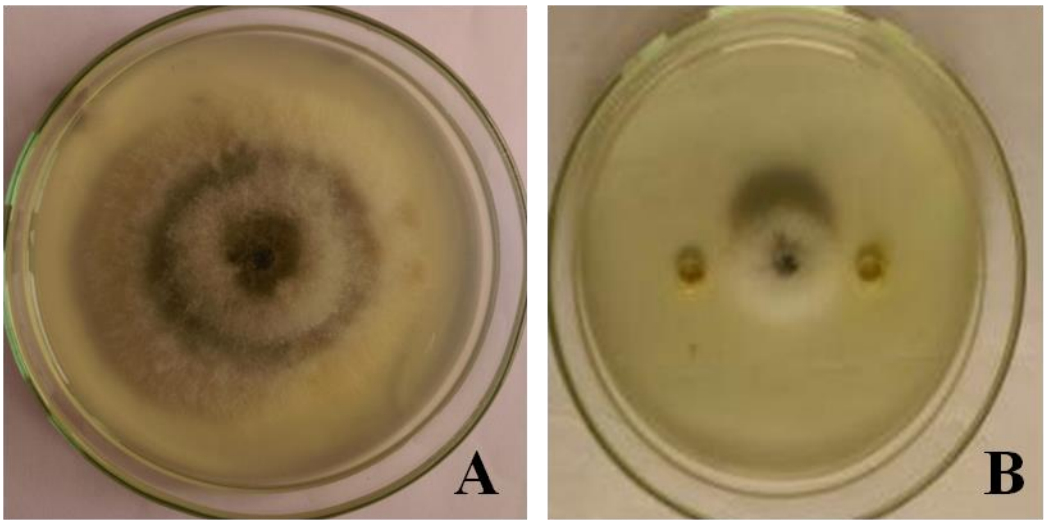


Figure 56: Radial growth bioassay of Ethyl acetate extracts of untreated control and treated inoculated rice leaves (cv. Black Nuniya). (A) Untreated Control and (B) Treated with bioinoculants [*T. harzianum* (NAIMCC-F-03288), *R. fasciculatus* and *B. altitudinus* (NAIMCC-B01485)] and inoculation with *D. oryzae*.

4.17.4. Cellular localization of Glucanase

Cellular localization of glucanase enzyme in leaves of rice plants was determined following indirect immunofluorescence test using FITC binding and treatment with PAb raised against glucanase. Leaf sections from untreated control plants and *T. harzianum* (NAIMCC-F-03288)+ *R. fasciculatus* + *Bacillus altitudinus* (NAIMCC-B01485) treated plants of rice cultivar Black nuniya was taken. Immunolocalization of glucanase in treated leaves sections of rice plants were observed using FITC after treatment with PAb raised against glucanase. Positive reaction with FITC was observed in cellular localization which gave indication of the induction of glucanase in rice leaf tissues (Fig.57). Bright apple green fluorescence was observed in treated leaves.

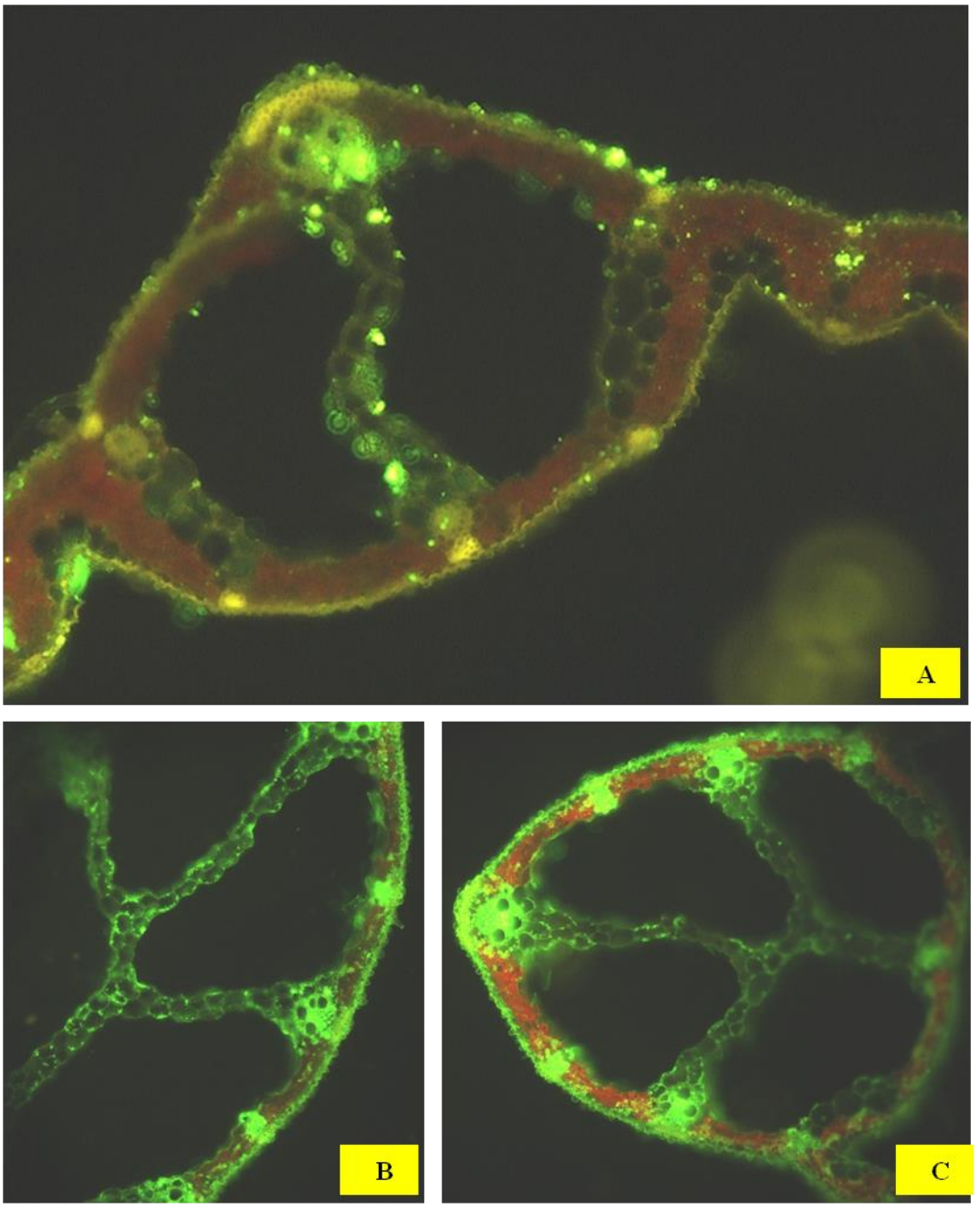


Figure 57: Cellular localization of glucanase in leaf tissue of rice (cv. Black Nuniya) following combined treatment of bioinoculants and challenge inoculation with fungal pathogen, probed with PAb of glucanase and labelled with FITC. (A) Control and (B&C) Treated.

4.17.5. Cellular localization of Chitinase

Cellular localization of chitinase enzyme in leaves of rice plants was determined following indirect immunofluorescence test using FITC binding and treatment with PAbraised against chitinase. Leaf sections from untreated control plants and

T. harzianum (NAIMCC-F-03288) + *R. fasciculatus* + *Bacillus altitudinus* (NAIMCC-B01485) treated plants of rice cultivar Black nuniya was taken. Immunolocalization of chitinase in treated leaves sections of rice plants was observed using FITC after treatment with PAb raised against chitinase. Positive reaction with FITC was observed in cellular localization which gave indication of the induction of chitinase in rice leaf tissues (Figure 58). Bright apple green fluorescence was observed in treated leaves which testified the increased accumulation of chitinase enzyme in treated leaves.

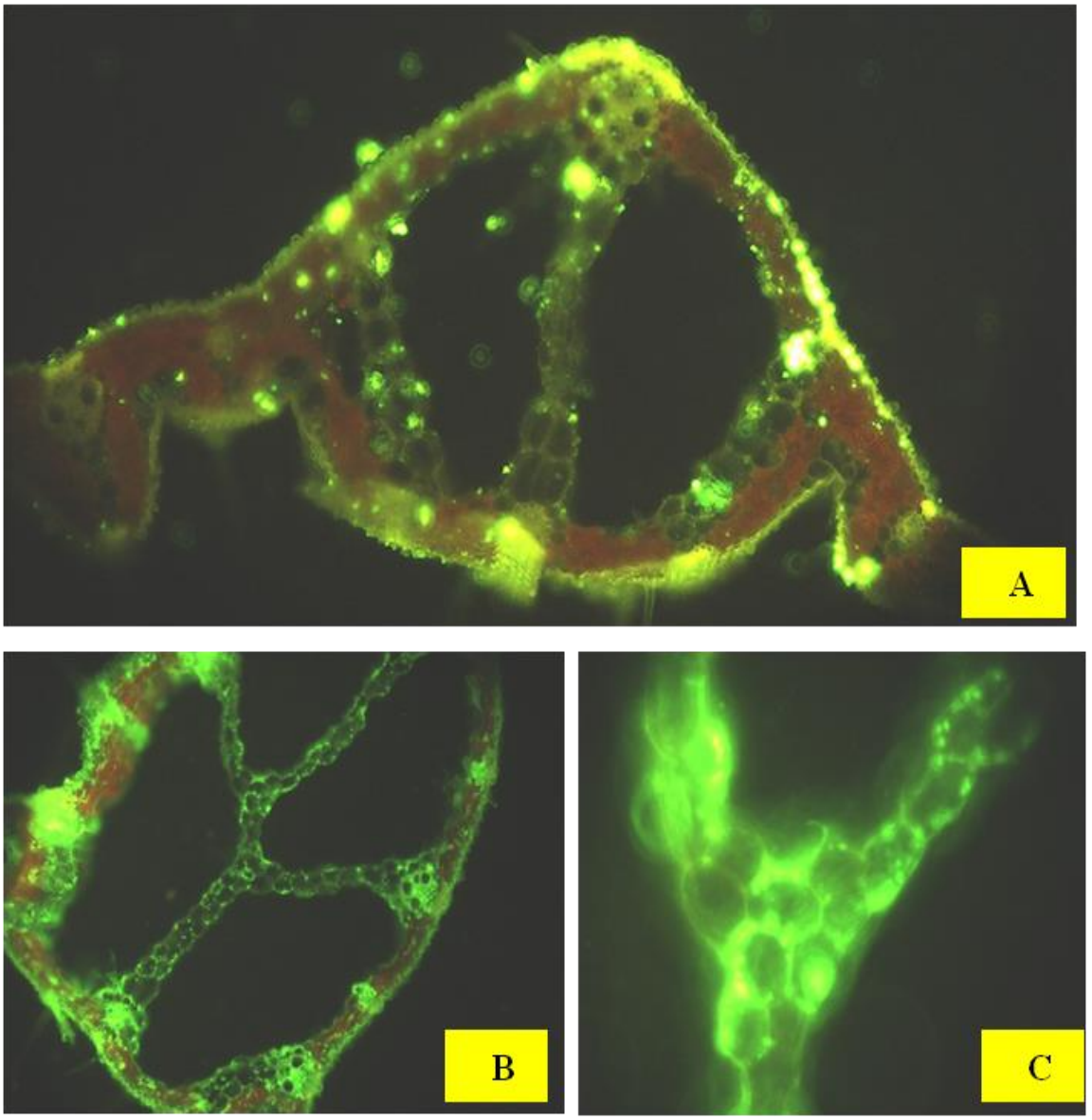


Figure 58: Cellular localization of chitinase in leaf tissue of rice (cv. Black Nuniya) following combined treatment of bioinoculants and challenge inoculation with fungal pathogen, probed with PAb of chitinase and labelled with FITC. (A) Control and (B&C) Treated.

4.17.6. HPLC analysis of phenolics

The changes in phenolic compounds in the leaves of Untreated inoculated and treated *T. harzianum* (NAIMCC-F-03288) + *R. fasciculatus* + *Bacillus altitudinus* (NAIMCC-B01485) inoculated samples of rice cultivar Black Nuniya showing the least PDI were measured using HPLC analysis. Analysis of the samples revealed the presence of different peaks showing a variety of phenolic acids present in treated and control rice cultivar Black Nuniya (Fig.60; Table 34). Both the control and treated leaves revealed the presence of seven main peaks. However the absorbance value of all the seven peaks was increased in treated samples in comparison to the control. Comparison with standards (Fig. 59; Table. 33) revealed the presence of phenols such as Galleic acid, Ferulic acid, Salicylic acid and Phloroglucinol. Increase in the absorbance value in treated samples confirm enhancement of phenolics contents following treatment with bioinoculants.

Table 33: Peak Value of Standard Phenolics

Sl No.	Compounds	Retention time(min)	Height (mV)
1	Phloroglucinol	4.540	76.290
2	Gallic acid	5.340	763.961
3	Pyrogallol	6.070	61.367
4	3,4- dihydroxybenzoic acid	9.170	371.707
5	Resorcinol	9.260	360.627
6	Catechol	11.580	436.976
7	Catechin	14.970	384.121
8	Chlorogenic acid	15.850	404.678
9	Caffeine	16.420	634.666
10	Caffeic acid	16.780	977.014
11	Vanillic acid	17.430	926.949
12	Ferulic acid	29.170	984.834
13	Salicylic acid	30.410	184.096
14	Cinnamic acid	39.740	798.840

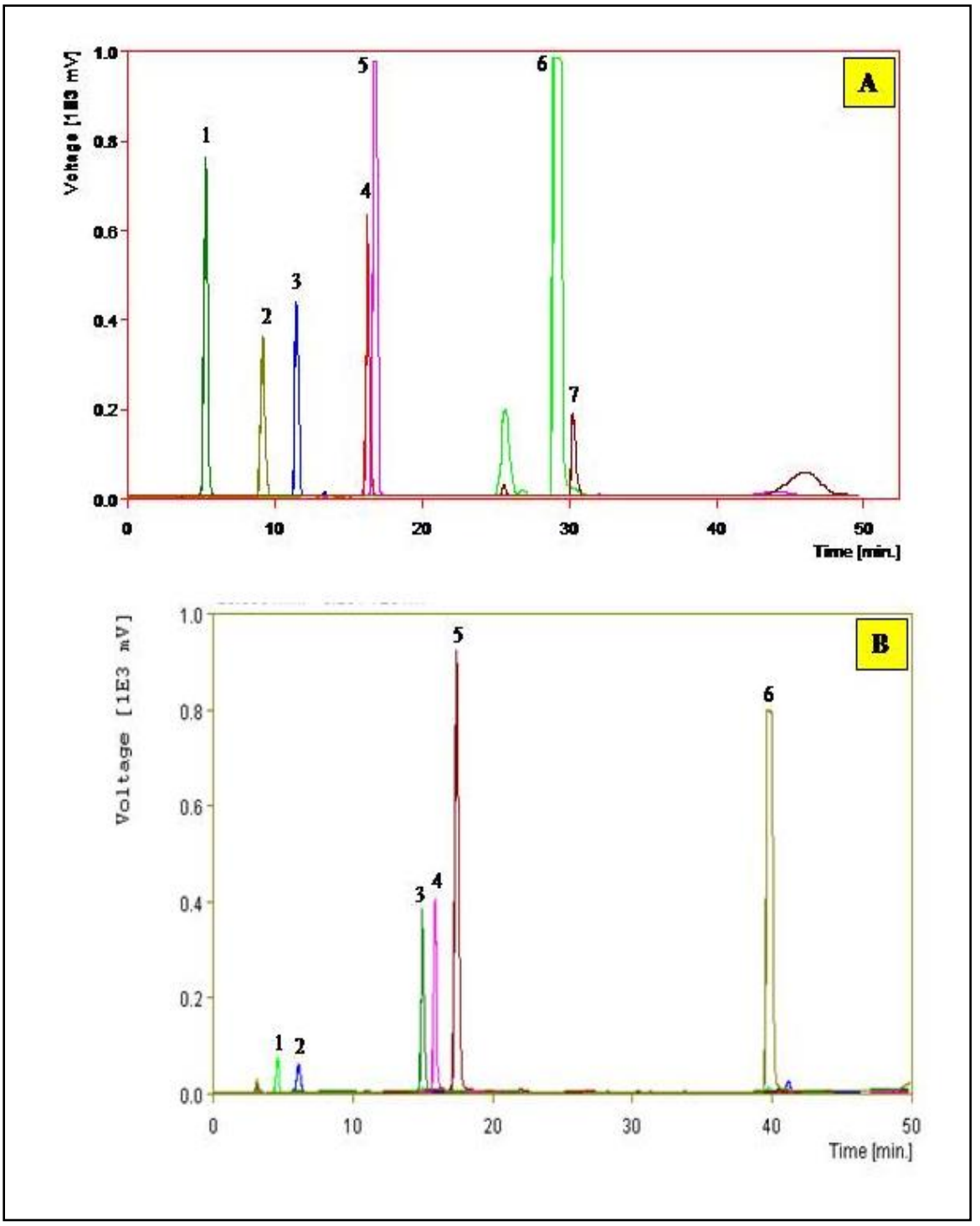


Figure 59: Standard phenolics detected through HPLC analysis. (A) 1: Gallic acid, 2: 3,4-dihydroxybenzoic acid(DHBA) , 3: Catechol, 4: Caffeine, 5: Caffeic acid, 6: Ferulic acid, 7: Salicylic acid. (B) 1: Phloroglucinol2: Pyrogallol3: Catechin4:Chlorogenic acid 5: Vanillic acid 6: Cinnamic acid.

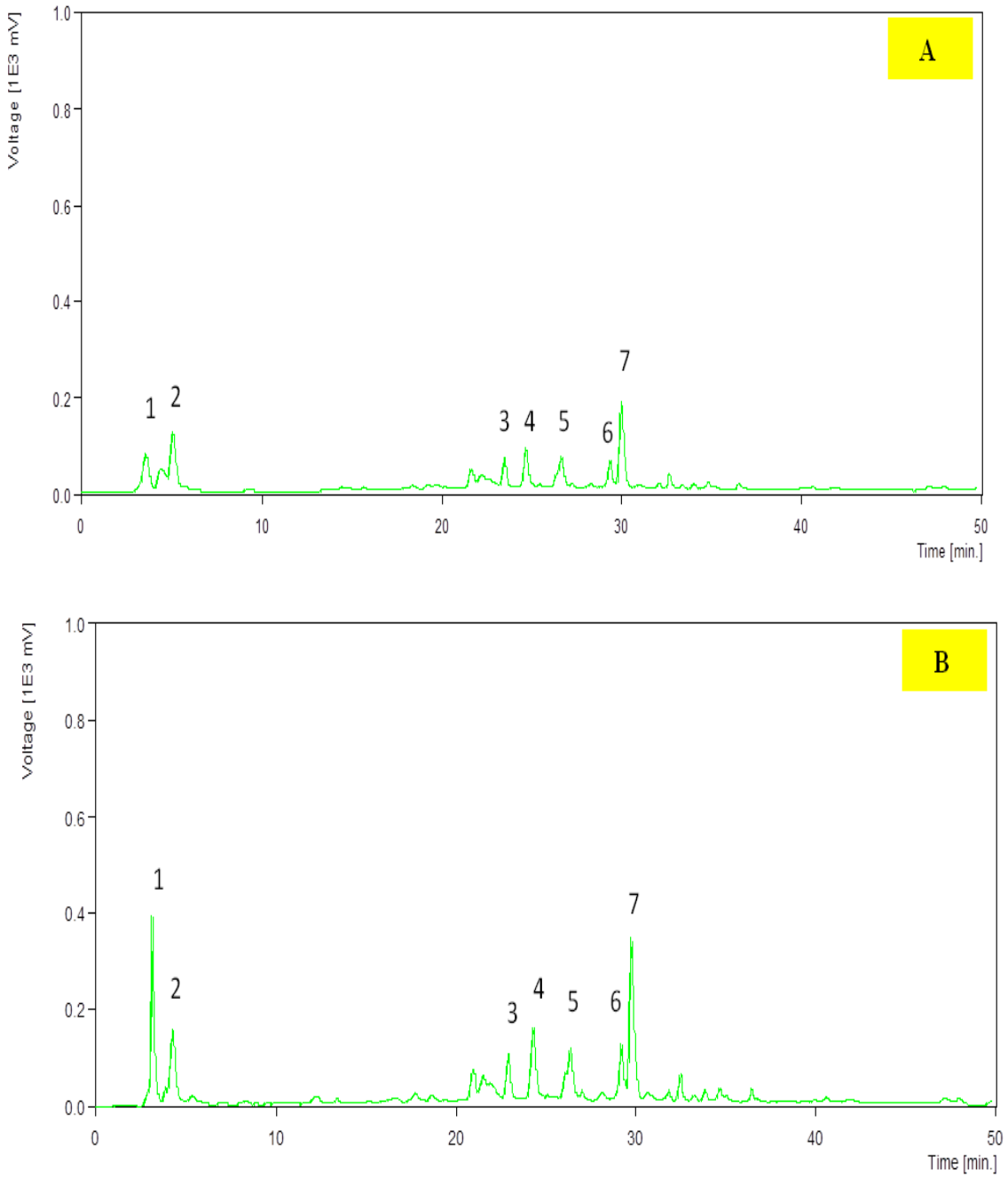


Figure 60: HPLC analysis of phenolic acid content from leaf extracts of (cv. Black Nuniya). (A) Control and (B) Treated with bioinoculants against pathogen challenge

Table 34. Peak results of Phenolics extracts from rice leaf of (cv. Black Nuniya) following treatment with bioinoculants and challenge inoculated with foliar fungal pathogen (*D. oryzae*)

Untreated control (UI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	3.580	80.511
2	5.070	124.973
3	23.600	68.934
4	24.760	89.719
5	26.770	72.170
6	29.500	63.284
7	30.120	188.013
Treated inoculated (TI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	3.190	394.599
2	4.300	158.840
3	23.040	109.145
4	24.380	163.667
5	26.510	118.813
6	29.350	126.748
7	29.890	350.685

4.17.7. HPLC analysis of phytoalexin

HPLC analysis was done for detecting the phytoalexin namely Phytocassanes from the leaves of rice cultivar Black nuniya in Untreated control and treated *T. harzianum*(NAIMCC-F-03288)+ *R. fasciculatus* + *Bacillus altitudinus* (NAIMCC-B01485) plants exhibiting the lowest PDI percentage. A total of 5 peaks were clearly visible in Untreated healthy as well as untreated plants infected with the pathogen. However the compounds increased markedly in treated infected plants as evident in all the peak (Figure 61; Table 35). A total of 10 peaks were clearly visible in treated healthy as well as treated plants infected with the pathogen. However the appearance of extra peaks in treated samples are clearly visible as a result of the treatment with bio inoculants which ultimately results in the better defense strategy. The compounds increased markedly in treated infected plants as evident in peak no. 2, 3, 5, 6 and 10. Increase in the absorbance value in treated samples confirm enhancement of phytocassanes contents following treatment with bio inoculants.

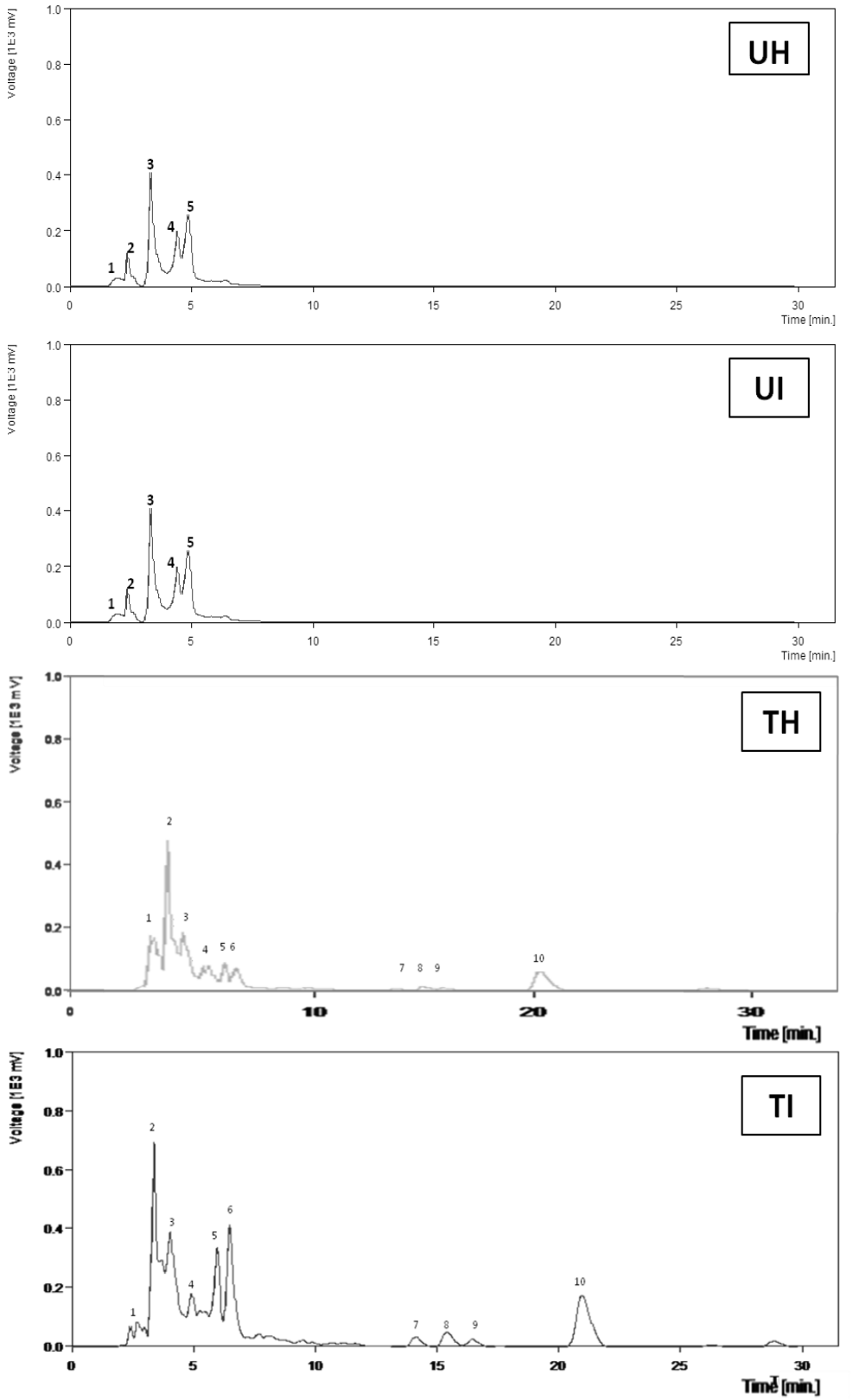


Figure 61: HPLC analysis of Phytocassanes from leaf extracts of (cv. Black Nuniya) treated with bioinoculants and challenge inoculation with foliar fungal pathogen (*D. oryzae*). UH= Untreated Healthy, UI= Untreated Inoculated, TH= Treated Healthy and TI= Treated Inoculated.

Table 35. Peak results of Phytocassanes extracts from rice leaves (cv. Black nuniya) following treatment with bioinoculants and challenge inoculation with foliar fungal pathogen (*D. oryzae*)

Untreated Healthy (UH)		
Peak no	**Retention time (min)**	**Height(mV)**
1	1.980	27.572
2	2.360	125.386
3	3.340	409.069
4	4.440	194.111
5	4.900	255.207
Untreated Inoculated (UI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	2.350	80.942
2	3.370	294.260
3	3.860	56.188
4	5.010	86.645
5	5.510	158.134
Treated Healthy (TH)		
Peak no	**Retention time (min)**	**Height(mV)**
1	2.480	176.814
2	3.290	486.390
3	4.010	186.293
4	5.200	080.168
5	5.880	087.571
6	6.430	073.021
7	13.770	006.343
8	15.030	011.698
9	15.950	008.085
10	20.370	070.437
Treated Inoculated (TI)		
Peak no	**Retention time (min)**	**Height(mV)**
1	2.670	061.453
2	3.590	796.210
3	4.290	401.150
4	5.592	267.876
5	6.421	301.020
6	6.970	345.021
7	12.667	045.697
8	15.142	068.020
9	16.076	030.210
10	20.510	268.436

Chapter 5
DISCUSSIONS

In our state West Bengal, agriculture plays a very vital role as a means of living in day to day life. Around 65% of population who live in village of our state are engaged in this occupation and about 95% are small and marginal farmers. Among the various other cereal crops cultivated in our state rice occupies the major area of farming about 53% (Adhikari *et al*., 2012). Although North Bengal is enriched with a huge varieties of rice cultivars, 15 rice cultivars were collected from different regions of North Bengal regions and Sikkim for our initial investigation. Morphological diversity among the seeds of all 15 rice cultivars were observed and recorded, both quantitative and qualitative traits were taken into consideration such as kernel colour, seed coat colour, aroma, presence of awn, length of the seed (Semwal *et al*., 2014). Awn less seed is an improved trait and high diversity in seed shapes and pericarp color may be important for developing quality rice to meet diverse consumer demand. Awn length and distribution, seed length, thousand seed weight and germination rates are the most important traits influencing the variability among populations (Fogliatto *et al*., 2011). Local farmers are conserving these landraces for specific traits like aroma, good taste and their regional importance. Characterizing these landrace could be huge help for the breeders to make use of specific character for the improvement of rice in future. Further germination rate, growth rate and total protein content of all the rice seedlings were measured and recorded. Populations with higher germination percentages (more than 80%) and higher survival ability (more than 80%) are important characteristics for crop improvement.

Success of a crop improvement programme depends on the magnitude of genetic variability and the extent to which the desirable characters are heritable (Ravi *et al*., 2003). Analysis of genetic variability in landraces of traditional rice cultivars can help in identifying diverse parental combinations for further selection and to help introgressing desirable genes. The landraces are known for significant variability with respect to the seed morphological characters and adaptation to local environments (Frankel *et al*., 1995; Hore, 2005). Many of these landraces are poor yielder and grown only in restricted pockets in the area of collection. Special drive is desirable for their

collection and conservation. Local farmers are conserving these landraces for specific traits like aroma, good taste and their regional importance. Characterization of landraces could help breeders to utilize appropriate characters in rice improvement programme. In addition, proper understanding on seed germination ability and survival is essential for adopting efficient management practices.

The present study deals with a major foliar fungal disease in the rice cultivars i.e. brown spot. Among the various diseases that hamper the rice yield every year brown spot has been a serious cause of decline in rice yield since long time. This disease is mainly triggered with environment having scarcity of water in combination with an imbalance in the content of nutrition particularly nitrogen (Baranwal *et al*., 2013). It is reported that the decrease in the grain yield varies according to the cultivar and the stage of infection (Kulkarni *et al*., 1980). In order to study the effects of this disease in the environmental conditions of North Bengal, all the 15 rice cultivars were grown in the experimental field of Immuno Phytopathology Laboratory, Dept. of Botany, University of North Bengal for their screening against the brown spot disease occurring naturally.

The results obtained revealed that the Percentage Disease index (PDI %) was found to be very high among the three local cultivars - Black Nuniya, Brimful and Champasari, collected from Bijanbari and lowest in two hybrid rice cultivars - Loknath 505 and UBKV-1. Following this observation the defence enzyme activity of phenylalanine ammonia lyase (PAL), peroxidase (POX), chitinase (CHT) and β-1, 3 glucanase (GLU) was measured which also revealed that the accumulation of all these defence enzyme was low in these three cultivars in comparison to the other cultivars showing its susceptibility towards the disease. However according to Abeles *et al*., 1970 and Pegg., 1988, chitin and β-1, 3-glucanase are major components in the cell wall of many fungi and there is possibility of plant chitinase and β-1, 3- glucanase enzymes to target fungi cell wall components as substrate and has anti fungal function. Therefore in the present study three different rice cultivars viz. Black nuniya, Brimful and Champasari which showed susceptibility towards the brown spot disease were taken into consideration for further experimental work.

It is observed in the present investigation that total phenol content increased in infected plants, more in plants with less infection. Infection by *Venturiain equalis* in apple caused an accumulation of phenolic compounds wherein Folin-Ciocalteu values

increased by 1.4 to 2.4 fold (Petkovsek *et al*., 2008). Taware *et al.* (2004) studied that there was significant increase in total phenolic content of grape leaves due to foliar powdery mildew infection. These results are in accordance with the observations made in our investigations.

At the onset of the present study growth and sporulation of brown spot pathogen, *Drechslera oryzae*, collected from the infected leaf samples was studied on various media and it was found that the maximum growth occurred in Potato Dextrose Agar media with an incubation period of 10d. Conidial colonies and mycelium characters of *D. oryzae* was observed and recorded. Microscopic observation of the conidia and conidiophores of the pathogen was also observed which is in accordance with the observations made by Motlagh *et al*., 2008.

Antifungal activity of the compounds (phytoalexin) collected from the healthy and infected leaves of two resistant cultivars (Loknath 505 and UBKV-1) and three susceptible cultivars (Black Nuniya, Brimful and Champasari) were conducted and it was seen that the mycelial growth of the pathogen was inhibited markedly in the medium supplemented with the ethyl acetate extracts of inoculated leaves of resistant cultivars (Loknath 505 and UBKV-1) in comparison to the susceptible cultivars in relation to their respective control. Our results are in accordance to Werder and Kern (1985) where they have shown that the phenolic metabolism plays a role in the resistance mechanism based on the different total phenolic accumulation between resistant and susceptible responses of maize to *Helminthosporium carbonum*. Fungitoxic compounds were found in both healthy and infected tissue, but the accumulation of these inhibitory components was greater in resistant inbreds, as compared to noninoculated control and infected susceptible maize inbreds. Also Purkayastha *et al*., 1983 proved that semi- dwarf rice cultivars are highly susceptible while the tall ones are resistant to sheath rot disease caused by *Acrocylindrium oryzae*. The role of momilactone in the differential resistant cultivars of tall and semi-dwarf rice cultivars was ascertained. Results indicated that resistant cultivars contain relatively higher amount of momilactone A than susceptible ones irrespective of coleoptiles or leaf sheaths.

In the present study varietal resistance of three susceptible rice cultivars against the fungal pathogen *D. oryzae* was carried out by detached leaf and whole plant inoculation techniques. Responses exhibited by both the techniques were almost

similar. Chakraborty *et al*. (1995) tested the pathogenecity of three different isolates of *Pestalotiopsis theae* on 12 tea varieties with detached leaf inoculation technique to reveal the susceptible and resistance variety of tea to grey blight disease. Chakraborty *et al.* (1996) also tested pathogenecity of *Glomerella cingulata* towards tea varieties using both detached leaf and cut shoot method.

Studies have been undertaken for detection of fungal pathogens in host tissues by immunological methods by Chakraborty and Chakraborty (2003). The development of serological techniques has produced a number of highly sensitive methods for identifying microorganisms in diseases plant tissues. These rely on solid or soluble antigenic materials by antibodies raised against the organisms and subsequent use of an enzyme labelling system. The possible involvement of cross reactive antigens (CRA) in determining the degree of compatibility has been reported by several workers in different host-pathogen systems, viz., potato- *Phytophthora infestans* (Alba and De Vay, 1985), soybean – *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1983), tea – *Bipolaris carbonum* (Chakraborty and Saha, 1994), groundnut - (Purkayastha and Pradhan, 1994), tea- *Ustulina zonata* (Chakraborty *et al.*, 2002b) and tea – *Exobasidium vexans* (Chakraborty *et al.*, 2009).

Optimization of ELISA was done considering two variables – dilution of antigen and antiserum. The PTA-ELISA format was employed for the detection of pathogen in artificially inoculated rice cultivars using PAb-Cg and PAb-Pt. Absorbance value (A405) was always higher in infected leaf extracts than healthy ones thereby allowing easy and early detection of infection, as early as 24 hrs of inoculation. Chakraborty *et al.* (2009) reported that PTA-ELISA format could easily detect pathogen *Exobasidium vexans* in susceptible variety of *Camellia sinensis* (AV-2) as early as 24h after artificial inoculation whereas the disease symptoms were not visible before 12 days. The results of PTA ELISA were confirmed by Dot-Immunobinding assay in which intensity of dots widely varied among different cultivars of rice plants artificially inoculated with the pathogens. Early and rapid diagnosis of red rot disease in sugarcane caused by *Colletotrichum falcatum* was also performed using DIBA technique where infected samples depicted dark blue precipitate on the nitrocellulose membrane due to the antigen-antibody reaction (Hiremath *et al*., 2004). Early detection of grey blight pathogens in som morphotypes using PTA-ELISA format and DIBA has also been reported by Acharya *et al.* (2015).

Effectiveness of mycelial antigen of pathogens in raising antibodies was assessed using DIBA. Development of deep violet colour following homologous reaction with antigen and antibody confirmed its identity. Western blot analysis using PAb-Do (Anti-*Dreschlera* antibody) was also carried out in the present study to develop strategies for rapid detection of pathogens. Here the bands on SDS-PAGE gel were compared with bands on nitrocellulose membrane. Bands of varying molecular weights were seen in SDS-PAGE out of which some bands were also seen on nitrocellulose membrane suggesting these to be the respective epitopes of the antibodies. Chakraborty *et al.* (2012) raised polyclonal antibodies against mycelial antigens of *Macrophomina phaseolina* and further used them in immunological formats such as immunodiffusion, PTA-ELISA, dot immunobinding assay, Western blot analysis and indirect immunofluorescence for quick and rapid detection of the pathogen.

In the present investigation indirect immunofluorescence study of young mycelia was carried out with homologous antibody labelled with FITC. Strong apple green fluorescence was seen in mycelia which confirmed homologous reaction of the pathogen and antibody. The present study also reports the use of indirect immunofluorescence tests using PAb-Do as a suitable technique for localization of the pathogen and could be employed for immunodetection of pathogen in rice leaf tissues. Kratka *et al* (2002) reported the use of a polyclonal antibody IgG K91 to detect a quarantine pathogen of strawberry, *Colletotrichum acatatum* using four different immunotechniques, PTA-ELISA, dot-blot, immune print and immunofluorescent microscopy.

Increasingly, molecular biology techniques have been used to explore genetic variability in fungi (Caligiorne *et al*., 1999).The polymerase chain reaction is undoubtfully the most important technique in diagnostics and has found wide application as a powerful molecular tool mostly due to the development of thermotolerant DNA polymerases and automated thermocyclers.PCR is preferred over classical or other molecular techniques in the diagnosis of plant pathogens for a number of advantages that makes it very popular. Since high quality of DNA is not generally required, there is no need for culturing the target pathogen. PCR cycles are completed in much shorter time than other molecular techniques, thus allowing a very fast screening of a large number of samples. Because of its high sensitivity, minute amounts of the target DNA are required. The ribosomal DNA gene cluster (rDNAs) is an

extensively used target sequence for PCR detection of fungal plant pathogens because of a number of useful features. rDNAs bear common sequences found in the nucleus and the mitochondria of eukaryotes. The nuclear rDNA cluster is present as tandem repeats of several hundred copies in cell, which allows high sensitivity of detection. The rDNA gene is consisted of three subunits: a large (LSU) of 28S and a small (SSU) of 18S that are separated by a much smaller gene of 5.8S. The three subunits are connected together with two internal transcribed spacers (ITS1 and ITS2). This whole gene cluster is repeated in the genome many times thus being an appealing target for PCR amplification (Paplomatas, 2006). ITS sequences have gained popularity for being more variable regions and therefore allow selective detection of closely related organisms. Universal primers designed on conserved sequences found on the small and large subunits, have been extensively used for the amplification of ITS regions. The amplified sequences are between 500-800 bp, a relatively small amount of target DNA is required for PCR, while the PCR products have been used as species-specific probes (Bruns *et al*., 1992; Gardes and Bruns, 1993; White *et al*., 1990). Moreover, determination of ITS sequences after amplification by universal primers, has allowed the detection, identification and taxonomy of unculturable or unknown fugal species.

In the present study, ITS regions of ribosomal genes for construction of primers were used to identify *D. oryzae*. ITS region of rDNA of the pathogens was amplified using genus specific ITS1 and ITS4 primers. Amplified products of size in range of 572bp were produced by the primer pairs. These PCR products were used for sequencing of 18S rDNA region of both the pathogens. The sequence information of the pathogens was then analysed through BLASTn program one at time. The information generated for *D. oryzae* isolates indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *D. oryzae* with 100% similarity.

Identified *D. oryzae* rDNA gene sequences obtained from NCBI Genbank of various host plants were selected for comparisons of rDNA gene sequences of *D. oryzae* isolate of rice plant. Phylogenetic tree was constructed for pathogens of rice plants to infer the evolutionary history of these isolates. Therefore the *D. oryzae* isolate in the present investigation could be identified at the genus and species level only using the molecular identification tools.

Population of different species of AM fungi was isolated from the rhizosphere of 15 rice cultivars. Spores were identified upto species level using the help of standard keys (Walker, 1992) and website of INVAM. Among the AM fungi *Glomus fasciculatum* currently known as *Rhizophagus fasciculatus* was found to be predominant, followed by other genera such as Gigaspora, Scutellospora, Acaulospora and Enthropospora (Khati and Chakraborty, 2015). Observation has it that plant roots emit a volatile signal that stimulates directional growth of the fungi towards them (Koske, 1982). AM fungi have weak cellulose and endopolygalacturonase activities which have the capacity to catalyze to the release of oligosaccharides or oligosaccharins from the plant cell wall (Fry *et al.,* 1993). The latter could trigger the colonization and spread of the fungus which are all controlled by the host.

Root colonization in rice plants varied according to the rice cultivars. Presence of abundant vesicles was evident. Organisms of AMF have a bimodal pattern of differentiation (Morton, 1990). The vegetative thallus consists of arbuscules, intraradical vesicles (shared only by species in the suborder Glomineae) and intraradical and extraradical hyphae (Smith and Read, 1997; Morton and Benny, 1990). Arbuscules are finely branched structures in close contact with the cell plasma membrane, functioning in exchange of nutrients between host and fungal cells (Smith and Read, 1997). Hyphae are important in nutrient acquisition and as propagules to initiate new root colonization (Graham *et al.,* 1982; Friese and Allen, 1991). Vesicles are globose structures arising from swelling of the hyphae and filled with glycogen granules and lipids and are considered to be storage structures (Bonfante-Fasolo and Grippiolo, 1984; Brundrett, 1991). AM effects the evolution of the plant, microbial communities, soil nutrient status and structure at long term.

The next phase of our study was to check for antagonistic activity of selected PGPR and PGPF against the fungal pathogen *D. oryzae* of rice plant. Ten different PGPR namely *Bacillus pumilus* (NAIMCC-B01483) (JF836847), *Bacillus pumilus* (NAIMCC- B01487) (JQ765579), *Bacillus pumilus* (NAIMCC- B01488) (JQ765580), *Burkholderia symbiont* (NAIMCC-B01489) (JQ765578), *Bacillus aerophillus* (NAIMCC-B01490) (KC603894), *Paenibacillus polymyxa* (NAIMCC-B01491) (KC703775), *Bacillus methylotrophicus* (NAIMCC-B01492) (JQ765577), *Bacillus altitudinis* (NAIMCC-B01484) (HQ849482), *Bacillus altitudinis* (NAIMCC-B01485) (JF899300), *Enterobacter cloacae* (NAIMCC-01486) (KC703974) was taken to check

their *in vitro* antagonistic activity against the pathogen. *Bacillus altitudinis* (NAIMCC-B01485) showed the maximum antagonism towards the pathogen (Khati *et al*., 2016). Similarly three different isolate of *T. harzianum* (NAIMCC-F-03288), (NAIMCC-F-03289), (NAIMCC-F-03290) and three different isolates of *T. asperellum* (NAIMCC-F-03291), (NAIMCC-F-03292), (NAIMCC-F-03293) was used for *in vitro* antagonistic study where *T. harzianum* (NAIMCC-F-03288) showed the maximum antagonism against the pathogen. Our results are similar to the findings of Khalili *et al*., 2012 who suggested the *Trichoderma* sp. significantly inhibited the mycelia growth of *D. oryzae* in many ways and the most effective ones belonged to *T. harzianum*. Hence these PGPR and PGPF were further utilized for their *in vivo* assay against the pathogen.

In the present study, growth promotion and biochemical changes in rice cultivar following the application of PGPR was observed. Results revealed that growth was affected by the different bacterial treatments. Maximum growth was observed in plants treated with *Burkholderia symbiont* in cultivar Black nuniya, *Bacillus altitudinis* (NAIMCC-B01485) in cultivar Champasari and in case of cultivar Brimful plants treated with *Bacillus altitudinis* (NAIMCC-B01484) and *Enterobacter cloacae* showed maximum growth (Khati *et al*., 2016). There was also a considerable decrease in the level of total soluble sugar in certain treatments and increase in chlorophyll, protein and phenol content of the rice cultivars following treatments with PGPR. Our results are in accordance to the findings of Ashrafuzzaman *et al*., 2009 who clearly suggested the effect of PGPR in the enhancement of rice growth.

Activation of defence response in rice cultivars against *D. oryzae* following application of PGPR was also observed. Disease index were prepared accordingly which showed higher amount of PDI percentage in control set of plant (76.19%) in comparison with the plants treated with *Bacillus altitudinis* (NAIMCC-B01485) (9.83%) in case of Black nuniya, *Bacillus pumilus* (BRHS/C1) (28.80%) in comparison to control set with (31.08%) in case of Champasari and *Bacillus pumilus* (BRHS/C1) (13.33%) in comparison to control set with (69.33%). Changes in levels of different defence related enzymes, viz. Phenylalanine ammonia lyase (PAL), Peroxidase (POX), Chitinase (CHT) and β-1,3-Glucanse (GLU) following infection with *D. oryzae* was also studied during this investigation. Significant increase in enzymatic activity were found in plants treated with *Bacillus altitudinis* (NAIMCC-B01485), *Burkholderia symbiont*, R72- *Paenibacillus polymyxa* which corresponds to the results obtained for

PDI%. Correlation of this result was also made with the study undertaken by Parihar *et al.* (2012) where it was seen that biochemical analysis of genotypes of *Brassica juncea* infected with *Alternaria* blight revealed an increase in PAL, PPO and POX activity. Singh *et al.* (2014) reported that preformed phenolic compounds as well as Peroxidase enzyme play important role in resistance of Chili against *Colletotrichum capsici.* Significant increase in enzymatic activity were found in plants treated with *Bacillus altitudinis* (NAIMCC-B01485), *Burkholderia symbiont*, R72- *Paenibacillus polymyxa* which corresponds to the results obtained for PDI%. Concomitant increase in defence enzymes following inoculation with PGPR was correlated with the induction of resistance in rice plants using bioinoculants (Khati *et al*., 2016).

Phytoalexins are known to play a very vital role in defence mechanism of plant system under stress, keeping this in mind rice leaf samples from cultivar Black nuniya treated with *Bacillus altitudinis* (NAIMCC-B01485) and which showed the least PDI % was taken for the extraction of rice phytoalexin viz. Phytocassanes. The samples of both untreated control and treated infected was analysed by HPLC at 280nm. The results revealed that the accumulation of the compound was in much higher amount in treated infected samples than in control which clearly indicated that the compound had some antifungal activity towards the pathogen which ultimately resulted in the control of disease (Khati *et al*., 2016). Differential response of rice leaves to some abiotic elicitors of phytoalexin was observed. GA_3 reduced sheath rot disease of rice significantly and also enhanced phytoalexin (momilactone A) level in treated leaf sheaths. Penicillin and sodium azide also induced the momilactone biosynthesis in rice plants. Significant change in antigenic pattern observed by NaN_3 and GA_3 treatment indicates that the common antigen relationship between the host and parasite could be altered by these abiotic elicitors, suggesting that a close relationship might exist between phytoalexins, plant antigens and disease resistance of rice plants (Ghosal and Purkayastha, 1987).

In the second trial of our study 6 different PGPF belonging to genus *Trichoderma* (*T. harzianum*, viz. NAIMCC-F-03288, NAIMCC-F-03289, NAIMCC-F-03290 and the other three *T. asperellum*, viz. NAIMCC-F-03291, NAIMCC-F-03292 and NAIMCC-F-03293) was taken under consideration for field trial in rice cultivars against pathogen challenge. The effects of these bioinoculants on the growth rate of rice cultivars showed that the treated rice cultivars were considerably of more height in

comparison to the control set which is in accordance to the results obtained by (Harman *et al*., 2004).

Various research works has proved that PGPR confers induction of biochemical changes thus playing a role in defence response against the pathogens (Kuc, 1995).Brown spot of rice is a high sugar disease. Resistant rice cultivars are known to produce low sugar content in response to the susceptible cultivars (Mishra and Prasad, 1964). This is in support of our results where the sugar contents in control samples are more than the treated ones. Soluble protein content for control and treated inoculated sets were also studied and it was found that the levels of protein content was increased in all the treated samples in comparison to their control sets. It was also seen that in all the three rice cultivars *T. harzianum* (NAIMCC-F-03288) showed better results showing its efficacy towards the pathogen, the results obtained is supported by (Biswas *et al*. 2010a; Kumawat *et al*., 2008).

Further total phenol for control and treated samples of rice cultivars were studied and similar results were obtained, the treated sets had considerably higher content of phenol in comparison to their control sets. *T. harzianum* (NAIMCC-F-03288) showed the best results in all the cultivars. Pre seed treatment with *T. harzianum* was found to induce resistivity against the brown spot disease with a considerably good amount of increase in the soluble protein and total phenol contents (Kumawat, 2006). Our results are in accordance with the above investigation.

The next phase of our work was to check the activation of defence response in plants following application of PGPF against *D. oryzae*. At first degree of disease suppression in all three rice cultivars following different treatments were observed and recorded after 48hrs of inoculation. It was found that symptoms appeared in untreated inoculated leaves much faster than the treated ones. All the treatments were significant in decreasing the disease severity. The lowest disease severity was observed in *T. harzianum* (NAIMCC-F-03288) treatment in rice cultivar Black nuniya which matches with the results given by Kumawat *et al*., 2008. Results of percent disease index correlates with results obtained from analysis of defence enzymes where the enzyme activity of PAL, POX, CHT and GLU was much higher in treated inoculated leaves indicating their increased defence against the pathogen. But the healthy leaves showed more levels of enzymes suggesting that the inoculated leaves are prone to disease development where the enzyme levels were less. Our results are similar to the

results given by Harman *et al*., 2004. Further HPLC analysis of rice phytoalexin viz. Phytoassanes in the rice cultivar Black Nuniya in untreated inoculated and PGPF (*T. harzianum* NAIMCC-F-03288) treated and inoculated plants was done. Accumulation of Phytocassanes was found to be more in inoculated samples than the healthy ones and maximum in treated inoculated sample.

In the third trial of our experiment AMF (*Rhizophagus fasciculatus*) was selected for observing its role in growth promotion and biochemical changes in rice cultivars following its application and pathogen challenge. The growth enhancement in terms of height was evaluated in all three rice cultivars and it was observed after every 20d interval of time. It was observed that the height of the plants was more in treated ones in comparison to its control. Maximum height was attained by rice cultivar Brimful after 80d.Further experiment was conducted to access the effect of single application of AMF on plants and a series of biochemical changes such as sugar content where AMF could reduce the sugar content up to certain limit. Further considerable increase in the amount of total chlorophyll, protein and phenol content was observed in treated plants with respect to the control.

Activation of defence response in rice cultivars following application of AMF and pathogen challenge was also accessed. Disease suppression following the pathogen inoculation was observed and recorded which showed the decrease in the amount of disease upto some level not only that enhancement in the accumulation of defence enzyme activity such as PAL,POX,CHT and GLU was also measured and found positive results. Increased activity of chitinase, β-1, 3-glucanase and peroxidase were also determined in tea plants following treatments with Josh - a bioformulation of AMF (Chakraborty *et al.*, 2007).

In the final trail experiments with the bioinoculants experiments were conducted on growth promotion and biochemical changes in rice cultivar following dual and combined application of PGPF (*T. harzianum*,NAIMCC-F-03288), AMF (*R. fasciculatus*) and PGPR (*B. altitudinus*,NAIMCC-B01485) against *D. oryzae*. Effect of dual and combined treatments on the growth promotion in terms of height of the plant was observed and recorded. It was found that the growth was much more enhanced in combined treatments than dual ones in case all three rice cultivars. Changes in biochemical activities such as total sugar, total chlorophyll, total protein and total

phenol content was also observed and the results revealed that the combined treatments showed much more content than the dual ones in comparison to the control.

Activation of defence response of *Rice* following dual and combined application of PGPF, AMF and PGPR against *D. oryzae* was also observed. PDI% was noted for all the treatments in three rice cultivars. It was noted that symptoms appeared in untreated inoculated leaves much faster than in treated inoculated plants and the combined application of bioinoculants showed better results than the dual application. Results of percent disease index correlate with the results obtained from the analysis of defence enzymes where the enzyme activity of PAL, POX, CHT and GLU was much higher in treated inoculated leaves indicating their increased defence against the pathogen. But the healthy leaves showed more levels of enzymes suggesting that the inoculated leaves are prone to disease development where the enzyme levels were less. Defence enzymes, chitinase, glucanase and peroxidase showed enhanced activities during disease suppression (Allay and Chakraborty, 2010). Chakraborty *et al*., (2016b) reported that dual application of *Bacillus pumilus* and *Rhizophagus fasciculatus* caused induction of resistance in *Camellia sinensis* against *Sclerotium rolfsii*. It is now clear that microbes in small consortia enhance the defence signalling cascades leading to enhance transcriptional activation of several metabolic pathways (Sarma *et al*., 2015). Analysis of peroxizyme was also conducted for rice cultivar Black nuniya with combined application of bioinoculants showing the least PDI %. Presence of an extra band in treated inoculated sample in comparison to the control was observed. Presence of new peroxidase isozyme in infected leaf samples was also recorded (Chakraborty *et al.*, 2016a). Analysis of peroxidase isozymes by polyacrylamide gel electrophoresis showed four isozymes in healthy tea leaf samples and five in tea leaves infected with *Exobasidium vexans*. They suggested that the appearance of new bands following infection can be correlated with the induction of the catalytic activity of more isozymes, leading also to an overall increase in peroxidase activity (Chakraborty *et al.*, 2002a).

Radial growth bioassay of antifungal compound (Phytocassanes) from ethyl acetate fractions taken from treated and untreated samples of rice leaves of cv. Black Nuniya showed that the growth of the pathogen (*D. oryzae*) was restricted in the sample containing the extracts of treated inoculated leaves in comparison to that of sample containing the extracts of untreated control leaves clearly indicating the presence of antifungal compound (Phytocassanes) which directly showed its potentiality by

restricting the growth of the pathogen. Therefore the antifungal compound (Phytocassanes) that was extracted from the rice leaves fulfils all the criteria given by Subba Rao and Strange (1994) suggesting it to be phytoalexin that actively played a role in rice defence system against brown spot pathogen. Also Grayer and Kokubun (2001) showed that leaves of rice plants produce a wide variety of both preformed and induced antifungal compounds. Many genes involved in rice resistance to the blast fungus have been identified, and it is likely that at least some of these genes code for a quick response by the plant defence system and the production of high concentration of phytoalexins. Phytoalexin production could be an important factor in the resistance against the rice blast pathogen.

The cellular localization of two defence enzymes glucanase and chitinase in leaf samples of rice cultivar Black Nuniya following combined application and inoculation by *D. oyzae* through fluorescent microscopy. When plants were colonized with AMF(*R. fasciculatus*) and then treated with *T. harzianum* (NAIMCC-F-03288) *and B. altitudinus*(NAIMCC-B01485) followed by challenge inoculation with *D. oryzae,* elicitation of chitinase and glucanase was evident as strong bright apple green fluorescence which activate the defence response in the plants against brown spot pathogens. Roots and leaves of mandarin plants treated with *T. asperellum* were reacted with PAb of Chitinase (Chakraborty *et al.*, 2004) followed by labelling with FITC. Strong bright apple green fluorescence was observed in the epidermal and homogenously in mesophyll tissues in leaves and homogenously in cortical cells and epidermal cells in roots. Observed plant health improvement and disease suppression in rice plants may be due to a combination of at least two mechanisms- direct inhibition of the pathogen or induction of resistance in the host by bioinoculants.

In the last phase of the study HPLC analysis of phenolics and antifungal compound (i.e. Phytoalexin) namely Phytocassanes in the rice leaves samples of Black Nuniya with combined application of bioinoculants was carried over. Results revealed that the amount of phenolics accumulated in treated inoculated samples was present in higher amount than the untreated control. Comparison with standards revealed the presence of phenols such as Galleic acid, Ferulic acid, Salicylic acid and Phloroglucinol. Chakraborty *et al*., (2016a) studied that *Colletotrichum gloeosporioides* triggered the production of resorcinol, catechol, chlorogenic acid, ferulic acid and salicylic acid in the muga host plant as biochemical defence strategy against the

pathogen. Presence of salicylic acid and ferulic acid in infected leaves and not in healthy leaves indicate the role of this phenolic acid in defence against pathogen. When biochemical characterization of maize plants infected with *Drechslera dactylidis* was done, it was found that salicylic acid increased 2-fold in infected leaf samples (Ghany, 2012). The presence of two cinnamic acid derivatives in the leaf blade cell walls of blast resistant and susceptible rice cultivars was reported. Two phenolic compounds *p*-coumaric and ferulic acids were obtained from the cell wall extracts from two resistant rice cultivars (Carreon and IR-8) artificially inoculated with the blast fungus *Pyicularia grisea*. The cell wall extracts from resistant cultivars possessed greater amounts of both *p*-coumaric and ferulic acids than those from the blast susceptible cultivars (Kumar and Sridhar, 1985).

Similarly the accumulation of Phytocassanes was higher in treated inoculated samples than the untreated control. Extra peaks were observed in the treated samples in comparison to the untreated ones hence clearly indicating the activation of antifungal compound by the application of bioinoculants. Our results are in accordance to the findings of Sinha and Das (1972) where they have shown that an earlier induction with the spore suspension of a mildly virulent race of *Helminthosporium oryzae* induced considerable resistance in two rice cultivars to a challenge inoculation with its virulent race. The treated plant becomes protected simply by absorbing certain metabolites of the mild race that are toxic to the virulent race. Some diffusible substances, indigenous to the spores and hyphae of the mild race, induce the production of a resistance factor, perhaps phytoalexin-like, in host tissue. Accumulation of such a substance in the plants first inoculated with the mild race of *H. oryzae* will afford them protection against the virulent race. Also it was observed by Purkayastha (1973) that phytoalexin production probably depends on the combination of host-parasite proteins or on specific antigens or interferon (i.e. a protein or peptide which protects the cell against all foreign nucleic acids). Antigens are not always proteins and therefore it is not desirable to consider them all as proteins. As antibody is not formed in plants, the combination of host-parasite antigens or proteins appear to be responsible for the alteration of metabolism and for the activation or formation of a protective substance (i.e. Phytoalexins) in the host from a substance originally non inhibitory to the pathogen. This protective substance takes an active part in the defence mechanism of the plants.

The above findings indicate that a multicomponent, coordinated defence mechanism is also operative in rice plants after *Drechslera oryzae* infection. The pre-inoculation with bio agents sensitized paddy seedling to increase elevated level of soluble proteins and total phenol content up to a certain level resulting induction of resistance against brown spot pathogen .It has been possible to enhance the defence response to some extent by bio control agents (AMF, PGPR, PGPF) which is evident from the higher production of phytoalexin, PR-proteins and higher activity of PAL in treated than in untreated plants. Thus biocontrol agents [*R. fasciculatus*, *T.harzianum* (NAIMCC-F-03288) and *B. altitudinus* (NAIMCC-B01485)] can be well exploited in future for the effective management of brown spot disease.

Chapter 6
CONCLUSION

- Fifteen different rice cultivars from different regions of North Bengal and Sikkim were collected and their seed morphological diversity, germination ability, growth rate, total protein content and were determined for initial screening.
- Percent Disease Index was measured following natural infection with brown spot disease. Occurrence of the disease was much higher in local cultivars viz. Black nuniya, Brimful and Champasari. Accumulation of defence enzymes such a PAL, POX, CHT and GLU and total phenol was also found to be in much lesser quantity in these cultivars. Hence they were selected for further experimental purpose.
- Brown spot causing pathogen was isolated and following completion of Koch's postulate.
- Growth characters and spore morphology of the pathogen was studied.
- Pathogenecity test of the three susceptible rice cultivars was studied using two techniques- detached leaf and whole plant inoculation technique. Both the techniques gave similar result where it was observed that Black nuniya was highly susceptible followed by Brimful and Champasari.
- Immuno detection of foliar fungal pathogens in healthy and artificially inoculated leaf tissues was carried out using PTA-ELISA as well as Dot immunobinding assay. The occurrence of infection was seen as early as 24 hrs and increased by time. Greater colour intensity was noted in Black Nuniya followed by Brimful and Champasari with the IgG which showed susceptible reaction to the pathogen in pathogenecity tests.
- Polyclonal antibody was raised separately against mycelial antigen of the fungal pathogen. These antibodies were used for serological characterization of the fungal pathogens by PTA-ELISA, Dot-blot, Western blot and Indirect immunofluorescence of mycelia and spore. Western blot analyses using polyclonal antibody of *D. oryzae* revealed that the PAb could show different levels of homologous reactions with the antigens of *D. oryzae* respectively. Sharp and intense bands were produced on the nitrocellulose membrane after enzymatic reaction with NBT BCIP. Efficacy of polyclonal antibodies raised against the

mycelial antigen was further tested with the help of indirect immuno fluorescence of young mycelia of *D. oryzae.* The mycelia treated with PAbs and labeled with FITC showed apple green fluorescence.

- Molecular detection of foliar fungal pathogen *D. oryzae* (R1.DO.01) was carried out using 18S rDNA sequencing using ITS1/ITS4 primers. The BLAST query of the 18S rDNA sequence of the isolates against GenBank database confirmed the identity of the isolate R1.DO.01 as *D. oryzae.* The sequences have been deposited to NCBI, Genbank database under the accession number KT768092.
- A multiple sequence alignment of ITS gene sequences of the above sequenced isolate was also conducted. Phylogenetic analysis of the isolate was carried out with the Extype strain sequences obtained from NCBI Genbank Database which showed maximum homology with the respective isolate
- Association of Arbuscular Mycorrhizal Fungi (AMF) in rice cultivars were observed and it was found that *Glomus* was the most dominant genera in the rice rhizosphere followed by *Gigaspora* and *Scutellospora.*
- Characterization of the species belonging to genus *Glomus* and *Gigaspora* was done. *Rhizophagus fasciculatus* was found to be most abundant in the rice rhizosphere, they were isolated and mass multiplied in maize plants.
- Histopathology and root colonization with AMF in rice cultivars was studied.
- Ten selected PGPR and six PGPF were evaluated for their antagonistic effect against the fungal pathogen and it was recorded that these bio inoculants could easily prevent the growth of fungal pathogen *in vitro*.
- Ten selected PGPR were screened for plant growth promotion and changes in biochemical activity. Among all the bacterial isolates *B. altitudinus* (NAIMCC-B01485) showed better results than the others.
- Percent Disease Index and defence enzyme activity of PAL,POX,CHT and GLU following treatment and pathogen infection was also measured and it was found that maximum activity was shown by treated samples in comparison to their control.
- HPLC analysis of rice leaf samples of rice cultivar Black nuniya treated with *B. altitudinus* (NAIMCC-B01485) showing lowest PDI% was also done which clearly

indicated the increased accumulation of Phytocassanes in treated samples than the control.

- Six selected PGPF were also screened for its role in plant growth and changes in biochemical activity following pathogen challenge in rice cultivars. *T. harzianum* (NAIMCC-F-03288) treated plants showed the maximum enhancement in the growth and biochemical parameters in comparison to the control.
- Disease suppression and increase in the level of all four defence enzyme viz. PAL, POX, CHT and GLU was also observed. Rice cultivars treated with *T. harzianum* (NAIMCC-F-03288) showed better results in all the cultivars.
- HPLC analysis of Phytocassanes was done for Black nuniya treated with *T. harzianum* (NAIMCC-F-03288). Enhancement of the compound in the treated samples was clearly visible which proves its role in suppression of the disease.
- Rice cultivars were treated with *R. fasciculatus* and challenged with the pathogen. Considerable increase in the height and biochemical activity was observed.
- A decrease of Percent Disease Index in the treated samples in comparison to the control was observed and considerable increase of defence enzymes was also observed in treated samples.
- Growth promotion and biochemical changes was observed following dual and combined application of PGPR (*B. altitudinus*, NAIMCC-B01485), AMF (*R. fasciculatus*)and (PGPF *T. harzianum* ,NAIMCC-F-03288). Enhancement in the growth and biochemical activities was observed in all treated samples in comparison to their control sets. Combined application gave better results than the dual ones.
- Decrease in disease incidence and increase in the level of defense enzymes was observed in treated samples. Combined application gave better results than the dual application. Isozymes of peroxidase were also checked using native PAGE and it was revealed that appearance of new peroxyzyme was seen in infected leaf samples.
- Cellular localization of two important defence enzymes – Glucanase and Chitinase was studied using indirect immunofluorescence. Expressions of these enzymes were noted in treated leaf sections, confirming earlier results obtained.

- HPLC analysis of phenolics in the leaf samples of rice cultivar Black nuniya treated with PGPR (*B. altitudinus*, NAIMCC-B01485), AMF (*R. fasciculatus*) and (PGPF *T. harzianum* ,NAIMCC-F-03288) was also done. Increase in the level of phytoalexin in comparison to the control was clearly seen. Presence of phenols such as Galleicacid, Ferulic acid, Salicylic acid and Phloroglucinol was observed in both control and treated samples but intensity increased in treated infected samples. Hence it was evident that Galleic acid, Ferulic acid, Salicylic acid and Phloroglucinol play important role in the defence of rice plants against brown spot pathogen.
- HPLC analysis of Phytocassanes was done for Black nuniya treated with PGPR (*B. altitudinus*, NAIMCC-B01485), AMF (*R. fasciculatus*) and (PGPF *T. harzianum* , NAIMCC-F-03288). Enhancement of the compound in the treated samples was clearly visible which proves its role in suppression of the disease.
- The application of bio inoculants promotes growth and bio primes the rice plant against brown spot pathogen by up-regulation of defence activities. These findings could be helpful in protecting rice plants against fungal pathogens and improving its health status that would in turn provide quality yield.

Chapter 7
BIBLIOGRAPHY

Abadi R, Perl-Treves R and Levy Y. Molecular variability among *Exserohilum turcicum* isolates using RAPD (random amplified polymorphic DNA). *Can. J. Plt. Pathol.* **18:** 29-34, 1996.

Abdel-Fataah GM, Shabana YM, Ismail AE and Rashad YM. *Trichoderma harzianum*: a biocontrol agent against *Bipolaris oryzae*. *Mycopathol*, **164:** 81-99, 2007.

Abeles FB, Bosshart RT, Forrense LE and Habig WH. Preparation and purification of glucanase and chitinase from bean leaves. *Plant Physiol.* **47:** 129-134, 1970.

Acharya A, Ghosh S and Chakraborty BN. Serological detection of *Pestalotiopsis disseminata* in *Persea bombycina* causing grey blight disease. *Int. J. Adv. Bio. Res.* **5(4):** 327-333, 2015.

Adhikari B, Bag MK, Bhowmick MK and Kundu C. Rice in West Bengal-rice knowledge management portal (www.rkmp.co.in), Directorate of Rice Research, Rajendranagar, Hyderabad, Andhra Pradesh, **pp.** 88, 2012.

Adlakha KL Wilcoxson RD and Raychaudhuri SP. Resistance of wheat spot blotch caused by *Bipolaris sorokiniana. Pl. Dis*. **68:** 320-321, 1984.

Akatsuka T, Kodama O, Kono Y and Takeuchi S. 3-Hydroxy-7-oxosandaracopimaradiene (oryzalexin A), a new phytoalexin isolated from rice blast leaves. *Agric. Biol. Chem*. **47:** 445–447, 1983.

Akiyama K, Matsuoka H and Hayashi H. Isolation and identification of a phosphate deficiency-induced C-glycosylflavonoid that stimulates arbuscular mycorrhiza formation in melon roots. *Mol. Pl.Mic. Int*. **15:** 334–340, 2002.

Alba APC and Devay JE. Detection of cross-reactive antigens between *Phytophthorainfestans* (Mont.) de Barley and *Solanum* species by indirect enzyme-linked immunosorbent assay.*Phytopathol.***112:** 97-104, 1985.

Alcorn JL. The taxonomy of "Helminthosporium" species. *Ann. Rev. Phytopathol*. **26:** 37-56, 1988.

Allay S and Chakraborty BN. Activation of defense response of mandarin plants against Fusarium root rot disease using *Glomus mosseae* and *Trichoderma hamatum. J. Myco. Plt. Pathol.* **40 (4):**499-511. 2010.

Aloui A, Recorbet G, Robert F, Schoefs B, Bertrand M, Henry C, Gianinazzi-Pearson V, Dumas-Gaudot E and Aschi-Smiti S. Arbuscular mycorrhizal symbiosis elicits shoot proteome changes that are modified during cadmium stress alleviation in Medicago truncatula. *BMC Plt. Biol*. **11:**75, 2011.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Res.***25:** 3389-3402, 1997.

Aluko MO. Crop losses caused by brown leaf spot disease of rice in Nigeria. *Plt. Dis. Rep*. **59:** 609–613, 1975.

Amadioha AC. Controlling Rice Blast in Vitro and in-Vivo with Etracts of Azadirachta indica. *Crop Protec.* **19:** 287-290, 2000.

Araujo AEDS, Baldani VLD, Galisa PDS, Pereira JA and Baldani JI. Response of traditional upland rice varieties to inoculation with selected diazotrophic bacteria isolated from rice cropped at the Northeast region of Brazil. *Applied Soil Ecol*. **64:** 49-55, 2013.

Arnon DI. Copper enzymes in isolated chloroplasts. Polyphenoloxydase in Beta vulgaris. *Plant Physiol*. **24:** 1-15, 1949.

Artursson V, Finlay RD and Jansson JK. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environ. Microbiol*. **8:** 1–10, 2006.

Ashrafuzzaman M, Hossen FA, Ismail MR , Hoque MA, Islam MZ, Shahidullah SM and Meon S. Efficiency of plant growth-promoting rhizobacteria (PGPR) for the enhancement of rice growth. *African J. Biotech.* **8 (7):** 1247-1252, 2009.

Ausubel FM. Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol*. **6:** 973–979, 2005.

Azcon-Aguilar C and Barea JM. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - An overview of the mechanisms involved. *Mycorrhiza*, **6:** 457–464, 1997.

Bae H, Sicher RC, Kim MS, Kim S, Strem MD and Melnick RL. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. *J. Expt. Bot.* **60:** 3279–3295, 2009.

Bagyaraj DJ and Varma V. Interaction between arbuscular mycorrhizal fungi and plants. Their importance in sustainable agriculture and in arid and semiarid tropics. In: Advances in microbial ecology, Academic Press, London, **pp.** 119–142, 1995.

Bais HP, Park SW, Weir TL, Callaway RM and Vivanco JM. How plants communicate using the underground information superhighway. *Trends Plant Sci*. **9:** 26–32, 2004.

Baker R. *Trichoderma* spp. as plant-growth stimulants. *Biotechnol*. **7:** 97–106, 1988.

Bakonyi J, Pomazi A, Fischl G and Hornok L.Comparison of selected species of *Bipolaris, Drechslera* and *Exserohilum* by random amplification of polymorphic DNA. *Acta. Microbiol. Immun. Hungarica* .**42:**355-366, 1995.

Ballhorn DJ, Kautz S, Heil M and Hegeman AD. Cyanogenesis of wild lima bean (*Phaseolus lunatus* L.) is an efficient direct defence in nature. *Plt. Sign. Behavior*. **4:** 735-745, 2009.

Baranwal MK, Kotasthane A, Magculia N, Mukherjee PK, Savary S, Sharma AK, Singh HB, Singh US, Sparks AH, Variar M and Zaidi N. A review on crop losses, epidemiology and disease management of rice brown spot to identify research priorities and knowledge gaps. *Eur. J. Plant Pathol*. **136:** 443-457, 2013.

Barea JM, Pozo MJ, Azcon R and Azcon-Aguilar C. Microbial co-operation in the rhizosphere. *J. Exp. Bot*. **56:**1761–1778, 2005.

Beckers GJ and Conrath U. Priming for stress resistance: from the lab to the field. *Curr. Opin. Plant Biol*. **10:** 425–431, 2007.

Bedi KS and Gill HS. Losses caused by the brown leaf spot disease in the Punjab. *Indian Phytopathol.* **13:** 161–164, 1960.

Bell CR, Dickie GA, Harvey WLG and Chan JWYF. Endophytic bacteria in grapevine. *Can. J. Microbiol*. **41:** 46–53, 1995.

Bera S and Purkayastha RP. Multicomponent coordinated defence response of rice to *Rhizoctonia solani* causing sheath blight: *Curr. Sci* .**76 (10):**1376-1384, 1999.

Berta G, Fusconi A and Hooker JE. Arbuscular mycorrhizal modifications to plant root systems: scale, mechanisms and consequences. In S. Gianinazzi, H. Schüepp, J. M. Barea, and K. Haselwandter (eds.), Mycorrhizal Technology in Agriculture. From Genes to Bioproducts. Birkhaeuser, Basel. **pp.**71–85, 2002.

Bever JD. Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests**.** *New Phyt.* **157:** 465–473, 2003.

Bigirimana J, De Mayer G, Poppe J, Elad Y and Hofte M. Induction of systemic resistance on bean (*Phaseolus vulgaris*) by *Trichoderma harzianum*. *Med. Fac. Landbouww. Univ. Gent*. Probably the first paper to clearly show induced systemic resistance resulting from an interaction between a plant and a *Trichoderma* strain. **62:** 1001–1007, 1997.

Biswas C, Srivastava SSL and Biswas SK. Biochemical changes associated with induction of resistance by *Trichoderma* spp. in paddy against brown spot disease. *Indian Phytopath.* **63 (3):** 269-272, 2010a.

Biswas C, Srivastava SSL and Biswas SK. Effect of biotic, abiotic and botanical inducers on crop growth and severity of brown spot in rice. *Indian Phytopathol.* **63:** 187–191, 2010b.

Blilou I, Ocampo JA and García-Garrido JM. Resistance of pea roots to endomycorrhizal fungus or Rhizobium correlates with enhanced levels of endogenous salicylic acid. *J. Exp. Bot*. **50:** 1663–1668, 1999.

Bloemberg GV and Lugtenberg BJJ. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr. Opin. Plant Biol*. **4:** 343–350, 2001.

Boddey RM, Moraes JCDS, Alves BJR and Urquiaga S. The contribution of biological nitrogen fixation for sustainable agricultural systems in the tropics. *Soil Biol. Biochem*. **29:** 787-799, 1997.

Boller T and He SY. Innate immunity in plants: An arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science*, **324:** 742–744, 2009.

Boller T and Mauch F. Colorimetric assay for chitinase. *Meth. In. Enzymol.***161:** 403-435, 1988.

Bonfante-Fasolo P and Grippiolo R. Cytochemical and biochemical observations on the cell wall of the spore of *Glomus epigaeum*. *Protoplasm.* **123:** 140-151, 1984.

Bonfante-Fasolo P and Perotto S. Plant and endomycorrhizal fungi: the cellular and molecular basis of their interaction In: Vermad,ed, *Molecular signals in plant –microbe communications.* Boca Raton, Florida: CRS Press, 445-470, 1992.

Boue SM, Cleveland TE, Carter-Wientjes C, Shih BY, Bhatnagar D and McLachlan JM. Phytoalexin-enriched functional foods. *J. Agric. Food Chem*. **57:** 2614–2622, 2009.

Bray HG and Thorpe WV. Analysis of phenolic compounds of interest in metabolism. *Methods. Biochem.Anal.***1:** 27-52, 1954.

Breuillin F, Schramm J, Hajirezaei M, Ahkami A, Favre P, Druege U, Hause B, Bucher M, Kretzschmar T, Bossolini E, Kuhlemeier C, Martinoia E, Franken P, Scholz U and Reinhardt D. Phosphate systemically inhibits development of arbuscular mycorrhiza in Petunia hybrid and represses genes involved in mycorrhizal functioning. *Plant J*. **64:**1002–1017, 2010.

Broglie K, Chet I, Holliday M, Cresmann R, Biddle P, Kowlton S, Mauvais C and Broglie R. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science*, **254:** 1194-1197, 1991.

Brooks CJW and Watson DG. Terpenoid phytoalexins. *Nat. Prod. Rep*. **8:** 367-389, 1991.

Brundrett M. Mycorrhizas in natural ecosystems. *Adv. Eco. Res.* **21:** 171-313, 1991.

Bruns TD, Vilgalys R, Barns SM, Gonzalez D, Hibbert DS, Lane DJ, Simon L, Stickel S, Szaro TM, Weisburg WG and Sogin ML. Evolutionary relationships within fungi: analysis ofnuclear small subunit rRNA sequences. *Mol. Phy. Evo.* **1:** 231-24, 1992.

Caligiorne RB, Resende MA, Dias–Neto E, Oliveira SC and Azevedo V. Dematiaceous fungal pathogens: analysis of ribosomal DNA gene polymorphism by polymerase chain reaction - restriction fragment length polymorphism. *Mycoses*. **42:**609-614, 1999.

Callaway RM, Thelen GC, Barth S, Ramsey PW and Gannon JE. Soilfungi alter interactions between the invader Centaurea maculosa and NorthAmerican natives**.** *Eco.* **85:**1062–1071, 2004.

Cameron RK. Salicylic acid and its role in plant defense responses: what do we really know? *Physiol. Mol. Plant Pathol*. **56:** 91-93, 2000.

Campos-Soriano L, García-Martínez J and Segundo BS. The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defense-related genes in rice leaves and confers resistance to pathogen infection. *Mol. Plant Pathol*. **10:** 1364-3703, 2012.

Cartwright D, Langcake P, Pryce RJ, Leworthy DP and Ride JP. Chemical activation of host defence mechanisms as a basis for crop protection. *Nat.* **267:** 511–513, 1977.

Chabot R, Antoun H and Cescas MP. Growth promotion of maize and lettuce by phosphatesolubilizing *Rhizobium leguminosarum* biovar. *phaseoli. Plt. Soil*. **184:** 311-321, 1996.

Chakrabarti NK. Epidemiology and disease management of brown spot of rice in India. In: Major Fungal Disease of Rice: Recent Advances. Kluwer Academic Publishers. **pp.** 293–306, 2001.

Chakraborty BN and Purkayastha RP. Serological relationship between *Macrophomina phaseolina* and soybean cultivars. *Physiol. Plant Pathol.* **23:** 197–205, 1983.

Chakraborty BN and Chakraborty U. Immunodetection of plant pathogenic fungi. In. Frontiers of Fungal Diversity in India.Editors, Rao, G.P., Manoharachari, C., Bhatt, D.J., Rajak, R.C. and Lakhanpal, T.N. International Book Distributing Company, India**, pp.** 23–41, 2003.

Chakraborty BN and Saha A. Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum*.*Physiol. Mol. Plant Pathol*.**44:** 403–416, 1994.

Chakraborty BN, Basu P, Das R, Saha A and Chakraborty U. Detection of cross reactive antigens between *Pestalotiopsis theae* and tea leaves and their cellular location. *Ann. Appl. Biol.* **127:** 11–21, 1995.

Chakraborty BN, Chakraborty U, Barman BC, Bhutia L and Ghosh PK. Induction of systemic resistance in tea plants against root rot pathogens upon field application of VAM, phosphate solubilizing fungus and bacterium. In: *Rhizosphere Biotechnology/Microbes in relation to plant health: Retrospects & Prospects*. Eds.A.K.Roy, B.N. Chakraborty, D.S. Mukadam and Rashmi, Scientific Publishers (India), Jodhpur, **pp.**61-71, 2007.

Chakraborty BN, Chakraborty U, Chakraborty AP and Sashankar P. Serological and molecular detection of *Bipolaris sorokiniana* Sacc. causing Spot blotch disease of wheat. *J. Mycopathol. Res.* **54(1):** 117-125, 2016a.

Chakraborty BN, Chakraborty U, De UK and Chakraborty AK. Induction of resistance in *Camellia sinensis* against *Sclerotium rolfsii* by dual application of *Rhizophagus fasciculatus* and *Bacillus pumilus. Arch. Phytopathol. Pl. Protect*. DOI: 10.1080/03235408.2016.1140607. 1-16,2016b.

Chakraborty BN, Chakraborty U, Das R, Basu P and Saha A. Serological relationship between *Glomerella cingulata* (Stoneman) Spaulid & Schrenk and *Camellia sinensis* (L.) O. Kuntze. *J. Plant. Crops.* **24:** 205-211, 1996.

Chakraborty BN, Chakraborty U, De U, Chakraborty AP and Rai K. Dual application of *Glomus mosseae* and *Bacillus pumilus* to enhance growth of tea and suppress sclerotial blight disease. *J Myco. Plant Pathol*. **39:**565-566, 2009.

Chakraborty BN, Chakraborty U, Rai K, Sunar K and Dey PL. Serological and molecular detection of *Macrophomina phaseolina* causing root rot of *Citrus reticulate*. *NBU J. Pl. Sci.* **6(1):** 77-86, 2012.

Chakraborty BN, Chakraborty U, Sengupta D, Deb D, and Das J. Development of immunodiagnostic kits for detection of *Ustulina zonata* in soil and tea root tissues. *J. Basic App. Myco.***1:** 58-61, 2002a.

Chakraborty BN, Dutta S and Chakraborty U. Biochemical responses of tea plants induced by foliar infection with *Exobasidium vexans*. *Indian Phytopath*.**55 (1):** 8-13, 2002b.

Chakraborty U, Basnet M, Bhutia L and Chakraborty BN. Plant growth promoting activity of *Bacillus pumilus* and *Bacillus megaterium* from tea rhizosphere. In:*Proceedings of 2004 International Conference on O-Cha (tea) Culture and Science (ICOS),* Shizuoka, Japan, **pp.**102-105, 2004.

Chakraborty U, Chakraborty BN, Kapoor M. Changes in the level of peroxidase and phenyl alanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. Folia *Microbiol*. **38:**491-496, 1993.

Chakraborty U, Sarkar B and Chakraborty BN. Protection of soybean root rot by *Bradyrhizobium japonicum* and *Trichoderma harzianum*, associated changes in enzyme activities and phytoalexins production. *J. Myol. Pl. Pathol.* **33:** 21-25, 2003.

Chakravorty A and Ghosh PD .Characterization of Landraces of Rice from Eastern India. *Ind. J. Plant Genet. Resour*. **26(1):** 62-67, 2013.

Chakravorty A, Ghosh PD and Sahu PK .Multivariate analysis of phenotypic diversity of landraces of rice of West Bengal. *Am. J. Exp. Agri*. **3:** 110-123, 2013.

Chandanie W, Kubota M and Hyakumachi M. Interactions between plant growth promoting fungi and arbuscular mycorrhizal fungus Glomus mosseae and induction of systemic resistance to anthracnose disease in cucumber. *Pl. Soil,* **286:** 209–217, 2006.

Chatterjee SD, Adhikari B, Ghosh A, Ahmed J, Neogi SB and Pandey N .The rice bio-diversity in West Bengal. Department of Agriculture, Govt. of West Bengal. **pp.** 50, 2008.

Cipollini D, Rigsby CM and Barto EK. Microbes as targets and mediators of allelopathy in plants. *J. Chem. Ecol*. **38 (6):** 714-727, 2012.

Clausen J. Laboratory techniques in biochemistry and molecular biology. Vol 1, part-3, (ed. R. H. Burdon and P. H. Van Kinppenberg). **pp.** 64-65, 1988.

Coleman JJ, White GJ, Rodriquez- Carres M and Vanetten HD. An ABC transporter and a cytochrome P450 of Nectria haematococca MPVI are virulence factors on pea and are the major tolerance mechanisms to the phytoalexin pisatin. *Mol. Plt. Microbe Interact*. **24:** 368–376, 2011.

Compant S, Reiter B, Sessitsch A, Nowak J, Cle´ment C and Ait Barka E. Endophytic colonization of *Vitis vinifera* L. by a plant growth- promoting bacterium, *Burkholderia* sp. strain PsJN. *Appl. Environ. Microbiol*. **71:** 1685–1693, 2005.

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman MA, Pieterse CMJ, Poinssot B, Pozo M J, Pugin A, Schaffrath U, Ton J, Wendehenne D, ZimmerlI L and Mauch-Mani B. Priming: Getting ready for battle. *Mol. Plant-Microbe Interact*. **19:** 1062–1071, 2006.

Cook RJ. Advances in plant health management in the 20 century. *Ann. Rev. Phytopathol.* **38:** 95-116, 2000.

Coquoz JL, Buchala AJ, Meuwly P and Metraux JP. Arachidonic acid treatment of potato plants induces local synthesis of salicylic acid and confers systemic resistance to *Phytophthora infestans* and *Alternaria solani*. *Phytopathol.* **85:**1219-1224, 1995.

Cordier C, Pozo MJ, Barea JM, Gianinazzi S and Gianinazzi-Pearson V. Cell Defense responses Associated with Localized and Systemic Resistance to *Phytophthora parasitica* Induced in Tomato by an Arbuscular Mycorrhizal Fungus. *Mol. Plant Microbe Interact.***11:** 1017-1028, 1998.

Cosio EG, Frey T and Ebel J .Identification of a high-affinity binding protein for a hepta-P-glucoside phytoalexin elicitor in soybean. *Eur. J. Biochem*. **204:** 1115-1123, 1992.

Cruickshank IA and Perrin DR. The isolation and partial characterisation of Monilicolin A, a polypeptide with phaseollin inducing activity from Monilinia fructicola. *Life Sci.* **7:** 449-458, 1968.

Dangl J and Jones J. Plant pathogens and integrated defence responses to infection. *Nat*. **411:** 826–833, 2001.

Datta KN and Datta SK. Expression and function of PR-proteins genes in transgenic plants. In: Datta S, Muthukrishnan S, eds. Pathogenesis related proteins in plants. Boca Raton: CRC Press, **pp.** 261-277, 1999.

Davis BJ. Disc electrophoresis methods and application to human serum proteins. *Annl. N. Y. Acad. Sci.***121:** 404, 1964.

De La Noval B, Pérez E, Martínez B, León O, Martínezg Allardo N and Délano-Frier J. Exogenous systemin has a contrasting effect on disease resistance in mycorrhizal tomato (Solanum lycopersicum) plants infected with necrotrophic or hemibiotrophic pathogens. *Mycorr.* **17:** 449–460, 2007.

De Laat AMM and van Loon LC. The relationship between stimulated ethylene production and symptom expression in virus-infected tobacco leaves. *Physiol Plant Pathol*. **22:** 261-273, 1983.

De Meyer G , Bigirimana J , Elad Y and Hofte M. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. *Eur. J. Plant Pathol.* **104:** 279–286, 1998.

De Román M, Fernández I, Wyatt T, Sahrawy M, Heil M and Pozo MJ. Elicitation of foliar resistance mechanisms transiently impairs root association with arbuscular mycorrhizal fungi. *J. Ecol*. **99:** 36–45, 2011.

De Weger LA, van der Bij AJ, Dekkers LC, Simons M, Wijffelman CA and Lugtenberg BJJ. Colonization of the rhizosphereof crop plants by plant-beneficial pseudomonads. *FEMS Microbiol. Ecol*.**17:**221–228, 1995.

Deb D .Seeds of tradition, seeds of future: folk rice varieties from east India. Research Foundation for Science Technology & Ecology, New Delhi, India, p 136, 2005.

Dela Paz and Madonna Angelita G. Molecular characterization of Isolates Causing Brown Spot of Rice (*Oryza sativa* L.) in Rainfed Ecosystem in Phillippines. 2005.

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E and Rylas J. A central role of salicylic acid in plant disease resistance. *Sci.* **266:**1247-1249, 1994.

Dickens JSW and Cook RTA. *Glomerella cingulata* on *Camellia*. *Plant Pathol*. **38:**75-85, 1989.

Djonovic S, Vargas WA, Kolomiets MV, Horndeski M, Wiest A and Kenerley CM. A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. *Plant Physiol.***145:** 875–889, 2007.

Dobbelaere S, Vanderleyden J and Okon Y.Plant growth-promoting effects of diazotrophs in the rhizosphere. *Crit. Rev. Plant Sci*. **22:** 107– 149, 2003.

Drechsler C. Phytopathological and taxonomic aspects of *Ophiobolus, Pyrenophora, Helminthosporium*, and a new genus, *Cochliobolus*. *Phytopathol.* **24:** 953-985, 1934.

Dubey A, Mishra MK, Singh PK and Vyas D. Occurrence of AM fungi at varying stages of growth of rice plants. *Proc. Nat. Acad. Sci. Ind*. **78(1):** 51–55, 2008.

Dumas-Gaudot E, Gollotte A, Cordier C, Gianinazzi S and GianinazzI-Pearson V. Modulation of host defence systems, In Y. Kapulnick, and D. D. Douds Jr (eds.), Arbuscular Mycorrhizas: Physiology and Function. Kluwer Academic Press, Dordrecht. **pp**.173–200, 2000.

Dumas- Gaudot E, Slezack S, Dassi B, Pozo MJ, Gianinazzi- Pearson V and Gianinazzi S. Plant hydrolytic enzymes (chitinases and β-1,3-glucanases) in root reactions to pathogenic and symbiotic microorganisms. *Plant and Soil*. 185: 211-221, 1992.

Durrant WE and Dong X. Systemic acquired resistance. *Annu. Rev* . *Phytopathol*. **42:** 185-209, 2004.

Edreva A. A novel strategy for plant protection: induced resistance. *Journal of Cell and Mol. Biol.***3:** 61–69, 2004.

Effmert U, Kalderas J, Warnke R and Piechulla B. Volatile mediated interactions between bacteria and fungi in the soil. *J. Chem. Ecol*. **38 (6):** 665-703, 2012.

Farmer EE and Ryan CA. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plt. Cell*, **4:** 129-134, 1992.

Fester T and Hause G. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. *Mycorr*.**15:** 373–379, 2005.

Fester T and Sawers R. Progress and challenges in agricultural applications of arbuscular mycorrhizal fungi. *Crit. Rev. Plant Sci*. **30:** 459–470, 2011.

Fester T, Fetzer I, Bucher TS, Lucas R, Rillig M and Hartig C. Towards a systemic metabolic signature of the arbuscular mycorrhizal interaction. *Oecol.* **167:** 913–924, 2011.

Fiorilli V, Catoni M, Miozzi L, Novero M, Accotto GP and Lanfranco L. Global and cell-type gene expression profiles in tomato plants colonized by an arbuscular mycorrhizal fungus. *New Phytol*. **184:** 975–987, 2009.

Fischer KS, Barton J, Khush GS, Leung H and Cantrell R. Collaborations in rice. *Sci.* **290:** 279**–**280, 2000.

Fitter AH and Garbaye J. Interaction between mycorrhizal fungi and other soil microorganisms. *Plt. Soil.***159:**123-132, 1994.

Flob DS, Hause B, Lange PR, Küster H, Strack D and Walter MH. Knock-down of the MEP pathway isogene 1-deoxy-d-xylulose 5-phosphate synthase 2 inhibits formation of arbuscular mycorrhiza-induced apocarotenoids, and abolishes normal expression of mycorrhiza-specific plant marker genes. *Plant J*. **56:** 86–100, 2008.

Fogliatto S, Vidotto F and Ferrero A. Morphological characterization of Italian weedy rice(*Oryza sativa*) populations..*An International journal of Weed Biology, Ecol. Veg. Mang*. **pp.** 60-69, 2011.

Fomba SN and Singh N. Crop losses caused by brown spot disease in mangrove swamps of North Sierra Leone. *Trop. Pest Mang*. **36:** 387 –393, 1990.

Food and Agricultural Organization, 2012.Faostat.fao.org.

Frankel OH, Brown AHD and Burdon JJ. The conservation of plant biodiversity. Cambridge University Press, London.1995.

Friese CF and Allen MF. The spread of VA mycorrhizal fungal hyphae in the soil: Inoculum types and external hyphae architecture. *Mycol. Res.* **92:**317-321, 1991.

Fritz M, Jakobsen I, Lyngkjær MF, Thordal-Christensen H and Pons-Kuhnemann J. Arbuscular mycorrhiza reduces susceptibility of tomato to *Alternaria solani*. *Mycorr.* **16:** 413–419, 2006.

Fry SC, Aldington S, Hetherington PR and Aitken J. Oligosaccharides as signals and substrates in the plant cell wall. *Plant Physiol.* **103:** 1–5, 1993.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H and Ryals J. Requirement of salicylic acid for the induction of systemic acquired resistance. *Sci.* **261:** 754-756, 1994.

García-Garrido JM and Ocampo JA. Regulation of the plant defense response in arbuscular mycorrhizal symbiosis. *J. Exp. Bot*. **53:** 1377–1386, 2002.

Gardes M and Bruns TD. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Mol. Eco.* **2:** 113-118, 1993.

Gerdemann JW and Nicolson TH. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. *Trans. B. Mycol. Soc.* **46:** 235-244, 1963.

Gerhardson B. Biological substitutes for pesticides. *Trends Biotechnol*.**20:** 338–343, 2002.

Gernns H, Von alten H and Poehling HM. Arbuscular mycorrhiza increased the activity of a biotrophic leaf pathogen – Is a compensation possible? *Mycorr.* **11:** 237–243, 2001.

Ghany TM. Fungal leaf spot of maize: pathogen isolation, identification and host biochemical characterization. *Mycopath.* **10(2):**41-49, 2012.

Ghosal A and Purkayastha RP. Biochemical responses of Rice (*Oryza sativa* L.) leaves to some abiotic elicitors of Phytoalexin. *Ind. J. Expt. Biol.***25:** 395-399, 1987.

Gilreath P. *Manatee Vegetable Newsletter*, University of Florida, Manatee County Extension Service, January/February, 2002.

Giovannetti M and Mosse B. An evaluation of techniques to measure vesicular-arbuscular infection in roots. *New Phytol.* **84:** 489-500, 1980.

Glazebrook J and Ausubel F. Isolation of phytoalexindeficient mutants of Arabidopsis thaliana andcharacterisation of their interactions with bacterial pathogens. *Proc. Nat. Acad. Sci. USA*. **91:** 8955-8959, 1994.

Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol*. **43:** 205–227, 2005.

Glick B. The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol*. **41:** 109–117, 1995.

Goel RK, Bala R and Singh K. Genetic characterization of resistance to brown leaf spot caused by *Drechslera oryzae* in some wild rice (Oryza sativa) lines. *Ind. J. Agri. Sci.* **76:** 705–707, 2006.

Goellner K and Conrath U. Priming: It's all the world to induced disease resistance. *Eur. J. Plant Pathol*. **121:** 233–242, 2008.

Graham JH, Linderman RG and Menge JA. Development of external hyphae by different isolates of mycorrhizal *Glomus* spp. in relation to root colonization and growth of troyer citrange. *New Phytol.* **91:**183-189, 1982.

Gray EJ and Smith DL.Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biol. Biochem*. **37:** 395–412, 2005.

Grayer RJ and Kokubun T. Plant-fungal interactions: The search for phytoalexins and other antifungal compounds from higher plants. *Phytochem.* **56:** 253-263, 2001.

Greenberg JT, Silverman FP and Liang H. Uncoupling salicylic acid –dependent cell death and defense- related responses from disease resistance in the *Arabidopsis* mutant acd5. *Gen*.**156:** 341-350, 2000.

Guimil S , Chang HS, Zhu T, Sesma A, Osbourn A, Roux C, Ioannidis V, Oakeley EJ, Docquier M, Descombes P, Briggs SP and Paszkowski U. Comparative transcriptomics of rice reveals an ancient pattern of response to microbial colonization. *Proc. Nat. Acad. Sci. USA*, **102:** 8066–8070, 2005.

Gundlach H, Miiller MJ, Kutchan TM and Zenk MH. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. USA*, **89:** 2389-2393, 1992.

Haas D, Keel C and Reimmann C. Signal transduction in plantbeneficial rhizobacteria with biocontrol properties. *Ant. Leeu*.**81:** 385–395, 2002.

Haas D, Blumer C, and Keel C. Biocontrol ability of fluorescent pseudomonads genetically dissected: importance of positive feedback regulation. *Curr. Opin. Biotechnol*. **11:** 290–297, 2000.

Hallman J, Quadt-Hallman A, Mahafee WF and Kloepper JW. Bacterial endophytes in agricultural crops. *Can. J. Microbiol*. **43:** 895–914, 1997.

Hammerschmidt R and Kuc J. Induced Resistance to Disease in Plants. Kluwer Academic Publishers, Dordrecht, Netherlands.**pp**.182, 1995.

Hammerschmidt R. Induced disease resistance: how do induced plants stop pathogens? *Phy. and Mol. Plant Pathol*. **55:**77-84, 1999a.

Hammerschmidt R. Phytoalexins: what have we learned after 60 years? *Annu.Rev. Phytopathol.* **37:** 285-306, 1999b.

Hammond-Kosack KE and Jones JDG. Resistance gene dependent plant defence responses. *Plt. Cell*. **8:** 1773-1791, 1996.

Harborne JB. Phytochemical methods.Chapman and hall Ltd. London- **pp.** 278, 1973.

Harborne JB. The comparative biochemistry of phytoalexin induction in plants. *Biochem. Syst. and Eco*. **27:** 335–367, 1999.

Harish S, Saravavakumar D, Radjacommar R, Ebenezar EG and Seetharaman K. Use of plant extracts and biocontrol agents for the management of brown spot disease in rice. *Biocontrol.* **53(3):** 555-567, 2007.

Harman GE, Howell CR, Viterbo A, Chet I and Lorito M. *Trichoderma* species — opportunistic, avirulent plant symbionts. *Microbiol.* **2:** 43-56, 2004.

Harman GE. Trichoderma—not just for biocontrol anymore. *Phytoparasi*. **39(2):**103–108, 2011.

Harrier LA and Watson CA. The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Manag. Sci*. **60:** 149–157, 2004.

Harrison M and Dixon R . Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular arbuscular mycorrhizal

associations in roots of *Medicago truncatula. Mol. Plant-Microbe Interact*. **6:** 643–659, 1993.

Harrison MJ. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **50:** 361–389, 1999.

Hause B and Fester T. Molecular and cell biology of arbuscular mycorrhizal symbiosis. *Planta*, **221:**184–196, 2005.

Hause B, Mrosk C, Isayenkov S and Strack D. Jasmonates in arbuscular mycorrhizal interactions. *Phytochem.* **68:**101–110, 2007.

Hawksworth DL. Fungal genera in urgent need of taxonomic work. *Microbiol. Sci*.**3:**58, 1986.

Herrera-Medina MJ, Gagnon H, Piche Y, Ocampo JA, García-Garrido JM and Vierheilig H. Root colonization by arbuscular mycorrhizal fungi is affected by the salicylic acid content of the plant. *Plant Sci*. **164:** 993–998, 2003.

Hiremath L and Naik GR. Rapid diagnosis of sugarcane red rot by Dot-immunobinding assay (DIBA) technique. *Ind. J .Biotech.* **3:** 542-545, 2004.

Holland KW and O'Keefe SF. Recent applications of peanut phytoalexins. *Rec.Pat. on Food, Nut. & Agri.* **2:** 221–232, 2010.

Hore DK. Rice diversity collection, conservation and management in north-eastern India. *Genet Resour. Crop Evol*. **52:** 1129-1140, 2005.

Hossain M, Khalequzzaman KM, Mollah MRA, Hussain MA and Rahim MA. Reaction of breeding lines/ cultivars of rice against brown spot and blast under field condition. *Asian J. Plt. Sci.* **3:** 614–617, 2004.

Huffaker A, Kaplan F, Vaughan MM, Dafoe NJ, Ni X and Rocca JR. Novel acidic sesquiterpenoids constitute a dominant class of pathogen-induced phytoalexins in maize. *Plt. Physiol.* **156:** 2082–2097, 2011.

Hunt MD, Neuenschwander UH, Delaney TP, Weymann KB, Friedrich LB, Lawton KA, Steiner HY and Ryals JA. Recent advances in systemic acquired resistance research-a review. *Gene*. **179:** 89-95, 1996.

Igawa T, Tokal T, Kudo T, Yamagushi I and Kimura M. A wheat xylanase inhibitor gene, Xip-I, but not Taxi-I, is significantly induced by biotic and abiotic

signals that trigger plant defense. *Biosci. Biotechnol.and Biochem.* **69:** 1058–1063, 2005.

Ito S and Kuribayashi. Cochliobolus miyabeanus. *Ind. J. Agric. Sci*. **12:** 733, 1942.

Iwai T, Miyasaka A, Seo S and Ohashi Y. Contribution of ethylene Biosynthesis for resistance to blast fungus infection in young rice plants. *Plt. Physiol*. **142:** 1202-1215, 2006.

Jackson AO and Taylor CB. Plant microbe interactions:life and death of the interface. *The Plt. Cell.* **8:** 1651-1668, 1996.

Jahangir M, Kim HK, Choi YH and Verpoorte R. Health-affecting compounds in Brassicaceae. *Comp. Rev. in Food Sci. and Food Saf.* **8:** 31–43, 2009.

Jones JDG and Dangl JL. The plant immune system. *Nat.* **444:** 323–329, 2006.

Jones LR. The relation of environment to disease in plant. *Ann. J. Bot*. **11:** 601 – 609, 1924.

Joshi N, Brar KS, Pannu PPS and Singh P. Field efficacy of fungal and bacterial antagonists against brown spot of rice. *J. of Bio. Cont*. **21:** 159–162, 2007.

Kachroo A and Kachroo P. Fatty Acid–Derived Signals in Plant Defense. *Annu. Rev. Phytopathol*. **47:** 153–76, 2009.

Kanno H, Satoh H, Kimura T and Fujita Y. Some aspects of induced resistance to rice blast fungus, *Magnaporthe grisea*, in rice plant infested by white-backed planthopper, *Sogatella furcifera*. *Appl. Entomol. and Zoo.***40:** 91–97, 2005.

Kaschuk G, Kuyper TW, Leffelaar PA, Hungria M and Giller KE. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biol. Biochem*. **41:** 1233–1244, 2009.

Katiyar RS, Das PK, Chowdhury PC, Ghosh A, Singh GB and Datta RK. Response of irrigated mulberry (Morus alba L.) to VA mycorrhizal inoculation under graded doses of phosphorus. *Plt. Soil*, **5:** 369–373, 1994.

Kato T, Kodama O, Akatsuka T and Hirukawa T. Oryzalexin E, a diterpene phytoalexin from UV irradiated rice leaves. *Phytochem.* **33:** 79–81, 1993.

Kerry BR. Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. *Annu. Rev. Phytopathol*. **38:** 423–441, 2000.

Kessler A, Halitschke R, Diezel C and Baldwin I. Priming of plant defense responses in nature by airborne signalling between Artemisia tridentata and Nicotiana attenuata. *Oecol*. **148:** 280–292, 2006.

Khush GS. Origin, dispersal, cultivation and variation of rice. *Plant Mol. Biol*. **35:** 25-34, 1997.

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O,Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A, Palmer TM,West SA,Vandenkoornhuyse P, Jansa J and Bucking H. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Sci.* **333:** 880–882, 2011.

Kilic-Ekici O and Yuen GY. Comparison of strains of *Lysobacter enzymogenes* and PGPR for induction of resistance against *Bipolaris sorokiniana* in tall fescue. *Biol. Cont*. **30:** 446–455, 2004.

Klessig DF, Vlot CA and Dempsey DA. Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopath*. **47:** 177–206, 2009.

Kloepper JW and Schroth MN. Plant growth-promoting rhizobacteria on radishes, In Station de pathologie vegetale etphyto-bacteriologie (ed.), Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, vol. II. Gilbert-Clarey, Tours, France, **pp.** 879–882, 1978.

Kloepper JW, Rodriguez-Ubana R, Zehnder GW, Murphy JF, Sikora E and Fernandez C. Plant root-bacterial interactions in biological control of soil borne diseases and potential extension to systemic and foliar diseases. *Austral. Plant Pathol*. **28:** 21–26, 1999.

Kloepper JW, Leong J, Teintze M and Schroth MN. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nat.* **286:** 885-886, 1980.

Kloepper JW, Schippers B and Bakker PAHM. Proposed elimination of the term endorhizosphere. *Phytopathol*. **82:**726–727, 1992.

Kloppholz S, Kuhn H and Requena N. A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. *Curr. Biol*. **21:**1204–1209, 2011.

Kodama O, Miyakawa J, Akatsuka T and Kiyosawa S. Sakuranetin, a flavanone phytoalexin from ultraviolet-irradiated rice leaves. *Phytochem.* **31:** 3807–3809, 1992.

Koga J, Ogawa N, Yamauchi T, Kikuchi M, Ogasawara N and Shimura M. Functional moiety for the antifungal activity of phytocassane E, a diterpene phytoalexin from rice. *Phytochem.* **44:** 249–253, 1997.

Koga J, Osima K, Ogawa N, Ogasawara N and Shimura M. A bioassay for measuring elicitor activity in rice leaves. *Ann. Phytopathol. Soc. Jpn*. **64:** 97-101, 1998.

Koga J, Shimura M, Oshima K, Ogawa N, Yamauchi T and Ogasawara N. Phytocassanes A, B, C and D, novel diterpene phytoalexins from rice, *Oryza sativa* L. *Tetrahedron*, **51:** 7907–7918, 1995.

Koide RT and Schreiner RP. Regulation of the vesicular arbuscular mycorrhizal symbiosis. *Ann. Rev. of Plt. Physiol. Plt. Mol. Biol*. **43:** 557-581, 1992.

Kono Y, Takeuchi S, Kodama O and Akatsuka T. Absolute conˆguration of oryzalexin A and structures of its related phytoalexins isolated from rice blast leaves infected with *Pyricularia oryzae*. *Agric. Biol. Chem.* **48:** 253–255, 1984.

Koricheva J, Gange AC and Jones T. Effects of mycor- rhizal fungi on insect herbivores: a meta-analysis. *Ecol.* **90:** 2088–2097, 2009.

Koske RE. Evidence for a volatile attractant from plant roots affecting germ tubes of a VA mycorrhizal fungus. *Trans. Brit. Mycol. Soc*. **79:** 305–310, 1982.

Kratka J, Kynerova BK, Kudlikova I, Slovacek J and Zemankova M. Utilisation of immunochemical methods for detecton of Colletotrichum spp. in Strawberry.*Plant Protec. Sci.* **38(2)**: 55-63, 2002.

Kravchuk Z, Vicedo B, FlorsV, Camañes G, González-Bosch C and García-Agustín P. Priming for JA-dependent defenses using hexanoic acid is an effective mechanism to protect Arabidopsis against *B. cinerea*. *Plant Physiol*. **168:** 359–366, 2011.

Krishna KR and Bagyaraj DJ. Influence of vesicular-arbuscular mycorrhiza on growth and nutrition of Arachis hypogea. *Legume Res*. **5:**18–22, 1982.

Kuc J. Increasing crop productivity and value by increasing disease resistance through non-genetic techniques. In: *Forest Potentials, Productivity and value, Weyerhauser Science Symp.* (Eds., R. Ballard *et al.*,) Weyerhauser Co. Press, pp. 147-190, 1985.

Kuc J. Phytoalexins, stress metabolism, and disease resistance in plants. *Ann. Rev. of Phytopahol*. **33:** 275-297, 1995.

Kulkarni S, Ramakrishnan K and Hegde RK. Incidence of brown leaf spot of rice caused by *Drechslera oryzae* (Breda de Haan) Subram. & Jain under different agroclimatic conditions of Karnataka. *Mysore J. Agri. Sci*. **14**: 321-322, 1980.

Kumar S and Sridhar R. Significance of cell wall phenols in the resistance of rice against blast. *Curr. Sci*. 54:874-876, 1985.

Kumawat GL, Biswas SK and Srivastava SSL. Biochemical evidence of defense response in paddy induced by bio-agents against brown leaf spot pathogen. *Indian Phytopath.* **61 (2):** 197-203, 2008.

Kumawat GL. Effect of *Trichoderma* on *Drechslera oryzae* and its role in defense against brown leaf spot of paddy. M.Sc. Thesis. *C.S.A. Univ. Agric. & Tech., Kanpur,* **pp.** 78, 2006.

Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nat.* **227:** 680-685, 1970.

Lange L, Heide M and Olson LW. Serological detection of *Plasmodiophora brassicae* by dot immunobinding and visualization of the serological reaction by scanning electron microscopy. *Phytopathol*. **79**: 1066-1071, 1989.

Larose G, Chenevert Moutoglis P, Gagne S and Piche Vierheilig H. Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. *J. Plant Physiol*.**159:** 1329–1339, 2002.

Lee CS, Lee YJ and Jeun YC. Observations of infection structures on the leaves of cucumber plants pre-treated with arbuscular mycorrhiza Glomus intraradices

after challenge inoculation with *Colletotrichum orbiculare*. *Plant Pathol. J.* **21:** 237–243, 2005.

Legrand M, Kauffmann S, Pierrette G and Fritig B. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis proteins are chitinases. *Proc. of the Nat.l Aca. of Sci.s of USA*. **84:** 6750-6754, 1987.

Lendzemo VW, Kuyper TW, Matusova R, Bouwmeester HJ and Ast AV. Colonization by arbuscular mycorrhizal fungi of Sorghum leads to reduced germination and subsequent attachment and emergence of Striga hermonthica. *Plant Sign. Behav*. **2:** 58–62, 2007.

Linderman RG. Role of VAM fungi in biocontrol. In FL. Pfleger and RG. Linderman (eds.), Mycorrhizae and Plant Health. APS Press, St. Paul, MN. **pp.** 1–26, 1994.

Liu J, Blaylock LA, Endre G, Cho J, Town CD,Vandenbosch KA and Harrison MJ. Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. *Plt. Cell*, **15:** 2106–2123, 2003.

Liu J, Maldonado-Mendoza I, Lopez-Meyer M, Cheung F, Town CD and Harrison MJ. Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. *Plant J*. **50:** 529–544, 2007.

Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M and van der Lelie D. Endophytic bacteria and their potential applications. *Crit. Rev. Plant Sci*. **21:** 583–606, 2002.

Lopez-Raez JA, Flors V, García JM.and Pozo MJ. AM symbiosis alters phenolic acid content in tomato roots. *Plant Sign. Behav*. **5:** 1138–1140, 2010a.

Lopez-Raez JA, Verhage A, Fernández I, García JM, Azcón-Aguilar C, Flors V and Pozo MJ. Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. *J. Exp. Bot*. **61:** 2589–2601, 2010b.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193:** 265-275, 1951.

Ludwig J, Moura AB, dos Santos AS and Ribeiro AS. Seed microbiolization for the control of rice brown spot and leaf scald. *Tropical Plt. Pathol*. **34:** 322–328, 2009.

Lugtenberg BJJ, Dekkers L and Bloemberg GV. Molecular determinants of rhizosphere colonization by *Pseudomonas. Annu. Rev. Phytopathol*. **39:** 461–490, 2001.

Lugtenberg BJJ and Dekkers LC. What make *Pseudomonas* bacteria rhizosphere competent? *Environ. Microbiol*. **1:** 9–13, 1999.

Luna E, Bruce TJA, Roberts MR, Flors V and Ton J. Next-generation systemic acquired resistance. *Plant Physiol*. **158:** 844–853, 2012.

Maclean JL, Dawe DC, Hardy B and Hettel GP. Rice almanac. Los Baños (Phillippines): *Int. Rice Res*. 2002.

Mahadevan N and Shridhar R. Methods in physiological plant pathology.2nd Ed. SivakaniPubl., India – **pp.** 242, 1982.

Maiti D, Variar M and Saha J. Colonization of upland rice by native VAM under rainfed monocropped ecosystem. In: Roy AK, Sinha KK (eds) Recent advances in phytopathological research, MD Publication, New Delhi, **pp.** 45–51, 1995.

Malamy J, Carr JP, Klessig DF and Raskin I. Salicylic acid: a likely endogenous signal in the resistance to tobacco to viral infection. *Sci.* **250:** 1002-1004, 1990.

Malik KA, Bilal R, Mehnaz S, Rasul G and Mirza MS. Association of nitrogen-fixing, Plant-Growth-Promoting Rhizobacteria (PGPR) with kallar grass and rice. *Plt. Soil*.**194**: 37-44, 1997.

Mansfield JW. Role of phytoalexins in disease resistance. In: Phytoalexins. Mansfield JW and Bailey J (Ed). Blackie, Glasgow.1982.

Mason JR and Mathew DN. Evaluation of neem as bird repellent chemical. *Int. J. Pest Mang*. **42 (1):** 47- 49, 1996.

Mathur A, Hong Y, Kemp BK, Barrientos AA and Erusalimsky JD. Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes. *Cardiovasc Res.***46 (1):**126-138, 2000.

Matsumura ATS, Moraes Fernandes MIB, Prestes AM, Zanettini MBB and Oliveira MAR. Relações fenéticas entre isolados naturais de Bipolaris sorokiniana: Resumo, XV Reunião Nacional de Pesquisa de Trigo, Passo Fundo, RS.p. 230, 1998.

Mauch F, Mauch-Mani B, Gaille C, Kull B, Haas D and Reimmann C. Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. *The Plt. J*.**25:** 67-77, 2001.

Mauch-Mani B and Metrau JP. Salicylic acid and systemic acquired resistance to pathogen attack. *Ann. of Bot.* **82:** 535-540, 1998.

Mayak S, Tirosh T and Glick BR. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiol. Biochem*. **42:** 565–572, 2004.

McInroy JA and Klopper JW. Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. *Can. J. Microbiol*. **41:** 895–901, 1995.

Meghvansi MK, Prasad K, Harwani D and Mahna SK. Response of soybean cultivars toward inoculation with three arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* in the alluvial soil. *Eur. J. of Soil Biol.* **44:** 316-323, 2008.

Mert-Turk F, Bennett MH, Glazebrook J, Mansfield J and Holub E. Biotic and abiotic elicitation of camalexin in Arabidopsis thaliana. 7th International Congress of Plant Pathology.Edinburgh, Scotland, UK. 1998.

Metraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W and Inverardi B. Increase in the salicylic acid at the onset of systemic acquired resistance. *Sci.* **250:**1004-1006, 1990.

Metraux JP, Nawrath C and Genoud T. Systemic acquired resistance. *Euphy.***124:** 237–243, 2002.

Metraux JP. Systemic acquired resistance and salicylic acid:current state of knowledge. *Eur.J. of Plt. Pathol*.**107:** 13-18, 2001.

Meuwly P, Molders W, Buchala A and Metraux JP. 1995. Local and systemic biosynthesis of salicylic acid in infected cucumber plants. *Plant Physiol*.**109:** 1107-1114, 1995.

Mew TW and Gonzales P. A Handbook of Rice Seed borne Fungi Science Publishers, Inc. 2002.

Mia MAB, Shamsuddin ZH and Mahmood M. Effects of rhizobia and plant growth promoting bacteria inoculation on germination and seedling vigor of lowland rice. *African J. Biotechnol*. **11:** 3758-3765, 2012.

Miransari M. Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. *Plant Biol*. **12:** 563–569, 2010.

Mishra AP and **Prasad Y**. The nature of resistance of paddy to *Helminthosporium oryzae* Bada de Haan. *Indian Phytopath.* **27:** 287-294, 1964.

Mohadevan A, Rahman N and Natarajan K. Mycorrhizae for green Asia. University of Madras, Madras. 1988.

Moller K, Kristensen K, Yohalem D and Larsen J. Biological management of gray mold in pot roses by coinoculation of the biocontrol agent Ulocladium atrum and the mycorrhizal fungus *Glomus mosseae*. *Biol Control*.**49:** 120–125, 2009.

Morandi D. Occurence of phytoalexins and phenolic compounds on endomycorrhizal interactions, and their potential role in biological control. *Plt. Soil*. **185:** 241–251, 1996.

Morton JB and Benny GL. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon.* **37:**471-491, 1990.

Morton JB. Evolutionary relationships among arbuscular mycorrhizal fungi in the Endogonaceae. *Mycologia*. **82:**192-207, 1990.

Motlagh MR and Kaviani B. Characterization of new *Bipolaris* spp.: the causal agent of rice brown spot disease in the North of Iran. *Int. J. Agri. Biol.* **10:** 638–42, 2008.

Mukerji K and Ciancio A. Mycorrhizae in the integrated pest and disease management, , in A. Ciancio and K. G. Mukerji (eds.), General Concepts in Integrated Pest and Disease Management. Springer Netherlands, Dordrecht, **pp.** 245–266, 2007.

Muller K. Studies on phytoalexins. In. The formation and the immunological significance of phytoalexin produced by Phaseolus vulgaris in response to infections with Sclerotinia fructicola and Phytophthora infestans. *Aust. J. Boil. Sci*. **11:** 275-300, 1958.

Muller KO and Borger H. Experimentelle Untersuchungen über die Phytophthora: Resistenz der Kartoffel. *Arb. Biol. Reichsanst. Land Forstwirtsch*. **23:** 189-231, 1940.

Muyanga SC, Nembaware V and Gehring C. The protection of *Pseudomonas aeruginosa* wheat seeds treated against powdery mildew and leaf blight correlates with up-regulated expression of a subtilisin-like gene in leaves. *South African J. of Sci.* **100:** 201–204, 2005.

Nagai I and Hara S. On the inheritance of variegation disease in a strain of rice plant. *Japanese J. of Gene*. **5:** 140–144, 1930.

Narwal S, Balasubrahmanyam A, Sadhna P, Kapoor H and Lodha ML. A systemic resistance inducing antiviral protein with N- glycosidase activity from Bougainvillea xbuttiana leaves. *Indian J. Exp. Biol*, **39:** 600, 2000.

Newsham KK, Fitter AH and Watkinson AR. Multi functionality and biodiversity in arbuscular mycorrhizas. *Trends Ecol. Evol*, **10:** 407–411, 1995.

Ng TB, Yi XJ, Wong JH, Fang EF, Chan YS, Pan W, Ye XY, Sze SC, Zhang KY, Liu F and Wang HX. Glyceollin, a soybean phytoalexin with medicinal properties. *Appl. Microbiol. Biotechnol*. **90:** 59–68, 2011.

Niki T, Mitsuhara I, Seo S, Ohtsubo N and Ohasi Y. Antagonistic effects of salicylic acid and jasmonic acid on the expression of pathogenesis- related (PR) protein genes in wounded mature tobacco leaves. *Plt. Cell Physiol.* **39:** 500-507, 1998.

Nowak J and Shulaev V. Priming for transplant stress resistance in in vitro propagation. *In Vitro Cell Dev. Biol. Plant*. **39:**107–124, 2003.

Okubara PA, Kornoely JP and Landa BB. Rhizosphere colonization of hexaploid wheat by *Pseudomonas fluorescens* strains Q8rl-96 and Q2-87 is cultivar-variable and associated with changes in gross root morphology. *Biol. Cont.* **30:** 392–403, 2004.

Olivieri F, Prasad V, Valbonesi P, Srivastava P, Ghosal- Chowdhury P, Barbieria L, Bolognesia A and Stirpe F. A systemic antiviral resistance-inducing protein isolated from Clerodendrum inerme Gaertn. is a polynucleotide:adenosine glycosidase (ribosome-inactivating protein).*FEBS Lett*, **396**: 132, 1996.

Ou SH. Rice disease (2nd ed.). Kew: Commonwealth Mycological Institute. **pp.** 380, 1985.

Padmanabhan SY. The great Bengal Famine. *Ann. Rev. of Phytopathol.* **11:** 11–26, 1973.

Pan SQ, Ye XS and Kuc J. A technique for detection of chitinase, β-1,3 glucanase andprotein patterns after a single separation using polyacrylamide gel electrophoresis orisoelectric focussing. *Phytopathol.* **81:** 970-974, 1991.

Paplomatas EJ. Molecular Diagnostics of Fungal Pathogens. *Arab. J. of Plt. Prot.***24:** 147-158, 2006.

Pari L and Latha M. Antihyperglycaemic effect of *Scopariadulcis*: effect of key metabolic enzymes of carbohydrate metabolim in streptozotocin-induced diabetes. *Pharm. Biol.* **42:** 570–576, 2004.

Pari L, Karamac M, Kosinska A, Rybarczyk A and Amarowicz R. Antioxidant activity of the crude extracts of the drumstick tree (*Moringaoleifera* Lam.) and Sweet Broomweed (*Scopariadulcis*L.) leaves. *Pol.J.Food.Nutr.Sci.* **57:** 203-208, 2007.

Parihar PS, Prakash O and Punetha H. Investigation on defensive enzymes activity of *Brassica juncea* genotypes during pathogenesis of *Alternaria blight*. *Nat. Sci.* **10:** 64-68, 2012.

Paroda RS and Malik SS .Rice genetic resources – its conservation and use in India. *Oryza.* **27:** 361-369, 1990.

Pastor A, Jones DM and Currie J. High-dose baclofen for treatment-resistant alcohol dependence. *J. Clin. Psychopharmacol*. **32:** 266–26810, 2012.

Paszkowski U. Mutualism and parasitism: the yin and yang of plant symbioses. *Curr. Op. Plant Biol*. **9:** 364–370, 2006.

Paul PK and Sharma PD. Azadirachta indica leaf extract induces resistance in barley against leaf stripe disease. *Physiol. Mol. Plant Pathol*. **61:** 3-13, 2002.

Pedras MSC and Seguin-Swartz G. The black-leg fungus: phytotoxins and phytoalexins. *Can. J. Plant Pathol*. **14:** 67-75, 1992.

Pedras MSC, Yaya EE and Glawischnig E. The phytoalexins from cultivated and wild crucifers: chemistry and biology. *Nat. Prod. Rep.* **28:** 1381–1405, 2011.

Pegg GF. Chitinase from *Verticillium alba-atrum*. *Methods in Enzymology.* **161:** 474-479, 1988.

Peng S, Biswas JC, Ladha JK, Gyaneshwar P and Chen Y. Influence of rhizobial inoculation on photosynthesis and grain yield of rice. *Agron. J.* **94:** 925-929, 2002.

Peret B, Svistoonoff S and Laplaze L. When plants socialize: Symbioses and root development. *Annu. Plant Rev*. **pp.** 209–238, 2009.

Petkovsek MM, Stampar F and Veberic R. Increased phenolic content in apple leaves infected with the apple scab pathogen. *J. Pl. Pathol*.**90 (1):** 49-55, 2008.

Pettersson M and Baath E. Effects of the properties of the bacterial community on pH adaptation during recolonization of a humus soil. *Soil Biol. Biochem.* **36:**1383–1388, 2004.

Phillips JM and Hayman DS. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection.*Trans.British. Mycol. Soc.* **55:** 157-160, 1970.

Picco AM and Rodolfi M. *Pyricularia grisea* and *Bipolaris oryzae*: a preliminary study on the occurrence of airborne spores in a rice field. *Aerobiol.* **18**(**2):** 163-167, 2002.

Pieterse CMJ, Leon-Reyes A, Van der ent S and Van Wees SCM. Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol*. **5:** 308–316, 2009.

Ping L and Boland W. Signals from the underground: bacterial volatiles promote growth in *Arabidopsis. Trends Plant Sci*. **9:** 263–269, 2004.

Plummer D. An introduction to plant biochemistry. Tata Mc Graw Hill Publication, New Delhi. 362, 1978.

Postma J, Montanari M and van den Boogert PHJF. Microbial enrichment to enhance the disease suppressive activity of compost. *Eur. J.Soil Biol*. **39:**157–163, 2003.

Pozo MJ and Azcón-Aguilar C. Unraveling mycorrhizainduced resistance. *Curr. Opin. Plant Biol*. **10:** 393–398, 2007.

Pozo MJ, Azcón-Aguilar C, Dumas-Gaudot E and Barea JM. Chitosanase and chitinase activities in tomato roots during interactions with arbuscular mycorrhizal fungi or Phytophthora parasitica. *J. Exp. Bot*. **49:**1729–1739, 1998.

Pozo MJ, Cordier C, Dumas-Gaudot E, Gianinazzi S, Barea JM and Azcón-Aguilar C. Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to Phytophthora infection in tomato plants. *J. Exp. Bot*. **53:** 525–534, 2002.

Pozo MJ, Dumas-Gaudot E and Barea JM. ß-1, 3-glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. *Plant Sci*. **141:** 149-157, 1999.

Pozo MJ, Jung SC, López-Ráez JA and Azcón-Aguilar C. Impact of arbuscular mycorrhizal symbiosis on plant response to biotic stress: The role of plant defence mechanisms. In H. Koltai and Y. Kapulnik (eds.), Arbuscular Mycorrhizas: Physiology and Function. Springer Netherlands, Dordrecht., **pp.** 193–207, 2010.

Pozo MJ, Van loon LC and Pieterse CMJ. Jasmonates - Signals in plant microbe interactions. *J. Plant Growth Reg*. **23:** 211–222, 2004.

Pozo MJ, Verhage A, García-Andrade J, García JM, and Azcón-Aguilar C. Priming plant defence against pathogens by arbuscular mycorrhizal fungi. In C. AzcónAguilar, J. M. Barea, S. Gianinazzi, and V. Gianinazzi-Pearson (eds.), Mycorrhizas - Functional Processes and Ecological Impact. *Springer*, Berlin Heidelberg. **pp.** 123–135, 2009.

Pueppke SG and Van Etten HD. The relation between pisatin and the development of Aphanomyces eluteiches in diseased Pisum sativum. *Phytopathol*. **66:** 1174-1185, 1976.

Purkayastha RP. Phytoalexins - Plant Antigens and Disease Resistance. *Sci. and Cul*.**39:**528-535, 1973.

Purkayastha RP, Ghosal A and Biswas S. Production of Momilactone associated with resistance of rice cultivars to Sheath Rot disease. *Curr. Sci.* **52:**131-132, 1983.

Purkayastha RP and Pradhan S. Immunological approach to study the etiology of *Sclerotium* rot disease of ground nut. *Proc. of Ind. Nat. Sci. Aca.***60 (2):** 157-165, 1994.

Ramamoorthy V, Raguchander T and Samiyappan R. Induction of defense related proteins in tomato roots treated with *Pseudomonas fluorescence* Pf1 and *Fusarium oxysporum* f. sp. *lycopersici* . *Plt. and Soil.* **239:** 55, 2002.

Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V and Samiyappan R. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. *Crop Protect*. **20:** 1-11, 2001.

Rao VP, Pawar SE and Singh SN. Production and application of vesicular-arbuscular mycorrhizal inocula for sustainable agriculture. In: Adholaya A, Singh S (eds) Mycorrhizae Biofertilizer for the Future. TERI Publication, New Delhi, **pp.** 424–428, 1995.

Raskin I. Role of salicylic acid in plants. *Annual Review of Plant Phyiology and Plant Mol. Biol.***43:** 439-463, 1992.

Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, Agrawal AA, Felton GW and Jander G. Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant Physiol*. **158:** 854–863, 2012.

Rasmussen JB, Hammerschmidt R and Zook MN. Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringe* pv *syringe*. *Plant Physiol*.**97:** 1342-1347, 1991.

Ravi M,Geethanjali S, Sameeyafarheen F and Maheswaran M. Molecular marker based genetical diversity in rice (*Oryza sativa* L.) using RAPD and SSR markers.*Euphy*. **133:** 243-252, 2003.

Reddy CS, Laha GS, Prasad MS, Krishnaveni D, Castilla NP and Nelson A. Characterizing multiple linkages between individual diseases, crop health syndromes, germplasm deployment and rice production situations in India. *Field Crops Res.***120:** 241–253, 2010.

Reddy MM and Garber ED. Genetic studies of variant enzymes.III.Comparative electrophoretic studies of esterases and peroxidise for species, hybrids and amphiploids in the genus *Nicotiana*. *Bot. Gaz*. **132:** 156-158.1971.

Reynolds HL, Packer A, Bever JD and Clay K. Grassroots ecology: plant– microbe–soil interactions as drivers of plant community structure and dynamics. *Eco.* **84:** 2281–2291**,** 2003.

Rovira AD. Interactions between plant roots and soil microorganisms. Annu Rev Microbiol. **19:** 241–266, 1965.

Roy JK, De RN, Ghorai DP and Panda A .Collection and evaluation of genetic resources of rice in India. *Phy. Tobreedon* .**1:** 1-9, 1985.

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW and Paré PW. Bacterial volatiles induce systemic resistance in Arabidopsis. *Plant Physiol*. **134:** 1017–1026, 2004.

Ryu CM, Murphy JF, Mysore KS and Kloepper JW. Plant growth-promoting rhizobacterial systemically protect *Arabidopsis thaliana* against *Cucumber mosaic virus* by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. *The Plant J*. **39:** 381–392, 2004.

Saha R, Saha J, Bhattacharya PM, Maiti D and Chowdhury S. Arbuscular mycorrhizal responsiveness of two varieties in nutrient deficient laterite soil (abstr.). In: Proc Nat Conf on Mycorrhiza, Barkhatullah University, Bhopal, **28:** 5–7, 1999.

Sahai AS and Manocha MS. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasitic interaction. FEMS. *Microbiol. Rev.***11:** 317-338, 1993.

Saharan BS and Nehra V. Plant growth promoting rhizobacteria: A critical review. Life Sci. Med. Res., **21:** 1-29, 2011.

Saitou N and Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. and Evo.* **4**:406-425, 1987.

Salzer P, Corbiere H and Boller T. Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhiza- forming fungus *Glomus mosseae* .*Planta*, **208:** 319-325, 1999.

Sarma BK, Yadav SK, Singh S and Singh HB. Microbial consortium-mediated plant defnse against phytopathogens: Readdressing for enhancing efficacy. *Soil Bio. and Biochem.* **87:** 25-33, 2015.

Sasaki T and Burr B. International rice genome sequencing project: the effort to completely sequence the rice genome. *Curr. Opin. Plant Biol*. **3:** 138**–**141, 2000.

Satija A, Chahal SS and Pannu PPS. Evaluation of rice genotypes against brown leaf spot disease. *Pl. Dis. Res.***20:** 163–164, 2005.

Savary S, Castilla NP, Elazegui FA and Teng PS. Multiple effects of two drivers of agricultural change, labour shortage and water scarcity, on rice pest profiles in tropical Asia. *Field Crops Res*. **91:**263–271, 2005.

Savary S, Nelson A, Sparks AH, Willocquet L, Duveiller E and Mahuku G. International agricultural research tackling the effects of global and climate changes on plant diseases in the developing world. *Pl. Dis.***48:** 1–40, 2011.

Savary S, Teng PS, Willocquet L and Nutter FW Jr. Quantification and modeling of crop losses: a review of purposes. *Ann. Rev. of Phytopathol*. **44:**89–112, 2006.

Savary S, Willocquet L, Elazegui FA, Teng PS, Du PV and Zhu D. Rice pest constraints in tropical Asia: characterization of injury profiles in relation to production situations. *Plt. Dis*. **84:** 341–356, 2000a.

Savary S, Willocquet L, Elazegui FA, Castilla N and Teng PS. Rice pest constraints in tropical Asia: quantification of yield losses due to rice pests in a range of production situations. *Plt. Dis.* **84:** 357–369, 2000b.

Schenck NC and Perez Y. Manual for the Identification of VA Mycorrhizal Fungi. Second Edition. International Culture Collection of VA Mycorrhizal Fungi (INVAM), University of Florida, Gainnesville, Florida. 1987.

Schippers B, Bakker AW and Bakker PAHM. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices .*Ann. Rev. of Phytopathol.* **25:** 339–358, 1987.

Schliemann W, Ammer C and Strack D. Metabolite profiling of mycorrhizal roots of Medicago truncatula. *Phytochem.***69:**112–146, 2008.

Schlumbaum A, Mauch F, Vogeli U and Boller T. Plant chitinases are potent inhibitors of fungal growth.*Nat.***324:** 365-367, 1986.

Schmelz EA, Kaplan F, Huffaker A, Dafoe NJ, Vaughan MM, Ni X, Rocca JR, Alborn HT and Teal PE. Identity, regulation, and activity of inducible diterpenoid phytoalexins in maize. *Proc. Natl. Acad. Sci. USA*, **108:** 5455–5460, 2011.

Schneider M, Schweizer P, Meuwly P and Metraux JP. Systemic acquired resistance in plants. In: Jeon KW.ed. *Int. Rev. of Cyt.* **168:** 303-340, 1996.

Schneider S and Ullrich WR. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. *Physiol. Mol. Plant Pathol*. **45:** 291–304, 1994.

Schroth MN and Hancock JG. Selected topics in biological control. *Annu. Rev. Microbiol*. **35:** 453–476, 1981.

Schubler A, Schwarzott D and Walker C. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol. Res*. **105:** 1413–1421, 2001.

Sekido H, Endo T, Suga R, Kodama O, Akatsuka T, Kono Y and Takeuchi S. Oryzalexin D (3,7-dihydroxy-(+)-sandaracopimaradiene), a new phytoalexin isolated from blast-infected rice leaves. *J. Pest Sci*. **11:** 369–372, 1986.

Semwal DP, Pandey A, Bhandari DC, Dhariwal OP and Sharma SK. Variability study in seed morphology and uses of indigenous rice landraces (*Oryza sativa* L.) collected from West Bengal, India. *Aust. J. of Crop Sci.* **8(3):**460-467, 2014.

Seo S, Seto H, Yamakawa H and Ohashi Y. Transient accumulation of jasmonic acid during the synchronized hypersensitive cell death in *tobacco mosaic virus*–infected tobacco leaves. *Mol. Plant-Microbe Interact*. **14:** 261-264, 2001.

Shibuya N, Kaku H, Kuchitu K and Maliarik MJ. Identification of a nove1 high-affinity binding site for N-acetylchitooligosac- charide elicitor in the membrane fraction from suspension-cul- tured rice cells. *FEBS Lett*, **329:** 75, 1993.

Shinbo Y, Nakamura Y, Altaf- Ul- Amin M, Asahi H, Kurokawa K, Arita M, Saito K, Ohta D, Shibata D and Kanaya S. KNApSAcK: a comprehensive species–metabolite relationship database. *Springer*, **57:** 165–181, 2006.

Shoemaker RA. "Nomenclature of *Drechslera* and *Bipolaris*, grass parasites segregated from *Helminosporium*". *Can. J. of Bot.* **37 (5):** 879–87, 1959.

Shoresh M, Harman GE and Mastouri F. Induced systemic resistance and plant responses to fungal biocontrol agents. *Ann. Rev. of Phytopathol.* **48:** 21–43, 2010.

Shrivastava D, Kapoor R, Shrivastava SK and Mukherji KA. Vesicular arbuscular mycorrhiza—an overview. In: Mukherji KG (ed) Kluwer Academic Publishers, Netherlands. **pp.** 1–39, 1996.

Shukla VD, Chauhan JS, Variar M, Maiti D, Chauhan VS and Tomar JB. Reaction of traditional rainfed rice accessions to brown spot, blast and sheath rot diseases. *Ind. Phytopath.***48:** 433–435, 1995.

Silverman P, Seskar M, Kanter D, Schweizer P and Metraux J. Salicylic acid in rice. *Plant Physiol.***108:** 633-639, 1995.

Singh DC. Scope of medicinal and aromatic plants in pest management. International Symposium, Allelopathy in sustainable Agriculture, Forestry and Environment, New Delhi. **pp.** 68, 1994.

Singh K, Khirbat SK, Chugh LK and Jain V. Biochemical changes in chilli against *Colletotrichum capsic i*. *Ind. Phytopath.* **67(2):** 187-189, 2014.

Singh RK, Singh CV and Shukla VD. Phosphorus nutrition reduces brown spot incidence in rainfed upland rice. *Int. Rice Res. Notes*, **30(2):** 31–32, 2005.

Singh RS. Plant Disease (8th edition)Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, **pp.** 439-444, 2005.

Sinha AK and Das NC. Induced resistance in rice plants to *Helminthosporium oryzae*. *Physiol. Plt. Pathol*. **2:** 401-410, 1972.

Slaughter A, Daniel X, Flors V, Luna E, Hohn B and Mauch-Mani B. Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. *Plt. Physiol*. **158:** 835–843, 2012.

Smith SE and Read DJ. Mycorrhizal Symbiosis (Academic, London). 2008.

Smith S, Facelli E, Pope S and Andrew Smith F. Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. *Plt. Soil*, **326:** 3–20, 2010.

Smith SE and Read DJ. *Mycorrhizal Symbiosis*. 2nd ed Academic Press, London. 605, 1997.

Smith SE, Jakobsen I, Gronlund M and Smith FA. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: Interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiol.* **156:** 1050–1057, 2011.

Smoliga JM, Baur JA and Hausenblas HA. Resveratrol and health – a comprehensive review of human clinical trials. *Mol. Nutr. Food Res*. **55:** 1129–1141, 2011.

Soto MJ, Domínguez-Ferreras A, Pérez-Mendoza D, Sanjuán J and Olivares J. Mutualism versus pathogenesis: the give-and-take in plant–bacteria interactions. *Cell. Microbiol.* **11:** 381–388, 2009.

Sticher L, Mauch-Mani B and Metraux JP. Systemic acquired resistance. *Ann. Rev. of Phytopathol.***35:** 235-270, 1997.

Strack D and Fester T. Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. *New Phytol.* **172:** 22–34, 2006.

Strack D, Fester T, Hause B, Schliemann W and Walter MH. Arbuscular mycorrhiza: Biological, chemical, and molecular aspects. *J. Chem. Ecol*. **29:** 1955–1979, 2003.

Sturz AV, Christie BR and Nowak J. Bacterial endophytes: potential role in developing sustainable systems of crop production. *Crit. Rev. Plant Sci*. **19:** 1–30, 2000.

Subamanian CV and Jain BL. A Revision of some Graminicolous Helminthosporia. *Curr.Sci*. **35:**352-355, 1966.

Sunar K, Chakraborty U and Chakraborty BN. Harnessing beneficial microorganisms from Darjeeling Hills and development of strategies for their utilization in management of root diseases. *J. Mycol. Plant Pathol*. **44(1):** 25-40, 2014.

Sunar K, Dey P, Chakraborty U and Chakraborty BN. Biocontrol efficacy and plant growth promoting activity of *Bacillus altitudinus* isolated from Darjeeling hills, India. *J. Basic Microbiol*. **53**:1-14, 2013.

Khati S, Bhattacharjee P, Sashankar P, Chakraborty U and Chakraborty B. Evaluation of plant growth promoting rhizobacteria on rice cultivars for management of brown spot. *J. Mycopathol Res*. **54(3):**401-408, 2016.

Khati S and Bishwanath Chakraborty. Morphological characterization of rice cultivars their root colonization with arbuscular mycorrhizal fungi and screening for field resistance caused by brown spot disease. *NBU J. of Plt. Sci.* **9:**78-86, 2015.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. and Evo.***30:** 2725-2729, 2007.

Taware PB, Dhumal KN, Oulkar DP, Patil SH and Banerjee K. Phenolic alterations

the neighbor-joining method. *Proc. of the Nat. Acad. of Sci.* (*USA*) **101**:11030-11035, 2004.

Thomashow LS. Biological control of plant root pathogens. *Curr. Opin. Biotechnol*.**7:** 343–347, 1996.

Thomma BPHJ, Nurnberger T and Joosten MHAJ. Of PAMPs and effectors: The blurred PTI-ETI dichotomy. *Plt. Cell*, **23:** 4–15, 2011.

Thompson JD and Clustal W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:**4673-4680, 1994.

Timmusk S and Wagner EGH. The plant-growth promoting rhizobacterium Paenibacillus polymyxa induces changes in Arabidopsis thaliana gene expression: A possible connection between biotic and abiotic stress responses. *Mol. Plant-Mic. Int.***12:** 951–959, 1999.

Ton J, D'aless Ro M, Jourdie V, Jakab G, Karlen D, Held M, Mauch-Mani B and Turlings TCJ. Priming by airborne signals boosts direct and indirect resistance in maize. *Plant J.* **49:** 16–26. 2007.

Ton J, Jakab G, Toquin V, Flors V, IavicolI A, Maeder MN, Métraux JP and Mauch-Mani B. Dissecting the ß-aminobutyric acid-induced priming phenomenon in Arabidopsis. *Plt. Cell*, **17:** 987–999, 2005.

Tsahouridou PC and Thanassoulopoulosh CC. Proliferation of *Trichoderma koningii* in the tomato rhizosphere and the suppression of damping off by *Sclerotium rolfsii*. *Soil Biol. Biochem.* **34:** 767-776, 2002.

Umemura K, Ogawa N, Shimura M, Koga J, Usami H and Kono T. Possible role of Phytocassanes, Rice Phytoalexin, in Disease Resistance of Rice against the Blast Fungus *Magnaporthe grisea*. *Biosci. Biotechnol. Biochem*. **67:** 899-902, 2003.

Vallad GE and Goodman RM. Systemic Acquired Resistance and Induced Systemic Resistance in Conventional Agriculture. *Crop sci.* **44:** 1920-1934, 2004.

van Der Ent S, Van Hulten M, Pozo MJ, Czechowski T, Udvardi MK, Pieterse CMJ and Ton J. Priming of plant innate immunity by rhizobacteria and ß-aminobutyric acid: Differences and similarities in regulation. *New Phytol*. **183:** 419–431, 2009.

van Etten HD. Differential sensitivity of fungi to pisatin and to phaseollin. *Phytopathol.***63:**1477-1482, 1973.

van der Putten WH. Plant defense belowground and spatiotemporal processes in natural vegetation. *Eco.* **84:** 2269–2280, 2003.

van Hulten M, Pelser M, Van Loon LC, Pieterse CMJ and Ton J. Costs and benefits of priming for defense in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **103:** 5602–5607, 2006.

van Loon LC and van Kammen A. Polyacrylamide disc electrophoresis of the soluble leaf protein from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN'II. Changes in protein constitution after infection with tobacco mosaic virus. *Virol.***40:**199-211, 1970.

van Loon LC, Bakker PAHM and Pieterse CMJ. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol*. 3**6:** 453–483, 1998.

van Loon LC, van Strien EA. The families of pathogenesis related proteins, their activities and comparative analysis of PR-1 type proteins. *Physiological and Mol. Plt. Pathol*.**55:** 85-97, 1999.

van Loon LC. Occurance and properties of plant pathogenesis- related proteins. In: Datta SK, Muthukrishnan S, eds.Pathogenesis related proteins in plants. Boca Raton: CRC Press, **pp.**1-19, 1999.

van Peer R , Punte HLM, de Weger LA and Schippers B. Characterization of root surface and endorhizosphere pseudomonads in relation to their colonization of roots. *Appl. Environ. Microbiol*. **56:** 2462– 2470, 1990.

van Wees SCM, Van Der Ent S and Pieterse CMJ. Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol*. **11:** 443–448, 2008.

Verhagen BWM, Glazebrook J, Zhu T, Chang HS, Van Loon LC and Pieterse CMJ. The transcriptome of rhizobacteria-induced systemic resistance in Arabidopsis. *Mol. Plant-Microbe Interact*. **17:** 895–908, 2004.

Verma SC, Ladha JK and Tripathi AK. Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *J. Biotechnol*. **91:** 127-141, 2001.

Vernooij B, Friedrich L, Morse A, Reist R, Kolditz-Jawhar R, Ward E, Uknes S, Kessmann H and Ryals J. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plt. Cell*. **6:** 959-965, 1994.

Vicedo B, Flors V, Leyva MO, Finiti I, Kravchuk Z and Real MD. Hexanoic acid-induced resistance against *Botrytis cinerea* in tomato plants. *Mol. Plant Microbe Interact.* **22:** 1455–1465, 2009.

Vierheilig H and Piché Y. Signalling in arbuscular mycorrhiza: Facts and hypotheses, in B. Buslig and J. Manthey (eds.), Flavonoids in Cell Functions. Kluwer Academic/Plenum Publishers, New York **pp.** 23–39, 2002.

Vierheilig H, Steinkellner S, Khaosaad T and Garciag Arrido JM. The biocontrol effect of mycorrhization on soilborne fungal pathogens and the autoregulation of the AM symbiosis: One mechanism, two effects? In A. Varma (ed.), mycorrhiza. *Springer,* Berlin, Heidelberg. **pp.** 307–320, 2008.

Vierheilig H. Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. *Plant Physiol*. **161:** 339–341, 2004.

Vinita P, Taneja N, Vikram P, Singh NK and Singh S. Molecular and morphological characterization of Indian farmers rice varieties (*Oryza sativa* L.). *Aust. J. Crop Sci* **7(7):** 923-932, 2013.

Vlot AC, Klessig DF and Park SW. Systemic acquired resistance: the elusive signal(s). *Curr. Opin. in Plant Biol.* **11:** 436–442, 2008.

Wakeham AJ and White JG. Serological detection in soil of *Plasmodophora brassicae* resting spores. *Physiol. Mol. Plant. Pathol*. **48:** 289-303, 1996.

Walker C. Systematics and taxonomy of the Arbuscular endomycorrhizal fungi (Glomales) a possible way forward. *Agronomie.* **12:** 887-897, 1992.

Walker W. *Acaulospora spinosa* sp. nov. With a key to the species of Acaulospora. *Mycotaxon*. **12:** 512-521, 1981.

Walters D and Heil M. Costs and trade-offs associated with induced resistance. *Physiol. Mol. Plant Pathol*. **71:** 3–17, 2007.

Wehner J, Antunes PM, Powell JR, Mazukatow J and Rillg MC. Plant pathogen protection by arbuscular mycorrhizas: A role of fungal diversity? *Pedobiologia*. **53:** 197-201, 2010.

Welbaum G, Sturz AV, Dong Z and Nowak J. Fertilizing soil microorganisms to improve productivity of agroecosystems. *Crit. Rev. Plant Sci*. **23:** 175–193, 2004.

Weller DM. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol*. **26:** 379–407, 1988.

Werder J and Kern H. Resistance of maize to *Helminthosporium carbonum*: Changes in host phenolics and their antifungal activity. *J. Plt. Dis. Protec.***92:** 477-484, 1985.

Whipps JM. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. *Can. J. Bot*. **82:** 1198–1227, 2004.

Whipps JM. Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot*. **52:** 487-511, 2001.

Whipps JM. Developments in biological control of soil-borne pathogens.In Advances in Botanical Research-Incorporating advances in Plant Pathology.**26:**1-30, 1997.

White TJ, Bruns T, Lee S and Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications. Eds: M.A. Innis, D.H. Gelgard, J.J. Sninsky and T.J. White, Academic Press, Inc. **pp.** 315-322, 1990.

Yamada A, Shibuya N, Kodama O and Akatuka T. Induction of phytoalexin formation in suspension-cultured rice cells. *Biosci. Biotechnol. Biochem*. **57:** 405-409.1993.

Yang L, Browning JD and Awika JM. Sorghum 3-deoxyanthocyanins possess strong phase II enzyme inducer activity and cancer cell growth inhibition properties. *J. of Agri. and Food Chem.* **57:** 1797–1804, 2009.

Yanni YG, Rizk RY, Corich V, Squartini A and Ninke K. Natural endophytic association between Rhizobium leguminosarumbv. trifolii and rice roots and assessment of its potential to promote rice growth. *Plt. Soil*. **194:** 99-114, 1997.

Yeasmin T, Zaman P, Rahman A, Absar N and Khanum NS. Arbuscular mycorrhizal fungus inoculum production in rice plants. *J. Exp. Bot*. **52:** 487–511, 2008.

Yi HS, Heil M, Adame-Álvarez RM, Ballhorn DJ and Ryu CM. Airborne induction and priming of plant defences against a bacterial pathogen. *Plt. Physiol*. **151:** 2152–2161, 2009.

Zadoks JC. On the political economy of plant disease epidemics—capita selecta in historical epidemiology. Wageningen: Wageningen Academic, **pp.** 249, 2008.

Zadoks JC. The role of epidemiology in modern Phytopathology. *Phytopath.***64:** 918–929, 1974.

Zamioudis C and Pieterse CMJ. Modulation of host immunity by beneficial microbes. *Mol. Plant-Microbe Interact*. **25:** 139–150, 2012.

Zanao Junior LA, Rodrigues FÁ, Fontes RLF, Korndorfer GH and Neves JCL. Rice resistance to brown spot mediated by silicon and its interaction with manganese. *J. Phytopathol.* **157:** 73–78, 2009.

Appendix A: List of thesis related publications

In Journals

1. **Khati S,** Bhattacharjee P, Sashankar P, Chakraborty U and Chakraborty BN. Evaluation of plant growth promoting rhizobacteria on rice cultivars for management of brown spot. *J Mycopathol Res*, **54(3):**401-408, 2016.

2. **Khati S** and Chakraborty BN. Morphological characterization of rice cultivars their root colonization with arbuscular mycorrhizal fungi and screening for field resistance caused by brown spot disease. *NBU J. Pl. Sci.*, **9:**78-86, 2015.

Appendix B: List of abbreviations

AMF – Arbuscular mycorrhizal fungi
APS – Ammonium per sulphate
Ar - Arbuscules
BLAST - Basic local alignment search tool
BR – Brown spot
BSA – Bovine serum albumin
$Ca_3(PO_4)_2$ – Tri- calcium phosphate
$CaHPO_4$ – Calcium phosphate
cfu – Colony forming unit
CHT – Chitinase
$CuSO_4$ – Copper sulphate
DEAE Cellulose – Di ethyl aminoethyl Cellulose
DIBA – Dot immunobinding assay
DMAB – Di methyl amino benzaldehyde
DNA – Deoxyribonucleic acid
EDTA – Ethylene diamine tetra acetic acid
ELISA – Enzyme linked immunosorbent assay
FAA – Formaline aceto alcohol
$FeCl_3$ – Ferric chloride
FITC – Fluoescein isothiocyanate
g – Gram
GlcNAc – N- acetyl glucosamine
H_2O_2 – Hydrogen peroxide
H_2SO_4 – Sulphuric acid
HCl – Hydrochloric acid
HCN – Hydroyanic acid
HPLC – High performance liquid chromatography
IgG – Immunoglobulin G
Ih – Intracellular hyphae
IR – Induced systemic resistance
Irs – Intraradicle spore
ITS-PCR – Internal transcribed sequence PCR
KCl – Potassium chloride
KH_2PO_4 – Potassium dihydrogen phosphate
kg – Kilo gram
L – Litre
mg – Mili gram
$MgCl_2$ – Magnesium chloride
ml – Mili litre
Na_2CO_3 – Sodium carbonate
Na_2HPO_4 – Di sodium hydrogen phosphate
Na_2MoO_4 – Sodium molybdate
NaCl – Sodium chloride
NaN_3 – Sodium azide
$NaNO_2$ – Sodium nitrite
NaOH – Sodium hydroxide
NB – Nutrient broth
NCBI – National Centre for Biotechnology Information

NBT/BCIP substrate – Nitro blue tetrozolium / (5- bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substance
NH_4Cl – Ammonium chloride
OA – Oats meal agar
PAb – Polyclonal antibody
PAL – Phenyl alanine ammonia lyase
PBS – Phosphate buffer saline
PCR – Polymerase chain reaction
PDA – Potato dextrose agar
PDB – Potato dextrose broth
PDI – Pecentage disease index
PGPF – Plant growth promoting fungi
PGPR - Plant growth promoting rhizobacteria
POX- Peroxidase
PTA- ELISA – Plate trapped antigen ELISA
PVPP – Polyvinyl pyrollidone phosphate
rpm – Round per minute
RA – Richards agar
SDS- PAGE – Sodium dodecyl sulphate poly acrylamide gel electrophoresis
TEMED – N,N,N',N'-Tetramethyl ethylene diamine
Tris HCl- Tris Hydrochloric acid
Vs - Vesicle
yr – Year
β-1,3- GLU – β-1,3- Glucanase
µl – Micro litre

Appendix C: List of chemicals

Ammonium chloride
Ammonium persulphate
Bovine serum albumin
Calcium phosphate
Carboxy methyl cellulose
Colloidal chitin
Copper sulphate
Di- methyl amino benzaldehyde
Di sodium hydrogen phosphate
Dinitro salicylic acid
Ethylene diamine tetra acetic acid
Ferric chloride
Fluorescein isothiocyanate
Helicase (3%)
Hydrochloric acid
Hydrocyanic acid
Hydrogen peroxide
Magnesium chloride
N,N,N',N'- Tetra methyl ethylene diamine
N- acetyl glucosamine
Nitro blue tetrazolium/(5- bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
O- dianisidine (5 mg/ml methanol)
Phosphate buffer saline- Tween
Poly vinyl – pyrollidone
Potassium chloride
Potassium dihydrogen phosphate
Sodium azide
Sodium carbonate
Sodium chloride
Sodium dodecyl sulphate
Sodium Hydroxide
Sodium molybdate
Sodium nitrite
Sulphuric acid
Tri- calcium phosphate
Tris Acetic Acid and EDTA buffer
Tris Hydrochloric acid
Water saturated phenol: Chloroform:Isoamyl alcohol
0.05(M) sodium phosphate buffer (pH 6.8)
0.1 (M) sodium phosphate buffer (pH 7.4)
0.1 (M) sodium acetate buffer (pH 5.0)
0.2 (M) sodium phosphate buffer (pH 5.4)
0.3 mM borate buffer (pH 8.0)
1M potassium phosphate buffer (pH 7.1)
1-Amino-cyclopropane-1- carboxylic acid hydrochloride
1M sodium acetate buffer (pH 4)
Sodium borate buffer (pH 8.8)

2 mM β- mercaptoethanol
1 M sodium borate buffer (pH 9.8)
2% L- phenylalanine
2,4- Diacetylphloroglucinol
4 mM H_2O_2
4% laminarin
2.5% glutaraldehyde
30%-90% absolute alcohol

Evaluation of plant growth promoting rhizobacteria on rice cultivars for management of brown spot disease

SWEATA KHATI, PRIYANKA BHATTACHARJEE, PUJA SASHANKAR, USHA CHAKRABORTY AND BISHWANATH CHAKRABORTY

J. Mycopathol, Res, 54(3) : 401-408, 2016;
ISSN 0971-3719

Department of Botany,
University of Calcutta,
Kolkata 700 019, India

J. Mycopathol. Res. ***54(3)*** *: 401-408, 2016;* (ISSN 0971-3719)

Evaluation of plant growth promoting rhizobacteria on Rice cultivars for management of Brown spot disease

SWEATA KHATI, PRIYANKA BHATTACHARJEE, PUJA SASHANKAR, USHA CHAKRABORTY AND BISHWANATH CHAKRABORTY*

Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal, Siliguri 734013, West Bengal

Received : 05.02.2016 RMS Accepted : 06.06.2016 Published : 31.10.2016

Effect of a number of rhizospheric bacteria on growth promotion of three Rice cultivars were tested. Results showed significant variations in plant growth according to the different PGPR treatments. The growth parameters such as plant height and number of leaves were observed at 20 days interval from the date of transplanting the seedlings to the experimental plot. Maximum enhancement of growth and dry biomass was observed in Rice plants treated with *Burkholderia symbiont*, *Bacillus altitudinis* and *Enterobacter cloacae*. In order to determine the potential of these bacteria on suppression of Brown spot disease of Rice, their antagonistic activities against *Drechslera oryzae* were tested *in vitro*. Seed bacterization as well as foliar application of *Bacillus altitudinis* (BRHS/ S 73) could reduce the natural occurrence of Brown spot disease markedly. Biochemical parameters such as total soluble protein, phenol, carbohydrate and chlorophyll content of leaf, activity of defense enzymes (chitinase and peroxidase) were also evaluated following treatment. HPLC analysis of treated Rice plants showed highest level of phytoalexin suggesting induction of resistance in Rice plants against Brown spot disease.

Key words: *Drechslera oryzae,* rice, PGPR , phytoalexin

INTRODUCTION

In modern agriculture where crop production has to be enhanced, preferably through the use of eco-friendly means specially by using biological fertilizers. Micro-organisms are important for agriculture in order to promote the circulation of plant nutrients and reduce the need for chemical fertilizers (Chakraborty *et al*, 2014). In this context, plant growth promoting rhizobacteria (PGPR) which are able to exert a beneficial effect upon plant growth, have been considered as an important strategy to increase production in sustained agricultural systems. Biological N fixation provides a major source of nitrogen for plants as a part of environmentally friendly agricultural practices. Research on Plant Growth-Promoting Rhizobacteria (PGPR) with non-legumes such as Rice have shown beneficial effects through biological nitrogen fixation and increased root growth as per (Mia *et al*, 2012) with plant growth enhancement stimulation by other beneficial bacteria and fungi according to (Saharan and Nehra, 2011). The beneficial effects of the selected rhizobial isolates could be due to their plant growth-promoting abilities namely biological Nitrogen fixation, phosphate solubilization and plant growth regulator or phytohormone similar to the known valuable effects of PGPR according to (Araujo *et al*, 2013). Elicitors have the property of inducing the production of phytoalexins in Rice plants, as well as to an agents for controlling rice diseases. Phytoalexins synthesized in the Rice plant bodies in response to the disease were extracted so as to check the level of the production

*Corresponding author : bncnbu@gmail.com

of phytoalexins following the treatment . This study was undertaken in order to investigate the effectiveness of novel bacterial strains on Rice cultivars. The objectives of this study were to determine the effect of different PGPR strains on total protein, total phenol and total soluble sugar content of rice plants, and to evaluate the effects on defense enzymes, establishment of natural disease, phytoalexin production and plant growth parameters.

MATERIALS AND METHODS

Plant material

Seeds of three cultivars of Rice (*Oryza sativa* L.), Black nuniya, Brimful and Champasari obtained from Bijanbari were selected. These were surface sterilized with 0.1% $HgCl_2$, washed thrice with sterile distilled water and then sown as per experimental design.

PGPR

Ten previously isolated, characterized and sequenced PGPR strains were taken for the study. The bacterial strains with NBAIM Acc. No. and NCBI(Gen Bank) Acc. No. are as follows *Bacillus pumilus* (NAIMCC-B01483) (JF836847), *Bacillus pumilus* (NAIMCC-B01487) (JQ765579),*Bacillus pumilus* (NAIMCC-B01488)(JQ765580), *Burkholderiasymbiont* (NAIMCC-B01489) (JQ765578), *Bacillus aerophillus* (NAIMCC-B01490) (KC603894), *Paenibacillus polymyxa* (NAIMCC-B01491) (KC703775), *Bacillus methylotrophicus* (NAIMCC-B01492) (JQ765577), *Bacillus altitudinis* (NAIMCC-B01484) (HQ849482), *Bacillusaltitudinis* (NAIMCC-B01485) (JF899300), *Enterobacter cloacae* (NAIMCC-01486) (KC703974) which are coded as (BRHS/C1), (BRHS/T382), (BRHS/T384), (BRHS/P92), (BRHS/ B104), (BRHS/R72), (BRHS/P91), (BRHS/ P22), (BRHS/S73), (BRHS/R71) accordingly.

Foliar Spray

The bacteria were grown in nutrient broth for 48 h at 28^0C and centrifuged at 12,000 rpm for 15 min. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer to obtain a final density of $3X10^6$ cfu ml^{-1}. The bacterial suspension after the addition of a few drops of Tween-20 was sprayed to the plants at the seedling stage of rice plants. Application was repeated four times at 15 days interval.

Biochemical analyses of leaves
Total Soluble Protein

Soluble proteins were estimated following the method as described by Lowry *et al*, (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% $CuSO_4$ and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% Na_2 CO_3 in 0.1 NaOH) was added. This was incubated for 15 min at room temperature and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for further 15 min following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

Total Sugar

One gm of leaf tissue were weighed and crushed with 95% ethanol. The alcoholic fraction was evaporated off on a boiling water bath. The aqueous fraction was centrifuged at 10,000 rpm for 15 min and the supernatant was collected. Total sugar content was determined following the Anthrone's method as given by Plummer (1978).

Phenol

One gm of leaf tissue was cut into small pieces and immersed in boiling alcohol (100%) in water bath and heated for 5-10 mins. Tissue was crushed using 80% alcohol and filtered in Whatman no. 1 filter paper in dark and phenol content was determined following the method as described by Mahadevan and Sridhar (1982) using caffeic acid as standard.

Quantification of chlorophyll content in leaves

Extraction of chlorophyll from leaves was done according to the method of Harbone (1973). 1g of leaf sample was homogenized in 80% acetone and filtered through Whatman No. 1 filter paper in a dark chamber. Addition of 80 % acetone from the homogenized sample was done repeatedly.The filtrate was collected and the total volume was made up to 10 ml using 80% acetone. Estimation of chlorophyll was done by measuring the OD of the filtrate at 663 nm and 645nm respectively in a UV-VIS spectrophotometer (UV-VIS spectropho-

tometer 118 systronics) against a blank of 80% acetone and calculated using standard.

Assessment of defense enzymes in leaves
Peroxidase Extraction

Extraction of peroxidse enzyme from the leaves were done by homogenizing 1g of the sample leaf in 5ml of ice-cold 50mM sodium phosphate buffer, pH 6.8, containing 1%(w/v) polyvinylpolypyrrolidone using liquid nitrogen in a pre-chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at -40^{0}C. The supernatant was taken out and used directly as crude extract for enzyme assays.

Estimation

Following the method of (Chakraborty *et al*, 1993) peroxidase (EC 1.11.17) activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2.

Chitinase
Extraction

Enzymes were extracted from leaf tissues using suitable buffers and liquid nitrogen. 0.1M sodium acetate buffer, pH 5 was used as extraction buffer for extraction of chitinase.

Estimation

Chitinase (CHT- EC. 3.2.1.39) activity was assayed following the method described by Boller and Mauch (1998). The enzyme activity was expressed as mg N-acetyl glucosamine (GlcNAc) released/ min/ g fresh tissue.

Disease Assessment

Establishment of natural brown spot disease caused by *Drechslera oryzae* (Breda de Haan) was observed and disease severity was assessed in terms of lesion number per leaf and percent disease index (PDI) was calculated following the formula - [(class rating x class frequency)/(total no. of leaves x maximum rating)] x 100.

Antifungal test of PGPR

The bacteria were streaked on one side of the Petri plate and 4mm fungal pathogen block was placed at the other side of the plate, incubation was undertaken for 5-7 days at 28^0±2^{0}C and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolates.

HPLC Analysis of phytoalexin

For phytocassanes extraction 2 g of Rice leaf sample was cut into small pieces and shaken with 20 ml of ethyl acetate and 20 ml. of Na_2CO_3 (pH 10.5) for 18 hour. After collecting the ethyl acetate fraction it was mixed with 0.02N HCl and centrifuged at 15,000 rpm for 30 min followed by evaporation in rotary evaporator. For HPLC analysis, the supernatant was collected ,loaded on a C-18 column and eluted with 45 % acetonitrile.(UV-VIS Detector and Liquid Chromatogram, SHIMADZU). Phytocassanes were monitored at 280 nm (Umemura *et al*, 2003).

RESULTS AND DISCUSSION

Effect of PGPR on growth of Rice plants

Plant growth in terms of height of plant was recorded at 20 days interval from the date of transferring seedlings to the experimental plot. Results revealed that growth was affected by the different bacterial treatments. Maximum growth was observed in plants treated with*Burkholderia symbiont* (BRHS/P 92) in variety Black nuniya, *Bacillus altitudinis* (BRHS/ S 73) in variety Champasari and in case of variety Brimful plants treated with *Bacillus altitudinis* (BRHS/P 22) and *Enterobacter cloacae* (BRHS/R 71) showed maximum growth (Figure 1). Similarly, dry biomass of root and shoot ratio of rice plants were also found to be enhanced by application of the PGPR treatments (Table 1). The present report is in agreement with the reports of Shirinzadeh *et al,* (2013) who found positive effect of seed priming with PGPR on agronomic traits and yield of Barley cultivars. Generation of salt tolerance Rice genotypes through the treatments of PGPR have been reported by some researchers (Adesemoye *et al,* 2013) which are in agreement with findings of current study that PGPR has a positive effect in development of plant health.

Effect of different treatments on biochemical components of Rice plants
Proteins, phenols, sugar and chlorophyll

Estimation of protein contents in all the Rice cultivars following various PGPR treatments revealed

Table 1 : Dry biomass of root and shoot of Rice plants per plot

Treatments		Root shoot ratio of dry biomass of rice plants*		
		Black Nuniya	Champasari	Brimful
Untreated Control		0.73	0.65	0.57
PGPR treated				
Bacillus pumilus	(BRHS/C1)	1.28	0.59	0.75
Bacillus altitudinis	(BRHS/P 22)	0.71	0.46	0.41
Bacillus altitudinis	(BRHS/ S 73)	0.47	0.54	0.42
Enterobacter cloacae	(BRHS/R 71)	0.80	0.57	0.54
Bacillus pumilus	(BRHS/T 382)	1.24	0.50	0.33
Bacillus pumilus	(BRHS/T 384)	1.13	0.73	0.57
Burkholderia symbiont	(BRHS/P 92)	0.72	0.33	1.56
Bacillus aerophilus	(BRHS/B 104)	0.49	0.40	0.74
Paenibacillus polymyxa	(BRHS/R 72)	0.50	0.46	0.53
Bacillus methylotrophicus	(BRHS/P -91)	0.73	0.61	0.69

*Average of ten plants

Table 2 : Protein content of Rice leaves following treatments with PGPR

Treatments		Protein content (mg/gm tissue)*		
		Black Nuniya	Champasari	Brimful
Control		23.90±0.34	37.25±0.93	31.19±0.67
Bacillus pumilus	(BRHS/C1)	45.50±0.67	53.86±0.29	50.17±0.54
Bacillus altitudinis	(BRHS/P 22)	55.25±0.27	50.53±0.54	46.41±0.96
Bacillus altitudinis	(BRHS/ S 73)	49.56±0.35	49.45±0.44	56.72±0.58
Enterobacter cloacae	(BRHS/R 71)	55.03±0.34	57.72±0.69	55.45±0.72
Bacillus pumilus	(BRHS/T 382)	66.77±0.56	57.22±0.82	59.42±0.60
Bacillus pumilus	(BRHS/T 384)	34.93±0.80	30.20±0.68	40.63±0.86
Burkholderia symbiont	(BRHS/P 92)	45.10±0.70	51.05±1.08	52.00±0.35
Bacillus aerophilus	(BRHS/B 104)	65.73±2.11	80.03±1.02	87.73±3.00
Paenibacillus polymyxa	(BRHS/R 72)	73.44±1.70	94.47±2.25	70.84±0.75
Bacillus methylotrophicus	(BRHS/P 91)	55.44±1.78	39.81±1.33	52.49±1.25

* Mean value of three replicates ± Standard error

Table 3 : Total phenol content of Rice leaves following treatments with PGPR

Treatments	Total phenol content(mg/gm tissue)		
	Black Nuniya	Champasari	Brimful
Control	2.71±0.08	3.50±0.20	3.60±0.23
Bacillus pumilus (BRHS/C1)	4.23±0.17	4.79±0.15	3.93±0.20
Bacillus altitudinis (BRHS/P 22)	4.70±0.07	4.83±0.27	5.06±0.12
Bacillus altitudinis (BRHS/ S 73)	4.93±0.20	6.22±0.15	5.76±0.08
Enterobacter cloacae (BRHS/R 71)	5.58±0.10	6.58±0.16	6.30±0.20
Bacillus pumilus (BRHS/T 382)	6.68±0.24	7.13±0.18	7.83±0.17
Bacillus pumilus (BRHS/T 384)	5.83±0.23	5.60±0.30	4.93±0.26
Burkholderia symbiont (BRHS/P 92)	7.10±0.15	6.80±0.20	6.72±0.13
Bacillus aerophilus (BRHS/B 104)	6.76±0.14	6.34±0.17	6.65±0.12
Paenibacillus polymyxa(BRHS/R 72)	8.26±0.14	7.06±0.12	7.33±0.21
Bacillus methylotrophicus (BRHS/P91)	5.86±0.26	5.63±0.31	5.96±0.14

Mean value of three replicates; ± Standard error

Table 4 : Total sugar content of Rice leaves following treatments with PGPR

Treatments		Total sugar content (mg/gm tissue)		
		Black Nuniya	Champasari	Brimful
Control		41.33±1.45	27.33±0.40	33.23±0.72
Bacillus pumilus	(BRHS/C1)	57.70±0.74	51.40±1.05	55.46±1.21
Bacillus altitudinis	(BRHS/P 22)	46.80±0.55	50.46±0.52	44.77±0.92
Bacillus altitudinis	(BRHS/ S 73)	46.39±0.48	46.83±0.60	40.54±0.89
Enterobacter cloacae	(BRHS/R 71)	57.65±0.70	59.42±0.67	57.53±0.29
Bacillus pumilus	(BRHS/T 382)	56.16±0.95	44.36±0.63	48.97±0.48
Bacillus pumilus	(BRHS/T 384)	34.68±0.15	38.13±0.85	35.80±0.33
Burkholderia symbiont	(BRHS/P 92)	47.63±0.20	42.20±0.49	41.00±3.01
Bacillus aerophilus	(BRHS/B 104)	64.48±1.05	47.33±0.48	59.49±0.44
Paenibacillus polymyxa	(BRHS/R 72)	56.30±0.45	49.20±0.41	51.73±0.93
Bacillus methylotrophicus	(BRHS/P-91)	59.86±0.75	58.47±0.86	62.87±0.73

Mean value of three replicates; ± Standard error

Table 5 : Total chlorophyll content of Rice leaves following treatments with PGPR

Treatments	Total chlorophyll content(mg/g tissue)		
	Black Nuniya	Champasari	Brimful
Control	12.17±0.10	11.35±0.06	12.93±0.23
Bacillus pumilus (BRHS/C1)	14.67±0.22	14.50±0.15	16.08±0.31
Bacillus altitudinis (BRHS/P 22)	12.81±0.21	13.60±0.07	13.50±0.11
Bacillus altitudinis (BRHS/ S 73)	12.84±0.07	11.98±0.17	12.60±0.18
Enterobacter cloacae (BRHS/R 71)	10.50±0.02	11.37±0.06	12.71±0.11
Bacillus pumilus (BRHS/T 382)	12.78±0.34	12.44±0.06	13.54±0.18
Bacillus pumilus (BRHS/T 384)	14.68±0.04	14.73±0.08	14.82±0.26
Burkholderia symbiont (BRHS/P 92)	14.87±0.17	15.64±0.23	15.80±0.10
Bacillus aerophilus (BRHS/B 104)	15.11±0.11	15.24±0.14	14.55±0.52
Paenibacillus polymyxa (BRHS/R 72)	11.69±0.45	13.07±0.19	12.68±0.43
Bacillus methylotrophicus (BRHS/P-91)	12.69±0.09	14.47±0.09	14.62±0.93

Mean value of three replicates; ± Standard error

Table 6 : Evaluation of Disease index for Brown spot in Rice plants following treatments with PGPR

Treatments	Black Nuniya		Champasari		Brimful	
	PDI(%)	Mean diameter of lesion(mm.)	PDI(%)	Mean diameter of lesion(mm.)	PDI(%)	Mean diameter of lesion(mm.)
Control	76.19	2.1	31.08	1.6	69.33	2.0
Bacillus pumilus (BRHS/C1)	26.18	1.7	28.80	0.6	13.33	2.1
Bacillus altitudinis (BRHS/P 22)	09.83	2.0	48.19	3.0	32.67	0.3
Bacillus altitudinis (BRHS/ S 73)	22.54	1.5	41.17	1.9	62.50	1.4
Enterobacter cloacae (BRHS/R 71)	16.92	0.6	34.54	2.2	16.54	1.6
Bacillus pumilus (BRHS/T 382)	19.73	1.8	36.17	0.9	24.50	1.5
Bacillus pumilus (BRHS/T 384)	32.94	0.8	44.14	0.5	47.05	1.0
Burkholderia symbiont (BRHS/P 92)	28.40	1.5	44.79	1.5	44.79	1.6
Bacillus aerophilus (BRHS/B 104)	54.42	2.0	46.30	1.0	25.8	1.8
Paenibacillus polymyxa (BRHS/R 72)	38.45	0.8	52.80	1.5	30.56	0.6
Bacillus methylotrophicus (BRHS/P-91)	37.95	0.5	61.12	1.5	14.47	0.4

PDI- Percentage of Disease Index

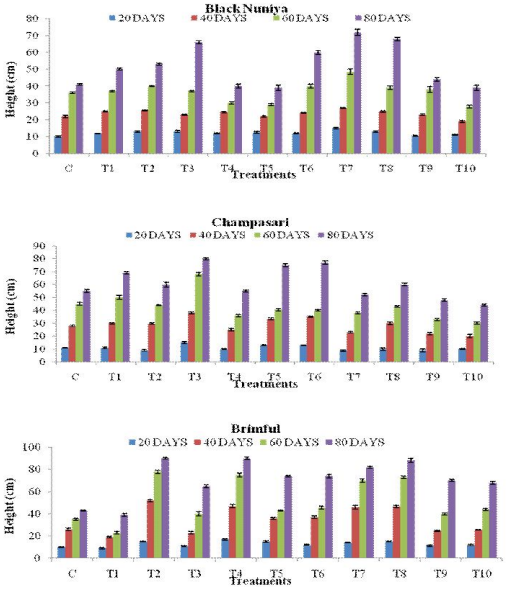


Fig.1: Increase in the height of Rice cultivars following different treatments at specific time intervals
[C-control,T1-*Bacillus pumilus* (BRHS/C1), T2-*Bacillus altitudinis* (BRHS/P 22), T3-*Bacillus altitudinis* (BRHS/ S 73), T4-*Enterobacter cloacae* (BRHS/R 71), T5-*Bacillus pumilus* (BRHS/T 382), T6-*Bacillus pumilus* (BRHS/T 384),T7- *Burkholderia symbiont* (BRHS/ P 92), T8-*Bacillus aerophilus* (BRHS/B 104), T9- *Paenibacillus polymyxa* (BRHS/R 72), T10-*Bacillus methylotrophicus* (BRHS/ P-91)]

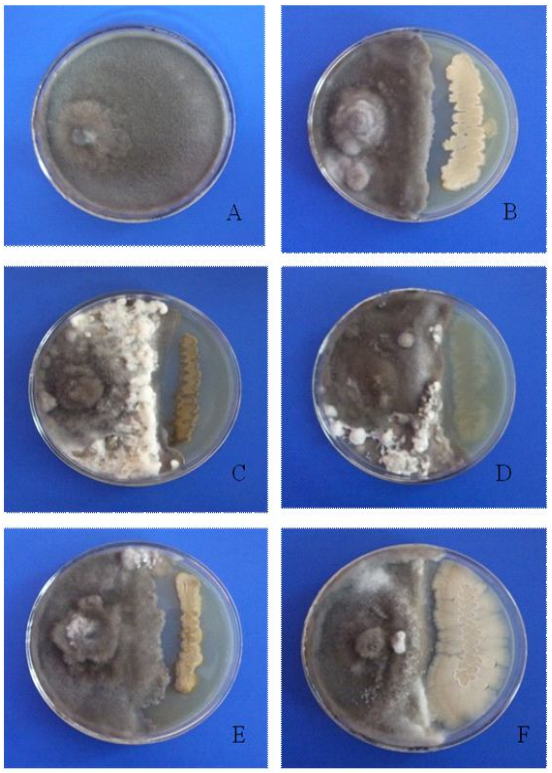


Fig.3: *In vitro* antifungal activities of PGPR against *Drechslera oryzae*
[Inhibition of *Drechslera oryzae* in dual plate culture assay by BRHS/ S 73 (B),BRHS/C 1 (C),BRHS/R 71 (D), BRHS/T 384 (E),BRHS/P 92 (F). Control (A)]

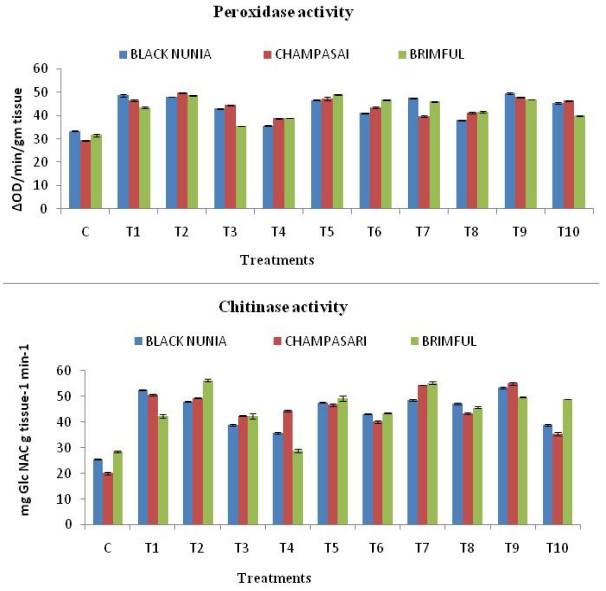


Fig.2: Activity of defense enzymes (Peroxidase and Chitinase) in Rice cultivars following different treatments
[C-control,T1-*Bacillus pumilus* (BRHS/C1), T2-*Bacillus altitudinis* (BRHS/P 22), T3-*Bacillus altitudinis* (BRHS/ S 73), T4-*Enterobacter cloacae* (BRHS/R 71), T5-*Bacillus pumilus* (BRHS/T 382), T6-*Bacillus pumilus* (BRHS/T 384),T7- *Burkholderia symbiont* (BRHS/ P 92), T8-*Bacillus aerophilus* (BRHS/B 104), T9- *Paenibacillus polymyxa* (BRHS/R 72), T10-*Bacillus methylotrophicus* (BRHS/ P-91)]

enhancement in protein content of which highest accumulation was obtained in treatment containing *Bacillus altitudinis* (BRHS/P 22). Maximum protein content in all the cultivars ranged between 71-95 mg/gm tissue (Table 2). Total phenols showed variations according to the treatments. Highest amount of total phenol was obtained in plants treated with *Bacillus altitudinis* (BRHS/P 22) in all the three cultivars. Total phenol content ranged between 7-8 mg/gm tissue (Table 3). In case of total sugar and chlorophyll content, results revealed that here also maximum accumulation occurred in treatment with *Bacillus methylotrophicus* (BRHS/P-91) and *Bacillus pumilus* (BRHS/C-1) (Table 4, 5). Total soluble protein, total phenol, total sugar and total chlorophyll content when estimated were found in increased amount in treated plant in comparison to control set of plant. Similar result was found in study of previous worker in the experiment on plant growth promoting rhizobacteria mediated improvement of health status of tea plants (Chakraborty *et al,* 2013).

Activity of defense enzymes in Rice plants

Defense enzymes activity when tested showed sig-

Table 7 : *In vitro* pairing of PGPR isolates with *Drechslera oryzae*

Interacting microorganisms		Diameter of fungal colony (cm)	% of inhibition
Drechslera oryzae		9.50±0.15	-
D. oryzae + Bacillus altitudinis	(BRHS/S73)	1.50±0.14	84±1.73
D. oryzae + Bacillus pumilus	(BRHS/C1)	1.98±0.21	79±1.63
D. oryzae + Enterobactor cloacae	(BRHS/R71)	2.10±0.23	77±1.73
D. oryzae + Bacillus pumilus	(BRHS/T384)	2.21±0.27	76±1.62
D. oryzae + Burkholderia symbiont	(BHRS/P92)	2.46±0.24	74±1.54
D. oryzae + Bacillus altitudinus	(BRHS/P22)	2.51±0.22	73±1.52
D. oryzae + Bacillus pumilus	(BRHS/T 382	2.52± 0.20	72±1.46
D. oryzae + Bacillus aerophilus	(BRHS/B 104)	2.53±0.23	72±1.45
D. oryzae + Paenibacillus polymyxa	(BRHS/R 72)	2.59± 0.25	71±1.43
D. oryzae + Bacillus methylotrophicus	(BRHS/P-91)	2.60±0.22	70±1.42

Mean value of three replicates; ± Standard error; Diameter of fungal colony after 7 days growth (cm)

nificant variation according to the treatment and higher activity was observed in treated rice plants rather than control set of plants. More enzymatic activity were found in plants treated with *Bacillus altitudinis*(BRHS/P 22), *Burkholderia symbiont* (BRHS/P 92), R72- *Paenibacillus polymyxa* (BRHS/R 72) (Figure 2). The results of our study agreed with the previous findings (Jha *et al,* 2013) where similar results were obtained on paddy plants inoculated with PGPR show better growth physiology and nutrient content under saline conditions.

Influence of PGPR on natural disease and antagonism

Rice plants were under observation from seedling stage to mature stage and data was collected for the establishment of natural disease caused by *Drechslera oryzae* under natural condition and disease index were prepared accordingly which showed higher amount of PDI percentage in control set of plant (76.19%) in comparison with the plants treated with PGPR (9.83%) (Table 6). *In vitro* pairing of PGPR isolates with *Drechslera oryzae* was also conducted as a result *Bacillus altitudinus* (BRHS/S73) showed the maximum percentage of inhibition followed by *Bacillus pumilus* (BRHS/C1), *Enterobacter cloacae* (BRHS/ R71), *Bacillus pumilus* (BRHS/T384) and *Burkholderia symbiont* (BRHS/ P92) (Table 7)(Figure 3).

HPLC analysis

HPLC analysis was done for detecting the phytoalexin namely Phytocassanes with the leaves of Rice cultivar Black nuniya in untreated inoculated and PGPR (*Bacillus altitudinus*) treated and inoculated plants. Treated plants had exhibited lowest PDI percentage. A total of 10 peaks were clearly visible in healthy as well as treated plants inoculated with the pathogen. However the compounds increased markedly in treated inoculated plants as evident in peak heights of nos. 2, 3, 5, 6 and 10 (Figure 4).

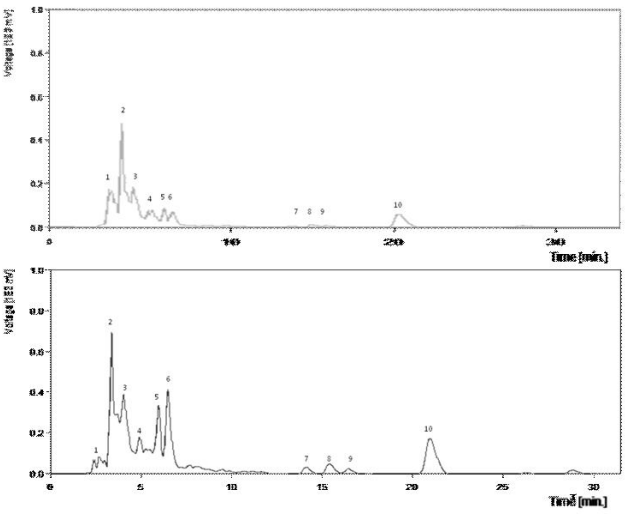


Fig. 4: HPLC analysis of Phytocassanes in Rice plant (cultivar Black nuniya) following treatment with *Bacillus altitudinus* (BRHS/ S73) [A. Treated healthy B. Treated infected]

ACKNOWLEDGEMENTS

Financial help received from Indian Council of Agricultural Research (ICAR) Govt. of India under AMAAS network project is gratefully acknowledged.

REFERENCES

Adesemoye A. O. and Egamberdieva D. 2013. Beneficial Effects of Plant Growth-Promoting Rhizobacteria on Improved Crop Production: Prospects for Developing Economies. Springer-Verlag Berlin Heidelberg .*Bacteria in Agrobiology*: *Crop. Prodct.*45-46.

Araujo A.E.D.S., Baldani V.L.D., Galisa P.D.S., Pereira J.A. and Baldani J.I. 2013. Response of traditional upland rice varieties to inoculation with selected diazotrophic bacteria isolated from rice cropped at the Northeast region of Brazil. *Applied Soil Ecol*. **64,** 49-55.

Boller T. and Mauch F.1998. Colorimetric assay for chitinase. *Methods . Enzymol.* **161,** 430-435.

Chakraborty, B.N.,Chakraborty, U., Sunar, K. and Dey, P.L.2014. *Harnessing beneficial microbial resources for crop improvement.* In : D.P.Singh and H.B.Singh (eds.) Trends in Soil Microbial Ecology, Studium Press, USA,pp.175-201.

Chakraborty U., Chakraborty B.N. and Kapoor M. 1993. Changes in the levels of peroxidase and phenyl alanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia. Microbiol*. **38,** 491-496.

Chakraborty U., Chakraborty B.N., Chakraborty A.P., Sunar K. and Dey P.L. 2013. Plant growth promoting rhizobacteria mediated improvement of health status of tea plants. *Ind. J. Biotech.* **12,** 20-31.

Harborne J.B. 1973. *Phytochemical Methods*, Chapman and Hall, Ltd., London, pp. 49-188.

Jha, Y. and Subramanian, R.B. 2013. Paddy plants inoculated with PGPR show better growth physiology and nutrient content under saline conditions. *Chil. J. of Agri. Res.* **73:,** 213-219.

Lowry O.H., Rosebrough N.J, Farr A.L. and Randall R. J.1951. Protein measurement with folin phenol reagent. *J. Bio. Chem*. **193,** 265-275.

Mahadevan A. and Sridhar R.1982. *Methods in physiological plant pathology*. 2nd Ed. Sivakami Pub. India.

Mia, M.A.B., Shamsuddin Z.H. and Mahmood M., 2012. Effects of rhizobia and plant growth promoting bacteria inoculation on germination and seedling vigor of lowland rice. *African J. Biotechnol.* **11,** 3758-3765.

Plummer D. 1978. *An introduction to plant biochemistry*. Tata Mc Graw Hill Pub., New Delhi. 326.

Saharan B.S. and Nehra V.2011. Plant growth promoting rhizobacteria: A critical review. *Life Sci. Med. Res*., **21,** 1-29.

Shirinzadeh A., Soleimanzadeh H. and Shirinzadeh Z. 2013. Effect of Seed Priming with Plant Growth Promoting Rhizobacteria(PGPR) on Agronomic Traits and Yield of Barley Cultivars. *W. App. Sci. J.* **21:** 727-731.

Umemura K.,Ogawa N.,Shimura M.,Koga J.,Usami H. and Kono T. 2003. Possible Role of Phytocassane, Rice Phytoalexin, in Disease Resistance of Rice against the Blast Fungus *Magnaporthe grisea*.*Biochem.***67,**899-902.

NBU Journal of Plant Sciences

Vol. 9, No. 1 March 2015 p. 78-86
ISSN No. 09746927

Morphological characterization of rice cultivars their root colonization with arbuscular mycorrhizal fungi and screening for field resistance caused by brown spot disease

Sweata Khati and Bishwanath Chakraborty*

Immuno Phytopathology Laboratory, Department of Botany, University of North Bengal, Siliguri-734013, West Bengal, India.

Abstract

Variability in seed morphology was studied in 15 rice cultivars using qualitative and quantitative characters. Germplasm of these indigenous rice landraces were collected from Bijanbari, Kalimpong, Sikkim, Malda, Siliguri and UBKV (Uttar Banga Krishi Viswavidyalaya). Data were recorded for traits such as Kernel colour, Seed coat colour, Aroma, Presence of Awn and Length of the seed. A total of 9 landraces had white kernel colour while 4 had brown and 2 had greyed-orange .The seed coat colour variation in different landraces ranged from Golden yellow, Yellow, Red and Black. 6 landraces were having aroma whereas 9 had no aroma and lastly 11 landraces were found to have awn and 4 were awnless.UBKV-4 was longest in length with 1.1 cm and Sano masuri being the smallest of 0.4 cm. Establishment of disease in naturally infected rice cultivars were observed and disease index was calculated. Arbuscular Mycorrhizal Fungi (AMF) were screened from rhizosphere of fifteen rice cultivars grown on experimental field using wet sieving and decanting method. Microscopical observation revealed the presence of different genus of AM fungi present in the roots as hyphae, spores and sporocarp. Among the different AM fungi species of *Glomus* sp.were found to be high in all the fifteen cultivars of rice plants followed by *Gigaspora*, *Scutellospora* and *Acaulospora*. Histopathological study of roots showed the presence of vesicles and arbuscules. AMF infection and total number of spores per 100 grams of soil were recorded. Present study evaluates the study of different AMF population and their histopathology harbouring in the rhizosphere of rice.

Keywords: Rice cultivars, Morphological traits, AM Fungi.

Introduction

Rice (*Oryza sativa* L., family Poaceae) is the leading staple food crop of India, grown in almost all the states, covering more than 30 per cent of the total cultivated area (Adhikari *et al.,* 2012; Chakravorty *et al.,* 2013). West Bengal is called as 'bowl of rice' with over 450 rice landraces (Deb D., 2005; Chatterjee *et al.,* 2008). Diversity studies in rice using morphological characters were done on improved and ancestral rice varieties of Philippines (Caldo *et al.,* 1997; Juliano *et a*l., 1998) and on Asian wild cultivated indigenous rice in Yunnan, China (Zeng *et al.,* 2003).

Agro-morphological traits, both qualitative and quantitative have been commonly and traditionally used to estimate relationships between genotypes (Goodman M.M., 1972). Variation due to adaptation to specific ecosystems selection and socio-economic condition resulted in differentiation in different named landraces of a region (Bajracharya *et al.,* 2006). Variability study for rice landraces from West Bengal was undertaken by (Chakravorty *et al.,* 2013). Keeping in view the under representation of rice landrace diversity from West Bengal and Sikkim 15 different rice cultivars were selected and studied for seed morphology, associated knowledge on local use of collected landraces was recorded to help in characterization of rice germplasm from this region.

Arbuscular Mycorrhizal Fungi (AMF) are vital components of the microbial soil community forming the most commonly occurring underground symbiosis between members of phylum *Glomeromycota* and roots of 80% of all terrestrial plant species (Wang *et al.,* 2008; Schüßler *et al.,* 2001). AMF are the key species groups that inter-connect plants into a functional web (Hegalson *et al.,* 1998), extending plant root systems and thereby, facilitates plants uptake of soil nutrients of poor mobility, especially phosphorus (Smith and Read, 2008).

*Corresponding Author:
Email: bncnbu@gmail.com

Besides, AMF improve plant fitness by improving seedling establishment, plant fecundity, tolerance to some root pathogens, water relations and formation and stability of soil aggregates (Read, 1999; Newsham *et al.,* 1995a). Efforts are being undertaken to develop a bio formulation which can reduce the disease occurrence.

Considering the importance of AMF for disease resistance in rice plants present investigation was made to assess the AMF population from fifteen different rice varieties collected from different areas of hilly and plain regions and were grown on experimental field of Immuno Phytopathology Lab, Department of Botany, NBU.

Materials and methods

Collection of Rice Seeds

Rice seeds were collected from different regions of West Bengal and Sikkim. Brimful, Champasari and Black Nuniya from Bijanbari. Kaberi 9090, Loknath 505 and Gouraknath 509 from Siliguri. Sano masuri and Adde from Sikkim. Attheu and Maiti from Kalimpong .Swarnamasuri and Tulai panji from Malda and finally UBKV-1, UBKV-4 and UBKV-5 from Uttar Bangha Krishi Viswavidyalaya (UBKV) respectively.

Morphological study and measurement of seeds

Seed morphology was recorded paying attention to Kernel colour, seed coat colour, aroma, presence of awn and finally the length of the seed was noted.

Disease Assessment

Establishment of naturally occurring brown spot disease was observed and disease severity was assessed in terms of lesion number per leaf and infection index calculated as described by Adlakha et al. (1984). For percent disease index (PDI) calculation, the following formula was used-[(class rating x class frequency)/(total no. of leaves x maximum rating)] x 100.

Isolation of AMF spores from soil

Arbuscular mycorrhizal fungal spores were screened from soil samples of fifteen rice varieties rhizosphere by the wet sieving and decanting method (Gerdeman & Nicholson, 1963). Soil samples (100gm each of the root zone) were collected, suspended in water (1 lt) in order to obtain a uniform suspension. Soil clusters are carefully dispersed in the water and is kept for 10 minutes to settle down the heavy particles. Aqueous suspension was passed through a set of sieves of different pore size (200, 170, 150, 80, 50 µm) arranged one below the other. The spores were picked by the help of bristles / brushes and transferred to grooved slides or vials and observed under dissecting microscope. Few spores were stained with Melzar's reagent and studied under sterio-microscope. Healthy spores are separated by fine brush and are stored in autoclaved glass vials either in sterile distilled water or Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in one litre of boiled distilled water) at 4ºC for further study and observation. It is evident from various studies that each plant has multiple AM fungi population.

Identification of AMF spores

Spore samples were separated according to their morphology size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachment etc. for the purpose of identification. The spores were identified with the help of standard keys (Walker, 1981; Schneck and Perez, 1987). Spores were critically examined with special reference to variation in vesicles (size, shape, wall thickness, wall layer, position and abundance), hyphal branching patterns, the diameter, structure (specially near entry points) and the staining intensity of hyphae.

Spore count

Rhizosphere soil (100gm) was taken and suspended in 250ml water. Wet sieving and decanting method was used for isolation of spores. Total number of spores was then counted and spore percentage of different genera was obtained.

Histo-pathological analysis

The root specimen were taken from field and washed with tap water. The roots were cut into pieces, after washing treated with 10% KOH added, kept in water bath for 1hr, then 1% HCl was added to neutralize the alkalinity. The root pieces were then washed with water (after 30 min) and staining was done by shimmering the roots in cotton blue: lactophenol (1:4) for 3-4 min with mild heating. Degree of contrast between fungal tissues and back ground plant cell was obtained according to the duration of storage of tissues. 1% HCl was added to acidify the tissues, as most histological stains are acidic. A little amendment in this process is noteworthy because it has been noticed that extraradical spore bearing hyphae and other extraradical fungal tissue with root segments are destroyed or dissolved when it is boiled in hot water bath at 90ºC twice with 2% KOH followed by 0.05 cotton blue and lacto glycerol for staining the internal structures of AMF inside the root segment i.e. arbuscules, vesicles, auxilliary cells etc. The total staining process can be done without heating but keeping the root fragments in 1-2% KOH for 24-48 hrs in a Petri dish and another 2-18 hrs in cotton blue and lactoglycerol with minimum movement of the samples yields remarkable results. In this method the spore bearing hyphal structures, auxiliary cells etc. are clearly visible and percent colonization can be determined with better accuracy. After preparing the roots the hyphal structures were viewed under dissecting stereo-microscope under 20X and 40X magnification.

Root colonization

Percent root colonization was estimated by using slide method by (Giovannetti and Mosse, 1980). All the infected and uninfected segments of root tissue and the percentage of infection was calculated as follows

AMF infection (%)=[infected root segments/total fragments of root taken] X 100.

Results and Discussion

Fifteen different rice cultivars were collected from different regions of West Bengal and Sikkim the cultivar name, type, origin and its GPS Location is given in (Table 1).

Table 1. Rice cultivars and its localization.

Sl.No.	Rice Cultivars	Cultivar type	Origin	GPS Location
1.	Brimful	Ethnic	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
2.	Champasari	Ethnic	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
3.	Black Nuniya	Local	Bijanbari	27° 02'N 88° 07'E/ 27.04° N 88
4.	Kaberi 9090	Commercial	Siliguri	26.7100° N,88.4300°E
5.	Loknath 505	Commercial	Siliguri	26.7100° N,88.4300°E
6.	Gouraknath 507	Commercial	Siliguri	26.7100° N,88.4300°E
7.	Sano Musuri	Ethnic	Sikkim	27.3300° N,88.6200°E
8.	Adde	Ethnic	Sikkim	27.3300° N,88.6200°E
9.	Attheu	Ethnic	Kalimpong	27° 04'N 88° 28'E/27.06,88.47°
10.	Maiti	Ethnic	Kalimpong	27° 04'N 88° 28'E/27.06,88.47°
11.	Swarnamasuri	Local	Malda	25.0000° N,88.1500°E
12.	Tulaipanji	Local	Malda	25.0000° N,88.1500°E
13.	UBKV-1	Research	UBKV	26° 24'15" N, 89° 23'5"E
14.	UBKV-4	Research	UBKV	26° 24'15" N, 89° 23'5"E
15.	UBKV-5	Research	UBKV	26° 24'15" N, 89° 23'5"E

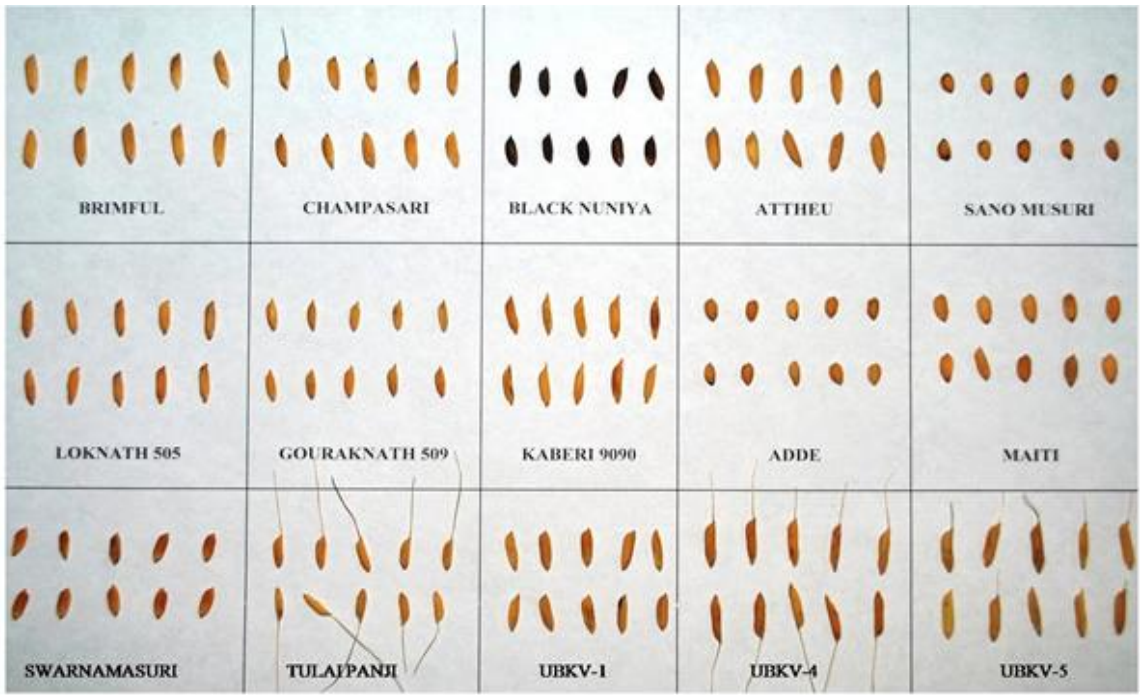


Fig. 1.Fifteen different rice cultivars.

Table 2: Morphological diversity of rice cultivars.

Sl. No.	Rice Cultivar	Area of collection	Kernel colour	Seed coat colour	Aroma	Presense of Awn	Length of the seed (cm)
1.	Brimful	Bijanbari	Brown	Red	Present	Absent	0.9
2.	Champasari	Bijanbari	White	Red	Absent	Present	0.8
3.	Black Nuniya	Bijanbari	Brown	Black	Present	Absent	0.7
4.	Attheu	Kalimpong	White	Yellow	Present	Absent	0.9
5.	Sano Masuri	Sikkim	White	Yellow	Absent	Absent	0.4
6.	Loknath 505	Siliguri	White	Golden Yellow	Absent	Absent	0.8
7.	Gouraknath 509	Siliguri	White	Golden Yellow	Present	Absent	0.7
8.	Kaberi 9090	Siliguri	White	Golden Yellow	Absent	Absent	0.9
9.	Adde	Sikkim	Brown	Yellow	Present	Absent	0.5
10.	Maiti	Kalimpong	Brown	Yellow	Absent	Absent	0.6
11.	Swarnamasuri	Malda	Greyed orange	Red	Absent	Absent	0.7
12.	Tulaipanji	Malda	Greyed orange	Golden Yellow	Present	Present	0.7
13.	UBKV-1	UBKV	White	Yellow	Absent	Present	0.9
14.	UBKV-4	UBKV	White	Red	Absent	Present	1.1
15.	UBKV-5	UBKV	White	Yellow	Absent	Absent	1.0

Table 3: Population count of AM Fungi in rhizosphere of fifteen different rice cultivars and percentage colonization in root

Sl. No.	Rice Cultivars	Percentage of VAM spore in soil (%)					Root colonization (%)
		Glomus	*Gigaspora*	*Scutellospora*	*Acaulospora*	*Entrophospora*	
1	Loknath 505	78.04	19.59	1.68	0.33	0.33	99 %
2	Gouraknath 509	83.85	15.09	-	1.04	-	91 %
3	Kaberi 9090	67.17	27.30	0.61	4.90	-	93%
4	Champasari	80.0	20	-	-	-	90%
5	Brimful	85.52	13.15	1.3	-	-	99%
6	Black Nuniya	83.33	13.88	-	2.77	-	94%
7	Adde	66.66	31.81	-	1.51	-	95%
8	Sano Masuri	65.94	33.74	-	.30	-	93%
9	Maiti	83.30	13.88	-	2.77	-	97%
10	Attheu	78.02	17.48	1.1	3.36	-	96%
11	Swarnamasuri	69.7	23.25	5.81	1.16	-	93%
12	Tulai Panji	65.19	33.77	1.04	-	-	95%
13	UBKV-1	90.39	5.64	1.12	2.82	-	98%
14	UBKV-4	60.30	36.43	0.75	2.51	-	100%
15	UBKV-5	50.07	41.07	2.52	6.31	-	98%

Seed Morphological Diversity of all the cultivar was observed (Fig. 1) and was seen that a total of 9 landraces had white kernel colour while 4 had brown and 2 had greyed-orange .The seed coat colour variation in different landraces ranged from Golden yellow, Yellow, Red and Black. 6 landraces were having aroma whereas 9 had no aroma and lastly 11 landraces were found to have awn and 4 were awnless. UBKV-4 was longest in length with 1.1 cm and Sano masuri being the smallest of 0.4 cm as shown in (Table 2). Table 3 shows the percentage of different AM fungi in the each soil samples and the maximum population was found to be of *Glomus* sp. followed by *Gigapora* sp., *Acaulospora* sp., *Scutellospora* sp. and

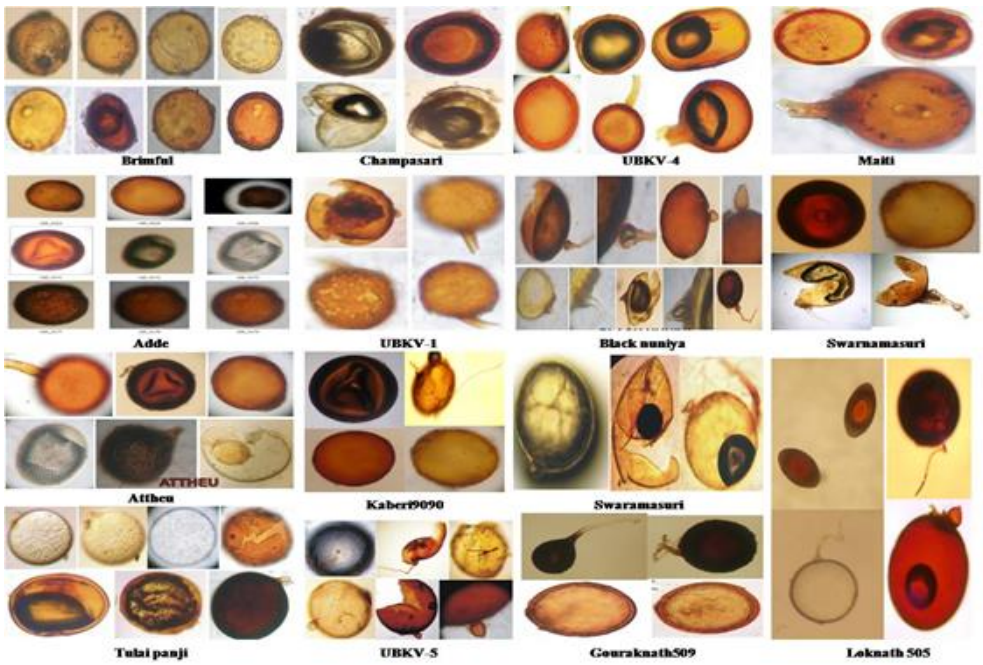


Fig. 2 AMF population collected from rhizospheric soils of rice

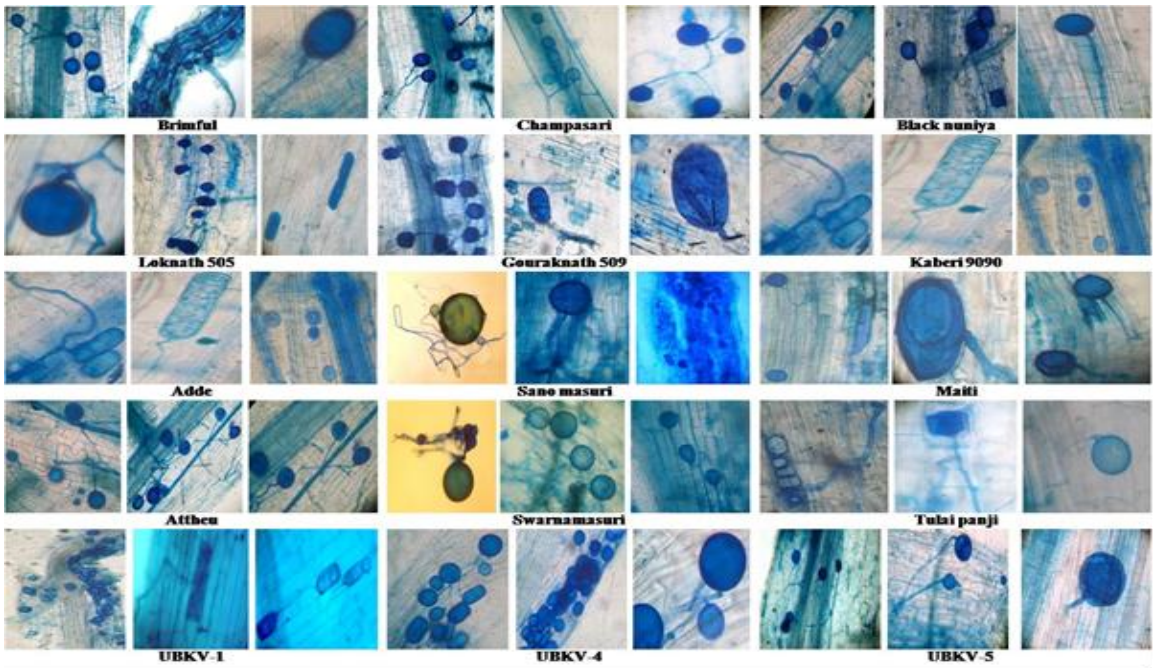


Fig. 3: Observation of rice root colonisation by AMF.

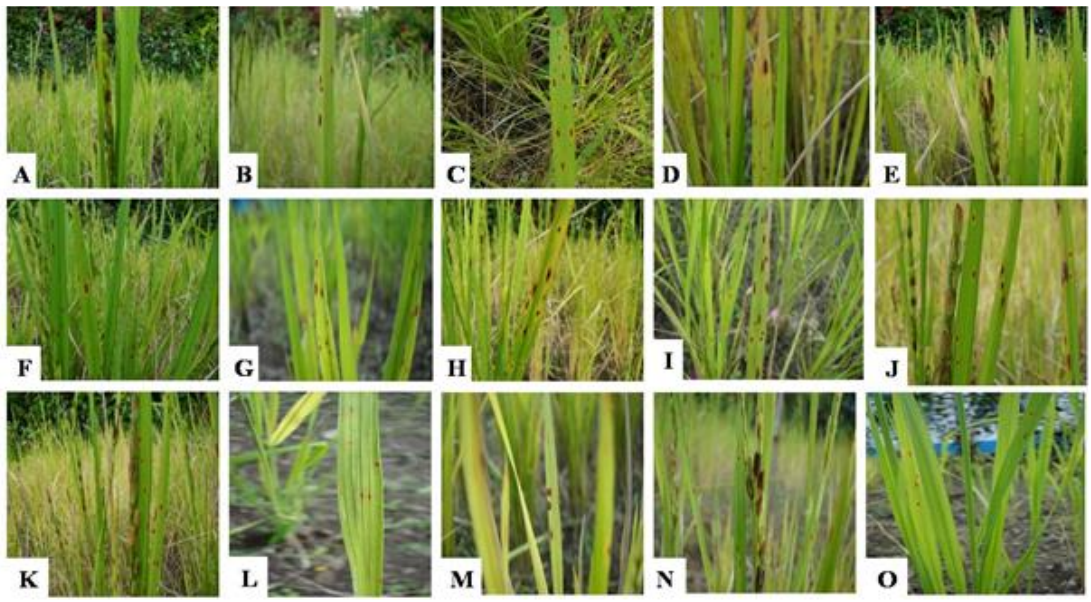


Fig. 4: Rice cultivars grown in experimental plots for study of AMF population and root colonisation. (A) Brimful,(B) Champasari, (C) Black nuniya, (D) Loknath 505, (E) Gouraknath 509,(F) Kaberi 9090 (G) Adde, (H) Sano masuri, (I) Maiti, (J) Attheu, (K) Swarnamasuri, (L) Tulai panji, (M) UBKV-1,(N) UBKV-4,(O)UBKV-5.

Enthropospora sp. was found in the rhizosphere of one of the cultivars.

Morphological and topographical characteristics of plant organs such as the shape and size of seeds and the structure of incidental

features have been useful weapons in identifying and classifying the plant and weed species (Noda *et al.,* 1985). Awn less seed is an improved trait and high diversity in seed shapes and pericarp color may be important for developing quality rice to meet diverse consumer demand.

Table 4: Disease index showing the establishment of natural disease.

Sl.No	Rice cultivars	Disease index (PDI %)
1.	Brimful	62.28
2.	Champasari	51.76
3.	Black Nuniya	52.72
4.	Kaberi 9090	58.47
5.	Loknath 505	41.66
6.	Gouraknath 507	50.05
7.	Sano Musuri	48.36
8.	Adde	59.82
9.	Attheu	49.44
10.	Maiti	47.89
11.	Swarnamasuri	53.33
12.	Tulaipanji	51.85
13.	UBKV-1	41.62
14.	UBKV-4	43.78
15.	UBKV-5	43.66

The role of below-ground soil organisms interacting with plant roots has gained increased attention in recent years (e.g. Reynolds et al., 2003; van der Putten, 2003; Callaway et al., 2004), and the interactions between beneficial and pathogenic organisms have been identified as being particularly relevant due to their important implications for plant fitness (e.g. Schippers et al., 1987; Fitter and Garbaye, 1994; Bever, 2003). Arbuscular Mycorrhizal Fungi were collected and screened from the rhizospheric soil of fifteen rice cultivars grown on experimental plots. The different types of spores which were observed in the rhizosphere of rice soil have been identified. On observation it was found that species of *Glomus* sp. and *Gigaspora* sp. dominated the AM population in all the soil sample (Fig. 2).

Histopathological study revealed the presence of vesicles and arbuscules in the root segments determining the fact that the rice roots has been infected by AMF spores (Fig. 3). Organisms of AMF have a bimodal pattern of differentiation (Morton 1990). The vegetative thallus consists of arbuscules intraradical vesicles (shared only by species in the suborder Glomineae), extra radical auxiliary cells (shared only by species in the suborder Gigasporineae), and intraradical and extra radical hyphae (Smith and Read, 1997; Morton and Benny, 1990). Arbuscules are finely branched structures in close contact with the cell plasma membrane, functioning in exchange of nutrients between host and fungal cells (Smith and Read, 1997). Hyphae are important in nutrient acquisition and as propagules to initiate new root colonization (Graham *et al*., 1982; Friese and Allen, 1991). Vesicles are globose structures arising from swelling of the hyphae and filled with glycogen granules and lipids are considered to be storage structures (Bonfante-Fasolo, 1984; Brundrett, 1991).

Under the natural condition the establishment of the brown spot disease was observed after four month growth of the rice plants grown on experimental plots (Fig.4) and Disease index (PDI%) was calculated. DI of rice cultivar Brimful was found to be the maximum with 62.28 and that of UBKV-1 to be minimum with 41.62 PDI% (Table 4).

Conclusion

The traits recorded during germplasm collection are listed on the basis of feedback from farmers and present data gives preliminary observations and require further validation after characterization /evaluation. Characterization of landraces could help breeders to utilize appropriate characters in rice improvement programme. The present investigation provides the base material for the rice breeders for exploitation of landraces possessing one or more desirable characters. The overall results of the present study have shown some of the important facts of the indigenous AM Mycorrhizal fungi present in experimental soils capable of infecting rice roots. Among the different types of AM fungi collected and observed *Glomus* sp. was found to be widely distributed in rhizosphere of rice plants in experimental plots. The present results also suggested that the rice plant may be considered as an initial stock plant which may be

used for inoculum production in departmental climatic condition. In future, the most modern and advanced technology should be considered for large-scale inoculum production of AM fungus under field condition.

Acknowledgement

Financial help received from Indian Council of Agricultural Research (ICAR) Govt. of India under AMAAS network project is gratefully acknowledged.

Reference

Adhikari, B., Bag, M.K, Bhowmick, M.K. and Kundu, C. (2012). Rice in West Bengal-rice knowledge management portal (www.rkmp.co.in), Directorate of Rice Research, Rajendranagar, Hyderabad, Andhra Pradesh, p 88.

Adlakha, K.L., Wilcoxson, R.D. and Raychaudhuri, S.P. (1984). Resistance of wheat spot blotch caused by *Bipolaris sorokiniana*. *Pl. Dis.,* 68:320-321.

Bajracharya, J., Steele, K.A., Jarvis, D.I., Sthapit, B.R. and Witcombe, J.R. (2006). Rice landrace diversity in Nepal: Variability of agro-morphological traits and SSR markers in landraces from a high-altitude site. *Field Crops Res* .95:327-335.

Bever, J.D. (.2003). Soil community feedback and the coexistence of competitors: conceptual frame works and empirical tests. *New Phytologist*. 157: 465–473.

Bonfante-Fasolo, P. (1984). Anatomy and Morphology of VA mycorrhiza. In: *VA Mycorrhizae*. C.L.I. Powell and D.J. Bagyaraj (Eds.).CRC Press, Boca Raton, Florida. Pp. 5-33.

Brundett, M.C. (1991). Mycorrhizas in natural ecosystems. *Advances in Ecological Research* 21:171-213.

Caldo, R.A, Sebastian, L.S. and Hernandez, J.E. (1997). Diversity of Philippines improved rice varieties and their progenitors using morphological and molecular markers. *Philip. J. Crop Sci.*, 22:11-26.

Callaway, R.M., Thelen, G.C., Barth, S., Ramsey, P.W., Gannon, J.E. (2004). Soilfungi alter interactions between the invader *Centaurea maculosa* and North American natives. *Ecol.* 85:1062-1071.

Chakravorty, A., Ghosh, P.D. and Sahu, P.K. (2013). Multivariate analysis of phenotypic diversity of landraces of rice of West Bengal. *Am. J. Exp. Agri.*, 3: 110-123.

Chatterjee, S.D., Adhikari, B., Ghosh, A., Ahmed, J., Neogi, S.B. and Pandey, N. (2008). The rice bio-diversity in West Bengal. Department of Agriculture, Govt. of West Bengal, p 50.

Das Gupta, A. (2013). Indigenous knowledge and women entrepreneurs among Rajbanshis: a case study. *Int. Res. J Soc. Sci* 2. 2: 12-20.

Deb, D. (2005). Seeds of tradition, seeds of future: folk rice varieties from east India. Research Foundation for Science Technology & Ecology, New Delhi, India, p 136.

Fitter, A.H. and Garbaye J. (1994). Interaction between mycorrhizal fungi and other soil microorganisms. *Pl. Soil.* 159:123-132.

Friese, C.F., and Allen, M.F. (1991). The spread of VA mycorrhizal fungal hyphae in the soil: Inoculum types and external hyphae architecture. *Mycol. Res.* 92:317-321.

Gerdeman, J.W., and Nicholson, T.H. (1963). Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Trans. Br. Mycol. Soc.* 46:235-244.

Giovannetti, M. and Mosse, B. (1980). An evaluation of techniques to measure vesicular-arbuscular infection in roots. *New Phytol*. 84: 489-500.

Goodman, M.M. (1972). Distance analysis in biology. *Syst Zool* .21:174-186.

Graham, J.H., Linderman, R.G., and Menge, J.A. (1982). Development of external hyphae by different isolates of mycorrhizal *Glomus* spp in relation to root colonization and growth of troyercitrange. *New Phytopathol.* 91: 183-189.

Helgason, T., Daniell, T.J., Husband, R., Fitter, A.H. and Young, J.P.W. (1998). Ploughing up the wood-wide web? *Nature*. 394:431.

Juliano, A.B., Naredo, E.B., Jackson, M.T. (1998). Taxonomic status of *Oryza glumaepatula* Steud. I. Comparative morphological studies of new world diploids and Asian AA genome species. *Genet. Resour. Crop. Evol* .45: 197-203.

Morton, J.B. (1990). Evolutionary relationships among arbuscular mycorrhizal fungi in the Endogonaceae. *Mycologia.* 82. 192-207.

Morton, J.B. and Benny, G.I. (1990). Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon.* 37:471-491

Newsham, K.K., Fitter, A.H. and Watkinson, A.R., (1995a). Arbuscular mycorrhiza protect an annual grass from root pathogenic fungi in the field. *J. Ecol*. 83: 991–1000.

Noda, K., Prakongvongs, C. and Chaiwiratnukul, L. (1985). Topography of the seeds and leaves of tropical weeds – with a scanning electron

microscope. National Weed Science Research Institute Project.p.158.

Read, D.J. (1999). Mycorrhiza-the state of the art. In: Varma, A., Hock, B. (Eds.), Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology. *Springer*. 3-34.

Schcnck, N.C. and Perez, Y. (1987).Manual for the Identification of VA Mycorrhizal Fungi. Second Edition. International Culture Collection of VA Mycorrhizal Fungi (INVAM), University of Florida, Gainnesville, Florida.

Schippers, B., Bakker, A.W. and Bakker, P.A.H.M. (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Ann. Rev. Phytopathol.* 25: 339–358.

Schüßler, A., Schwarzott, D. and Walker, C. (2001). A new fungal phylum Glomeromycota: phylogeny and evolution. *Mycol. Res.* 105: 1413–1421.

Smith, S.E. and Read, D.J. (1997). Mycorrhizal Symbiosis. 2nded Academic Press, London. 605pp.

Smith, S.E. and Read, D.J. (1997). Mycorrhizal Symbiosis. 2nded Academic Press, London. 605pp.

van der Putten, W.H. (2003).Plant defense belowground and spatiotemporal processes in natural vegetation.*Ecology* 84: 2269–2280.

Walker (1981). *Acaulospora spinosa* sp. nov. With a key to the species of *Acaulospora. Mycotaxon*. 12: 512-521.

Wang, C.X., Li, X.L., Zhou, J.C., Wang, G.Q. and Dong, Y.Y. (2008). Effects of arbuscular mycorrhizal fungi on growth and yield of cucumber plants*, Comm. Soil Sci. Pl. Anal.* 39: 499-509.

Zeng, Y., Shen, S., Li, Z., Yang, Z., Wang, X., Zhang, H. and Wen, G. (2003). Eco-geographic and genetic diversity based on morphological characters of indigenous rice (*Oryza sativa* L.) in Yun- nan, China. *Genet. Resour. Crop. Evol*. 50: 567-577.