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.