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Activation of defense response in rice cultivars against *Drechslera oryzae* following root colonization with AMF and combined application of PGPR and PGPF

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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. *Drechslera oryzae* the causal organism for brown spot disease of rice has been a threat out of many, which highly impacts on the productivity rates of rice. Bioinoculants such as PGPR(*Bacillus altitudinus*), PGPF(*Trichoderma harzianum*) and AMF(*Rhizophagus fasciculatus*) were applied in dual and in combined form for three rice cultivars (Brimful, Champasari and Black nuniya) and challenge inoculated with *Drechslera oryzae*. Seed coating and foliar spray of all the bioinoculants caused plant growth promotion but significant increase was obtained in case of combined treatment. Disease incidence was markedly reduced following application of bioinoculantswith a sharp increase in polyphenolic accumulations and activities of four major defense enzymes (peroxidase, phenylalanine ammonia lyase, chitinase and glucanase). Biochemical parameters such as total soluble proteins and reducing sugar and chlorophyll content were also evaluated following treatment. HPLC analysis of combined treated inoculated plants showed the highest level of phytoalexin accumulation suggesting induction of resistance in rice plants against brown spot disease.

Keywords : *Bacillus altitudinus, Drechslera oryzae,* HPLC, *Rhizophagus fasciculatus* rice, *Trichoderma harzianum*

INTRODUCTION

Among the various other cereal crops cultivated in our state rice occupies the major area of farming about 53% (Adhikari *et al*. 2012). 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).

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 (Gerhardson, 2002). Biological control is therefore being considered as an alternative way of reducing the use of chemicals in agriculture as reported by (Gerhardson, 2002;Postma *et al*. 2003; Welbaum *et al*. 2004).Morphological characterization of rice cultivars and their root colonization with arbuscular mycorrhizal fungi (AMF) have been studied (Khati and Chakraborty, 2015). Use of AMF for induction of resistance against *Drechslera oryzae* have also been reported (Khati and Chakraborty, 2019). 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. On the other hand,*Trichoderma harzianum* has also been well documented as potential biocontrol agent (BCA) for the management of plant diseases

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(Chakraborty and Chakraborty, 2020; Khati and Chakraborty, 2023).

The objectives of this study were to determine the effect of three potential beneficial microorganisms (PGPR, PGPF and AMF) alone and in combination on improvement of health status of rice plants and induction of resistance towards fungal pathogen causing brown spot disease

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.

Bioinoculants

Rhizophagus fasciculatus (AMF), *Trichoderma harzianum* (NAIMCC-F-03288) (PGPF) and *Bacillus altitudinus* (NAIMCC-B01485) (PGPR).

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 and combined treatments application was done accordingly. Growth parameters were finally recorded after two months of application of last treatment.

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% CuSO4 and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% Na_2CO_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). Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

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.

Assessment of defense enzymes in leaves
Extraction

By using suitable buffers and liquid nitrogen enzymes were extracted from life tissues. For extraction of chitinase and β-1,3-glucanase 0.1M sodium acetate buffer (pH=5) was used.Phenylalanine ammonia lyase was extracted using 0.1M sodium borate buffer (pH=8.8) and peroxidase was extracted using 0.1 M sodium phosphate buffer (pH=8.8).

Assay

Chitinase (CHT, EC 3.2.1.14) activity was measured according to the method described by

Boller and Mauch (1988). The enzyme activity was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg N-acetyl glucosamine (GlcNAc) released/min/ g fresh wt.

Assay of β-1, 3-glucanase (β- GLU, EC 3.2.1.38) activity was done by following the laminarin dinitrosalicylate method described by Pan *et al.* (1991). The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissue.

Phenylalanine ammonia lyase (PAL- EC.4.3.1.5) was assayed following the method described by Chakraborty *et al.* (1993) with modifications. PAL activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The enzyme activity was expressed as µg cinnamic acid produced min^{-1} g^{-1} fresh tissue

Assay of peroxidase (POX, EC1.11.1.7) activity was done following the method of (Chakraborty *et al*. 1993). The reaction mixture contained 1 ml of 0.2 Na-phosphate buffer (pH 5.4), 1.7 ml dH_2O, 100 µl crude enzyme, 100 µl O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidaseas described by Davis (1964).

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.

Localization of chitinase and glucanase by immunofluorescence

Indirect fluorescence staining of cross-section of tea leaves was done using FITC labelled goat antirabbit lgG following the method of Chakraborty and Saha (1994).

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).

HPLC Analysis of phytoalexin phytocassanes

For phytocassanes extraction 2gm. of rice leaf sample was cut into small pieces and shaken with 20ml. 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 following by evaporation in rotary evaporator. To measure the amount of Phytocassanes induced following the treatment, the supernatant was collected after the extraction procedure and put through High Performance Liquid Chromatography (HPLC) eluted with 45 % acetonitrile. (UV-VIS Detector and Liquid Chromatogram, SHIMADZU). Phytocassanes were monitored at 280 nm (Umemura *et al*. 2003).

RESULTS AND DISCUSSION

Effects of bioinoculants on growth of Rice plants

All the bioinoculants, PGPR (*B. altitudinus,* NAIMCC-B01485), PGPF (*T. harzianum*, NAIMCC-F-03288) and AMF (*R. fasciculatus*) 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.Growth enhancement in terms of height was measured after 20, 40, 60 and 80 days of final treatment. The results as shown in Table1revealed 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.

Changes in biochemical activity Sugar, Chlorophyll, Proteins and Phenols

Total sugar content and total chlorophyll content was quantified for all three rice cultivars following the treatment and it was observed that the content

Table 1 : 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	1.00	4.66	6.24	6.48
	Cultivars	0.77	3.61	4.84	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*)

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.2).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.3A) 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 2.

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 (*R. fasciculatus*)then the plots

Table 2: 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)
	Control	35.02±0.05	13.45±0.52	32.02±0.25	3.68±0.12
Brimful	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	3.85	0.88	2.29	0.27
	Cultivars	2.98	0.68	1.78	0.21

± Standard Error. Average of three replicates. PGPR- *Bacillus altitudinus,*PGPF- *T. harzianum*and AMF- *R. fasciculatus*;

Table 3: Effects of antifungal compound (Phytocassanes) from rice leaf extracts of (cv. Black Nuniya) following treatment with bioinoculants (*T. harzianum* ,*R. fasciculatus* and *B. altitudinus*) 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)

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.1).

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 Fig. 2revealed thatlevels of defense enzymes were increased in bioinoculants 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 which is in favour to the results obtained by Chakraborty *et al*. (2016 a & 2016b).

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

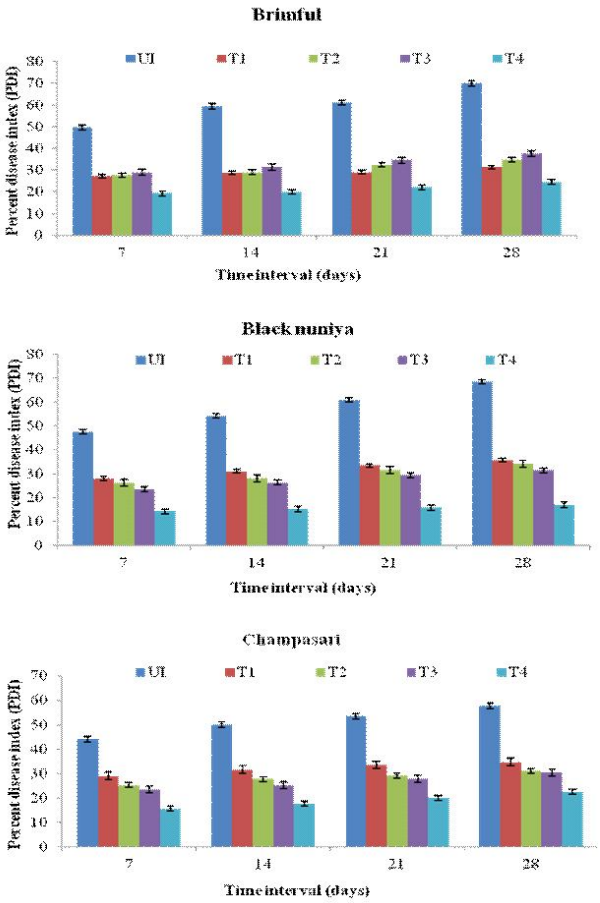

Fig. 1: Percent disease index in rice cultivars following treatment and artificial inoculation with *D.* ***oryzae.*** (UI- Untreated inoculated, T1- *Bacillus altitudinus* + *R. fasciculatus*, T2- *T. harzianum*+ *R. fasciculatus*, T3= *T. harzianum*+ *Bacillus altitudinus*and T4= *T. harzianum*+ *R. fasciculatus* + *Bacillus altitudinus*

samples(Fig.3B) and this is line with the findings of (Chakraborty *et al.* 2002a).

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.3, Fig. 4) 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 which correlates with the findings of Grayer and Kokubun (2001).

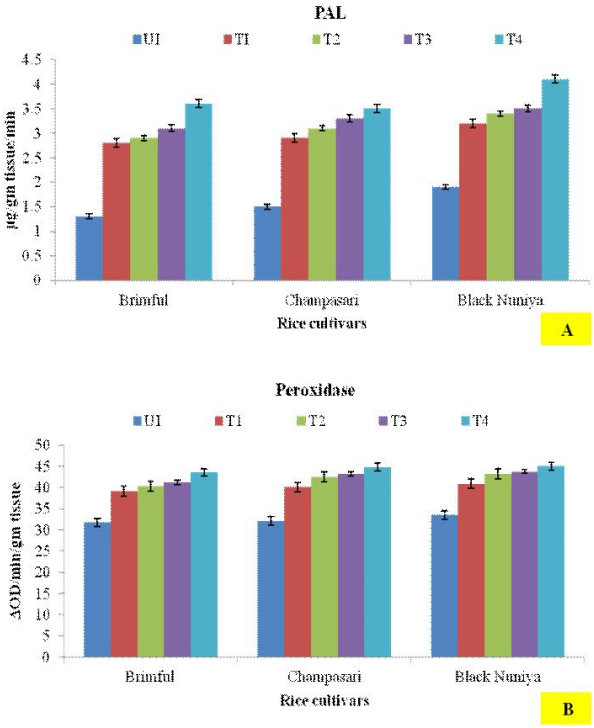

Fig.2: Defense enzyme activity of rice cultivars against *D. oryzae* following dual and combined application of bioinoculants. (A) PAL (Phenylalanine ammonia lyase) and (B) Peroxidase. (UI- Untreated inoculated, T1- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, T2- *T. harzianum* (NAIMCC-F-03288)+ *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)

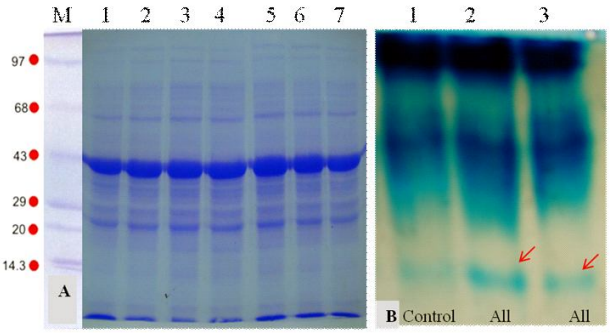

Fig. 3: SDS-PAGE (A) and Peroxizyme analysis (B) of leaf proteins of rice cultivar (Black nuniya) following treatment with bioinoculants and challenged inoculation with *D. oryzae*

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

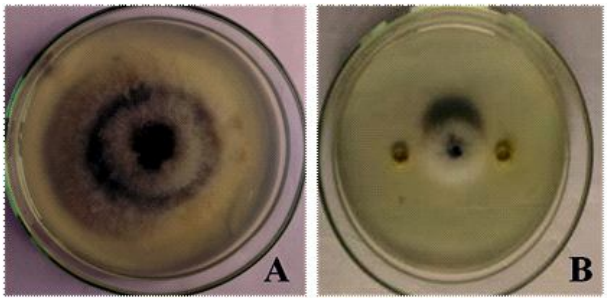

Fig.4: Radial growth bioassay of Ethyl acetate extracts of untreated control and treated inoculated rice leaves (cv. Black Nuniya). (A) Untreated Control and (B) Treated with bioinoculants (*T. harzianum*, *R. fasciculatus* and *B. altitudinus*) and inoculation with *D. oryzae*.

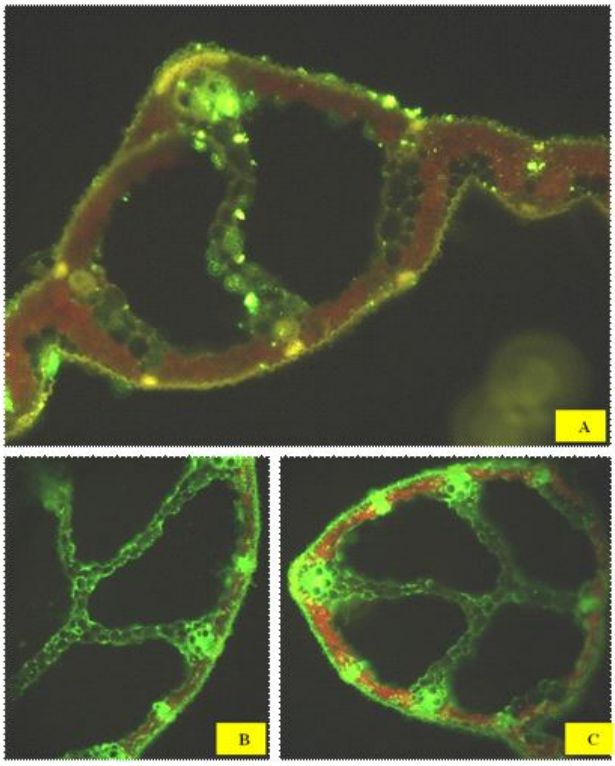

Fig. 5: Cellular localization of glucanase in leaf tissue of rice (cv. Black Nuniya) following combined treatment of bioinoculants and challenge inoculation with fungal pathogen, probed with PAb of glucanase and labelled with FITC. (A) Control and (B&C) Treated.

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.5). Bright apple green fluorescence was observed in treated leaves.

Cellular localization of Chitinase

Cellular localization of chitinase enzyme in leaves of rice plants was determined following indirect

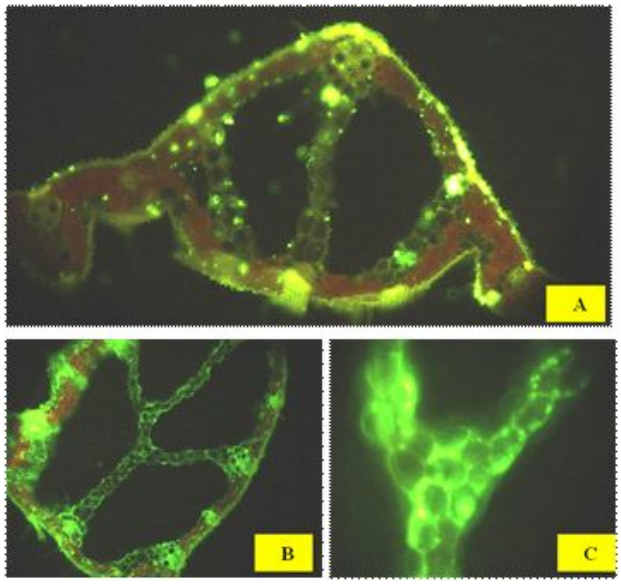

Fig. 6: Cellular localization of chitinase in leaf tissue of rice (cv. Black Nuniya) following combined treatment of bioinoculants and challenge inoculation with fungal pathogen, probed with PAb of chitinase and labelled with FITC. (A) Control and (B&C) Treated.

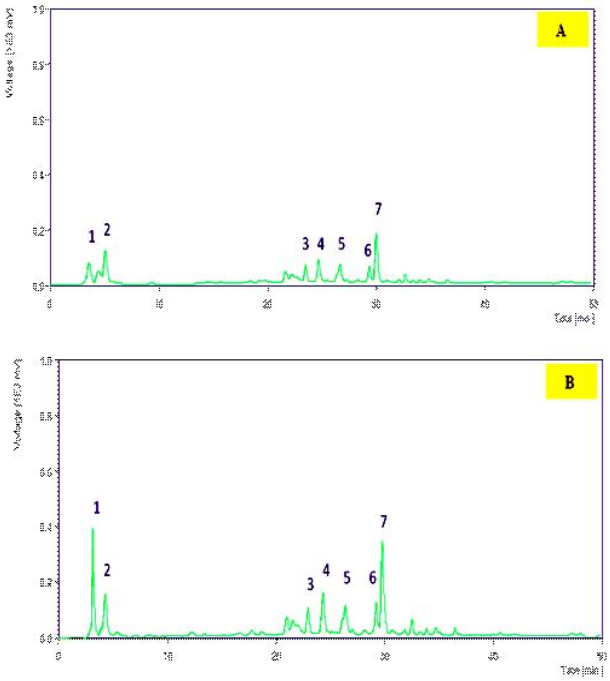

Fig.7 : HPLC analysis of phenolic acid content from leaf extracts of (cv. Black Nuniya). (A) Control and (B) Treated with bioinoculants against pathogen challenge

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

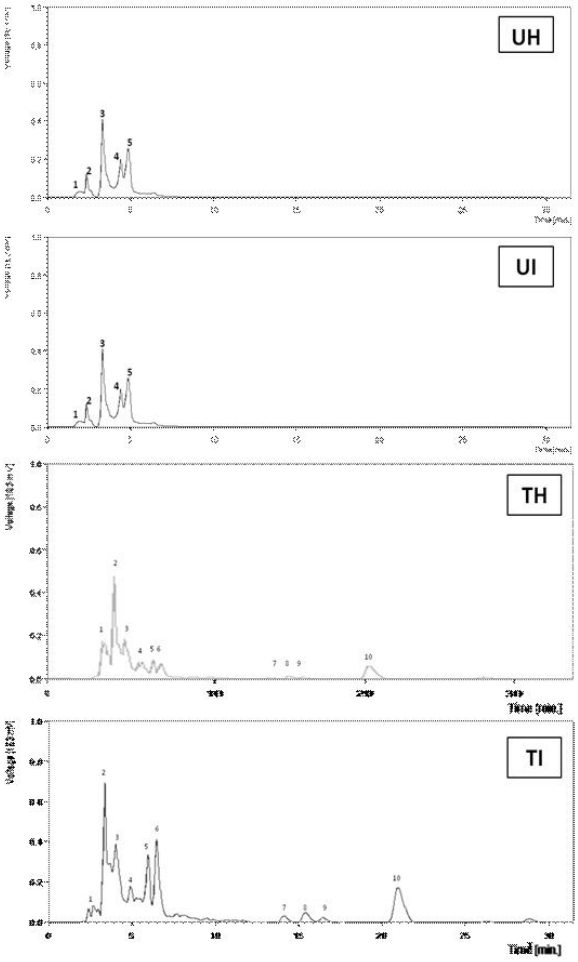

Fig. 8: HPLC analysis of Phytocassanes from leaf extracts of (cv. Black Nuniya) treated with bioinoculants and challenge inoculation with foliar fungal pathogen (*D. oryzae*). UH= Untreated Healthy, UI= Untreated Inoculated, TH= Treated Healthy and TI= Treated Inoculated.

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 (Fig.6). Bright apple green fluorescence was observed in treated leaves which testified the increased accumulation of chitinase enzyme in treated leaves which is in line with the findings of Chakrabortye*t al.* 2004.

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.7). 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.

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 the entire peak (Fig. 8) which is in favour of results obtained by previous workers. 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 is 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.

CONCLUSION

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 defense 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.

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DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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