# 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.

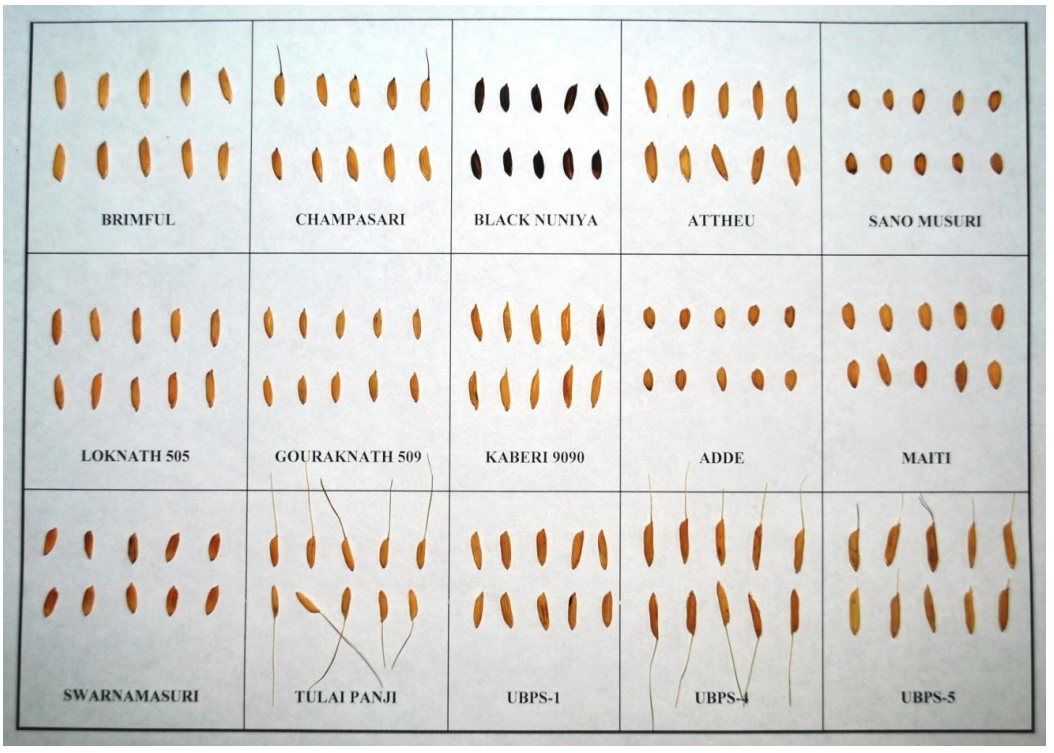



Figure 5: Fifteen different rice cultivars showing morphology

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 .

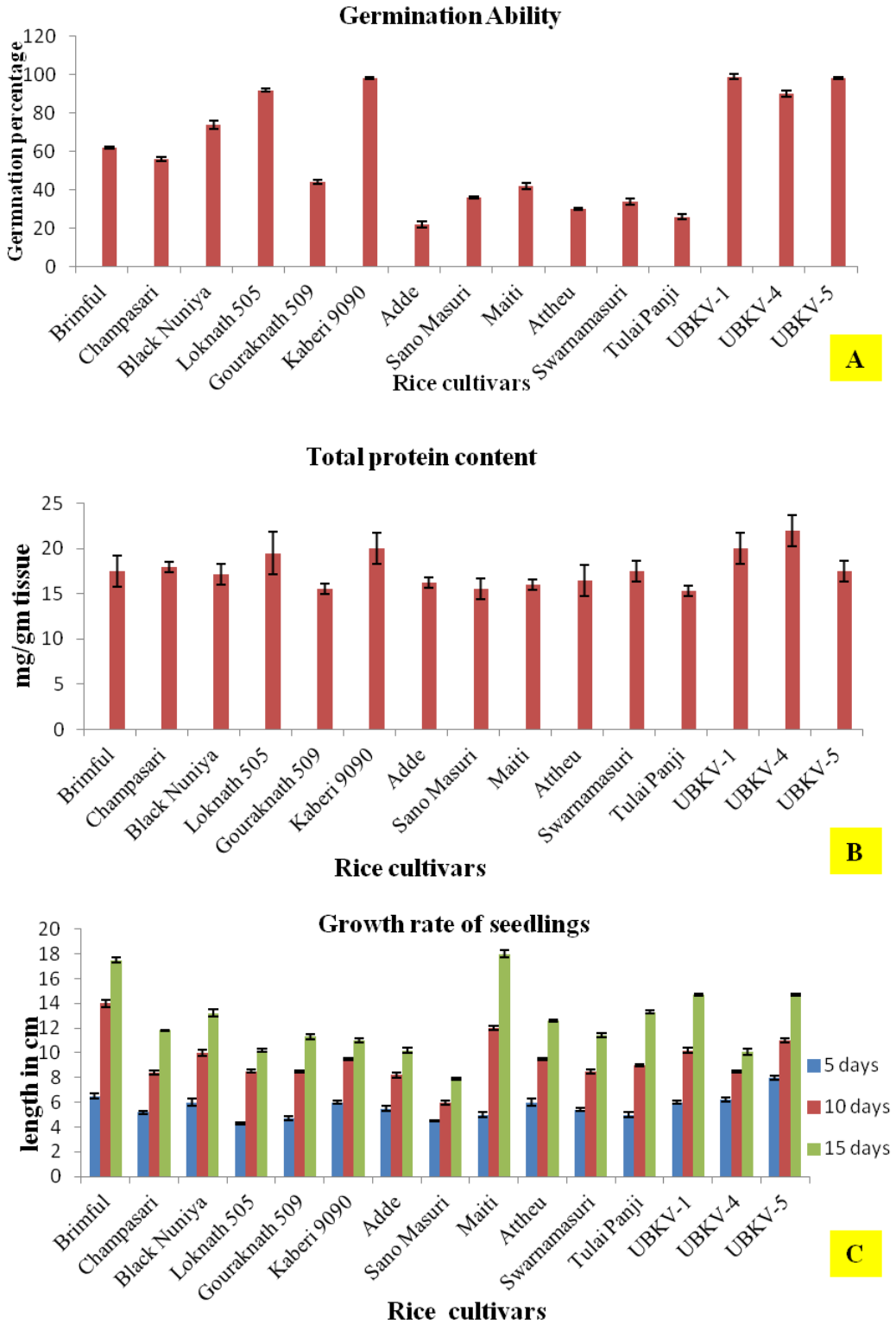


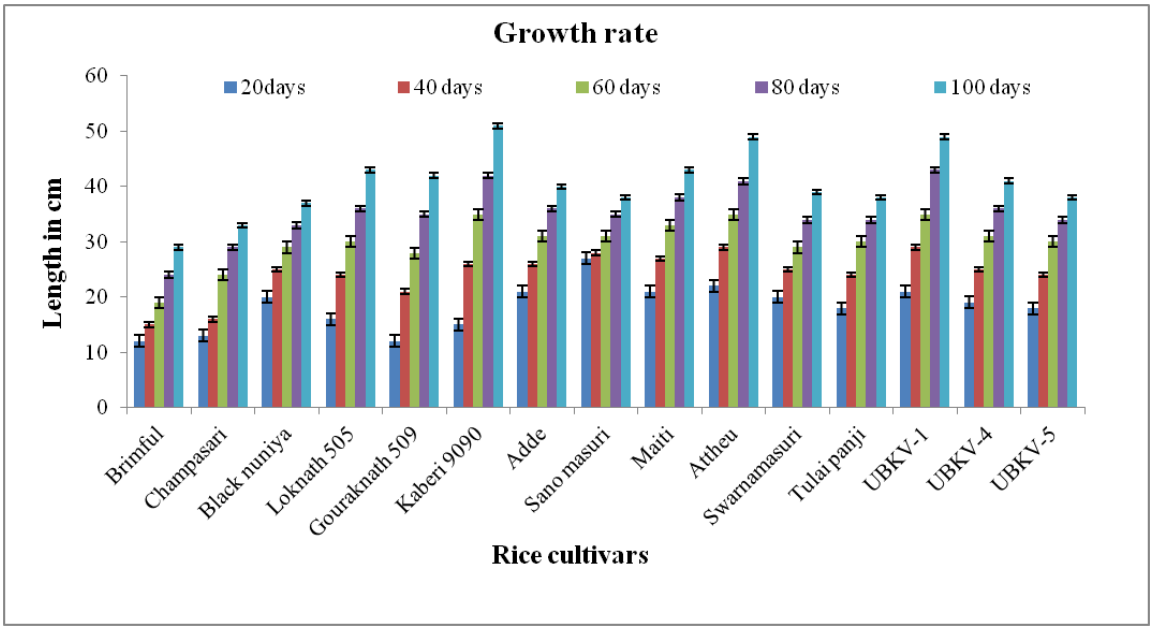

Figure 6: Initial screening of rice cultivars grown in petriplates. (A) Germination ability, (B) Protein content and (C) Growth rate.

### 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.

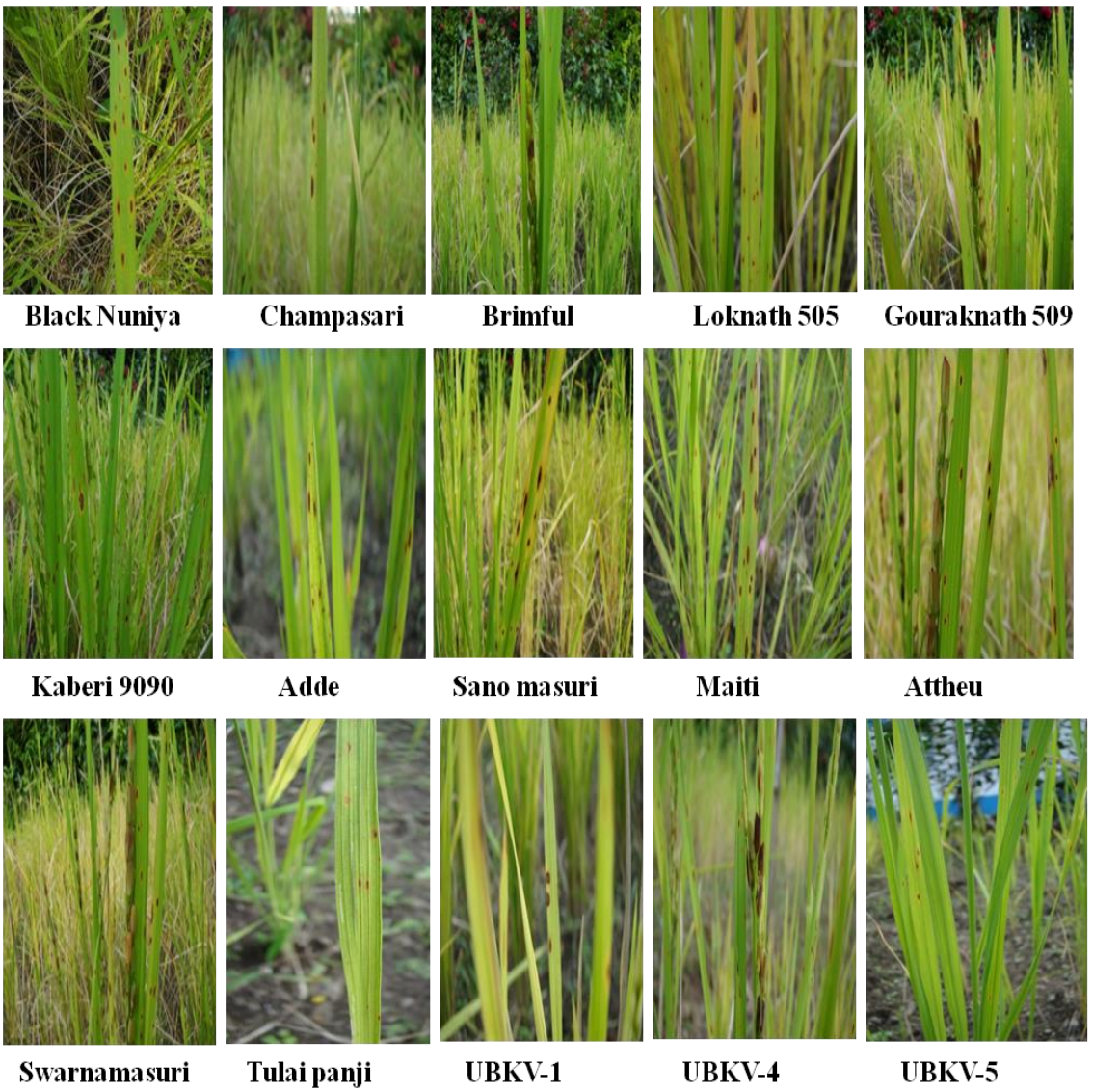



Figure 8: Rice cultivars grown in experimental plot showing natural infection (Brown Spot) caused by *D. oryzae*.

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

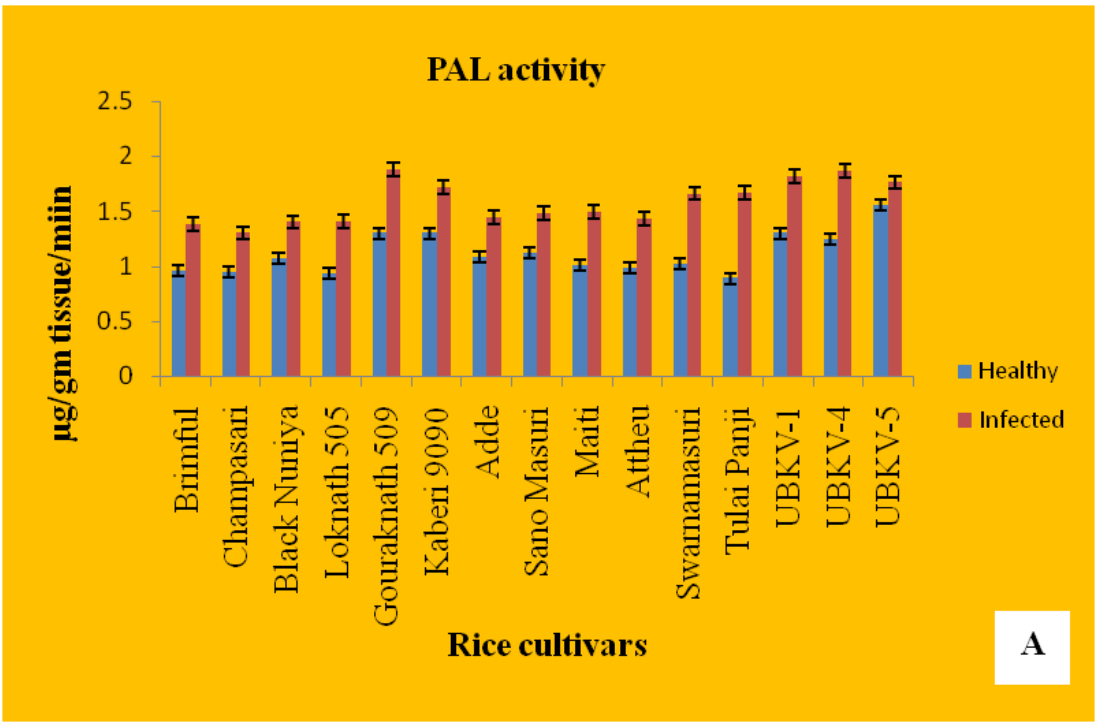
was more in infected samples than in healthy samples. Maximum ammount of PAL accumulation was observed in infected samples of rice cultivar Gouraknath 509(1.88µg/gm tissue/min) and the minimum accumulation was observed in infected samples of rice cultivar Champasari (1.3 µg/gm tissue/min) in comparison 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.



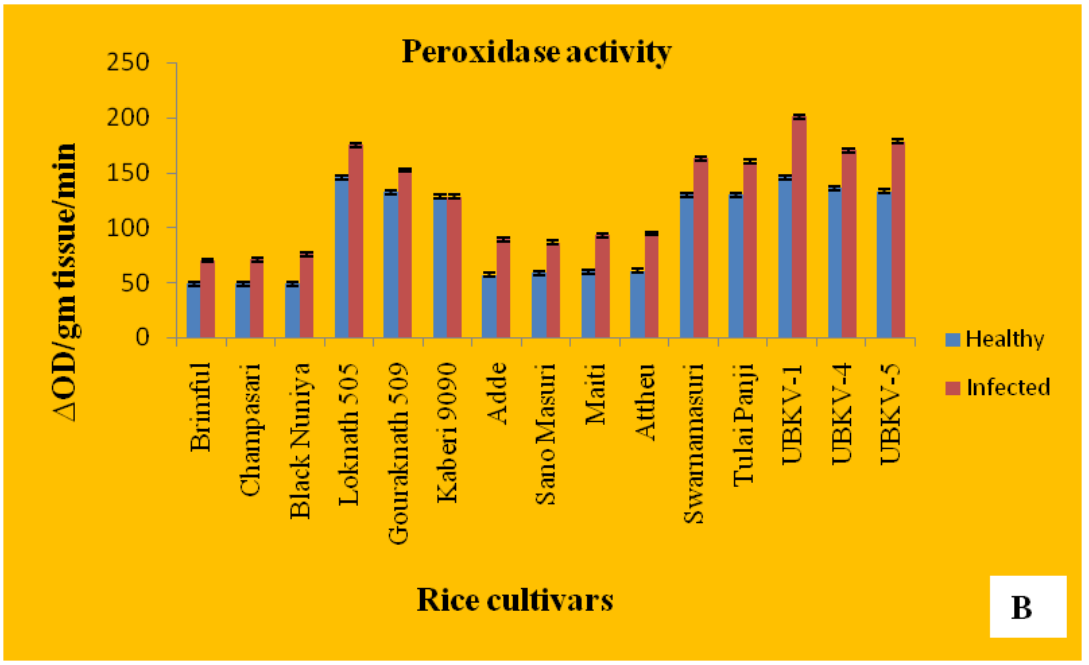
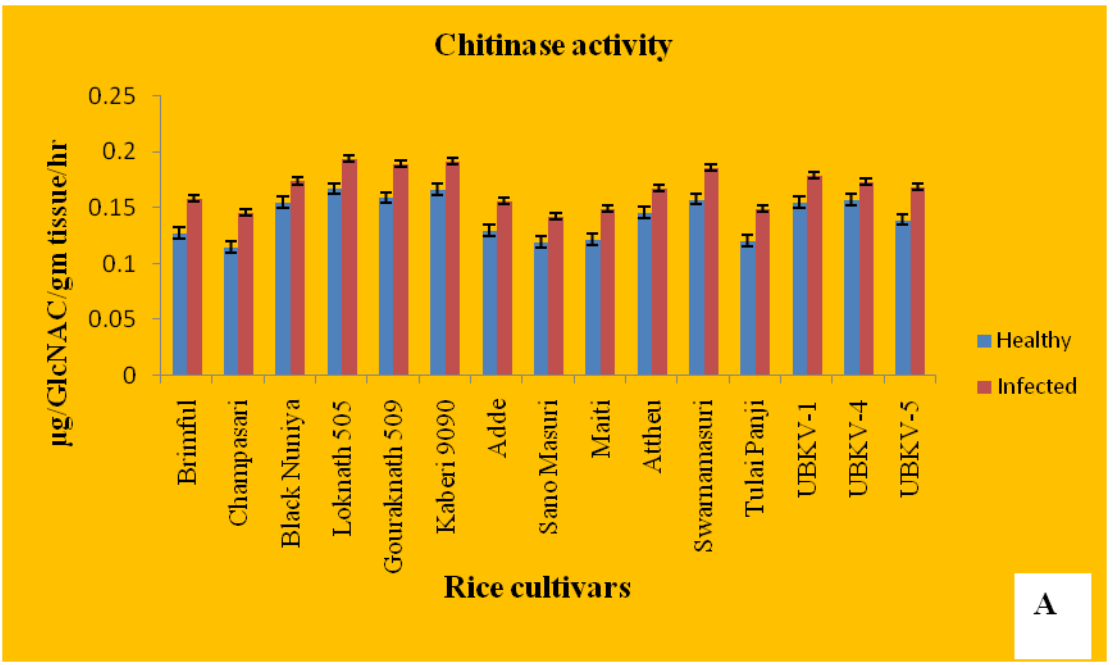




Figure 9: Phenylalanine ammonia lyase (A) and Peroxidase (B) activity in healthy and naturally infected rice leaves.



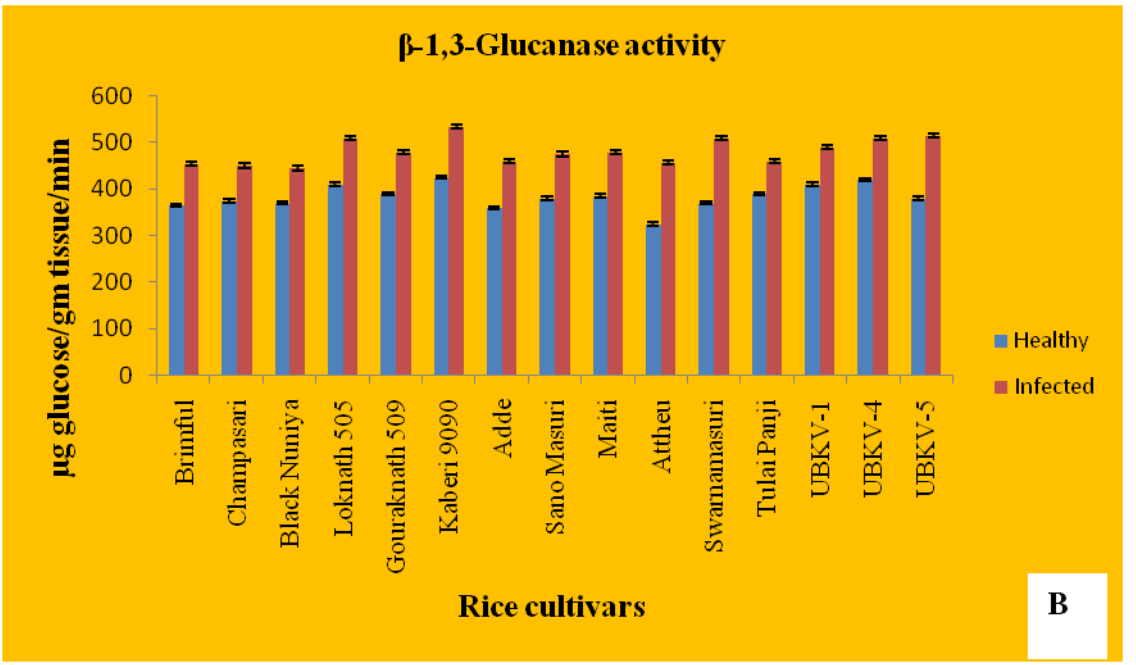




Figure 10: Chitinase (A) and β-1,3-glucanase (B) activity in healthy and naturally infected rice leaf samples

### 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).

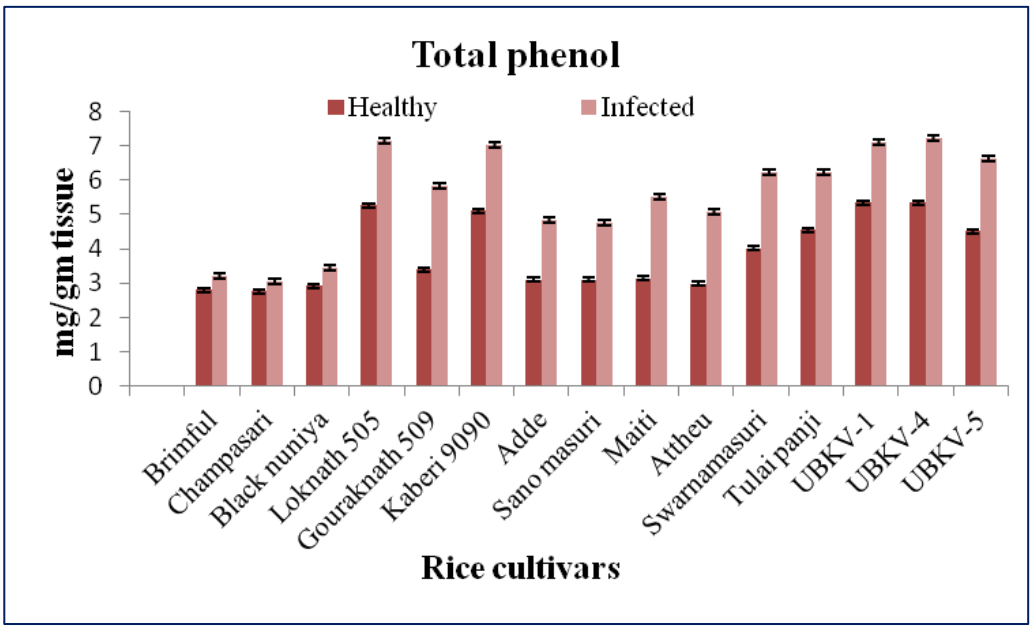



Figure 11: Total phenol content of healthy and naturally infected rice cultivars.

### 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 $28^0$C for further use. Pathogen was also taken for the completion of Koch's postulate for the conformation of disease.

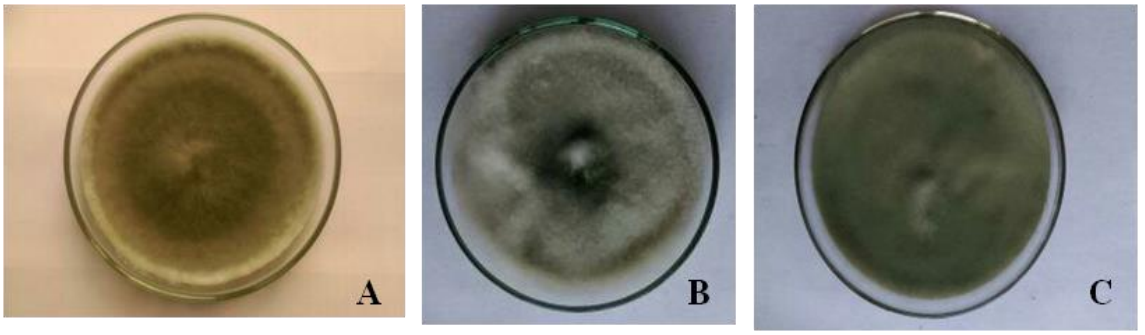



Figure 12: Radial growth pattern of fungi in different media (7-10 days old). (A) Potato dextrose agar, (B) Oats meal agar and (C) Richard's synthetic agar.

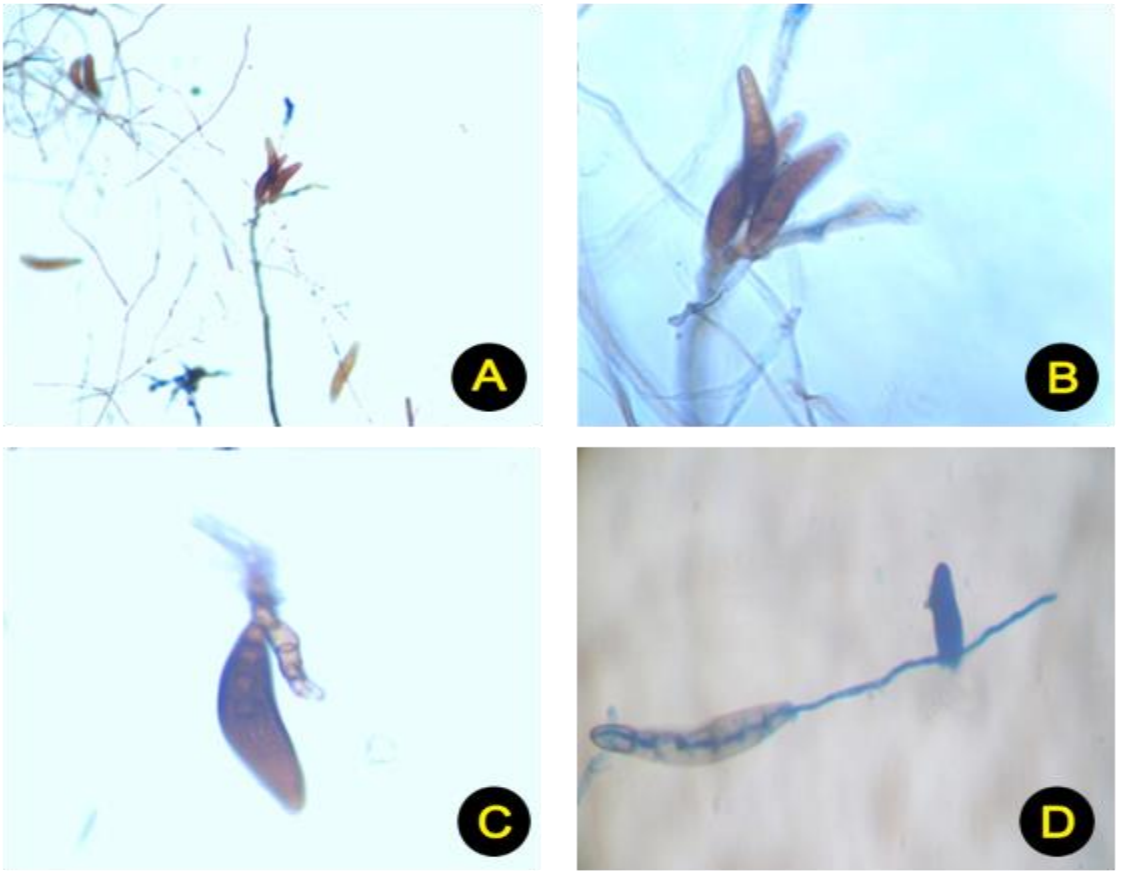



Figure 13: Microscopic view of fungal isolate from infected rice leaves. (A) Conidiophore bearing conidia (10X), (B) Enlarged view of (C 40X), (C) Conidium (40X) and (D) Germinating conidium (40X)

### 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.

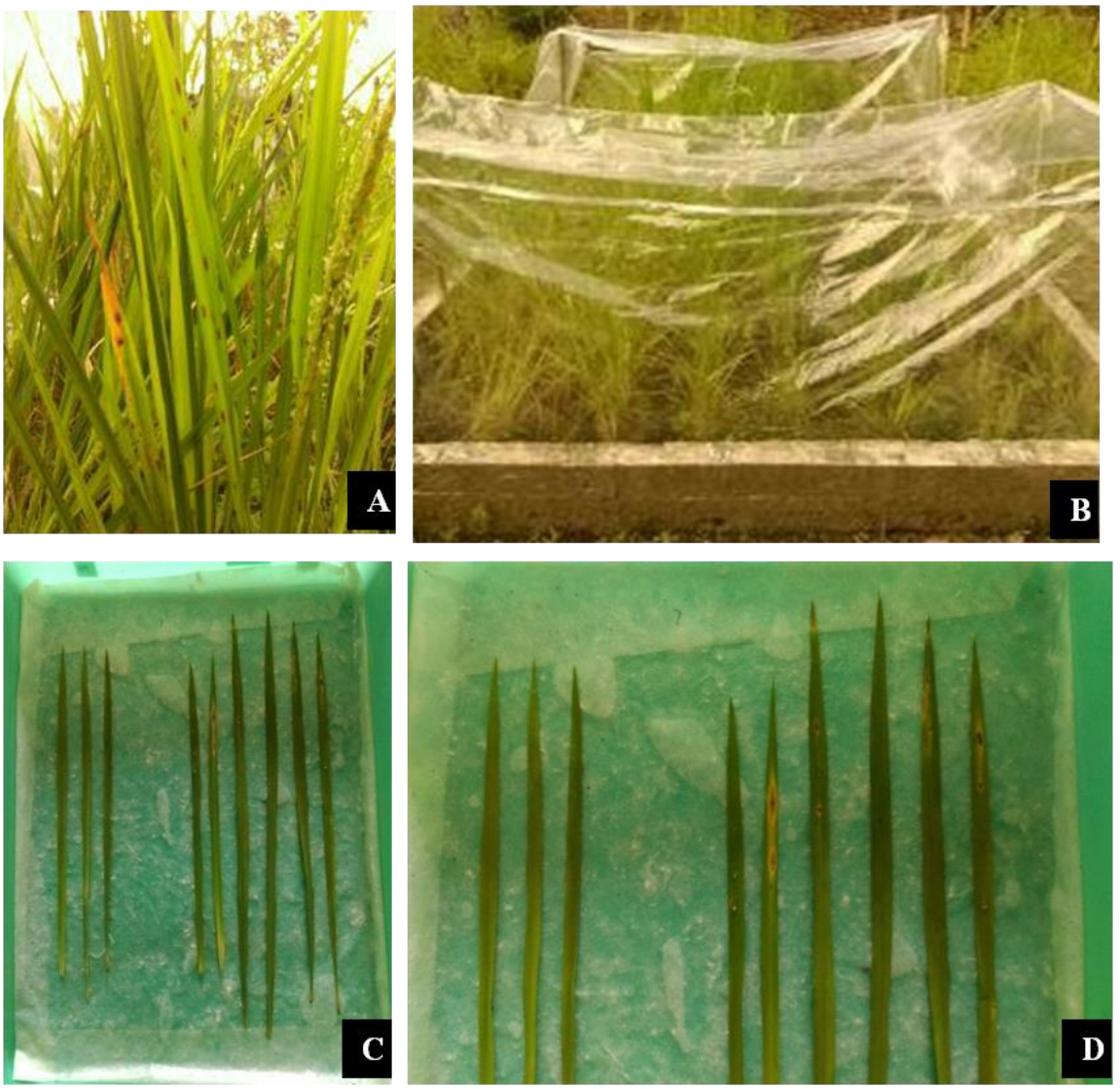



Figure 14: Pathogenecity test of rice cultivars against *D. oryzae*. (A&B) Whole plant (C&D) Detached leaf

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).

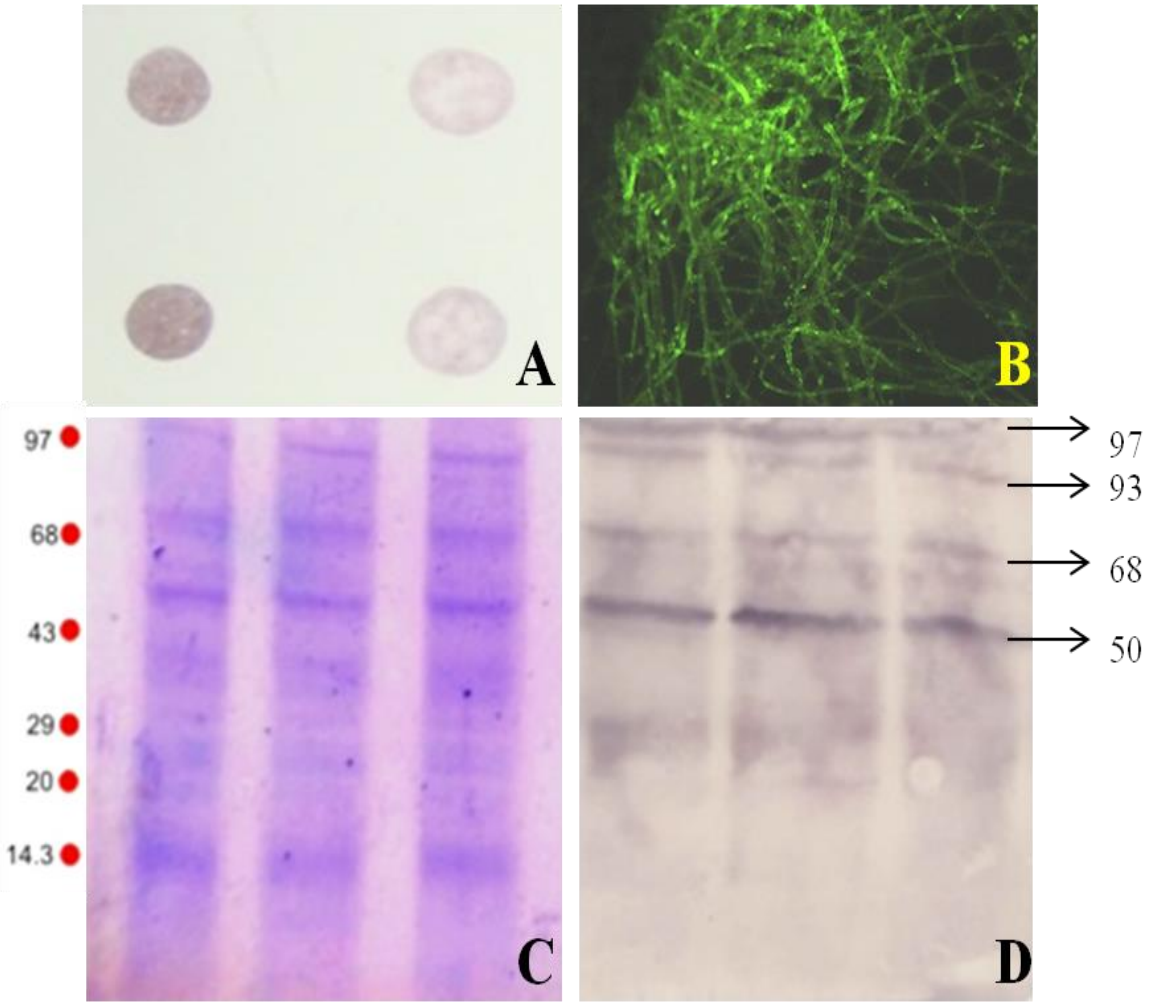



Figure 15: Serological assays of *Drechslera oryzae*; (A) Dot-blot, (B) Immuno-fluorescence of young mycelia treated with PAbs of *D. oryzae* and labelled with FITC, (C) SDS-PAGE and (D) Western blot analysis of *D oryzae*

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

#### 4.7.1. 18 S rDNA sequence analysis for identification of pathogens

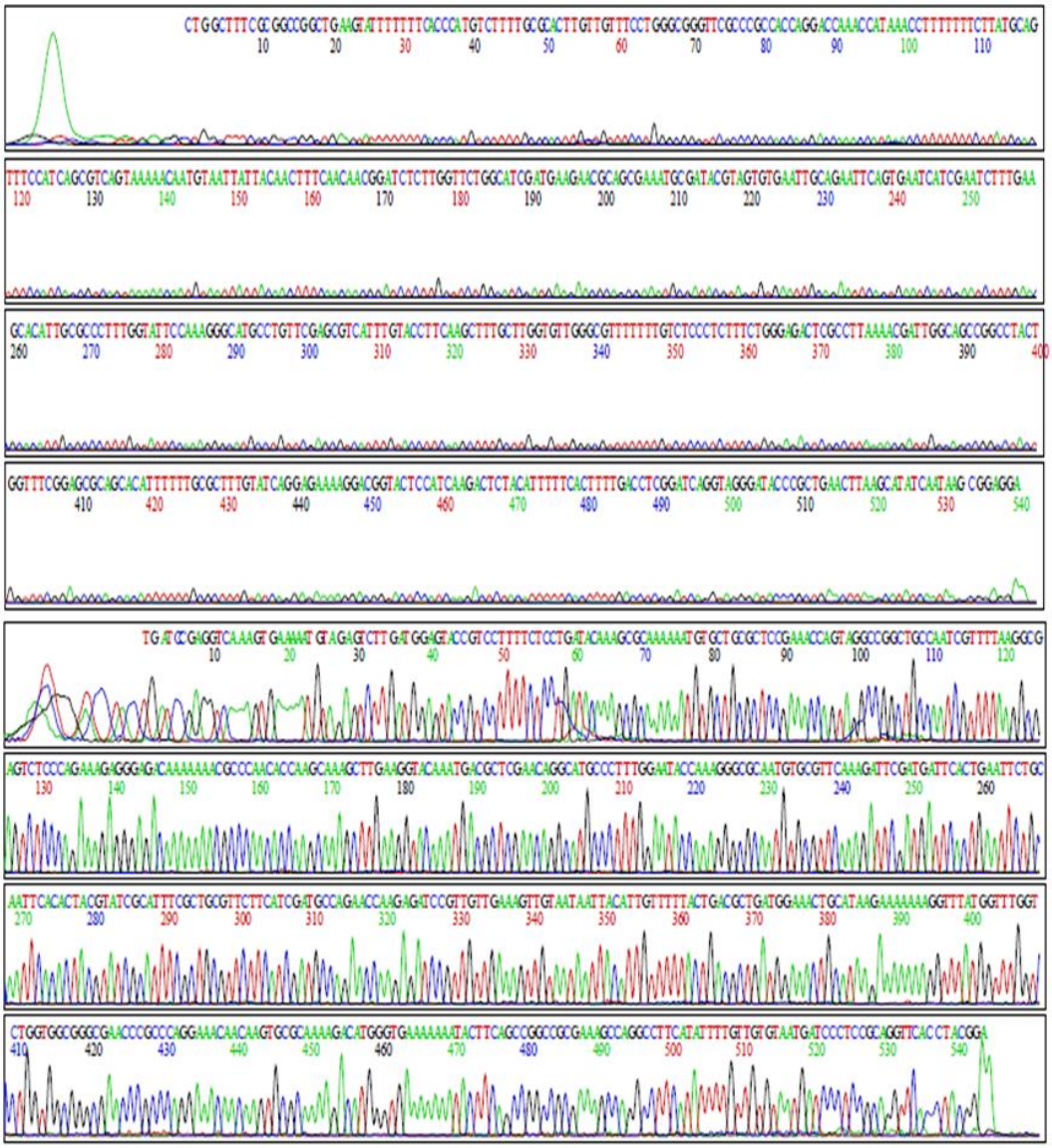
Genomic DNA of *D. oryzae* isolate -R1.DO.01 was isolated and purified and re-suspended in 1X TE buffer until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free. Purity of DNA evaluated in terms of the ratio between absorbance of $A_{260}$ and $A_{280}$ showed that 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).

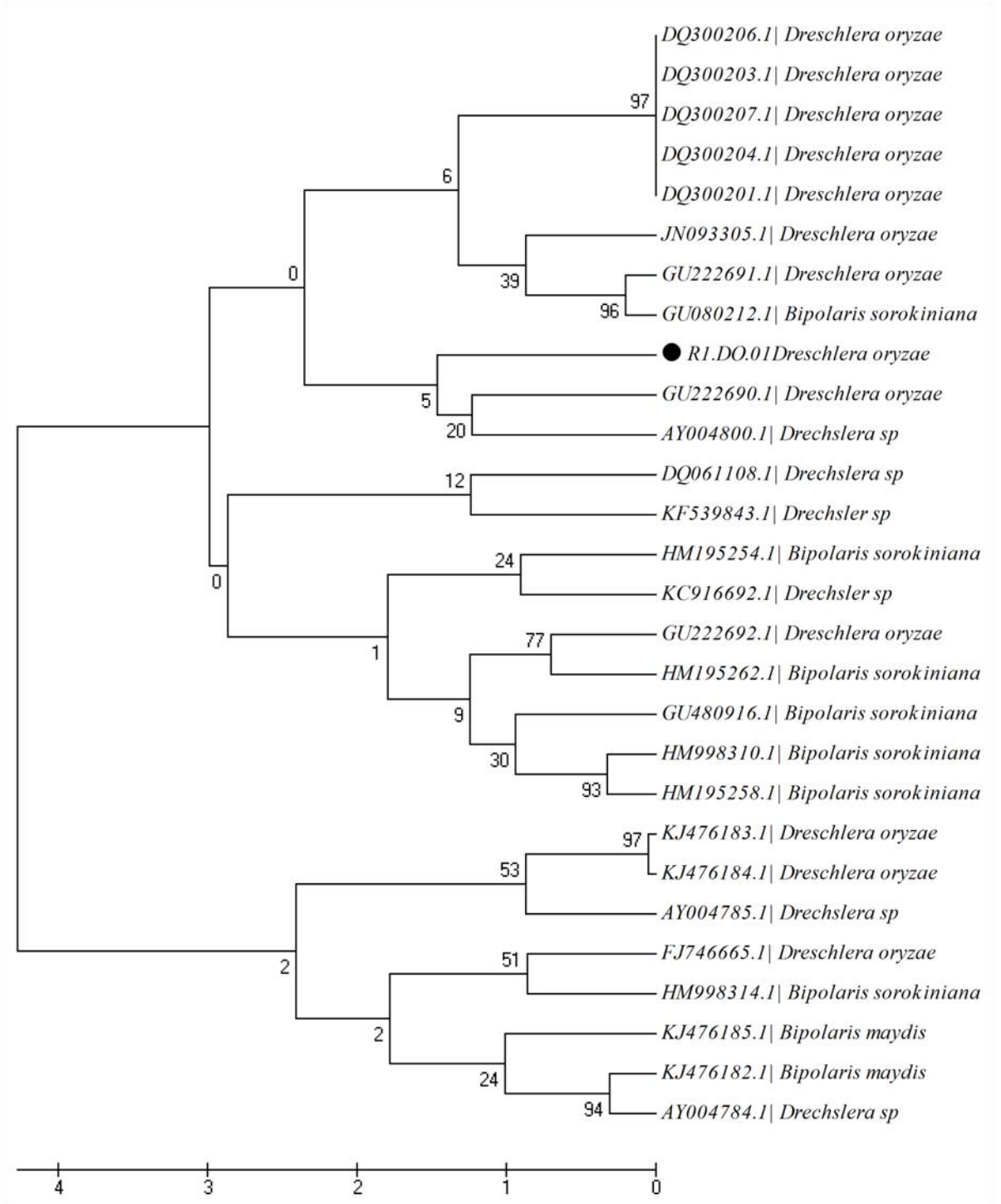



Figure18: Phylogenetic placement of *Drechslera oryzae* KT768092with other ex-type strains obtained from NCBI GenBank Database.

### 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.

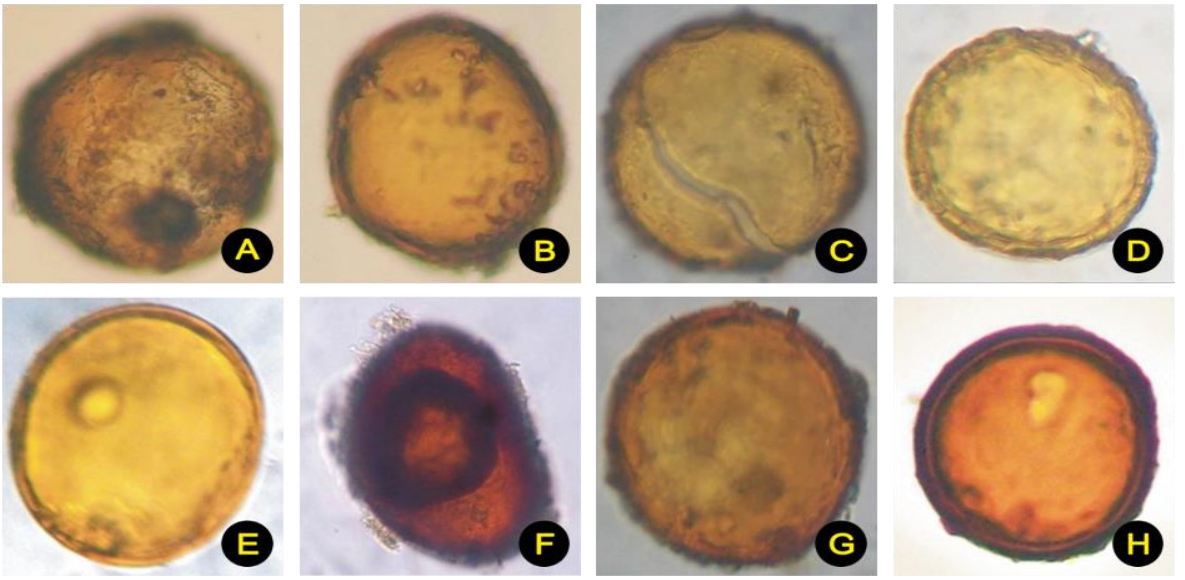



Figure 19:Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Brimful). (A) *Glomus* sp.; (B) *Glomus* sp.;(C) *Glomus* sp.;(D) *Glomus fasciculatum*; (E) *Glomus mosseae* (juvenile);(F) *Scutellospora* sp.;(G) *Glomus* sp.;(H) *Glomus mosseae* (mature).

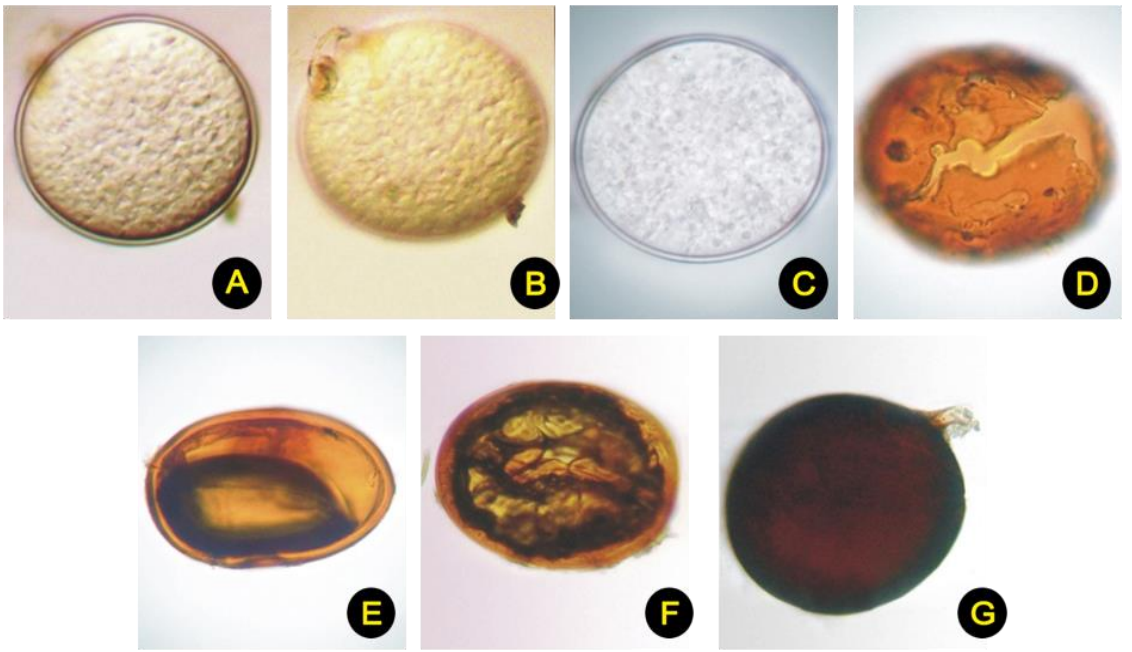



Figure 20: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Tulaipanji).(A) *Glomus* sp. ;(B)*Glomus* sp.;(C) *Glomus* sp.;(D) ruptured spore of *Glomus* sp.;(E) *Scutellospora* sp.;(F) mature *Glomus* sp.;(G) *Glomus constrictum*

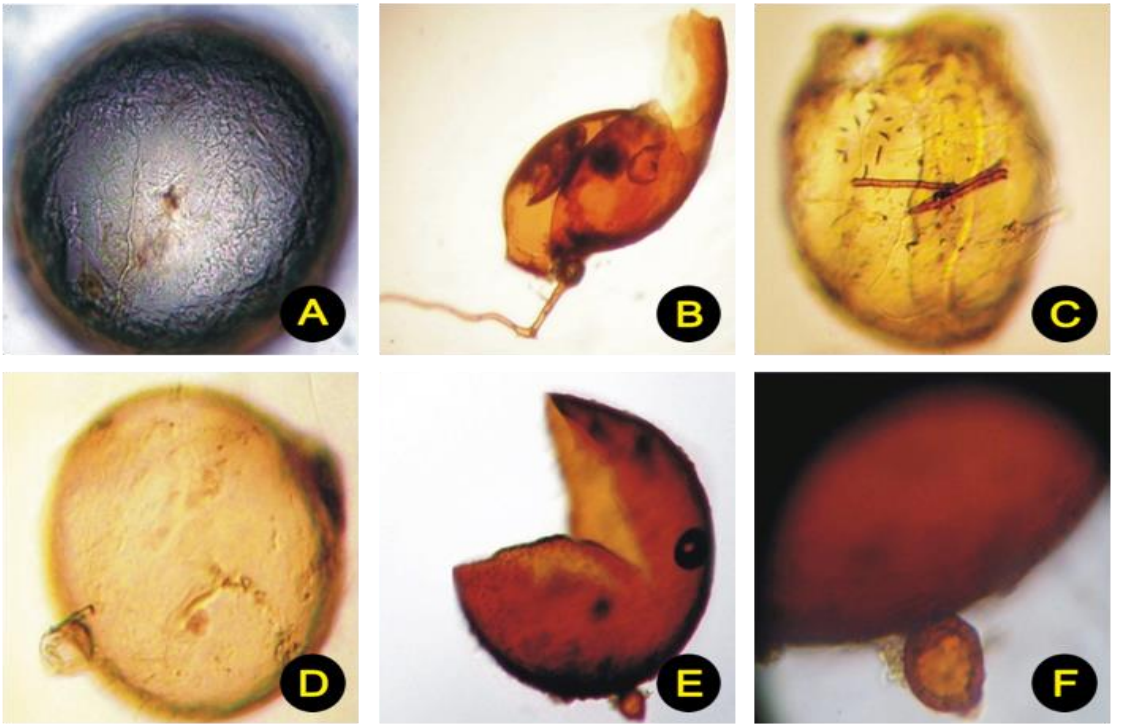



Figure 21: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-5).(A) *Acaulospora* sp.;(B) ruptured *Gigaspora* with conspicuous hyphal attachment;(C) *Scutellospora* sp.;(D) *Glomus* sp.;(E)*Scutellospora* sp.;(F) *Scutellospora* with hyphal attachment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

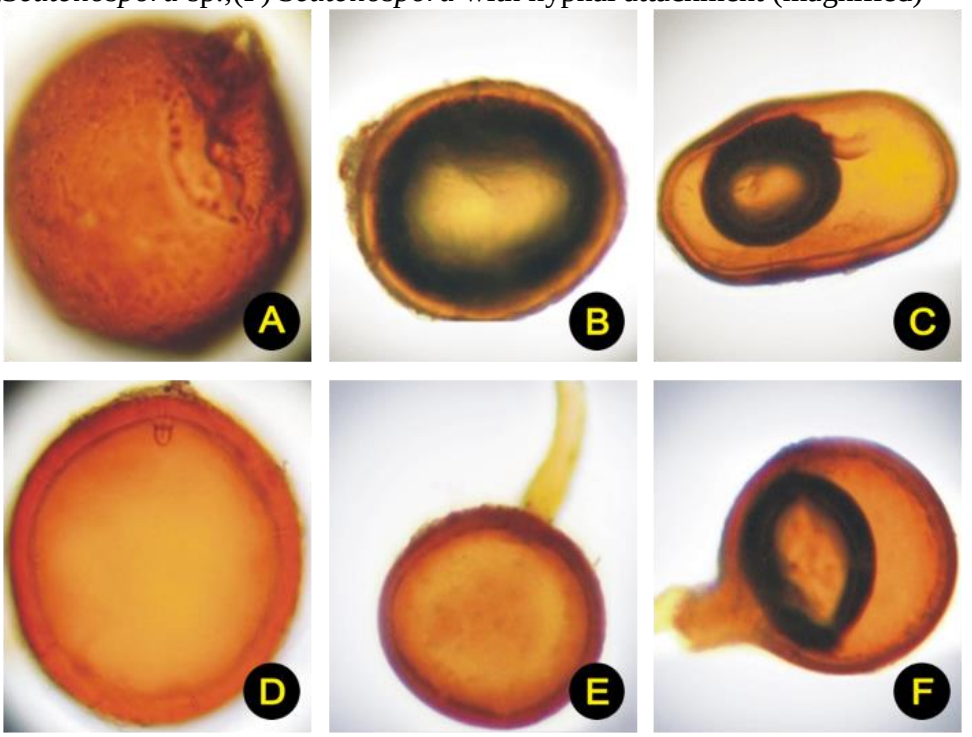



Figure 22: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-4).(A)*Scutellospora* sp.;(B) *Gigaspora* sp.;(C) *Glomus* sp.;(D)*Glomus* sp.;(E) Hyphal attachment of *Gigaspora* sp.;(F) *Scutellospora* sp.

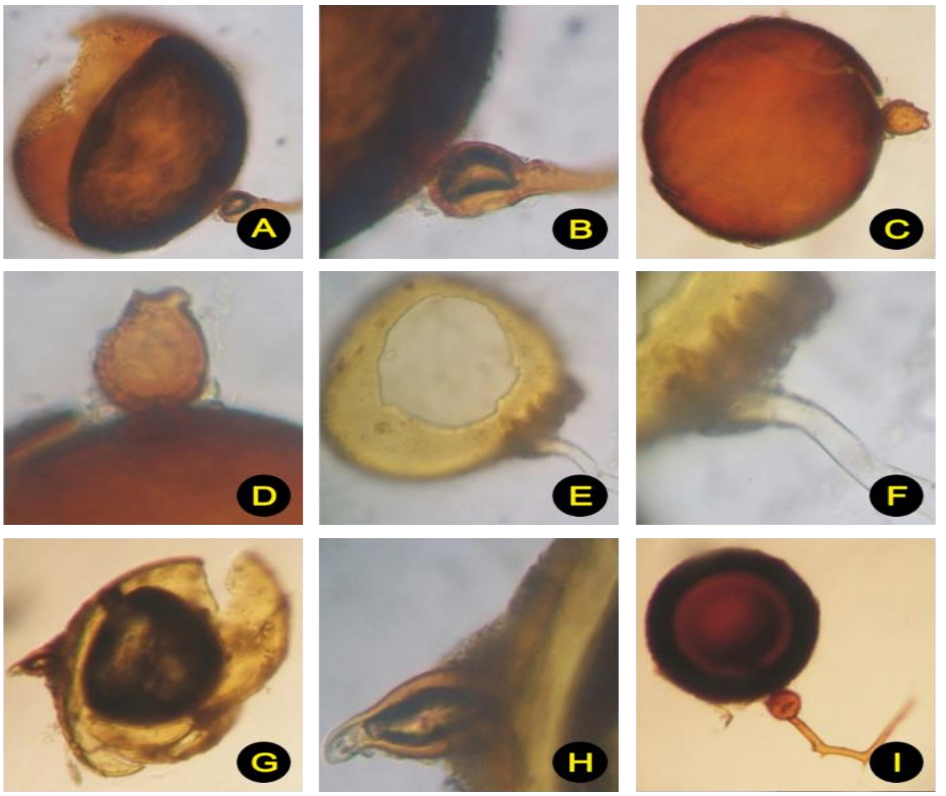



Figure 23: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Black Nuniya).(A) *Glomus* sp.;(B) *Glomus* sp.;(C) *Scutellospora* sp.;(D*) Glomus* sp.;(E) *Glomus* sp.; (F) *Acaulospora* sp.; (G) *Glomus* sp.;(H) *Glomus* sp.; (I) *Glomus* sp.

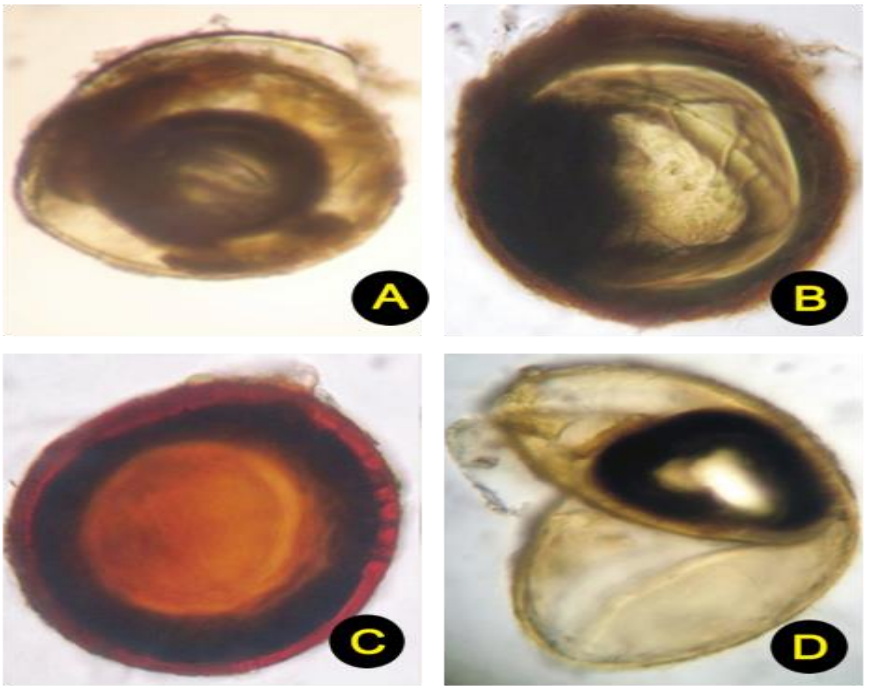



Figure 24: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Champasari).(A) *Gigaspora* sp.;(B) *Glomus* sp.;(C) Ruptured spores of *Scutellospora* sp.;(D) *Scutellospora* sp.(matured)

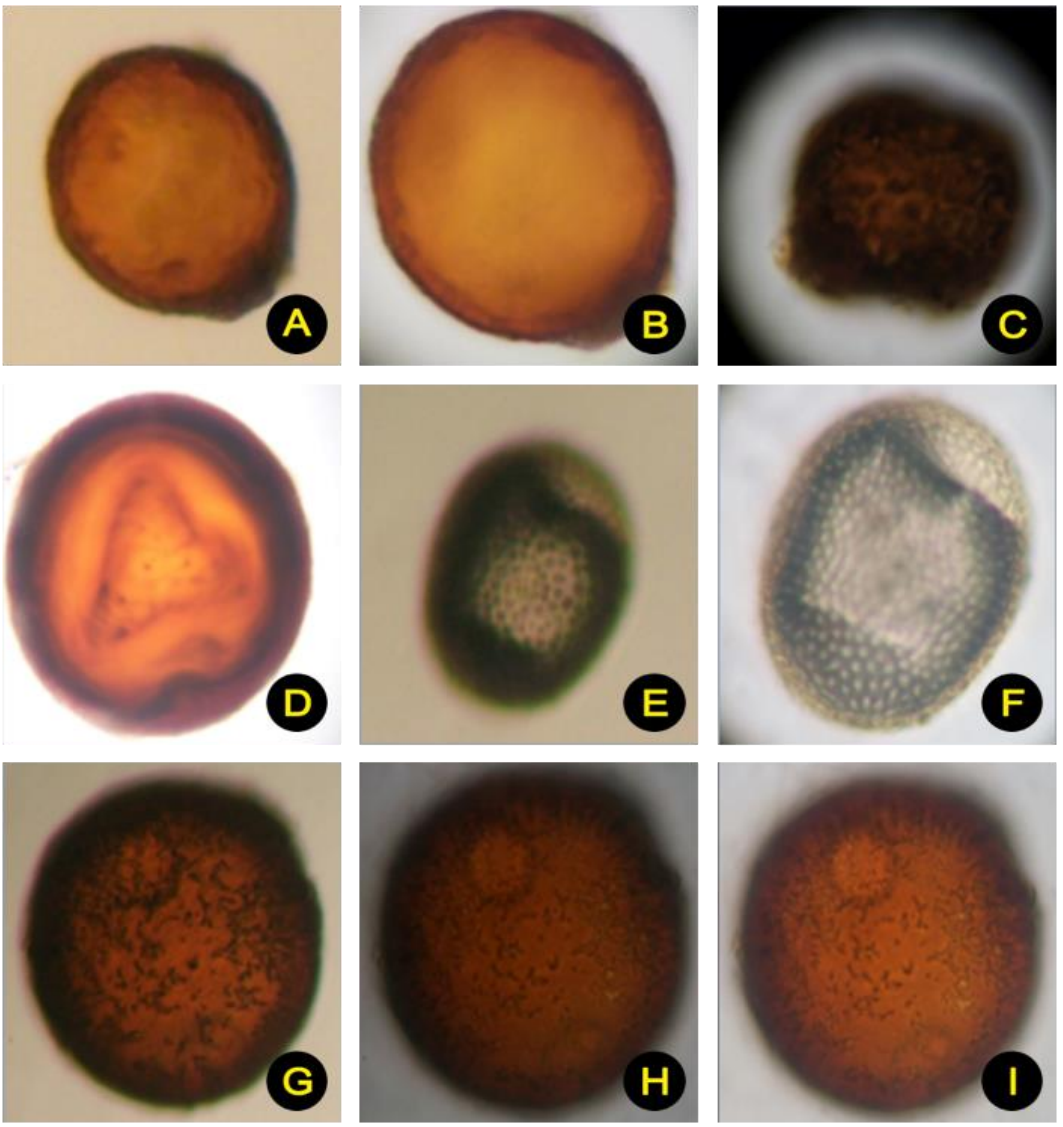



Figure 25: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Adde).(A) *Glomus constrictum*;(B) *Glomus constrictum*;(C) *Glomus mosseae*;(D) *Acaulospora* sp.;(E) *Acaulospora* sp.;(F) *Acaulospora* sp.;(G,H&I) *Glomus mosseae* in different angles

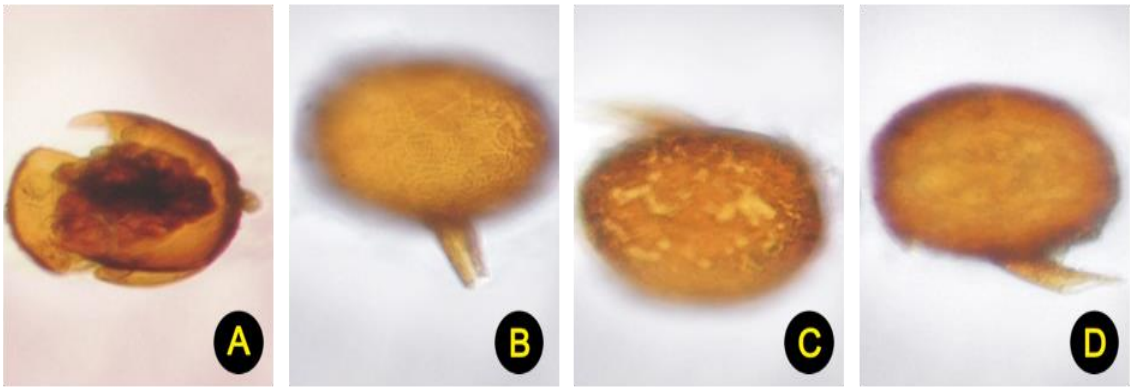



Figure 26: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (UBKV-1).(A) *Gigaspora* sp.;(B) *Glomus* sp.;(C)*Glomus* sp.;(D)*Glomus* sp.

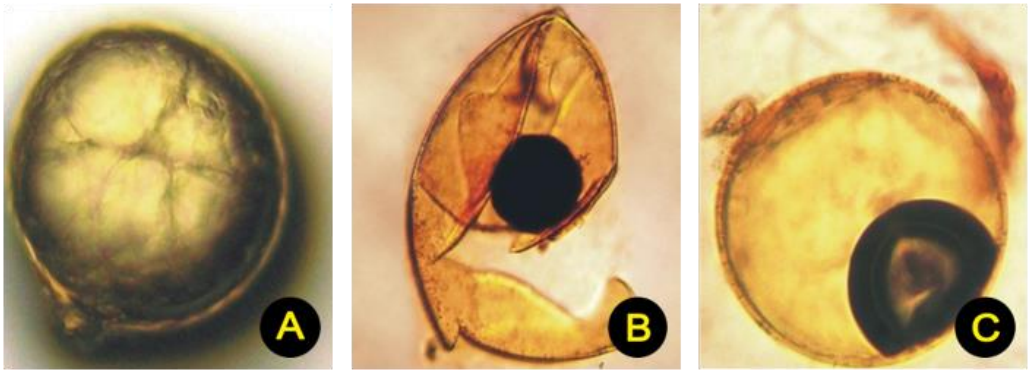



Figure 27: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Swarnamasuri).(A) *Scutellospora* sp.; (B) Ruptured *Glomus* sp.;(C) *Glomus* sp. with hyphae

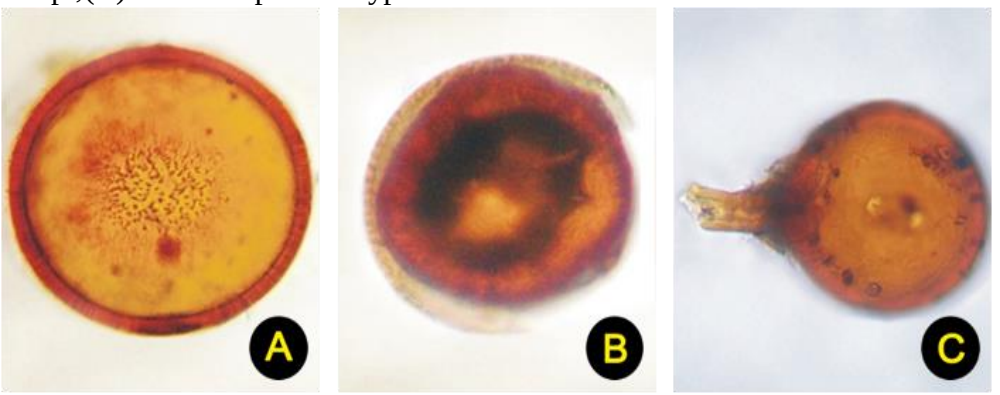



Figure 28: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Maiti). (A) *Scutellospora* sp.;(B) *Scutellospora* sp.;(C) *Gigaspora margarita*

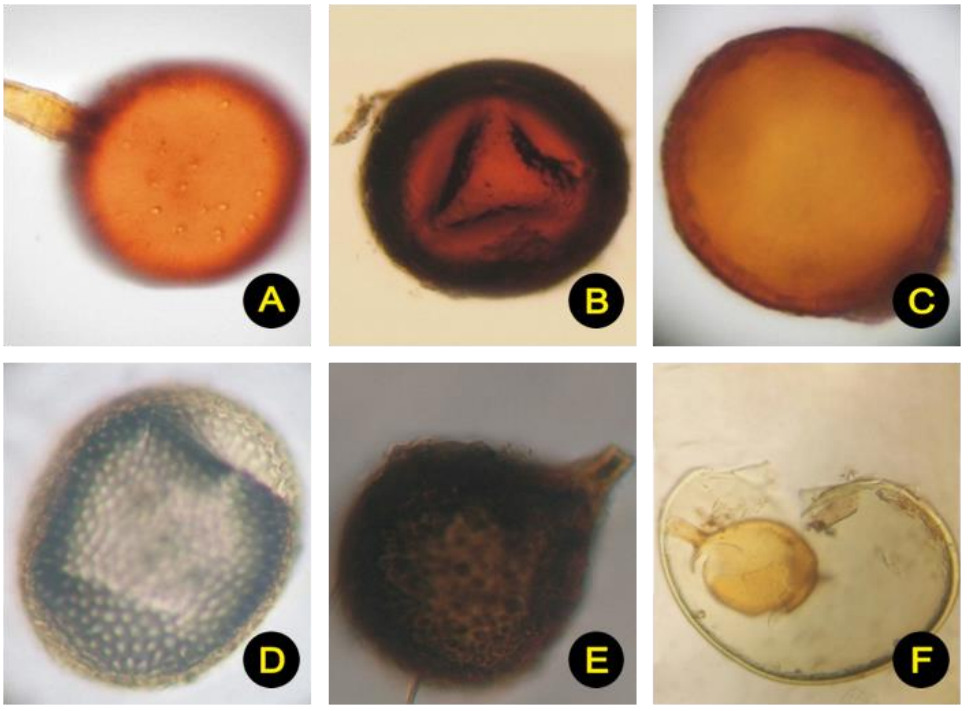



Figure 29: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Attheu).(A) *Glomus* sp.;(B) *Scutellospora* sp.;(C) *Glomus* sp;(D) *Acaulospora* sp.;(E) *Glomus constrictum*;(F)Ruptured AMF.

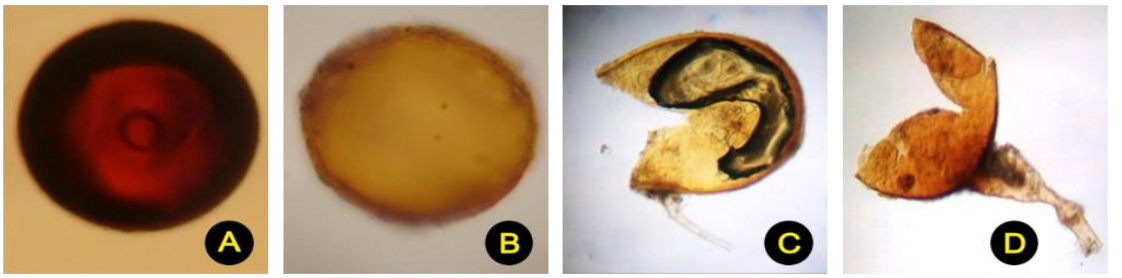



Figure 30: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Sanomasuri).(A) *Scutellospora* sp.;(B) *Scutellospora* sp.;(C) *Glomus* sp.;(D) *Gigaspora rosea*.

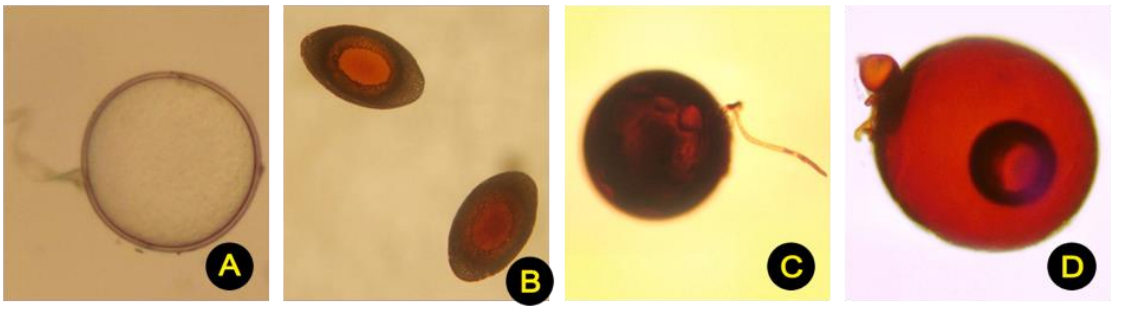



Figure 31: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Loknath 505).(A) *Glomus* sp. juvenile;(B) *Entrophospora* sp.;(C)*Glomus* sp.;(D) *Scutellospora* sp. with hyphal attachment

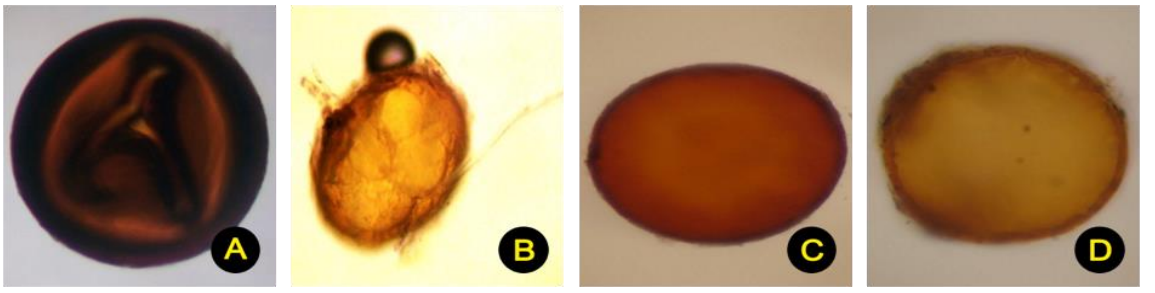



Figure 32: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Kaberi 9090).(A) *Glomus* sp.;(B) *Glomus* sp.;(C) *Glomus badium* ;(D) *Glomus badium*

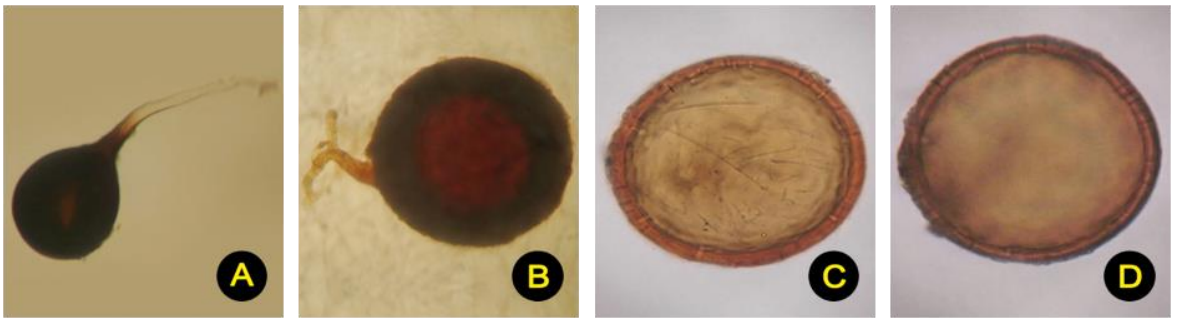



Figure 33: Compound microscopic observation of Arbuscular Mycorrhizal Fungal spores obtained from rice cultivar (Gouraknath 509).(A) *Glomus fasciculatum*;(B) *Glomus multicaule*;(C) *Glomus* sp.;(*Glomus* sp.)

### 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).

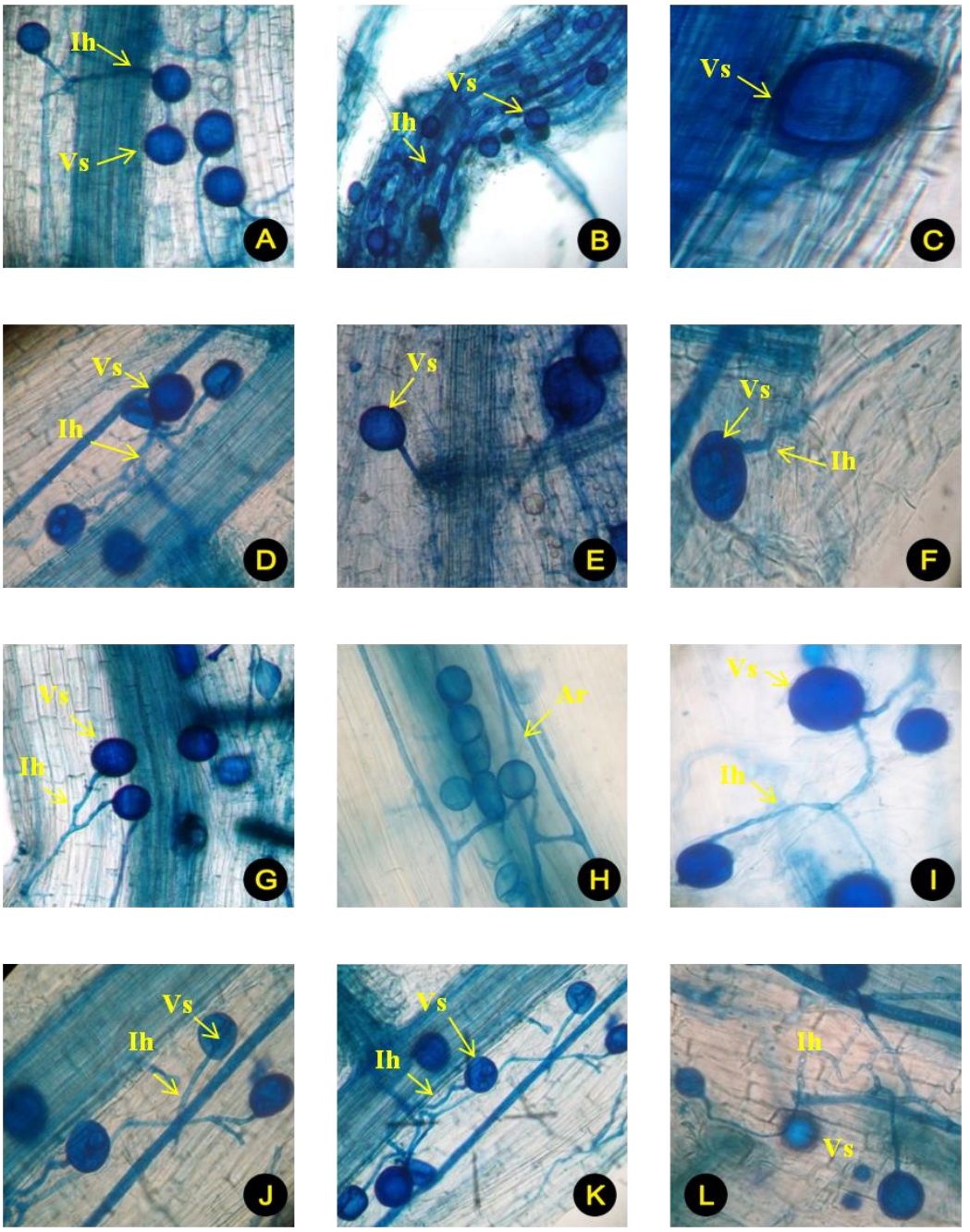



Figure 34: Root colonization of rice cultivars. (A-C) Brimful; (D-F) Black nuniya; (G-I) Champasari and (J-L) Attheu.(Ih- Intracellular hyphae, Vs- Vesicle, Ar- Arbuscule).

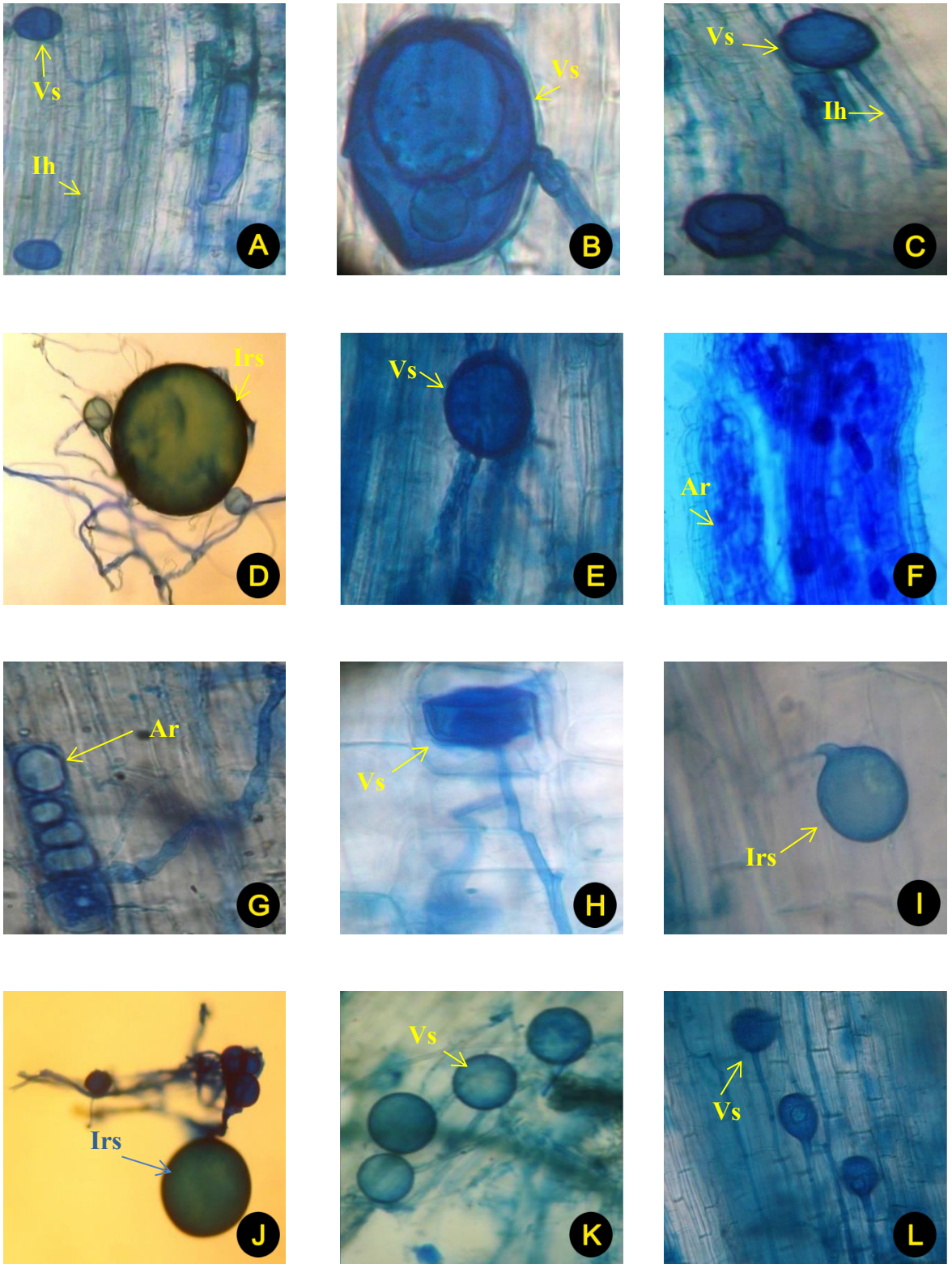



Figure 35: Root colonization of rice cultivars. (A-C)Maiti; (D-F) Sano masuri; (G-I)Tualaipanji and (J-L)Swarnamasuri.(Ih- Intracellular hyphae,Irs- Intra radicle spore, Vs- Vesicle, Ar- Arbuscule).

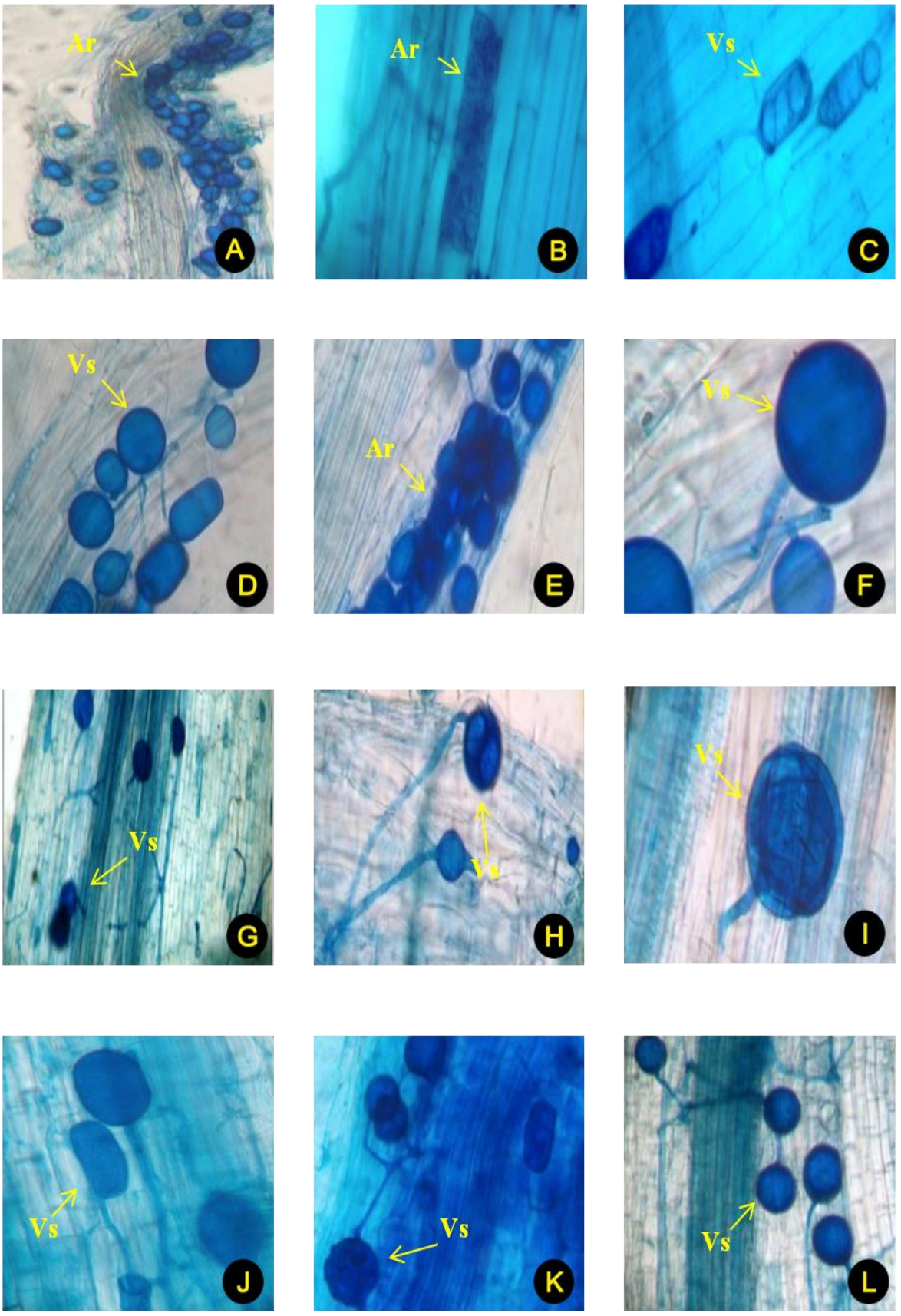



Figure 36: Root colonization of rice cultivars. (A-C)UBKV-1; (D-F) UBKV-4; (G-I) UBKV-5 and (J-L)Adde. (Ih- Intracellular hyphae, Vs- Vesicle, Ar- Arbuscule)

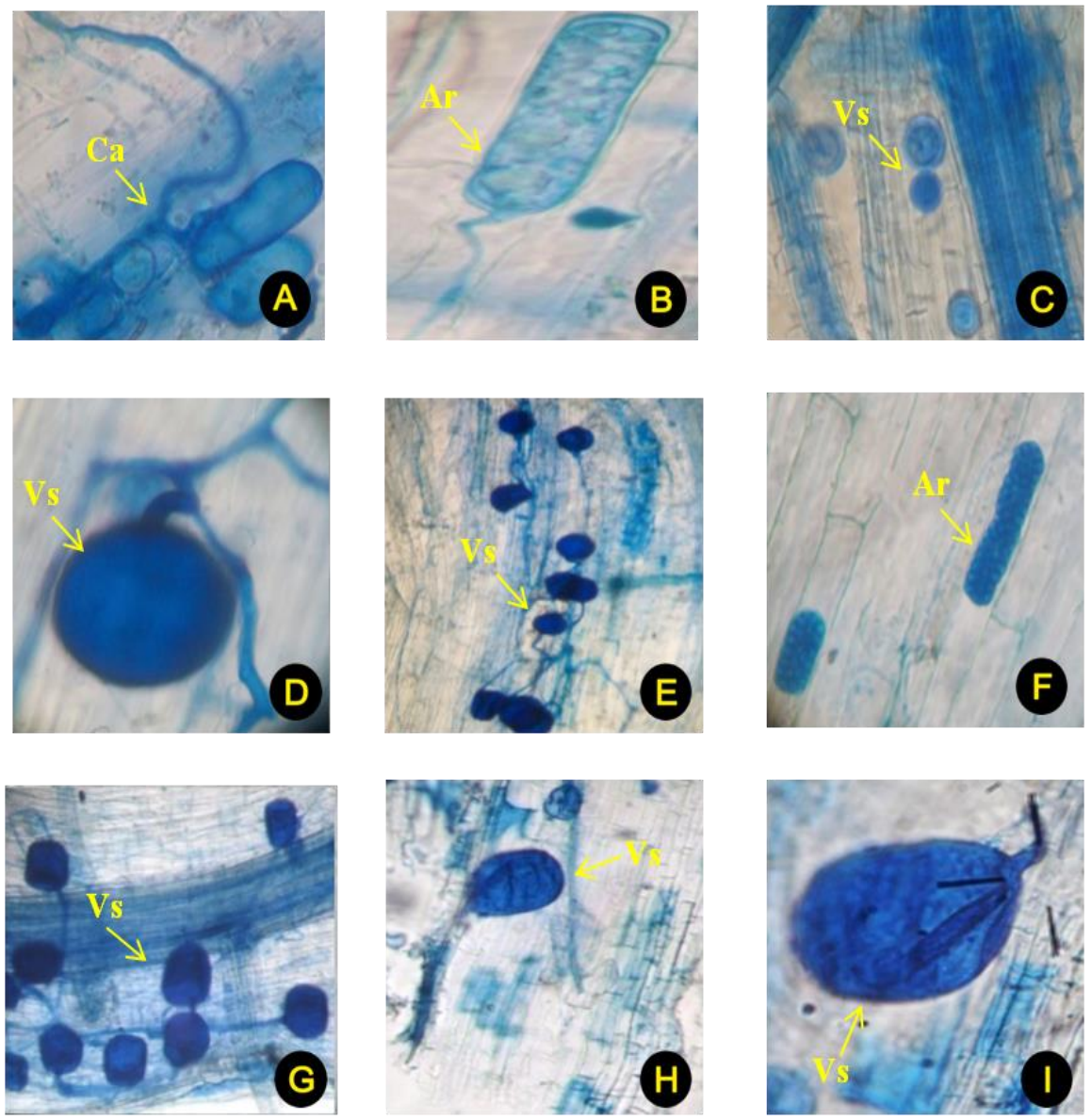



Figure 37: Root colonization of rice cultivars. (A-C)Kaberi 9090; (D-F)Loknath 505; (G-I)Gouraknath 509.(Ih- Intracellular hyphae, Ca- Coiled arbuscule, Vs- Vesicle, Ar- Arbuscule)

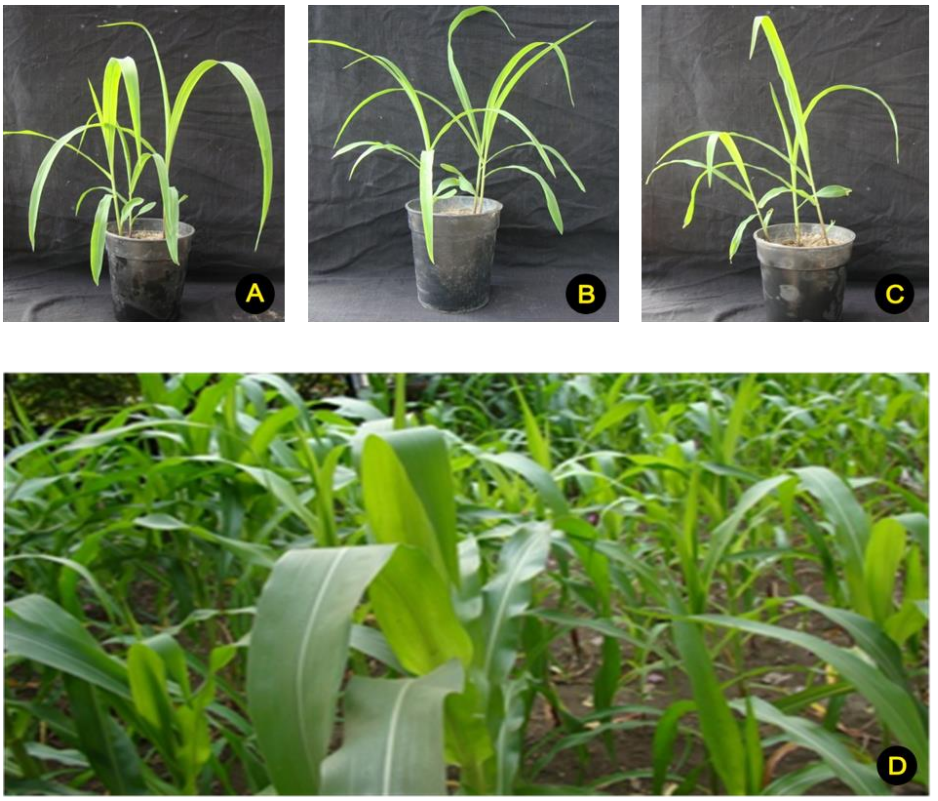



Figure 38: Mass multiplication of AMF spores in maize plant (A-C) Pots; (D) Experimental field

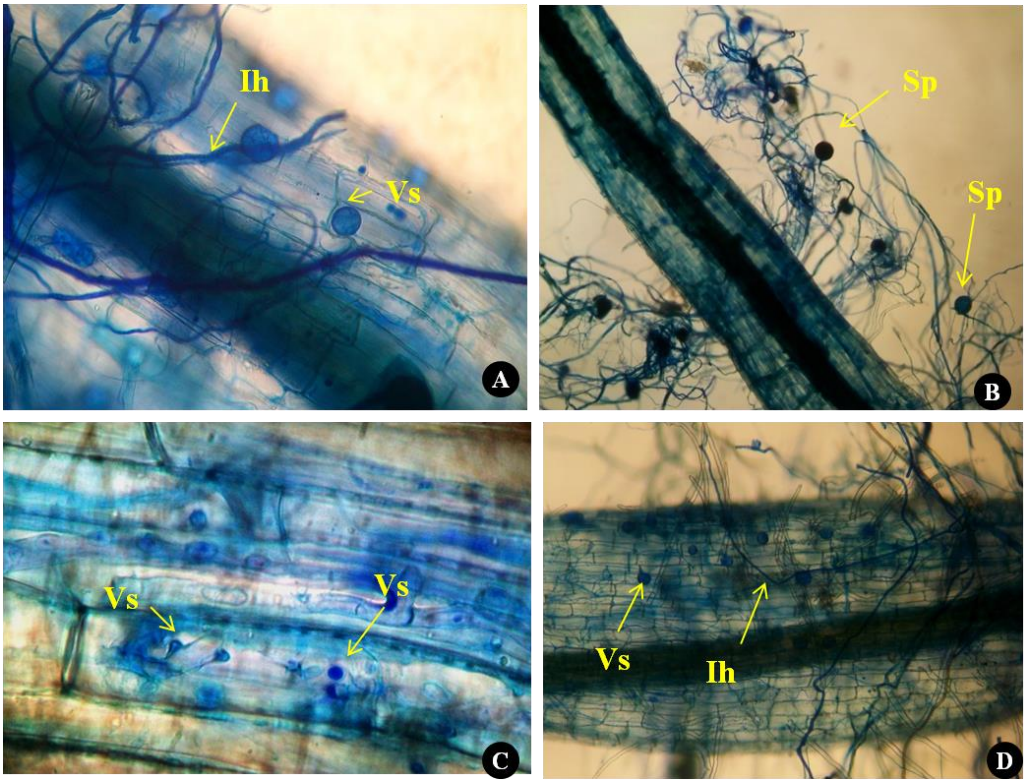



Figure 39: Histopathological study of AMF colonized maize roots. (A)Vesicles and intracellular hyphae, (B) Spores bearing hyphae,(C) Vesicles,(D) Vesicle and Intracellular hyphae.(Vs- Vesicle,Ih-Intracellular hyphae, Sp-Spore)

### 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)

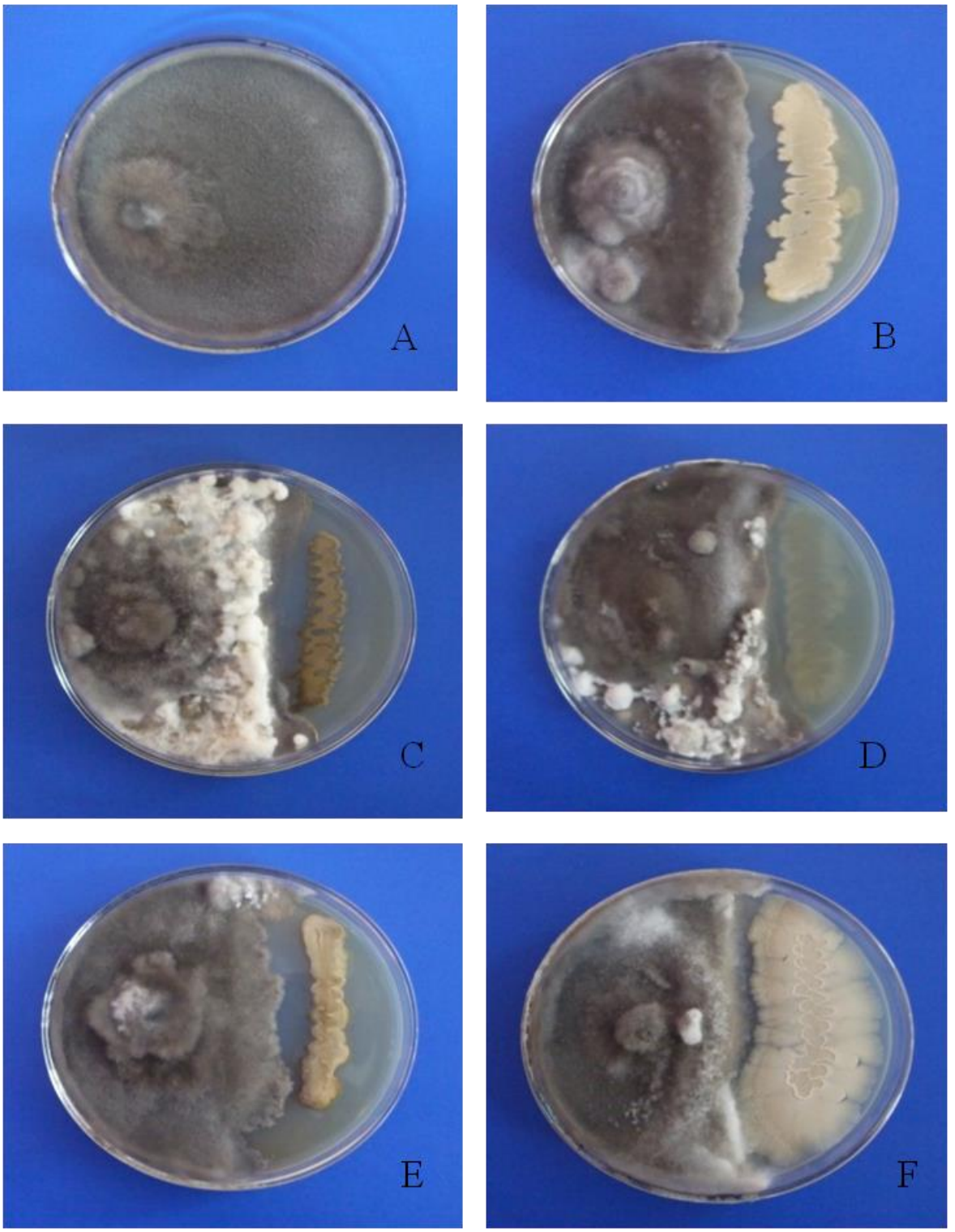



**Figure 40: *In vitro* antifungal activities of foliar fungal pathogen *Drechslera oryzae* against selected PGPR isolates.** Inhibition of *Drechslera oryzae* in dual plate culture assay by NAIMCC-B01485 (B), NAIMCC-B01483 (C), NAIMCC-B01486 (D), NAIMCC-B01488 (E), NAIMCC-B01489 (F) and Control (A)

### 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.

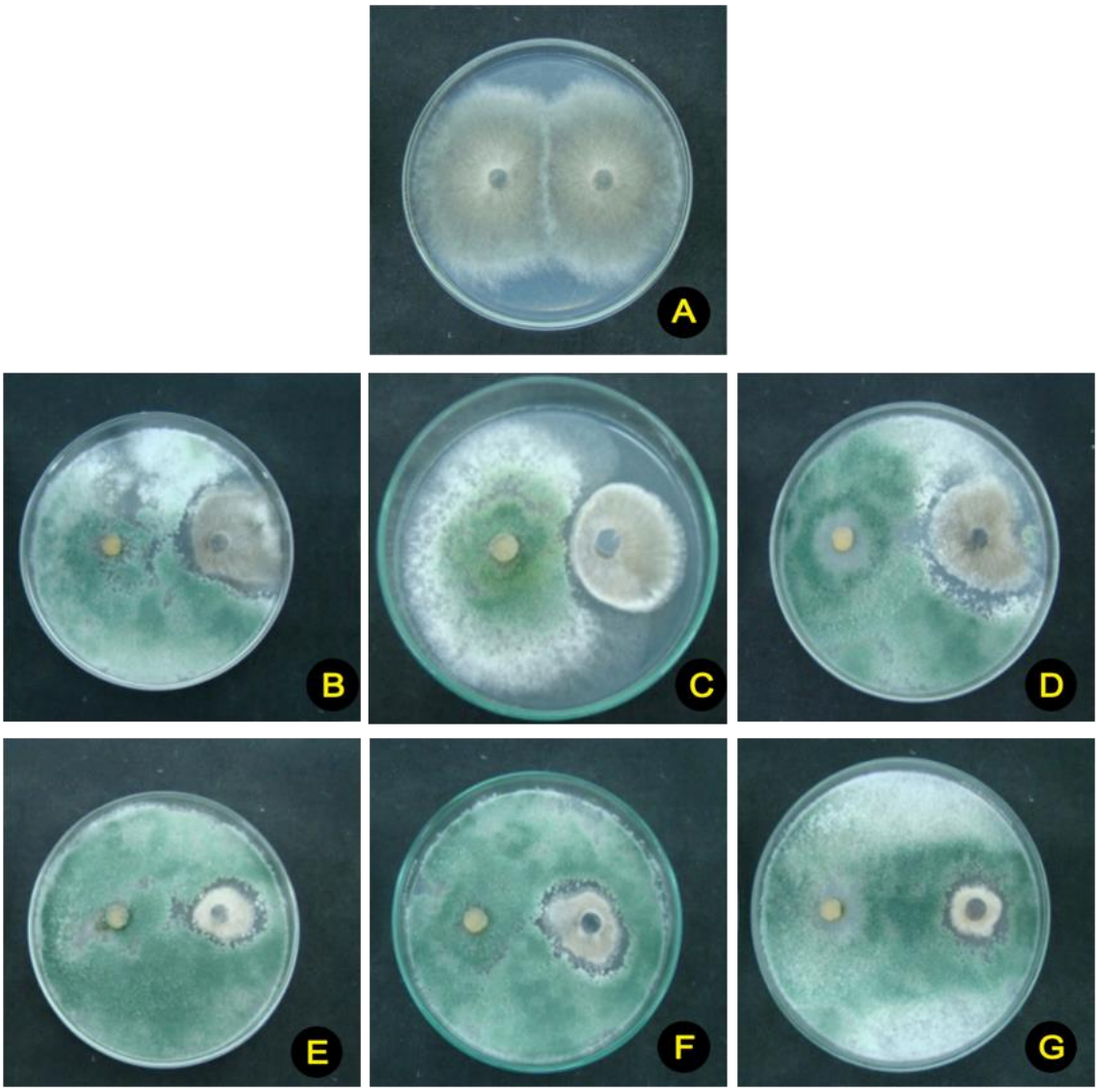



**Figure 41: *In vitro* antagonistic test of foliar fungal pathogen (*D. oryzae*) against *Trichoderma* isolates** .(B) *T. harzianum* (NAIMCC-F-03289), (C) *T. harzianum* (NAIMCC-F-03288), (D) *T. harzianum* (NAIMCC-F-03290), (E) *T. asperellum* (NAIMCC-F-03291), (F) *T. asperellum* (NAIMCC-F-03292),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

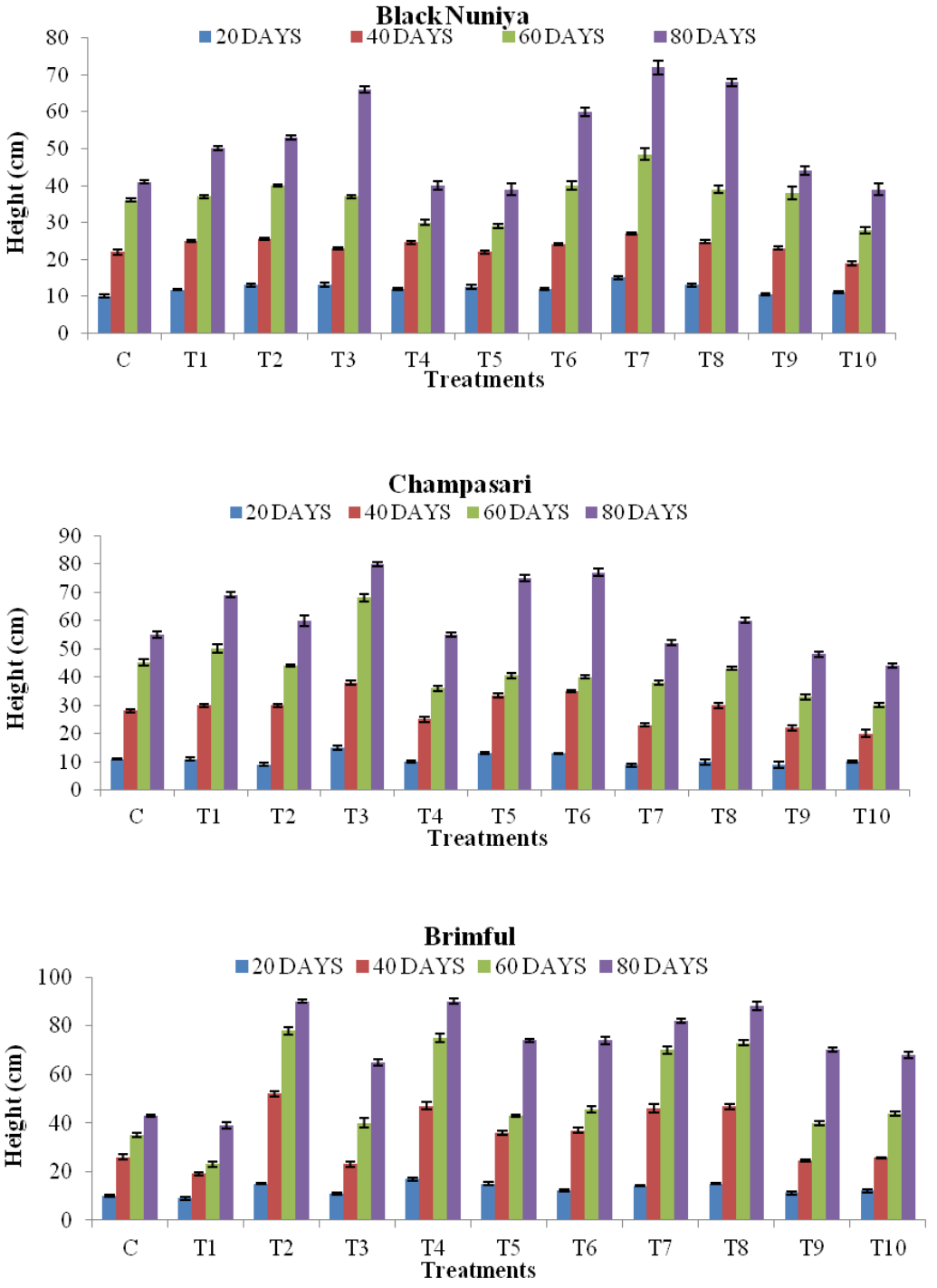



**Figure 42: Growth promotion of rice plants following treatment with different PGPR.**C-Untreated control (UI),T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01484), T3-*Bacillus altitudinis* (NAIMCC-B01485), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (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<br>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<br>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<br>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<br>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).

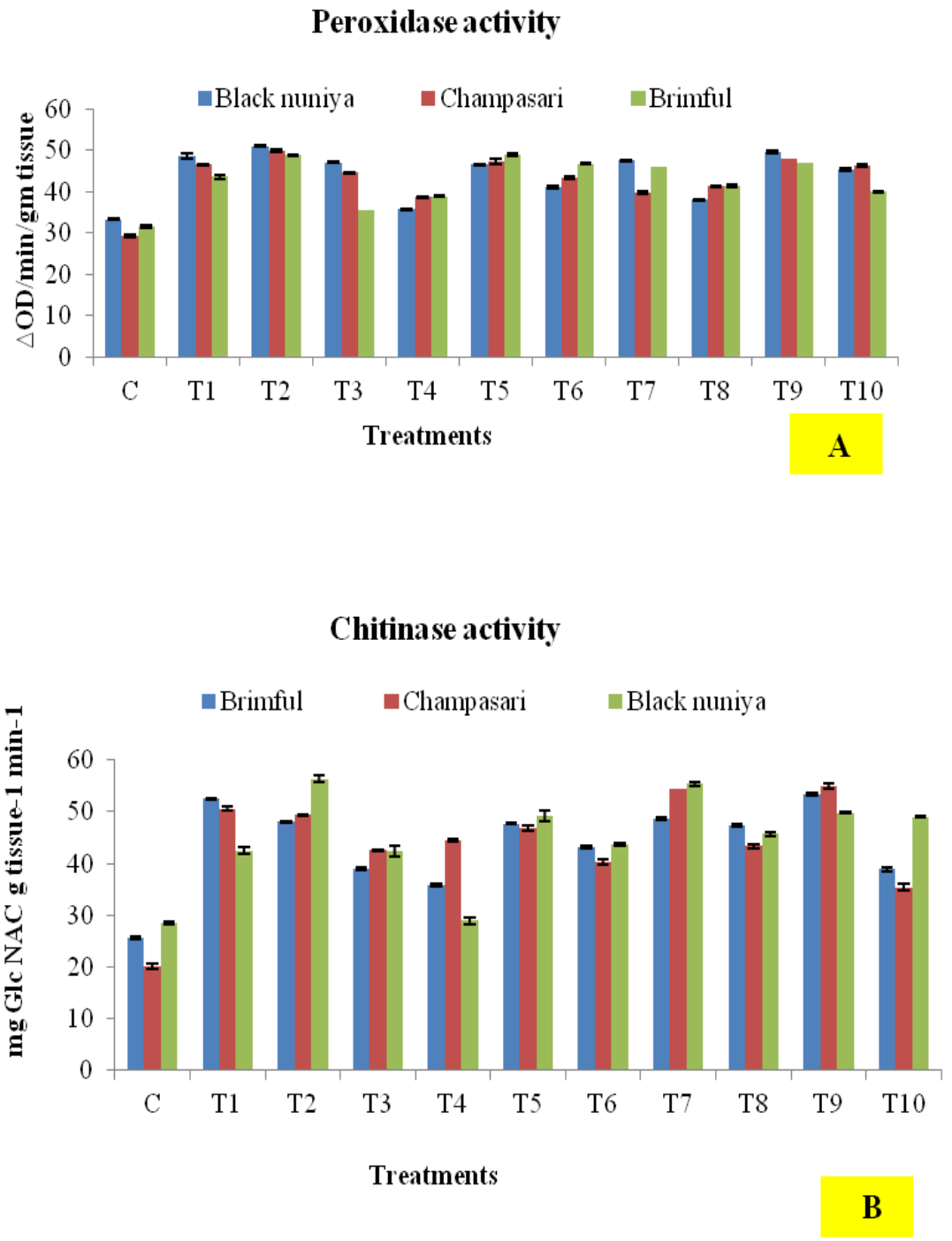



**Figure 43: Activity of defense enzymes in rice leaf samples (A) Peroxidase and (B) Chitinase.** C-Untreated control,T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01485), T3-*Bacillus altitudinis* (NAIMCC-B01484), T4-*Enterobacter cloacae* (NAIMCC-B01486), T5-*Bacillus pumilus* (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).

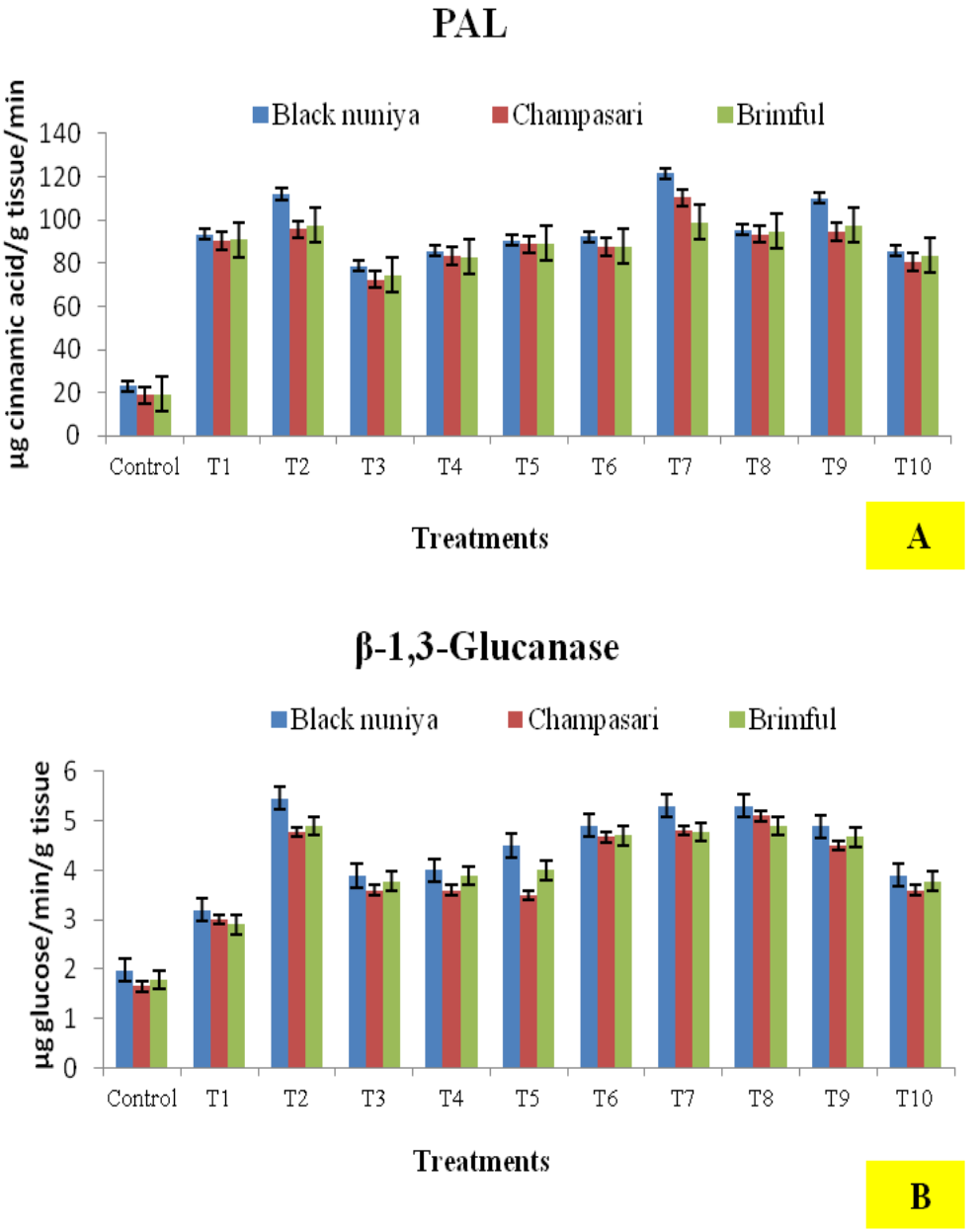



**Figure 44: Activity of defense enzymes in rice leaf samples (A) PAL (Phenyl alenine ammonia lyase) and (B)β-1,3-Glucanase.** C-Untreated control,T1-*Bacillus pumilus* (NAIMCC-B01483), T2-*Bacillus altitudinis* (NAIMCC-B01485), T3-*Bacillus altitudinis* (NAIMCC-B01484), T4-*Enterobacter cloacae* (NAIMCC-B01486), 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).

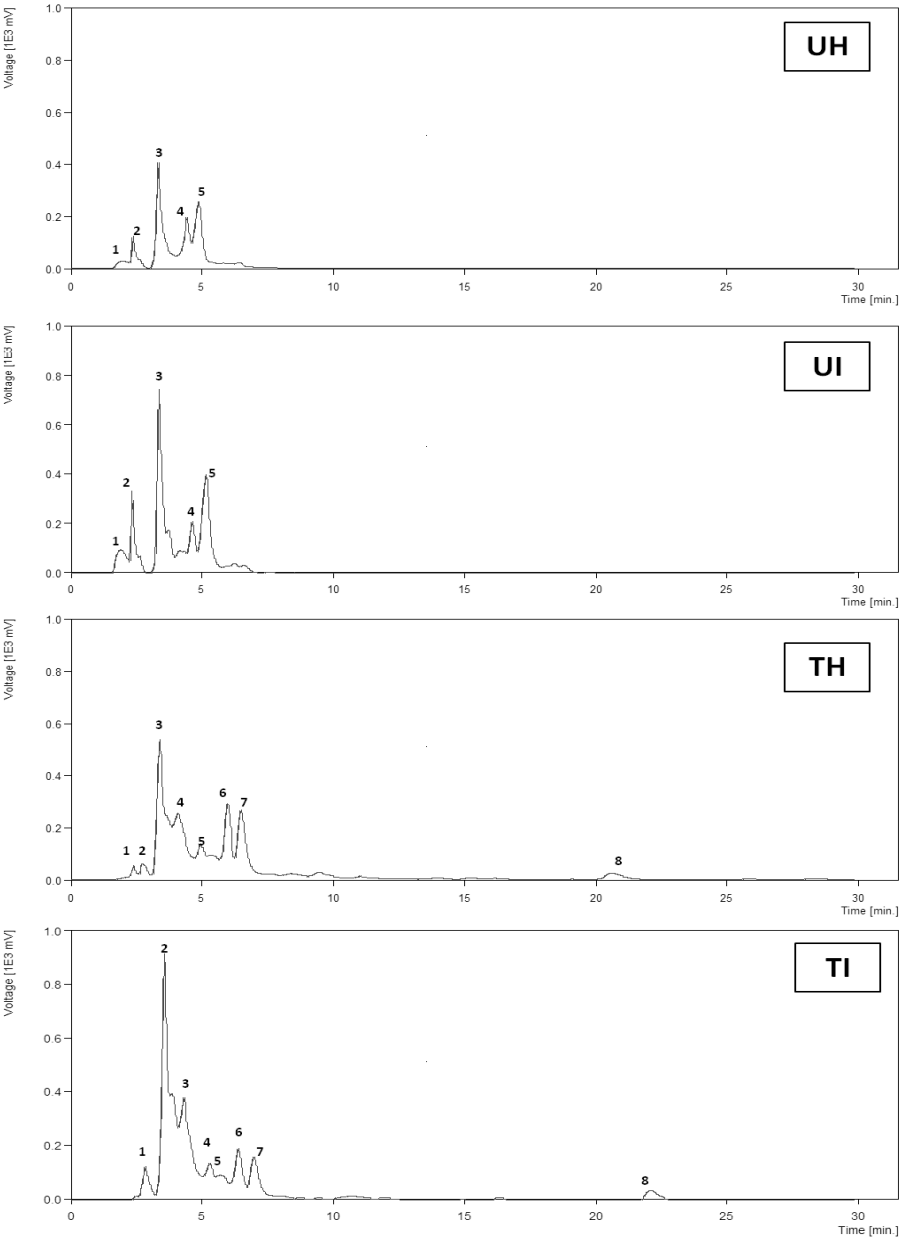



Figure 45: HPLC profile of Phytocassanes obtained from leaf extracts of rice plant (cv. Black nuniya) following treatment with *Bacillus altitudinus* (NAIMCC-B01485) and challenge inoculation with *D. oryzae*. (UH= Untreated Healthy, UI= Untreated Inoculated, TH= Treated Healthy and TI= Treated Inoculated)

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.

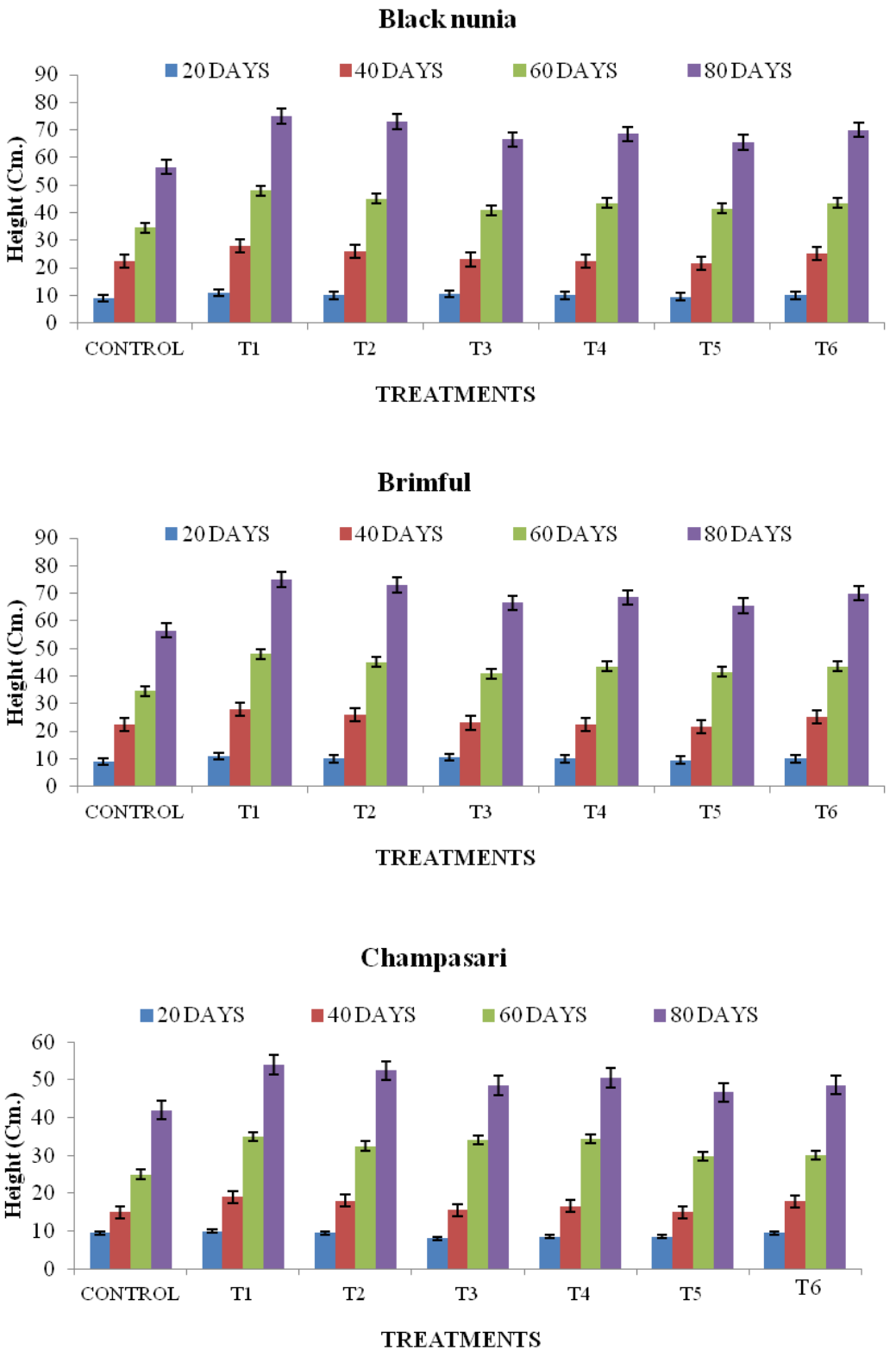



**Figure 46: Growth promotion of rice cultivars following treatment with different PGPF.** (C= Untreated Control, T1= *T. harzianum* NAIMCC-F-03288, T2= *T. harzianum* NAIMCC-F-03289, T3=*T.harzianum* NAIMCC-F-03290, T4= *T.asperellum* NAIMCC-F-03291, T5=*T. asperellum* NAIMCC-F-03292 and T6= *T. asperellum*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

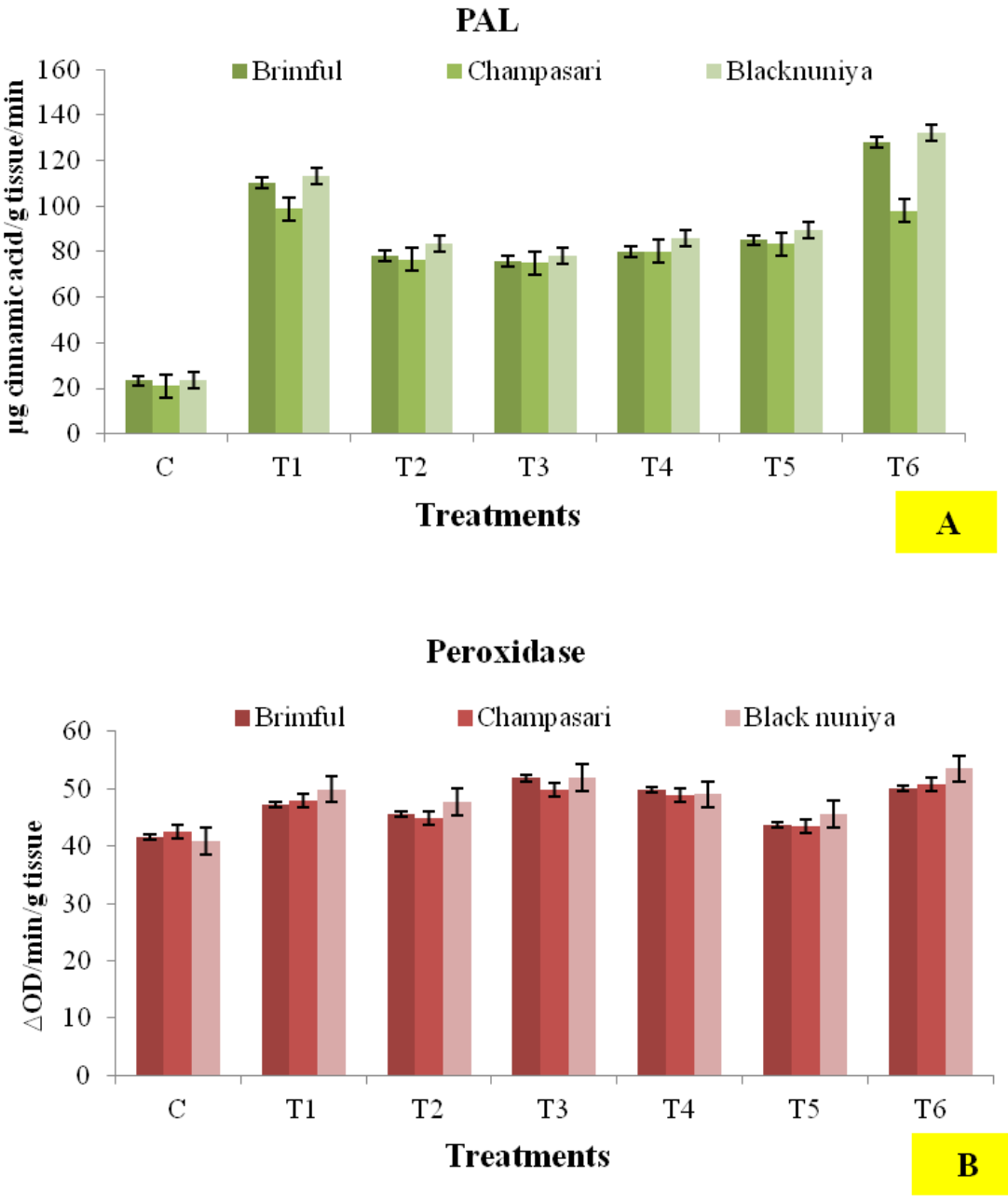



**Figure 47: Defense enzyme activity (A) Phenylalanine ammonia lyase and (B) Peroxidase of rice cultivars following treatment with PGPF and inoculated with pathogen (*D oryzae*).** C-Untreated Control, T1-T6=Treated Inoculated [T1-*T.asperellum* (NAIMCC-F-03293), T2-*T.harzianum* (NAIMCC-F-03289), T3-*T.harzianum*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

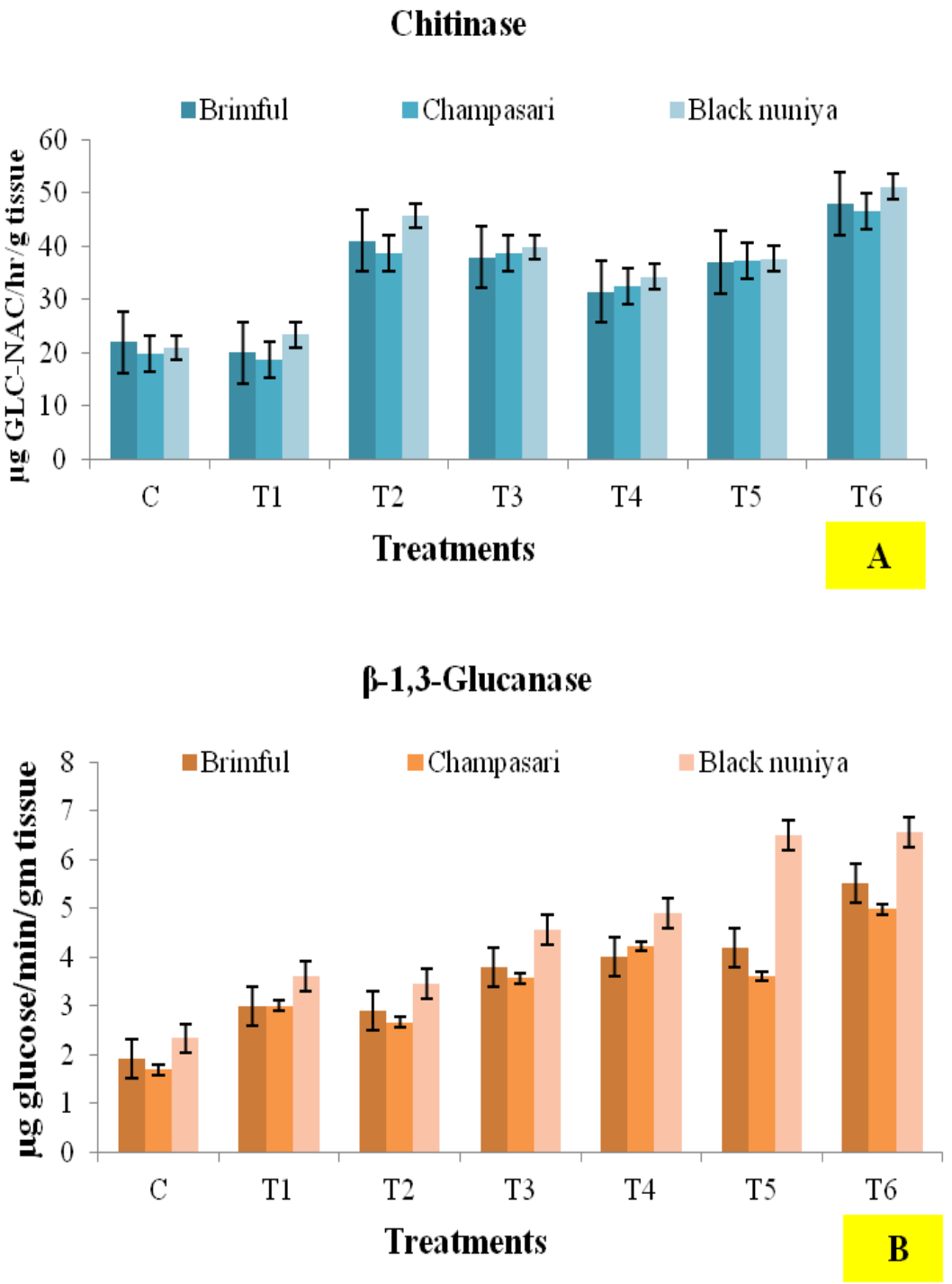



**Figure 48: Defense enzyme activity (A) Chitinase (B) β-1, 3-Glucanase of rice cultivars following treatment with PGPF and pathogen challenge.** C-Untreated Control,T1-T6=Treated Inoculated [T1-*T.asperellum* (NAIMCC-F-03293), T2-*T.harzianum* (NAIMCC-F-03289), T3-*T.harzianum* (NAIMCC-F-03290), T4-*T.asperellum*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

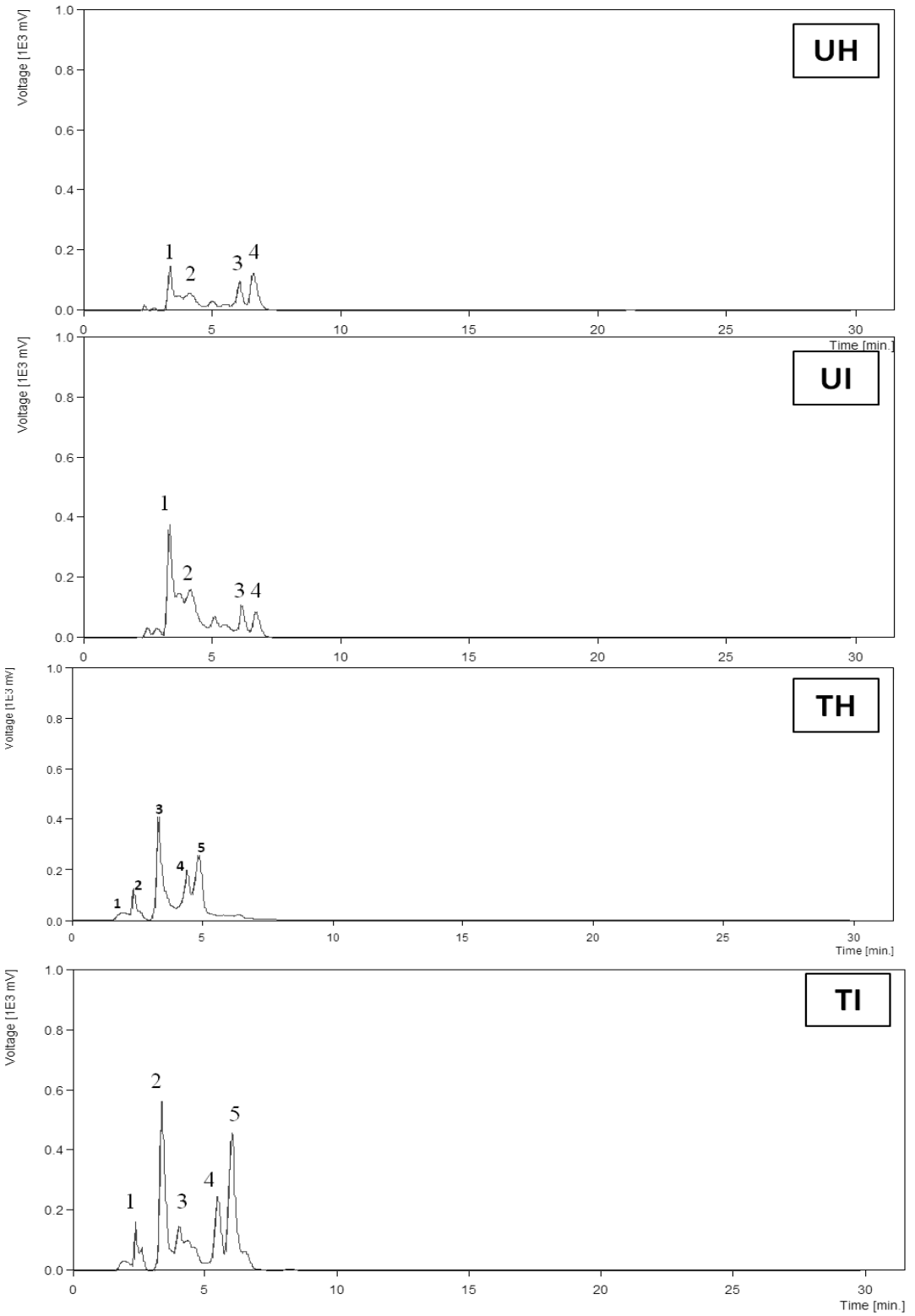



**Figure 49: HPLC analysis of Phytocassanes from leaf extracts of rice plant (cv. Black Nuniya) treated with *T. harzianum* (NAIMCC-F-03288) and pathogen challenge.** (UH- Untreated Healthy, UI- Untreated Inoculated, TH- Treated Healthy and TI- Treated Inoculated)

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.

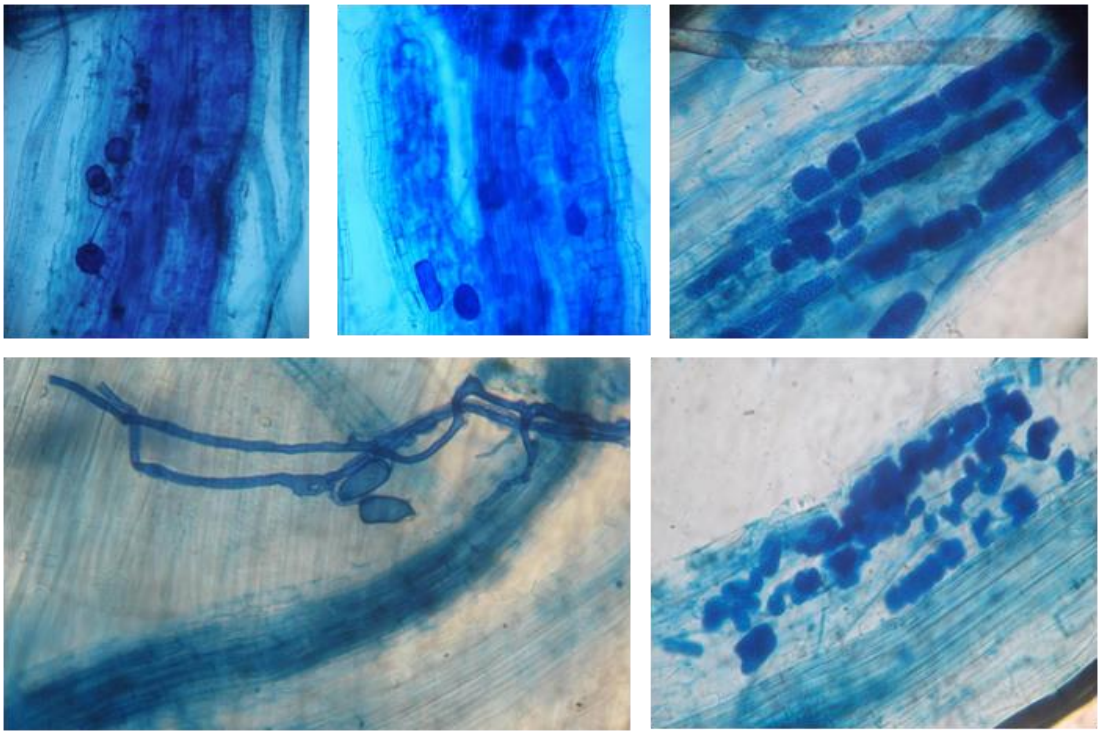


Figure 50 : Root colonization of rice following application of AMF (*R. fasciculatus*).

### 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).

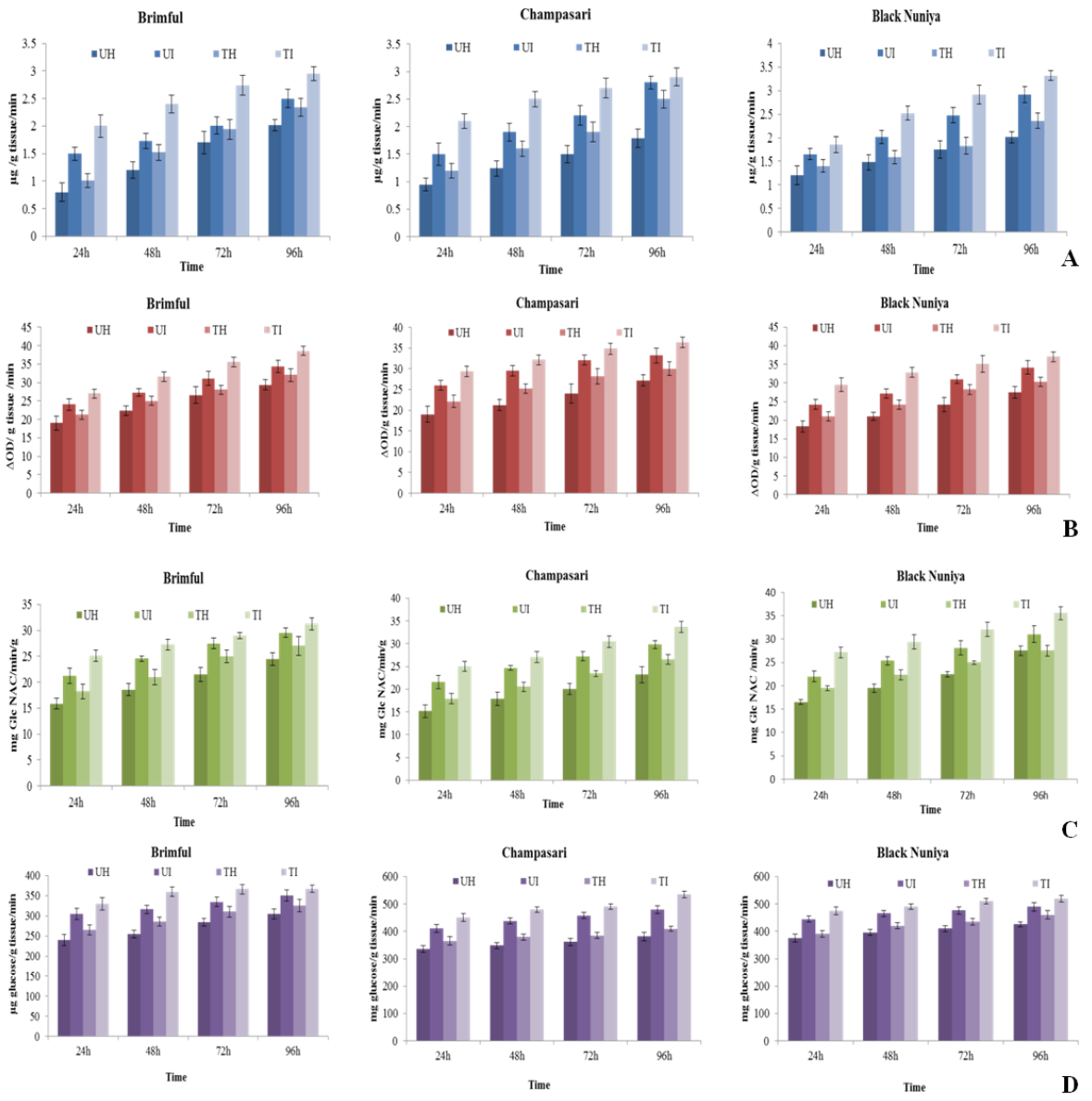



**Figure 51: Changes in the level of defense enzyme in rice cultivars treated with *R. fasciculatus* following artificial inoculation with *D. oryzae*.(A) PAL, (B) Peroxidase, (C) Chitinase and (D) β-1,3 Glucanase.** (UH- Untreated Healthy, UI- Untreated Inoculated, TH- Treated Healthy and TI- Treated 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**<br>**Cultivars** | **1.00**<br>**0.77** | **4.66**<br>**3.61** | **6.24**<br>**4.84** | **6.48**<br>**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<br>Cultivars | 3.85<br>2.98 | 0.88<br>0.68 | 2.29<br>1.78 | 0.27<br>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).

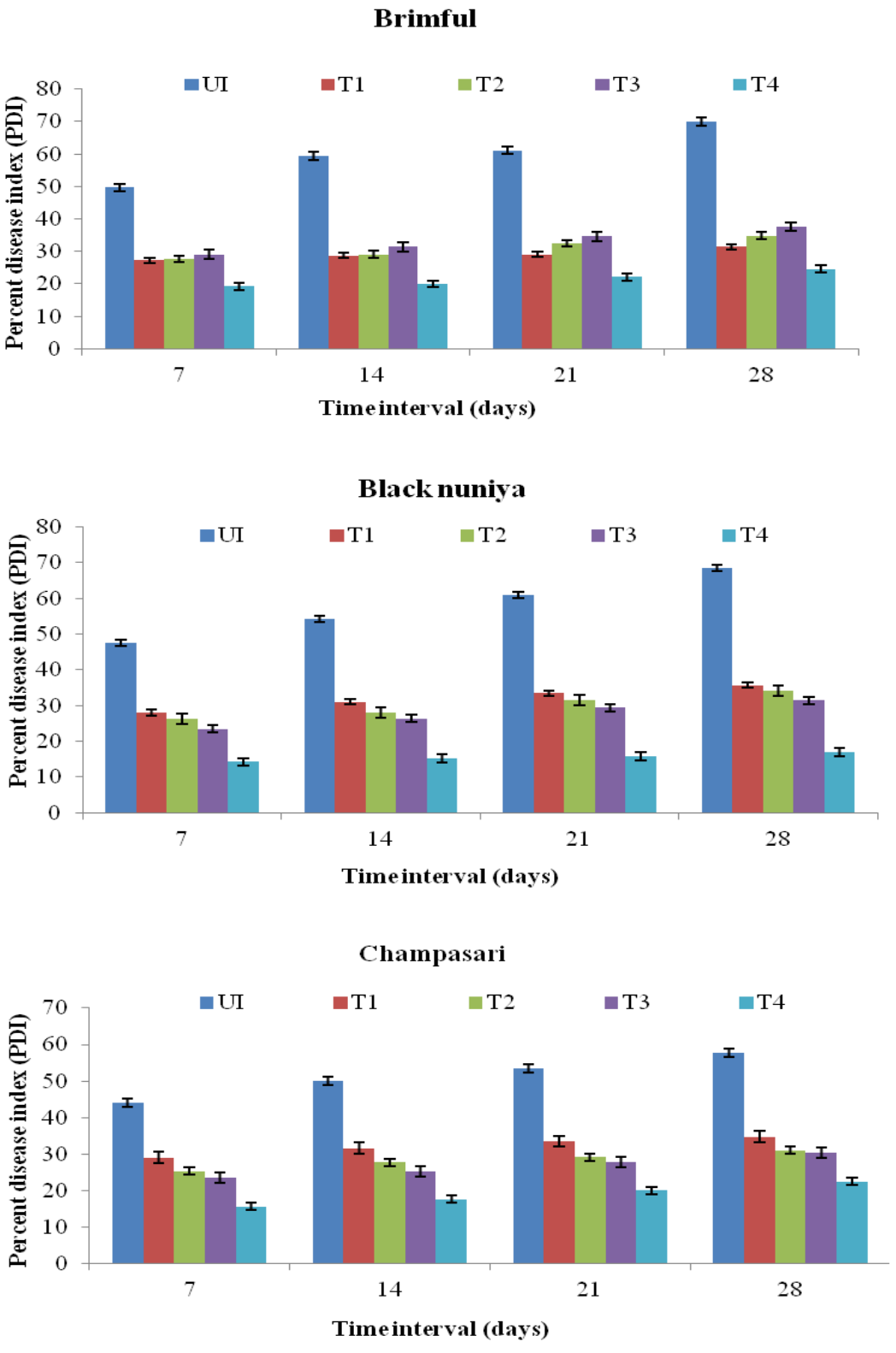



**Figure 52: Percent disease index in rice cultivars following treatment and artificial inoculation with *D. oryzae*.** (UI- Untreated inoculated, T1- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, T2- *T. harzianum* (NAIMCC-F-03288)+ *R. fasciculatus*, T3= *T. harzianum* (NAIMCC-F-03288)+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

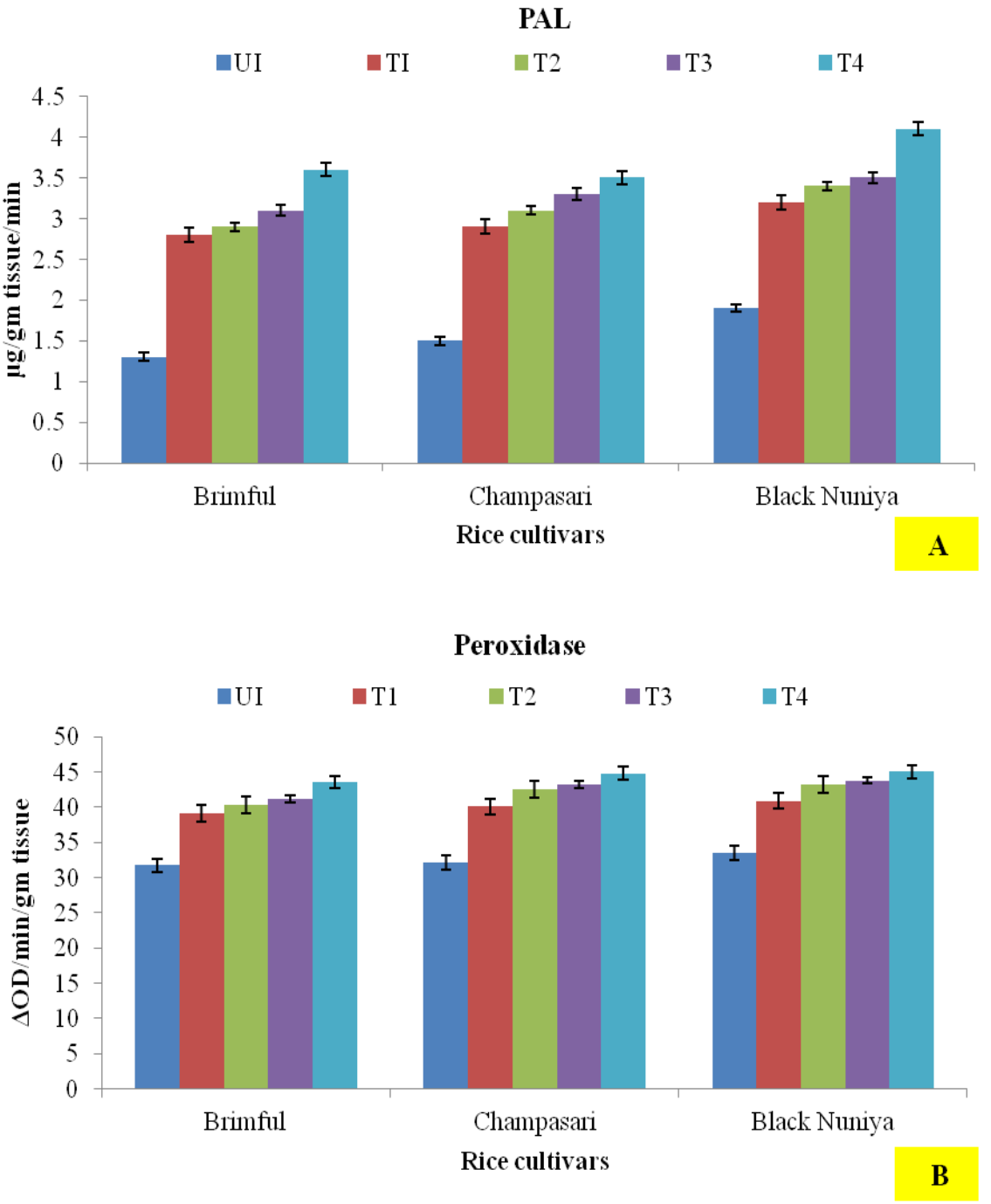



**Figure 53: Defense enzyme activity of rice cultivars against *D. oryzae* following dual and combined application of bioinoculants. (A) PAL (Phenylalanine ammonia lyase) and (B) Peroxidase.** (UI- Untreated inoculated, T1- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, T2- *T. harzianum*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

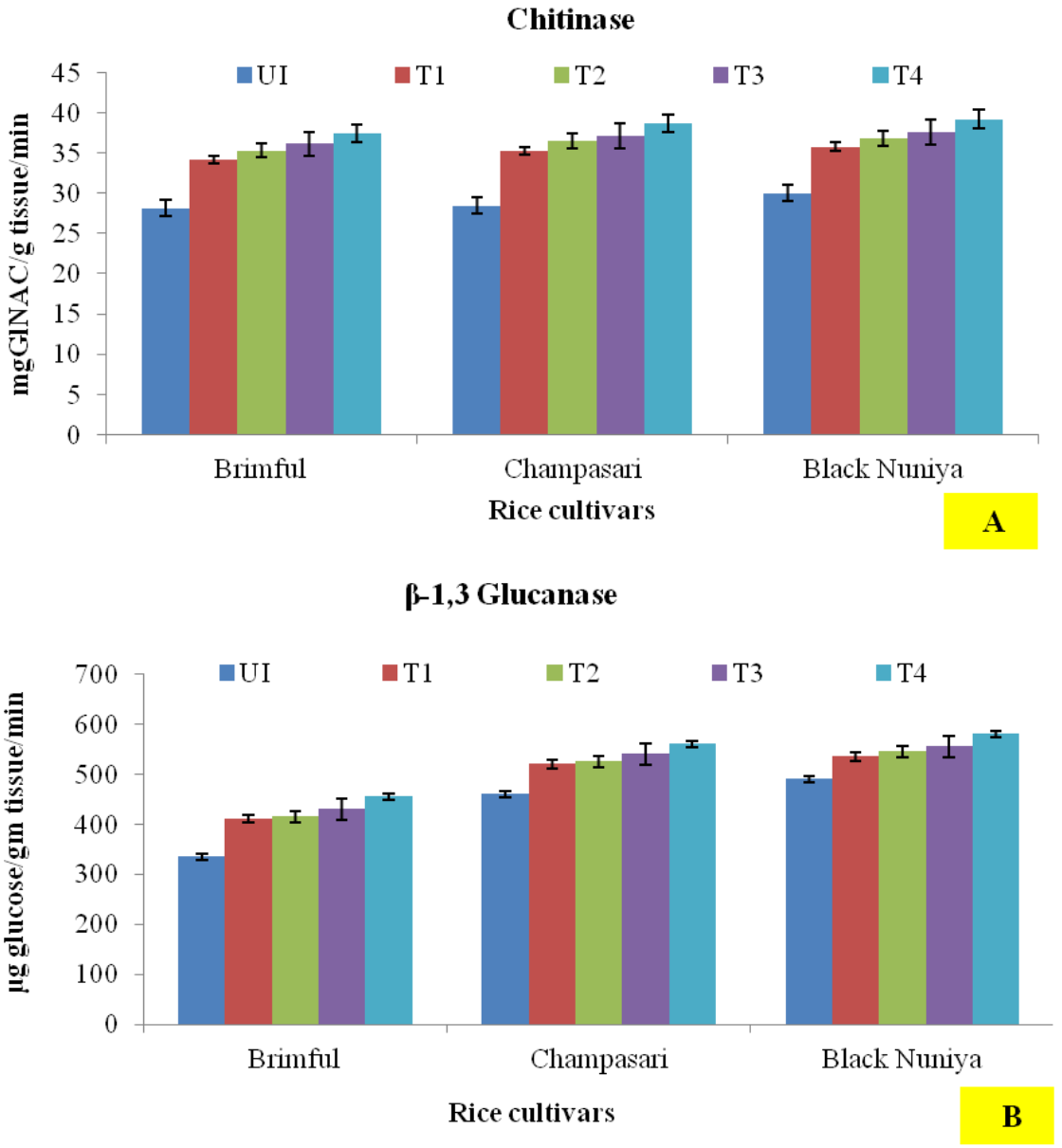



**Figure 54: Defense enzyme activity of rice cultivars against *D. oryzae* following dual and combined application of bioinoculants. (A)Chitinase and (B) β-1,3Glucanase.** (UI- Untreated inoculated, T1- *Bacillus altitudinus* (NAIMCC-B01485) + *R. fasciculatus*, T2- *T. harzianum* (NAIMCC-F-03288)+ *R.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

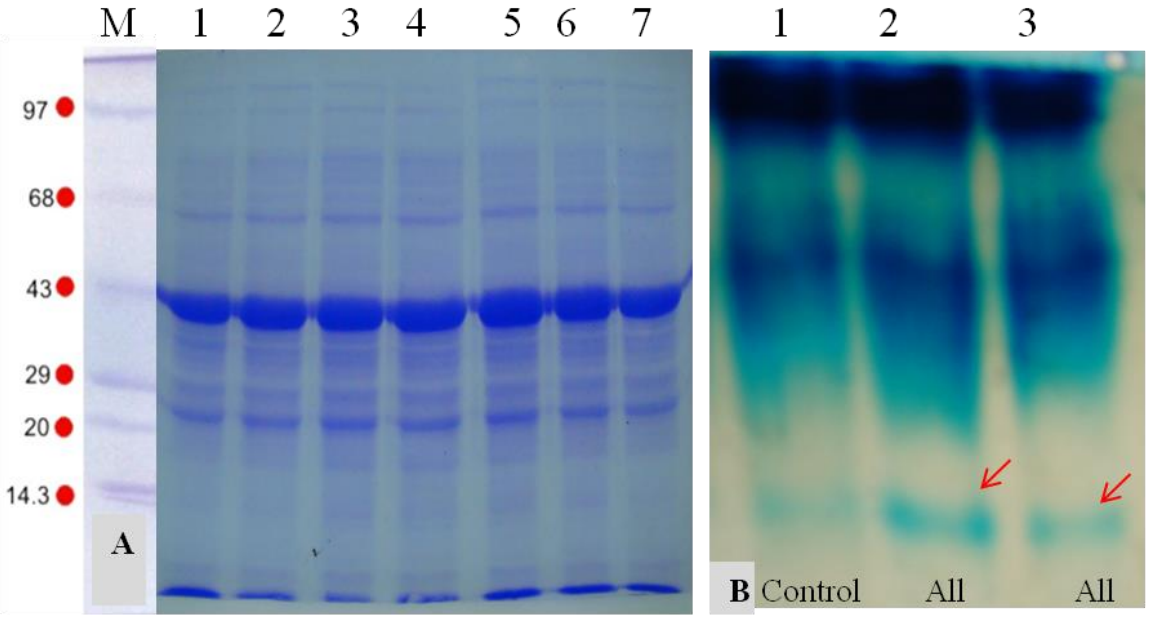



Figure 55: SDS-PAGE (A) and Peroxizyme analysis (B) of leaf proteins of rice cultivar (Black nuniya) following treatment with bioinoculants and challenged inoculation with *D. oryzae*

### 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)

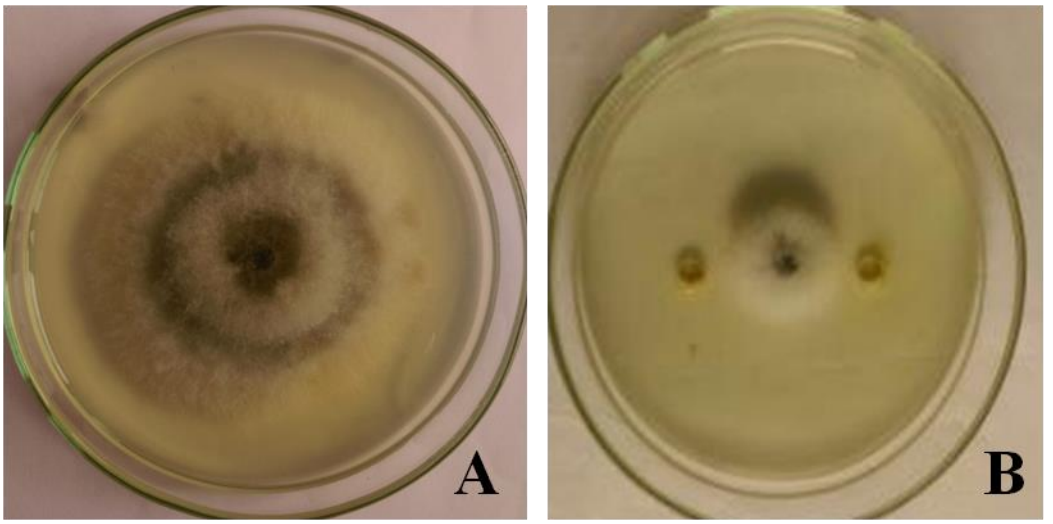



Figure 56: Radial growth bioassay of Ethyl acetate extracts of untreated control and treated inoculated rice leaves (cv. Black Nuniya). (A) Untreated Control and (B) Treated with bioinoculants [*T. harzianum* (NAIMCC-F-03288), *R. fasciculatus* and *B. altitudinus* (NAIMCC-B01485)] and inoculation with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

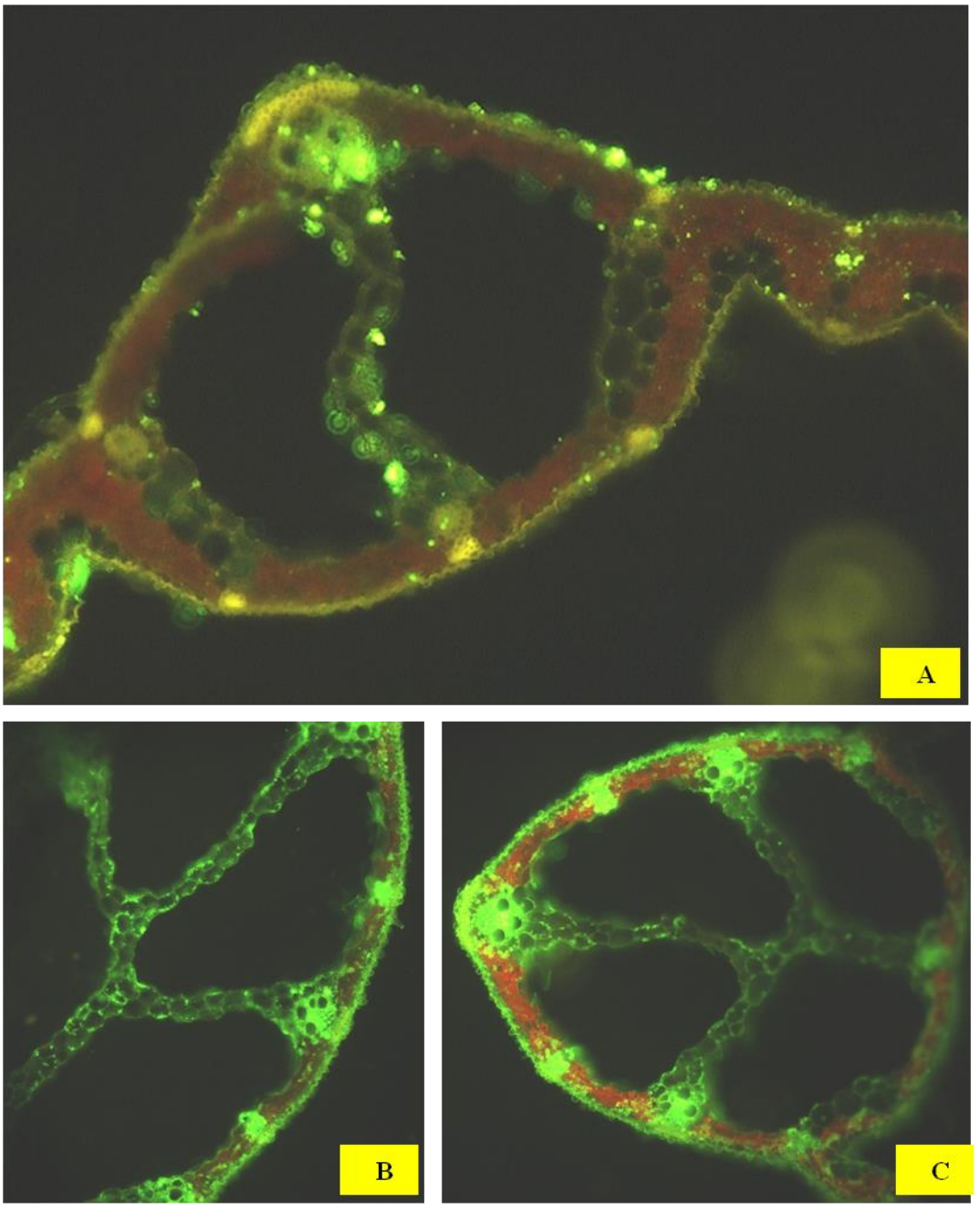



**Figure 57: Cellular localization of glucanase in leaf tissue of rice (cv. Black Nuniya) following combined treatment of bioinoculants and challenge inoculation with fungal pathogen, probed with PAb of glucanase and labelled with FITC.** (A) Control and (B&C) Treated.

### 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.

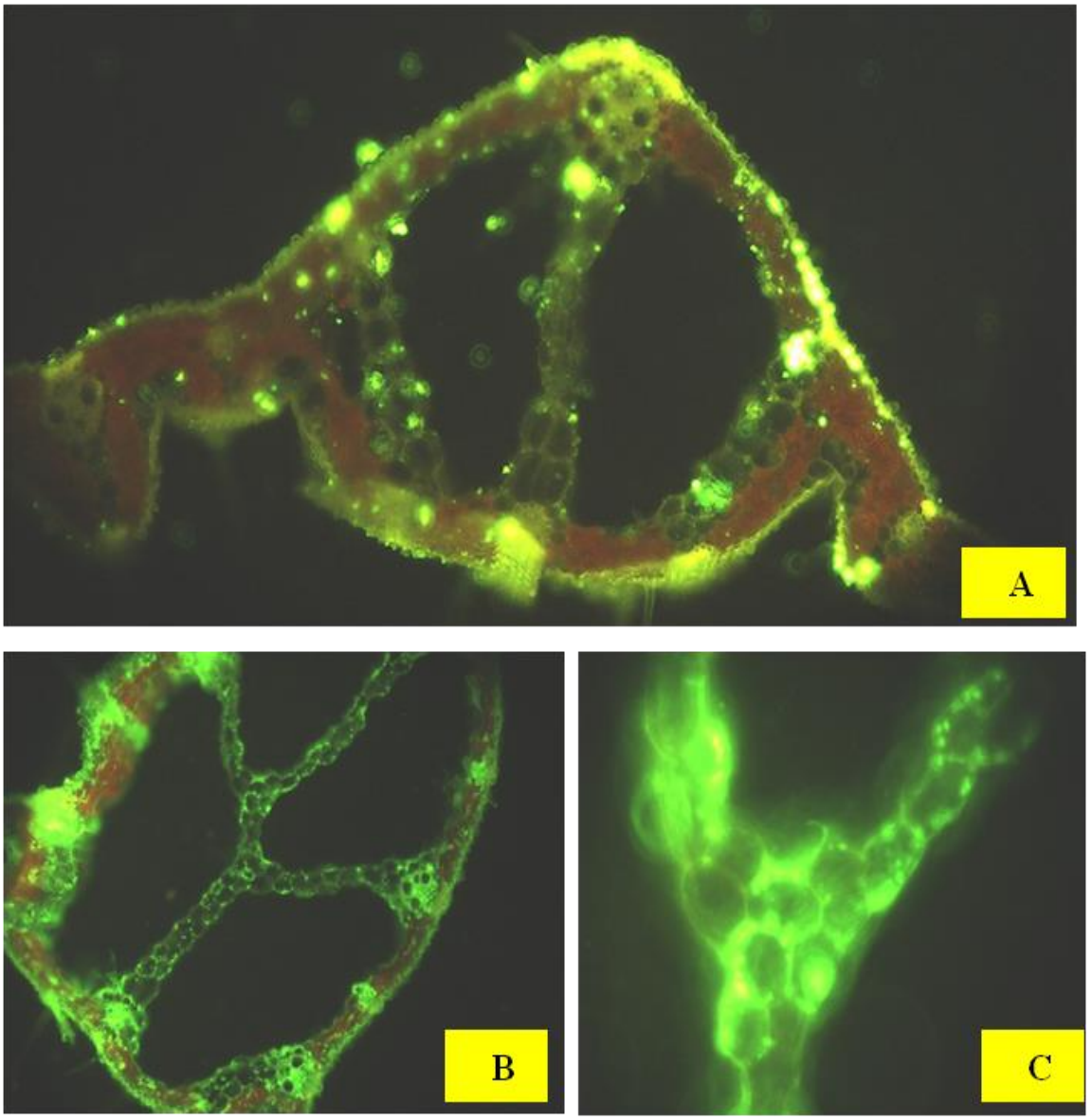



Figure 58: Cellular localization of chitinase in leaf tissue of rice (cv. Black Nuniya) following combined treatment of bioinoculants and challenge inoculation with fungal pathogen, probed with PAb of chitinase and labelled with FITC. (A) Control and (B&C) Treated.

### 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 |

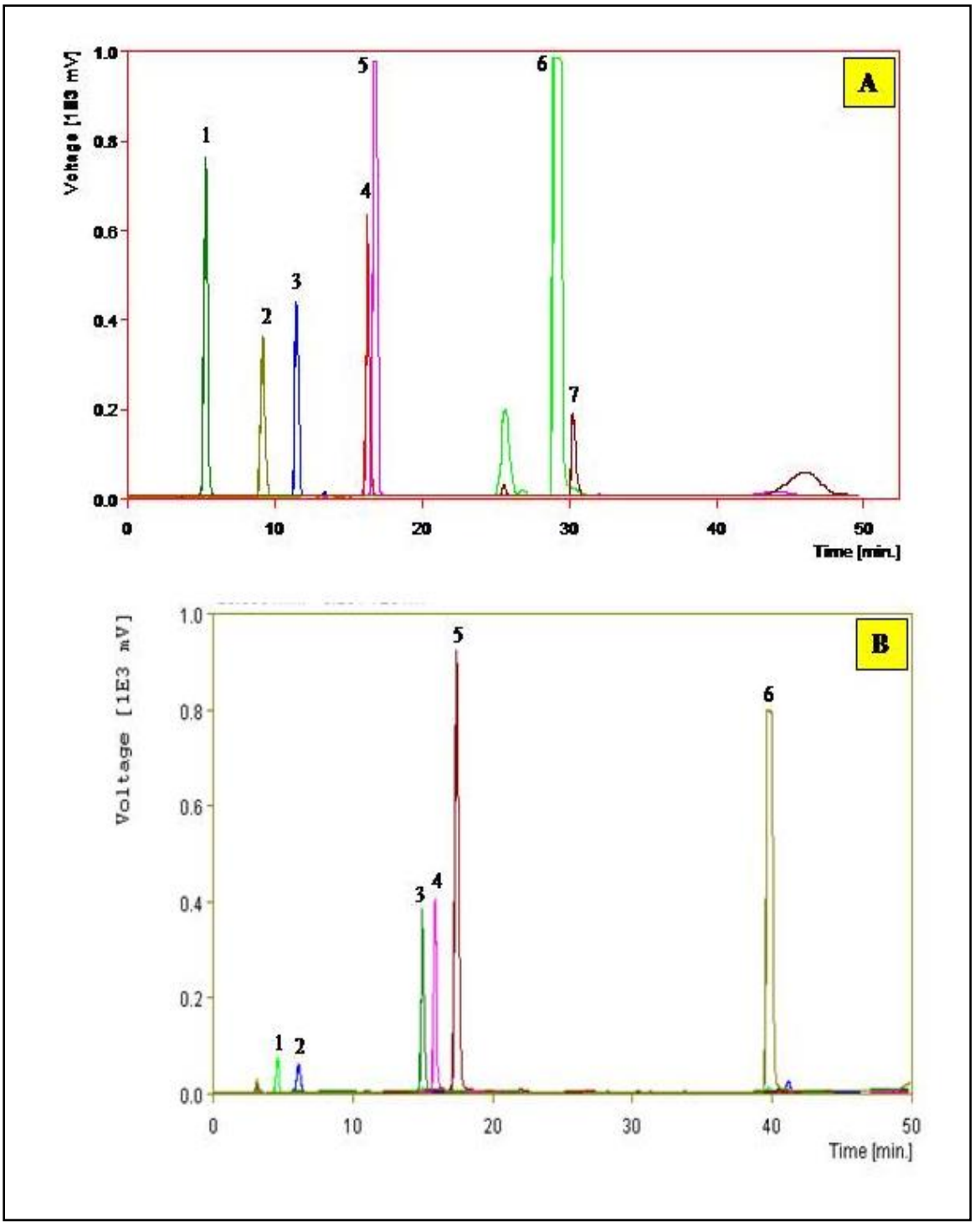



Figure 59: Standard phenolics detected through HPLC analysis. (A) 1: Gallic acid, 2: 3,4-dihydroxybenzoic acid( DHBA) , 3: Catechol, 4: Caffeine, 5: Caffeic acid, 6: Ferulic acid, 7: Salicylic acid. (B) 1: Phloroglucinol2: Pyrogallol3: Catechin4:Chlorogenic acid 5: Vanillic acid 6: Cinnamic acid.

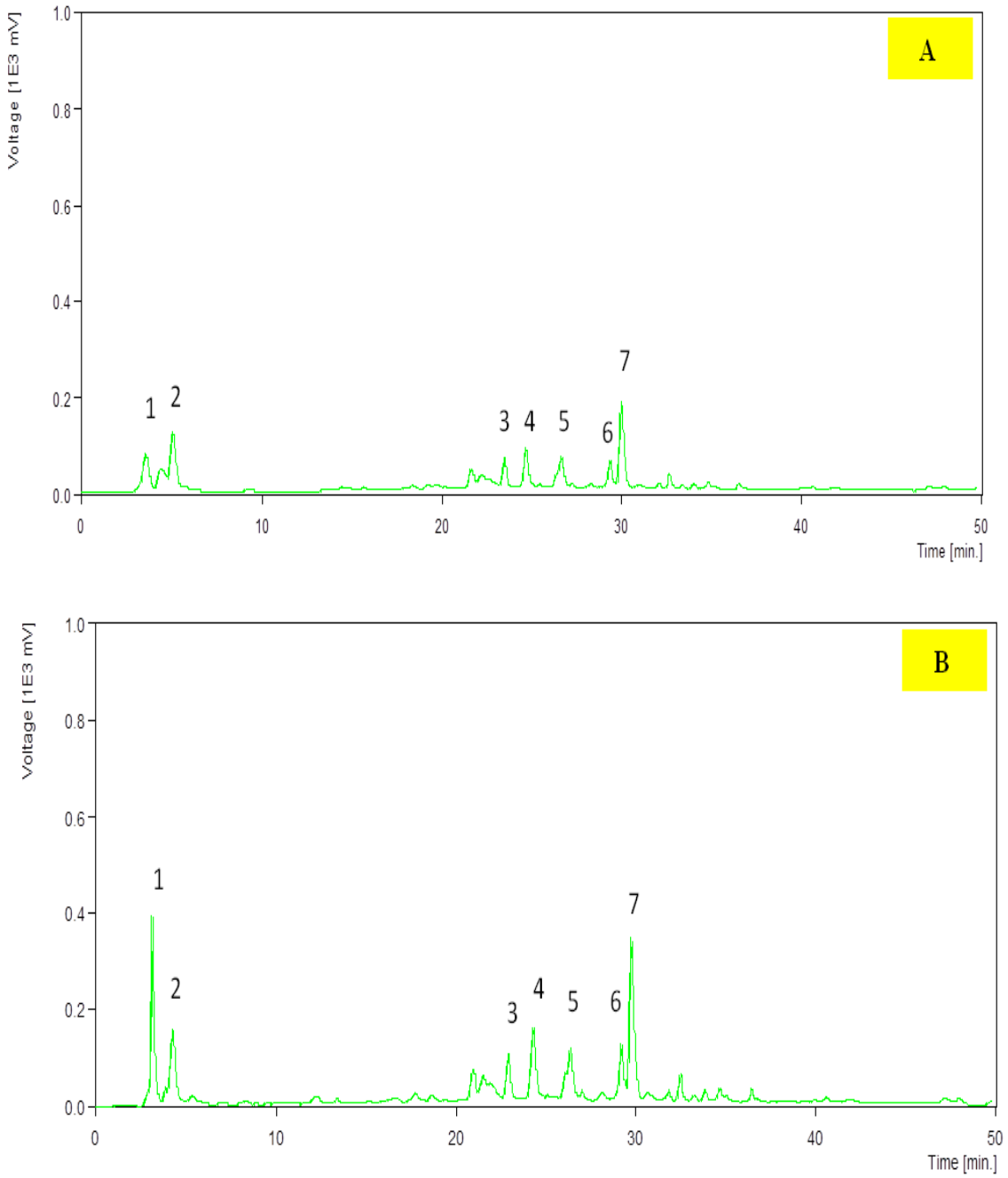



Figure 60: HPLC analysis of phenolic acid content from leaf extracts of (cv. Black Nuniya). (A) Control and (B) Treated with bioinoculants against pathogen challenge

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.

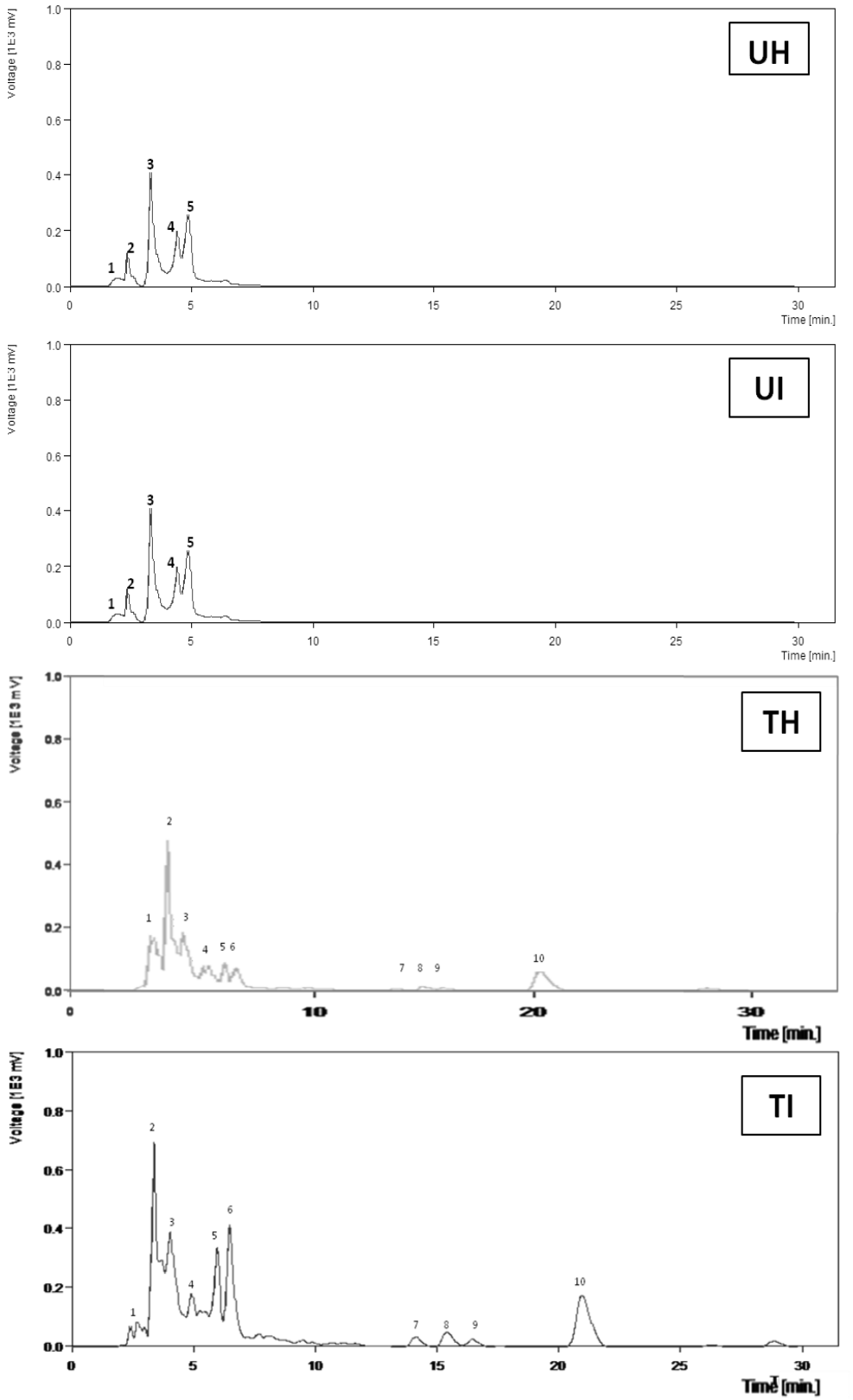



**Figure 61: HPLC analysis of Phytocassanes from leaf extracts of (cv. Black Nuniya) treated with bioinoculants and challenge inoculation with foliar fungal pathogen (*D. oryzae*).** UH= Untreated Healthy, UI= Untreated Inoculated, TH= Treated Healthy and TI= Treated Inoculated.

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 |