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Induced immunity in rice plants against *Dreschlera oryzae* following application of *Trichoderma* spp.

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Three strains each of *Trichoderma harzianum* and *Trichoderma asperellum* were evaluated for their growth promotion and induction of resistance in three cultivars of rice (*Oryza sativa*). Seed treatment and foliar spray application of all these six different strains of *Trichoderma* spp. caused plant growth promotion but significant increase was obtained in case of *T. harzianum*. All these strains exhibited *in vitro* antagonistic activity against *Dreschlera oryzae.* Disease incidence was markedly reduced following application of *Trichoderma* with a sharp increase in polyphenolic accumulations and activities of four major defense enzymes (peroxidase, phenylalanine ammonia lyase, chitinase and glucanase). Biochemical parameters such as total soluble proteins and reducing sugar were also evaluated following treatment. HPLC analysis of *Trichoderma* treated plants following inoculation with *D. oryzae* showed highest level of phytoalexin phytocassanes confirmed induction of resistance in rice plants against brown spot disease.

Keywords: *Dreschlera oryzae*, HPLC, phytocassanes, *T. asperellum, T. harzianum,* Rice

INTRODUCTION

Cereal crop plants, the most important members of the Poaceae, provide the bulk of the world's caloric intake. Rice being one of the most important cereal crop for all over the world is the seed of the grass species *Oryza sativa*. Almost half of the population of the world feeds on rice and it also adds for more than 50% of the regular calorie intake (Maclean *et al*.2002).

World rice production is forecast to rise by 1.3 percent in 2023/ 24 to 523.5 million tonnes, while international trade is expected to drop by 4.3 percent in volume terms to 53.6 million tonnes (FAO, 2023). Rice brown spot caused by *Drechslera oryzae* is the most aggressive and important rice disease in the world affecting millions of hectres of land every year (Chakrabarti 2001; Savary *et al.* 2000; Zanao *et al*. 2009). Brown spot prevails in almost every places where rice is grown specially in China (Singh, 2005).

Immunodetection of *D. oryzae*, its root colonization with arbuscular mycorrhizal fungi and their use for induction of resistance have been demonstrated by Khati and Chakraborty (2019). Practical use of natural compounds as control agents is receiving increased attention and this is partly due to their non-toxicity to humans, their systemicity and biodegradability. Free-living fungi such as *Trichoderma* spp. are common in soil and root ecosystems. Among various fungal and bacterial biocontrol agents, *Trichoderma* spp. was most frequently used against various plant diseases. Research during the previous two decades has led to the possibility of biological control as an increasingly realistic option for rice disease management (Tsahouridou and Thanassoulopoulosh, 2002). This organism has been shown to be efficient for the control of brown spot disease and the increase of plant growth on rice (Harish *et al*. 2007). Foliar application of *T. harzianum* was also found to reduce the disease severity and appreciably improve grain yield, total grain carbohydrate and protein content , in addition to a significant improvement in the total photosynthetic pigments in rice leaves (Abdel-Fattah *et al.* 2007). Genetic relatedness among *Trichoderma harzianum*, *T. asperellum* and *T. erinaceum* and their evaluation for management of Sclerotial blight of *Vigna radiata* has been

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demonstrated by Dey *et al.* (2020). Induced immunity developed by *Trichoderma* spp. in plants have been presented by Chakraborty *et al*. (2020). *Trichoderma* strains obtained from tree bark could also be considered to be utilized for the sustainable health management of rice crop has recently been reported (Mukherjee , 2023).

Plants respond to pathogen infection through a variety of defense responses that include the production of phytoalexins, antifungal secondary metabolites. Developments of phytoalexin research on cereal crops viz. rice, sorghum, wheat and maize belonging to family Poaceae with special emphasis on their types, biological activity, mechanisms of their synthesis, induction and their role in stress adaptation have been recently reviewed by Sashankar and Chakraborty (2023). In this investigation, attempts have been made for induction of resistance in rice plants against *D. oryzae* following application of *Trichoderma harzianum* and *T. asperellum.*

MATERIALS AND METHODS

Plant material

Seeds of three cultivars of rice (*Oryza sativa* L.), Black nuniya, Brimful and Champasari obtained from Bijanbari were selected. These were surface sterilized with 0.1% $HgCl_2$, washed thrice with sterile distilled water and then sown as per experimental design.

Plant growth promoting fungus (PGPF)

Six previously isolated, characterized and sequenced *Trichoderma* isolates were taken into consideration for this study. Out of these six, three were *T. harzianum*, viz RHS/S559 (NAIMCC-F-03288, HQ334997), RHS/S560 (NAIMCC-F-03289, HQ334995) and RHS/M511 (NAIMCC-F-03290, GQ995194), and the other three were *T. asperellum*, viz RHS/M512 (NAIMCC-F-03291, HQ265418), RHS/M517 (NAIMCC-F-03292, HQ334994) and RHS/M561 (NAIMCC-F-03293, HQ334996).

Seed treatment and foliar spray

The seeds of three rice cultivars were coated with PGPF spore mass prior of sowing to the field. After one month of sowing foliar spray with all six *Trichoderma* spore suspension were done to the rice plants three times at 10 days interval.

Biochemical analyses Total Soluble Protein

Soluble proteins were estimated following the method as described by Lowry *et al*., (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% CuSO4 and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% Na_2CO_3 in 0.1 NaOH) was added. This was incubated for 15 min at room temperature and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for further 15 min following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

Total Sugar

One gm of leaf tissue were weighed and crushed with 95% ethanol. The alcoholic fraction was evaporated off on a boiling water bath. The aqueous fraction was centrifuged at 10,000 rpm for 15 min and the supernatant was collected. Total sugar content was determined following the Anthrone's method as given by Plummer (1978).

Phenol

One gm of leaf tissue was cut into small pieces and immersed in boiling alcohol (100%) in water bath and heated for 5-10 mins. Tissue was crushed using 80% alcohol and filtered in Whatman no. 1 filter paper in dark and phenol content was determined following the method as described by Mahadevan and Sridhar (1982) using caffeic acid as standard.

Assessment of defense enzymes in leaves Extraction

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

Phenylalanine ammonia lyase was extracted using 0.1M sodium borate buffer (pH 8.8) and peroxidase was extracted using 0.1 M sodium phosphate buffer (pH 8.8).

Assay

Chitinase (CHT, EC 3.2.1.14) activity was measured according to the method described by Boller and Mauch (1988). The enzyme activity was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg N-acetyl glucosamine (GlcNAc) released/ min/g fresh wt.

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

Phenylalanine ammonia lyase (PAL- EC.4.3.1.5) was assayed following the method described by Chakraborty *et al.,* (1993) with modifications. PAL activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The enzyme activity was expressed as µg cinnamic acid produced min^{-1} g^{-1} fresh tissue Assay of peroxidase (POX, EC1.11.1.7) activity was done following the method of Chakraborty *et al*. (1993). The reaction mixture contained 1 ml of 0.2 Na-phosphate buffer (pH 5.4), 1.7 ml dH_2O, 100 µl crude enzyme, 100 µl O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2.

Disease Assessment

Establishment of natural brown spot disease caused by *Drechslera oryzae* (Breda de Haan) was observed and disease severity was assessed in terms of lesion number per leaf and percent disease index (PDI) was calculated following the formula - [(class rating x class frequency)/(total no. of leaves x maximum rating)] x 100.

Antifungal test of PGPF

Mycelial block (4mm) from advancing zone of different *Trichoderma* strain was placed at one side of the petri plate and fungal pathogen block was place at the other side of the plate, incubated at 28º±2ºC for 5-7 days and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the PGPF isolates.

HPLC Analysis of phytoalexin phytocassanes

For phytocassanes extraction 2gm. of rice leaf sample was cut into small pieces and shaken with 20ml. of ethyl acetate and 20 ml. of Na_2CO_3 (pH 10.5) for 18 hour. After collecting the ethyl acetate fraction it was mixed with 0.02N HCl and centrifuged at 15,000 rpm for 30 min following by evaporation in rotary evaporator. To measure the amount of Phytocassanes induced following the treatment, the supernatant was collected after the extraction procedure and put through High Performance Liquid Chromatography (HPLC) eluted with 45 % acetonitrile, (UV-VIS Detector and Liquid Chromatogram, SHIMADZU). Phytocassanes were monitored at 280 nm (Umemura *et al*., 2003).

RESULTS AND DISCUSSION

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 strains of *Trichoderma* treatments. Maximum growth was observed in plants treated with *T. harzianum* (NAIMCC-F-03288), *T. asperellum* (NAIMCC-F-03291,) and *T. asperellum* (NAIMCC-F-03293) in all the three rice varieties (Fig.1) Protein contents in all the rice cultivars following various PGPF treatments revealed enhancement in protein content of which highest accumulation was obtained in treatment containing *T.harzianum* (NAIMCC-F-03288), *T.asperellum* (NAIMCC-F-03293) which is in favour of results obtained by Chinmay *et al*. (2010). Maximum protein content in all the cultivars ranged between 70.60-73.40 mg/ gm.tissue (Table 1). Total sugar showed variations according to the treatments. Lowest amount of total sugar was obtained in plants treated with *T.harzianum* (NAIMCC-F-03288) in all the three cultivars. Total sugar content ranged between 65.84-68.87 mg/gm tissue (Table 2). In case of total phenol content, results revealed that here also maximum accumulation occurred in treatment with *T.harzianum* (NAIMCC-F-03288),

Table 1 : 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
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-F-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

Table 2: Total sugar content of rice leaves following treatments with PGPF

Treatments	Total sugar content (mg/gm tissue)		
	Black nuniya	Champasari	Brimful
Untreated Control (UI)	31.50±0.72	31.33±0.48	35.67±0.84
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-F-03291	48.90±1.03	47.33±1.21	64.67±0.83
T.asperellum NAIMCC-F-03292	48.78±0.80	45.67±0.87	50.45±1.28
T.asperellum NAIMCC-F-03293	59.94±0.94	56.70±0.40	56.16±1.22

Table 3: Total phenol content of rice leaves following treatments with *Trichoderma* spp.

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
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-F-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

Table 4 . Evaluation of Disease index for brown spot in rice plants following treatments with *Trichoderma* spp. against pathogen challenge

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	60.45	2.5	84.50	1.8	55.60	2.0
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

T.asperellum (NAIMCC-F-03291) and *T.asperellum* (NAIMCC-F-03293) in three rice varieties. (Table 3).

Defense enzymes activity when tested showed significant variation according to the treatment and higher activity was observed in treated rice plants rather than control set of plants. More enzymatic activity were found in plants treated with. *T.harzianum* (NAIMCC-F-03288), *T.asperellum* (NAIMCC-F-03291) and *T. asperellum* (NAIMCC-F-03293) (Fig. 3&4). From the above experimental results it is revealed that seed coating as well as foliar application of

Table 5. *In vitro* antagonistic tests of selected isolates of *Trichoderma* spp against *D.oryzae*

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

Isolates of *Trichoderma* testd against *D.oryzae

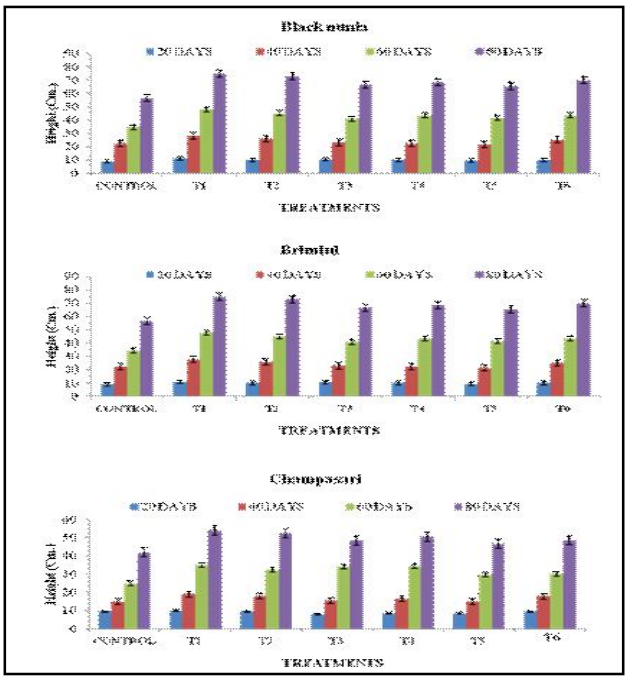

Fig.1: Growth promotion of rice cultivars following treatment with different PGPF. (C= Untreated Control, T1= *T. harzianum* NAIMCC-F-03288, T2= *T. harzianum* NAIMCC-F-03289, T3=*T.harzianum* NAIMCC-F-03290, T4= *T.asperellum* NAIMCC-

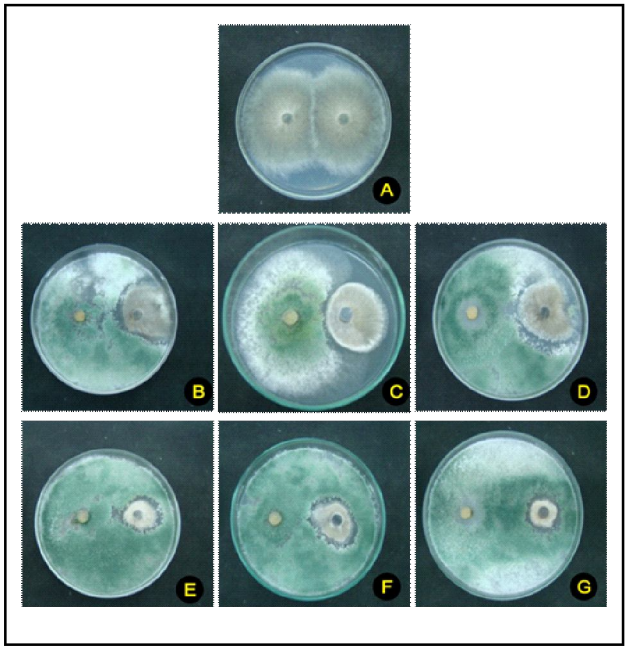

Fig. 2: *In vitro* antagonistic test of foliar fungal pathogen (*D. oryzae*) against different *Trichoderma* spp. (A) Control (*D. Oryzae*), paired with (B) *T. harzianum* (NAIMCC-F-03289), (C) *T.harzianum* (NAIMCC-F-03288), (D) *T. harzianum* (NAIMCC-F-03290), (E) *T. asperellum* (NAIMCC-F-03291), (F) *T. asperellum* (NAIMCC-F-03292), (G) *T. asperellum* (NAIMCC-F-03293).

different strain of *Trichoderma* (PGPF) in rice decreases disease severity and this is in line with the findings of Nzojiyobiri *et al.* (2003) who reported the efficacy of these bio-agents against blast and bacterial blight respectively. In case of untreated infected plants PDI was quite higher than PGPF treated infected plants. Application of *T.harzianum* (NAIMCC-F-03288) reduced disease index markedly compared to untreated control and other treated set of plants (Table 4). *In vitro* antagonistic activity of *Trichoderma* isolates against *D. oryzae* showed that *T. harzianum* (NAIMCC-F-03288) showed the maximum inhibition zone (Fig. 2, Table 5). HPLC analysis was done for detecting the phytoalexin namely Phytocassanes from the leaves of rice cultivar Black nuniya in untreated inoculated and PGPF (*T.harzianum* , NAIMCC-F-03288) treated

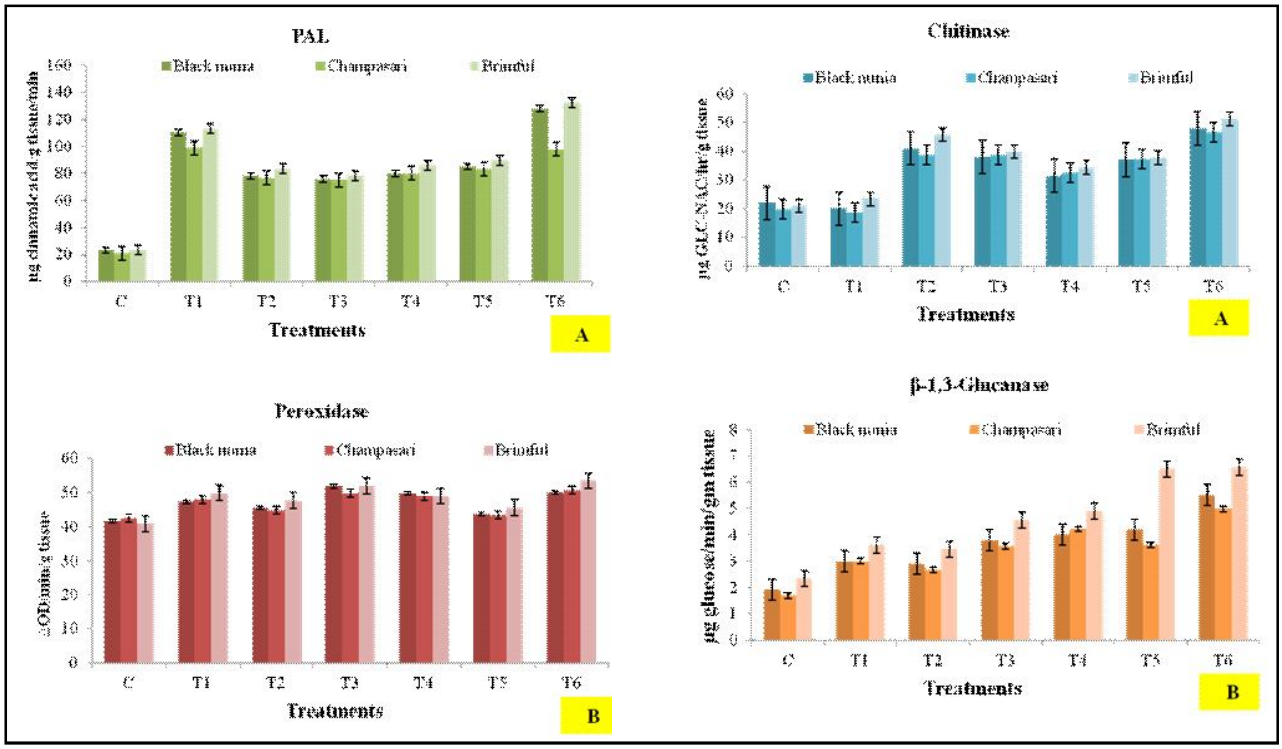

Fig. 3 : Defense enzyme activity (A) Phenylalanine ammonia lyase and (B) Peroxidase of rice cultivars following treatment with PGPF and pathogen challenge. C-Untreated Control,T1-T6=Treated Inoculated [T1-*T.asperellum* (NAIMCC-F-03293), T2-*T.harzianum* (NAIMCC-F-03289), T3-*T.harzianum* (NAIMCC-F-03290),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

Fig. 4: Defense enzyme activity (A) Chitinase (B) â-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)]

and inoculated plants. Treated plants exhibited lowest PDI percentage. A total of four peaks were clearly 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. The higher accumulation of phytocassanes in treated inoculated plants with lowest PDI percentage are in acceptance with the fact that phytocassanes play a significant role in disease resistance of rice plants which is in acceptance with the results given by Umemura *et al*. (2003) suggesting the involvement of phytocassanes in disease resistance of rice plants to the rice blast fungus.

It was reported that phytocassanes A, B, C and D are produced by induction mediated by infection with the pathogens *M. oryzae* and *Rhizoctonia solani*. However, the phytocassanes E are induced by the pathogen *Phytophthora infestans* and have antifungal action against *M. oryzae*. Furthermore, phytocassane accum-

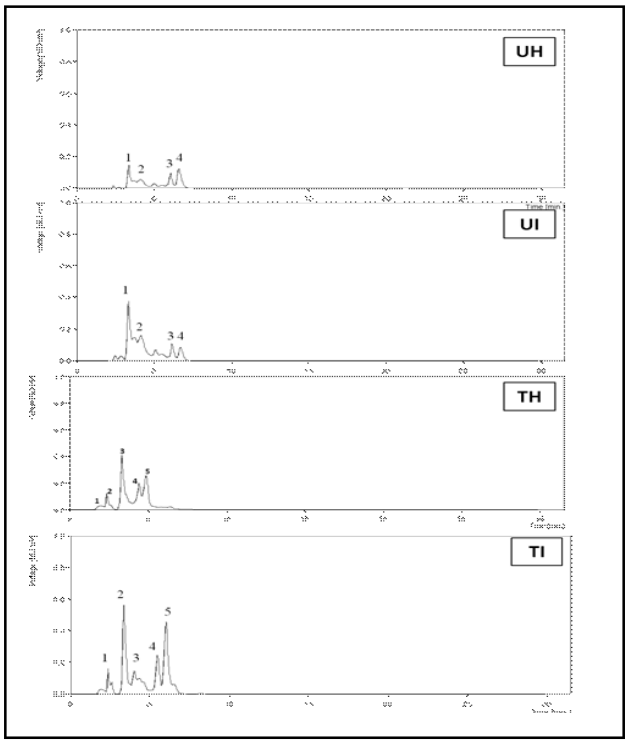

Fig. 5: HPLC analysis of Phytocassanes from leaf extracts of rice plant (cv. Black Nuniya) treated with *T. harzianum* (NAIMCC-F-03288) and pathogen challenge. (UH- Untreated Healthy, UI-Untreated Inoculated, TH- Treated Healthy and TI- Treated Inoculated)

ulation was most abundant at the edges of necrotic lesions, indicating that the phytoalexins prevent subsequent spread of the fungus from the infected site. Seed bacterization as well as foliar application of *Bacillus altitudinis* could markedly reduced the natural occurrence of Brown spot disease of rice plants caused by *D. oryzae*. HPLC analysis of treated rice plants showed highest level of phytoalexin namely Phytocassanes suggesting induction of resistance in rice plants (cultivar Black nuniya) against *D. oryzae* (Khati *et al*. 2016). Prospects of rice phytoalexins in food preservation and their role in stabilizing economic status and food security have also been discussed (Sashankar and Chakraborty, 2023).

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