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Biochemical responses of rice (*Oryza sativa* L.) as influenced by *Trichoderma* spp. under stresses like drought and blast disease

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Abstract
National food security systems largely depend on the production and productivity of rice which is challenged by biotic and abiotic stresses. Among biotic stresses, blast, a devastating fungal disease of rice is cosmopolite in its presence. A drought like situation also occurs at a similar temperature and moisture levels and aggravates blast disease. There are instances of occurrence of blast and drought on single crop that catapult the loss in yield. In the present study, the impact of isolates of endophytic fungus *Trichoderma* on rice response to drought and blast disease was investigated. Five different isolates of *Trichoderma* were used in the study, of which three [T-14, 94(A) and IRRI-2] were obtained from IRRI and two (OT-3 and OT-8) were isolated from native soil by serial dilution method. Seeds treated with five isolates were sown in different pots and further the crop was challenge inoculated with *Pyricularia grisea* and drought stress induced by withdrawing irrigation. The studies on biochemical parameters revealed an augmentation in CMS, phenol, peroxidase, chitinase and SOD which were known to alleviate these stresses through various mechanisms. The study also revealed a reduction in the level of oxidative chemicals like ascorbate peroxidase, proline, malondialdehyde and hydrogen peroxide due to interference of *Trichoderma* spp.

Keywords: *Trichoderma* spp, drought, blast, *Pyricularia grisea*

Introduction
The burgeoning rise in human population demands for higher production of rice as it provides staple food for more than half of the world's population (Huang *et al*., 2014) [9]. India being a populous country, contributing substantial to the world's population is a leading producer of rice, but its rice productivity remains unsatisfactory. Among the various reasons for unsatisfactory productivity, the susceptibility of crop to several biotic and abiotic stresses resulting in yield loss occupies a prime position. Blast caused by *Magnaporthe grisea* is the eminent biotic stress because of its wide distribution and destructiveness under favourable condition resulting in serious loss of yield. Besides rice ecosystem is also affected by abiotic stresses like drought, which is the major constraint in production of rice. Approximately, 50% of the world rice population is affected by drought stress (Bouman *et al*., 2005) [2]. There are many reports which depict an aggravation of blast disease under drought stress. Hence, different situations of stress during crop period can be combated by mitigating both drought and blast disease of rice using suitable bio-control agents to enhance the productivity of rice. *Trichoderma* spp. is well known bio-control agents against phytopathogens. *Trichoderma* releases a variety of compounds that induce resistance responses to biotic and abiotic stresses (Harman, 2004) [7]. *Trichoderma harzianum* increases the growth of roots, auxin production, siderophore formation, thereby increasing plant productivity and results in increased level of plant enzymes including various peroxidases, chitinases, β -1,3-glucanase, lipoxygenase-pathway hydro peroxide lyase and compatible solutes like proline, phenols to provide durable resistance against stress (Harman, 2006 and Gachomo *et al.*, 2008) [8, 5]. Its colonization modulates the endogenous plant hormones, plant enzymes, antioxidants and compatible solutes and compounds like phytoalexins and phenols level to confer drought tolerance (Chen *et al*., 2013) [4]. Superoxide dismutage (SOD) is one of the most effective components of the antioxidant defense system in plant cells against ROS (Reactive Oxygen Species) toxicity. *Trichoderma* enhances protection against ROS by increasing ROS scavenging antioxidant activities (Pandey *et al*., 2016) [10]. Production of malondialdehyde (MDA), which is an effective means of evaluating oxidative stress, increases as drought stress increases in plant and serves as an index of lipid peroxidation (Shukla *et al*., 2012) [13]. But *Trichoderma harzianum* treatment reduces the accumulation of this lipid peroxides in rice plants under

drought stress (Pandey *et al*., 2016) [10]. Hence, present study involved changes in biochemical constituents of rice as influenced by different isolates of *Trichoderma* spp. under stresses like drought and blast disease.

Materials and Methods

Isolation, purification of *Trichoderma* spp. and induction of stresses

Three different isolates of *Trichoderma harzianum* [T-14, 94 (A) and IRRI-2] were obtained from IRRI and two *Trichoderma* isolates (OT-3 and OT-8) were isolated from native soil by serial dilution method. *Trichoderma* isolates were grown on Potato Dextrose Agar (PDA) and incubated at 28±2ºC in BOD incubator. Morphologically different colonies appearing on the plates were purified in PDA and preserved at 4ºC. *Trichoderma* was applied in two schedules. 1) Through seed treatment @ 5 g per kg of seed, 2) foliar spray during tillering stage. Further the crop was challenged to biotic stress by inoculating *Pyricularia grisea.* For this conidial suspension of *P. grisea* was prepared by washing the culture plates with 10 ml of distilled water. The leaves were punctured with the help of sterile pin. The suspension was inoculated into the punctured leaf with the help of needle and immediately protected by cotton swab. The inoculated plants were covered with polythene bags moistened inside for 24 hr with a view to provide appropriate humid conditions during initial stages of infection. Then the crop was also challenged to abiotic stress by inducing drought by withholding water application for five consecutive days at the time of flowering stage of rice crop and a thatch was made to avoid the entry of rain water into the pots.

Assessment of biochemical parameters

All the biochemical parameters were taken after induction of stresses. The chitinase activity was assayed by using the method given by Giri *et al.* (1998) [6]. For enzyme extraction 0.5 g of infected and control leaf samples were macerated with 2 ml of 0.1 M sodium citrate buffer and the homogenate was centrifuged at 10,000 rpm for 10 min at 10°C. 0.5 ml of supernatant was added to 2 ml of chitin suspension containing 7.5 mg of BSA and was incubated in water bath at 37°C for 3 h. From that an aliquot of 0.1 ml was taken for the estimation of N-acetyl glucosamine as per the method of Nelson-Smogyi.

Enzyme extract for Ascorbate Peroxidase (APX) was prepared by grinding 0.5 g of leaf sample with 2 ml of 100 mM Potassium phosphate buffer (pH = 7.5). The homogenate was centrifuged at 15,000 rpm for 15 min at 4°C. The reaction mixture contained 2.3 ml phosphate buffer, 0.2 ml ascorbic acid, 0.2 ml EDTA, 50 µl enzyme extract, 50 µl H_2O_2 and 0.3 ml distilled water. The reaction was started with addition of 0.2 ml of hydrogen peroxide. Decrease in absorbance after 30 sec was measured at 290 n min UV–visible spectrophotometer. The activity was determined using molar extinction coefficient U = 2.8 mM^{-1} cm^{-1}.

Enzyme extract for SOD was prepared by grinding 0.5 g of leaf samples with 2 ml of 100 mM Potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 15,000 rpm for 15 min at 4°C. The reaction mixture contained, 1.5 ml phosphate buffer, 0.2 ml methionine, 0.1 ml Ethylene-diamine tetra acetic acid (EDTA), 0.1 ml sodium carbonate,0.1 ml enzyme extract, 0.1 ml NBT, 0.9 ml distilled water, and 0.1 ml riboflavin. The reaction was started by adding 0.1 ml of riboflavin and placing the tubes under two 15 W fluorescent lamps for15 min. A complete reaction mixture without enzyme, which gives the maximal colour, served as control. Switching off the lights and putting the tubes into dark stopped the reaction. The non-irradiated complete reaction mixture served as blank.

The assay of peroxidase activity was performed as described by Sadasivam and Manickam (1996) [11]. CMSI were assayed by estimating the electrolyte leakage from fresh leaf tissue into the distilled water using the method described by Sairam and Srivastava (2002) [12]. Proline content of leaf sample was estimated by using the method given by Bates *et al.,* 1973 [1]. Total phenolic content (TPC) was determined using the method described by Singleton *et al.* (1999) [14]. Lipid peroxidation was measured in terms of malondialdehyde (MDA) in the leaf based on reaction with thiobarbituric acid (TBA) following the procedure described by Cakmak and Horst (1991) [3].

Results and Discussion

Biochemical changes were brought about in plants upon invasion by *P. grisea* and abiotic agent like drought which are shown in Table 1. Enzyme chitinase is unanimously considered as a tool to strengthen plant immune response against a variety of pathogens by various workers owing to its property to lyse fungal cell wall. The data obtained in present investigation revealed an increase in level of chitinase activity in *Trichoderma* treated plants as compared to untreated plants. Among the treated plants highest amount of chitinase activity (48.93 µg NAG released min^{-1} mg^{-1} protein) was recorded in 94(A) treated plants and lowest (44.36 µg NAG released min^{-1} mg^{-1} protein) in OT-8 treated plants. This finding is in agreement with that of Van Aalter *et al.* (2000) [15]. SOD is one of the most effective components of the antioxidant defense system in plant cells against ROS (Reactive Oxygen Species) toxicity. *Trichoderma* enhances protection against ROS by increasing ROS scavenging antioxidant activities. *Trichoderma harzianum* isolate 94(A) was found to be more effective in the contest of SOD among all the isolates. Similarly, increased levels of peroxidase activity and phenol content were recorded in *Trichoderma* treated plants and among the isolates, both the parameters were found to be highest in 94(A) treated plants. Shukla *et al.* (2012) [13] also demonstrated positive influence of *Trichoderma* treatment on phenol content of plants under stress conditions. The enzyme APX is considered as a scavenger of H_2O_2 which is formed in plant cells under normal as well as stress conditions. A record of APX in the present study revealed a significant decrease in the APX level in plants treated with *Trichoderma* spp. Among the *Trichoderma* isolates, 94(A) induced least synthesis of APX (226.26 n moles ascorbates oxidized min^{-1} mg^{-1} protein) indicating more tolerance to the stress. This role of *Trichoderma* in reducing the stress due to oxidants and thereby reducing the production of enzymatic antioxidants like APX is a new line of study.

A stress condition causes severe loss in membrane stability in rice plants. In the present study the untreated plants showed severe loss in membrane stability to an extent of 25.57%. The maximum in CMSI value (38.71 %) was recorded by 94(A) treated plants. Proline is one of the best solutes whose protective function has been studied in the present experiment. In the present study, the proline content was increased in response to stress and the control plant showed highest proline content. The plants treated with *Trichoderma* spp. showed least increase in proline content as compared to control plants. Drought and disease stress increase MDA and

H_2O_2 content in the plants. The present investigation revealed highest contents of MDA and H_2O_2 in untreated control plants and lowest in *Trichoderma* treated plants. In all the biochemical studies, *Trichoderma harzianum* isolate 94(A) was found to be superior among all the isolates. Similar findings have been demonstrated by Shukla *et al*. (2012) [13] in the past.

Table 1: Influence of *Trichoderma* spp on biochemical constituents of rice plant under stresses like drought and blast disease

Trichoderma isolates	Chitinase content (µg NAG released min^{-1} mg^{-1} protein)	Ascorbate Peroxidase (APX)(n moles ascorbates oxidized min^{-1} mg^{-1} protein)	Superoxide Dismutase (SOD) (U mg^{-1} protein)	Peroxidase content (U g^1 of FW)	CMSI (%)	Proline Content (µmol g^{-1} of FW)	Phenol Content (µg g^{-1} of FW)	MDA Content (µmol g^{-1} of FW)	H_2O_2 Content (µmol g^{-1} of FW)
T-14	47.01	233.74	14.73	88.88	37.37	8.02	141.04	2.94	1.82
94(A)	48.93	226.26	12.80	92.72	38.71	6.85	152.08	2.70	1.56
IRRI-2	46.57	242.26	17.56	83.80	35.19	8.20	129.17	3.32	2.04
OT-3	45.35	253.56	19.19	79.28	32.50	8.88	117.26	3.84	2.37
OT-8	44.36	259.88	20.96	78.53	31.88	9.10	114.82	3.92	2.68
-	29.22	313.65	12.49	62.83	25.57	12.27	81.01	7.08	3.32
SE(m)±	0.28	5.20	0.27	0.33	0.39	0.12	0.89	0.05	0.02
C.D. ($p \leq 0.05$)	0.84	15.56	0.81	0.99	1.18	0.37	2.66	0.14	0.05

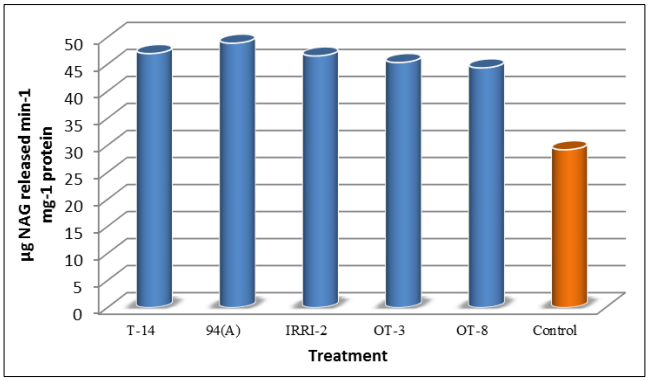

Fig 1: ascorbate peroxidase

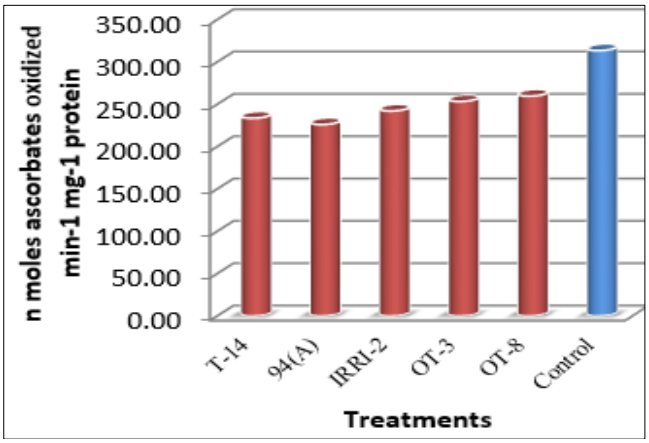

Fig 2: superoxide dismutase

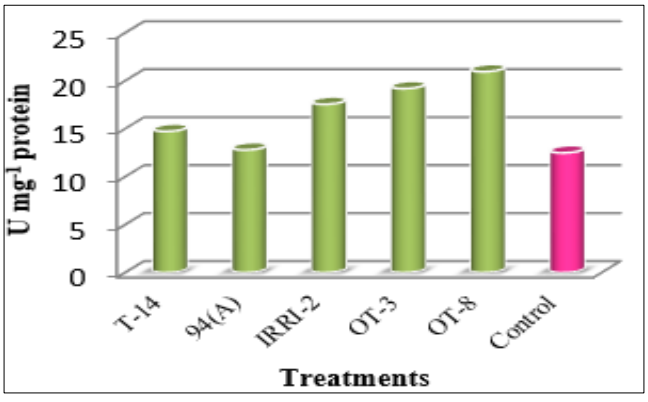

Fig 3: peroxidase

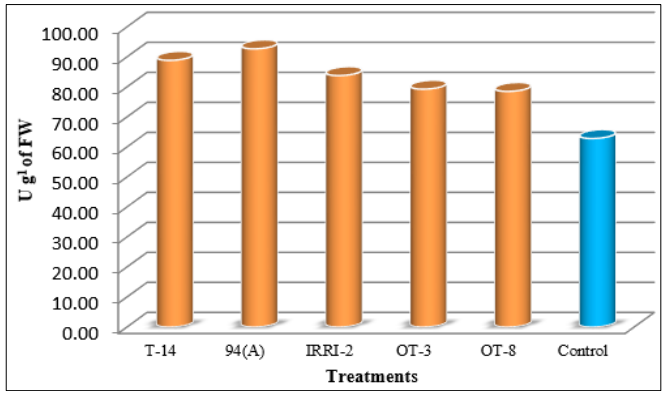

Fig 4: cell membrane stability

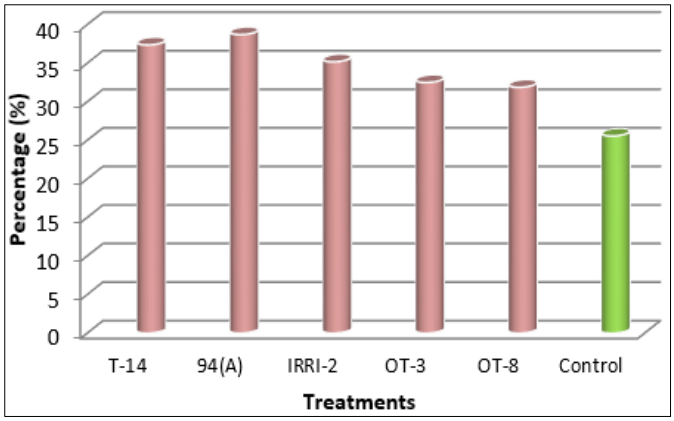

Fig 5: proline

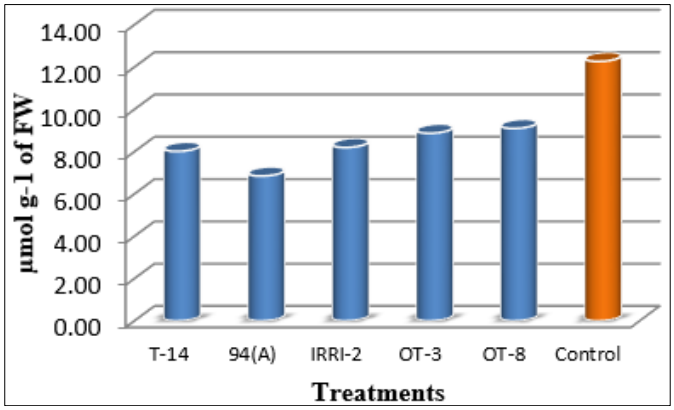

Fig 6: phenol

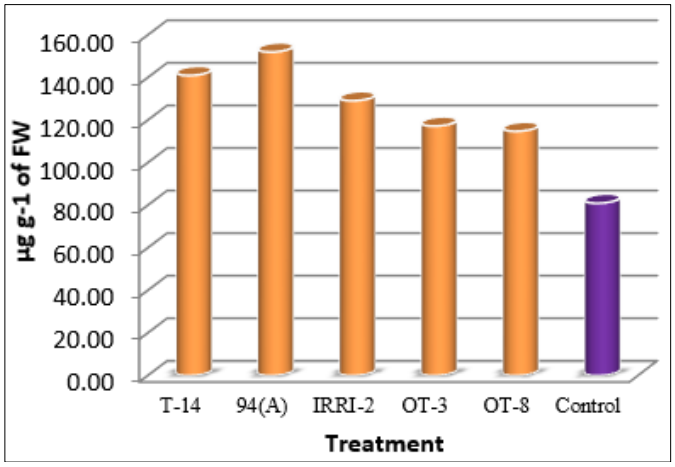

Fig 7: malondialdehyde

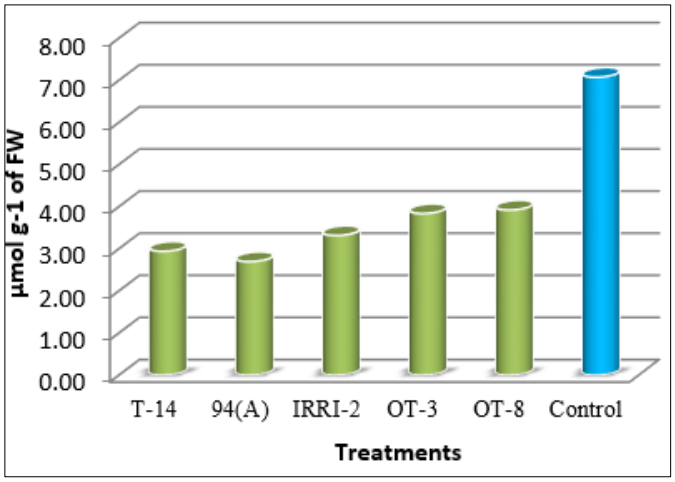

Fig 8: and hydrogen peroxide

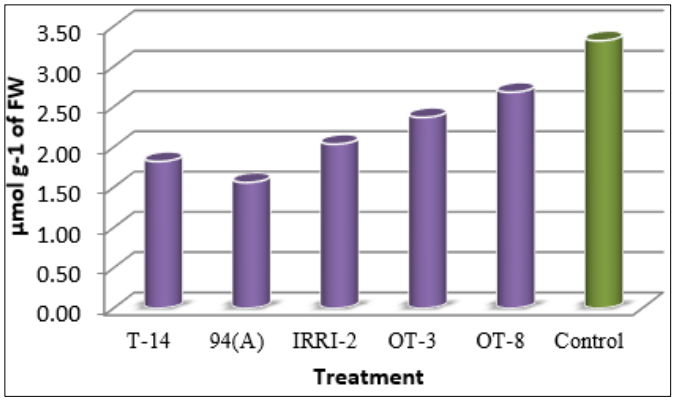

Fig 9: content due to *Trichoderma* treatment

Conclusion

The biochemical constituents of rice plant were found to be influenced by different isolates of *Trichoderma* spp. It can be concluded from the present study that *Trichoderma harzianum* has multiple benefits in plant. Their benefits in reducing disease and drought stress have been demonstrated separately. This study happens to be a novel approach to manage two stresses with single intervention. The effect of *Trichoderma* on disease incidence and drought stress demonstrated in the present experiment were correlated with biochemical characteristics which substantiated the findings. The use of different isolates and record of variations among them in rendering benefit is a new concept which should be further investigated.

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