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Bioprospecting Drought Tolerant *Trichoderma harzianum* Isolates Promote Growth and Delay the Onset of Drought Responses in Wheat (*Triticum aestivum* L.)

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Abstract The objectives of this study were to investigate whether seed biopriming through drought tolerant isolates of *Trichoderma harzianum* induces drought tolerance in wheat. Physiological and biochemical parameters were also monitored under greenhouse conditions to explore the mechanism underlying plant water stress resilience in response to *Trichoderma* inoculation.The impact of bioprospecting *Trichoderma harzianum* drought tolerant isolates, *Rani Th-14, Rani Th-21, Rani Th-25, Rani Th-30 and Rani Th-39* on wheat response to drought was studied. Measurements of the stomatal conductance, net photosynthesis, chlorophyll fluorescence and greenness of plants were performed. In addition, analysis of the total phenolic compounds, free proline, MSI (membrane stability index) as well as lipid peroxidation was carried out. With or without exposure to drought conditions, colonization by *Trichoderma* isolates promoted seedling growth, the most consistent effect being an increase in shoot and root growth. The primary direct effect of colonization was promotion of root growth, regardless of water status, and an increase in water content, which it is proposed, caused a delay in many aspects of the drought response of wheat. Colonization of *Trichoderma* by seed biopriming enhanced drought tolerance of wheat plants as they delayed drought induced changes like stomatal conductance, net photosynthesis, chlorophyll fluorescence and greenness of plants. Drought stress from 4 to 13 d of withholding water induced an increase in the concentration of many stress induced metabolites in wheat leaves, while *Trichoderma* colonization caused a decrease in proline, MDA and H_2O_2 contents and an increase in phenolics concentration. Among different *Trichoderma* isolates, *Rani Th-14* induced maximum drought tolerance as treated plants recorded only 20-40 per cent wilting even at 13 days drought stress (DDS). The study is important as the experiments confirmed that the drought tolerant isolates of *Trichoderma* through seed bio priming are critical in inducing tolerance to drought.

Keywords Drought stress; *Trichoderma harzianum*; *Triticum aestivum*; Free proline; Phenolics; Photosynthesis; Chlorophyll fluorescence

Introduction

Drought stress is the most widespread environmental stress, which affects growth and productivity and induces many physiological, biochemical and molecular responses in plants (Arora et al., 2002). Plant experiences drought stress either when the water supply to roots becomes difficult or when transpiration rate becomes very high. Available water resources for successful crop production have been decreasing in recent years. Furthermore, in view of various climatic change models suggested by scientists in many regions of world, crop losses due to increasing water shortage will further aggravate its impacts (Passioura, 2007). With respect to physiological aspects of drought tolerance in crop plants, a number of mechanisms have been studied that operate at the whole plant level (Blum, 1998). Although such

traits are likely to be associated with improved productivity, they are frequently genetically complex and therefore require considerable effort to manipulate in a drought tolerance program.

The permanent or temporary water deficit severely hampers the plant growth and development more than any other environmental factor. The first and foremost effect of drought is impaired germination and poor stand establishment (Harris et al., 2002). The incorporation of factors enabling plants to withstand drought stress would be helpful to improve crop production under drought conditions. Incorporation of *Trichoderma* during seed biopriming treatments in many cereal and vegetable crops has resulted in increased levels of plant growth hormones and improved seed performance (Howell, 2003). Biopriming is a process of biological seed treatment that refers to a combination of seed hydration and seed inoculation with beneficial organisms to protect seed. The technique helps seeds to evenly germinate even under adverse soil conditions (Singh et al., 2003). Some of the mechanisms used by *Trichoderma* to alter the drought response includes drought avoidance through morphological adaptations, drought tolerance through physiological and biochemical adaptations, and enhanced drought recovery (Malinowski et al., 2000).

Many studies have shown the decreased photosynthetic activity under drought stress due to stomatal or non-stomatal mechanisms (Del Blanco et al., 2000; Samarah et al., 2009). Under drought, the maintenance of leaf turgor may also be achieved by way of osmotic adjustment in response to the accumulation of proline, sucrose, soluble carbohydrates, glycine betaine, and other solutes in cytoplasm thereby improving water uptake from drying soil. The process of accumulation of such solutes under drought stress is known as osmotic adjustment and observed to enhance tolerance to water stress (Nayyar et al., 2003). Of these solutes, proline is the most widely studied because of its considerable importance in the stress tolerance. Proline accumulation is the first response of plants exposed to water-deficit stress in order to reduce injury to cells. Though proline is one of the best known solutes, however, its relative importance for tolerance and precise protective function during stress requires investigations (Bohnert et al., 1996). Drought induces oxidative stress in plants by generation of reactive oxygen species (ROS) (Farooq et al., 2009). Production of MDA, which is an indicative of oxidative stress, increases as drought stress increases in plant, serves as an index of lipid peroxidation. Peroxidation damage of the plasma membrane leads to leakage of contents, rapid desiccation and cell death (Scandalios, 1993). However, biocontrol agent, *Trichoderma*, releases a variety of compounds that induce resistance responses to biotic and abiotic stresses (Harman et al., 2004). Several studies have shown that root colonization by *Trichoderma harzianum* results in increased level of plant enzymes, including various peroxidases, chitinases, ß-1,3-glucanases, lipoxygenase-pathway hydro peroxide lyase and compounds like phytoalexins and phenols to provide durable resistance against stress (Harman, 2006; Hoitink et al., 2006; Gachomo et al., 2008).

Acclimatization of plants to water deficit is the result of different events, which lead to adaptive changes in plant growth and physio-biochemical processes, such as changes in plant structure, growth rate, tissue osmotic potential and antioxidant defenses (Duan et al., 2007). It has however become imperative to elucidate the responses and adaptation of crops to water deficit, and take actions to improve the drought resistance ability Recently, Rawat et al. (2011 & 20112) characterized the possibility of *Trichoderma* species inducing tolerance to abiotic stresses. Therefore, the objective of present investigation was to characterize effective drought tolerant *Trichoderma* strains and elucidate their effect on drought responses of wheat.

1 Results and Discussion

All *Trichoderma* isolates were evaluated for their growth response (cfu/g air dried substrate) at six moisture levels (5, 10, 20, 40, 70 & 90 %) and results are presented in Table 1. Growth of *Trichoderma* was significantly affected by both isolates and moisture regimes. At higher moisture regimes of 40 and 70 per cent, isolate *Rani Th-14* resulted in maximum population (cfu/g air dried substrate) followed by *Rani Th*-21 & *Rani Th*-25 while minimum population was recorded for *Rani Th*-40. At 90 per cent moisture content, *Rani Th*-39 showed maximum growth followed by

Rani Th-14. However, at 20 per cent moisture, maximum population was obtained for *Rani Th*-14 & *Rani Th*-39 followed by *Rani Th*-30, whereas minimum population was recorded for *Rani Th*-40 showing no growth. At lower moisture content, growth of the isolates was significantly lower than the growth at 40 per cent moisture. At 5 per cent moisture level only two isolates viz., *Rani Th*-14 and *Rani Th*-25 were able to grow putting 1.0 $x10^8$ and 0.7 $x10^8$ cfu/g air dried cow dung, respectively.

Glass house experiment on wheat plants revealed that prior to the shift in watering cycles at 35 d, wheat seedlings bioprimed with drought tolerant isolates of *Trichoderma* had larger shoot and root length (Figure 1). Seed biopriming of wheat with powdered formulation of drought tolerant isolates of *Trichoderma harzianum* enhanced tolerance against water stress condition. Isolates *Rani Th-14, Rani Th-25* and *Rani Th-30* performed better (< 60%) to stand the plants against drought stress even upto 13 DDS while plants treated with other isolates *viz.*, *Rani Th-21* and *Rani Th-39* recorded 65-80% and 81- 100% wilting respectively at 13 days Post watering. However, untreated wheat plants observed 100% wilting only after 7 days of water stress (Table 2). The interaction between drought and *Trichoderma* was not significant so means are presented in Table 2 for drought effect over both *Trichoderma* treatments (colonized or non- colonized) and for the *Trichoderma* effct over all drought treatment time points (4,7,10 and 13 d). A few recent reports demonstrated that these fungi alleviate abiotic stresses (Rawat et al., 2011). This is justified by the fact that they may confer tolerance to drought stress at least in part through promotion of deeper root penetration into the soil profile (Harman, 2000). In a recent report, *T. hamatum* increased tolerance of cocoa plants to water deficit through increasing root growth that provided greater water resources to treated plants and delayed the onset of water deficit in these plants. Colonization of cacao seedlings by endophytic *Trichoderma* resulted in a delay in many aspects of the drought response. Thus, it is proposed that this effect is mediated through enhanced root growth, resulting in an improved water status allowing cacao seedlings to tolerate drought stress (Bae et al., 2009). The present data, along with the frequent observation that the greatest advantage of *Trichoderma* treatments to plants occurs when they are under stress, gives credence to the concept that these beneficial fungi ameliorate abiotic plant stresses and improve nutrient uptake by the plants. Further studies demonstrated that *Trichoderma* also increases root development and crop yield, the proliferation of secondary roots, and seedling fresh weight and foliar area (Harman, 2000). Moreover, *T. harzianum* can solubilize several plant nutrients (Altomare et al., 1999); and the colonization of cucumber roots by *T. asperellum* has been shown to enhance the availability of P and Fe to plants, with significant increases in dry weight, shoot length and leaf area (Yedidia et al., 2001).

Environmental stresses have a direct impact on the photosynthetic apparatus, essentially by disrupting all major components of photosynthesis including the thylakoid electron transport, the carbon reduction cycle and the stomatal control of the CO_2 supply together with an increased accumulation of carbohydrates, peroxidative destruction of lipids and disturbance of water balance (Allen et al., 2001). In the present study, physiological responses like net photosynthesis, stomatal conductance, chlorophyll fluorescens and chlorophyll content were significantly reduced as the time without water increased from 4 d to 13 d post watering (PW) (Table 3). Treatments with *Trichoderma* isolates suppressed the reduction in photosynthetic rate, stomatal conductance, chlorophyll fluorescens and chlorophyll content. In drought conditions, the highest photosynthesis rates were detected for *Rani Th*-14, *Rani Th*-25 and *Rani Th*-30. Photosynthesis rate was reduced due to drought by 71.8 per cent 10 d PW and to 92.9 per cent 13 d PW. Values for stomatal conductance were reduced due to drought by 73.6 per cent 10 d PW and to 93.0 per cent 13 d PW. Non-colonized seedlings averaged a 63.7 per cent reduction in stomatal conductance and a 59.1% reduction in net photosynthesis across the 7, 10, and 13 d watering cycles compared with the regular watered plants. Minimum % reduction was recorded in isolate *Rani Th-14* followed by *Rani Th-25* and *Rani Th-30*. Shangguan et al., (1999) found that the photosynthesis process during drought is possible due to the osmoregulation which affects the state of the leaf stomata and adaptation of the photosynthetic apparatus to drought conditions.

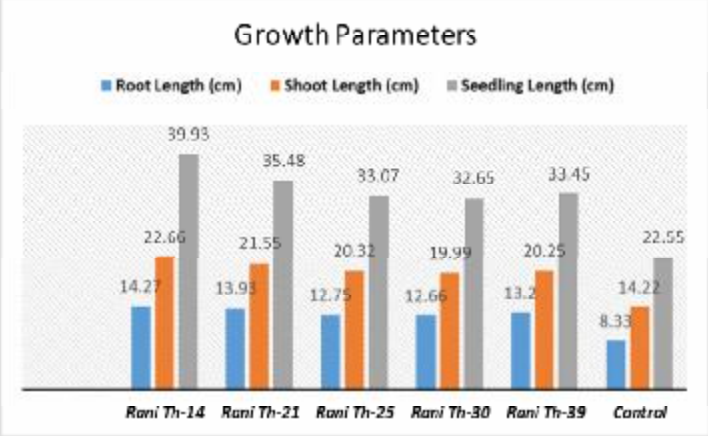

Figure 1 Effect of *Trichoderma* isolates on shoot length (a) and root length (b) of wheat plant

Note: The seeds were either bioprimed with selected drought tolerant isolates of *Trichoderma harzianum: Rani Th-14, Rani Th-21, Rani Th-25, Rani Th-30* and *Rani Th*-39 or non colonized (control). Before altering the water cycle root length and shoot length were measured for colonized and non colonized plants (35 days old seedlings)

In the present study, it was found that per cent reduction in chlorophyll content was significantly increased under drought in wheat plants. Selected drought tolerant *Trichoderma harzianum* isolates showed significantly delayed drought induced changes in total chlorophyll content and chlorophyll fluorescence (Table 3). Similar findings of Bae et al., 2009 also suggest enhanced greenness and chlorophyll content under drought tolerant isolate *Trichoderma hamatum* DIS 219b-colonized seedlings.

Relative greenness (SPAD value) was significantly decreased under drought stress conditions as the time without water increased from 4 to 13d (Figure 2). However, *Trichoderma* isolates viz.; *Rani Th*-14, *Rani Th*-21, *Rani Th*-25, *Rani Th*-30, *Rani Th*-39 suppressed the reduction in SPAD value. Untreated plants averaged 89 per cent reduction in relative greenness across the 4, 7, 10, 13 DDS compared with regular watering cycle i.e., control. Whereas, treated plants averaged only 14-34 per cent reduction in SPAD value, across the same watering cycles. Minimum per cent reduction in SPAD value was reported in *Rani Th*-14 and *Rani Th*-25 followed by *Rani Th*-30 at 13 DDS. It has also been demonstrated that *T. harzianum* strain T 22 enhanced leaf greenness in maize as demonstrated by measurement of chlorophyll (Mastouri et al., 2010); providing the growing plant with more energy and carbon source for their growth.

Limiting watering from 1 to 13 DDS caused a severe loss in membrane stability in untreated plants, approximately 51 per cent loss was found in untreated plants (Figure 3). However, significantly less fluctuation (20-37%) in MSI value were observed in *Trichoderma* treated plants. Minimum per cent loss in MSI value was recorded by *Rani Th-14* (20%) followed by *Rani Th 30* (22%) and *Rani Th 25* (23%). The higher leakage of solutes was probably due to enhanced H_2O_2 accumulation and lipid peroxidation under oxidative stress as also reported by (Dionisio-Sese et al., 1998). Harman, (2006) also concluded that root colonization by *T. harzianum* strain T 22 resulted in enhanced concentration of antioxidant enzymes (like peroxidases, chitinases, etc). These antioxidant enzymes act as scavengers of reactive oxygen species (ROS) and thus cause membrane stability. An important consequence of drought stress is the generation of ROS. These oxidants, formed under stress, cause membrane disorganization and metabolic toxicity, resulting in higher leakage of solutes. The plasma membrane is generally protected from desiccation-induced damage by the presence of membrane-compatible solutes, such as sugars and amino acids. Therefore, a link may exist between the capacity for osmotic adjustment and the degree of membrane protection from the effect of dehydration (Liley and Ludlow, 1996).

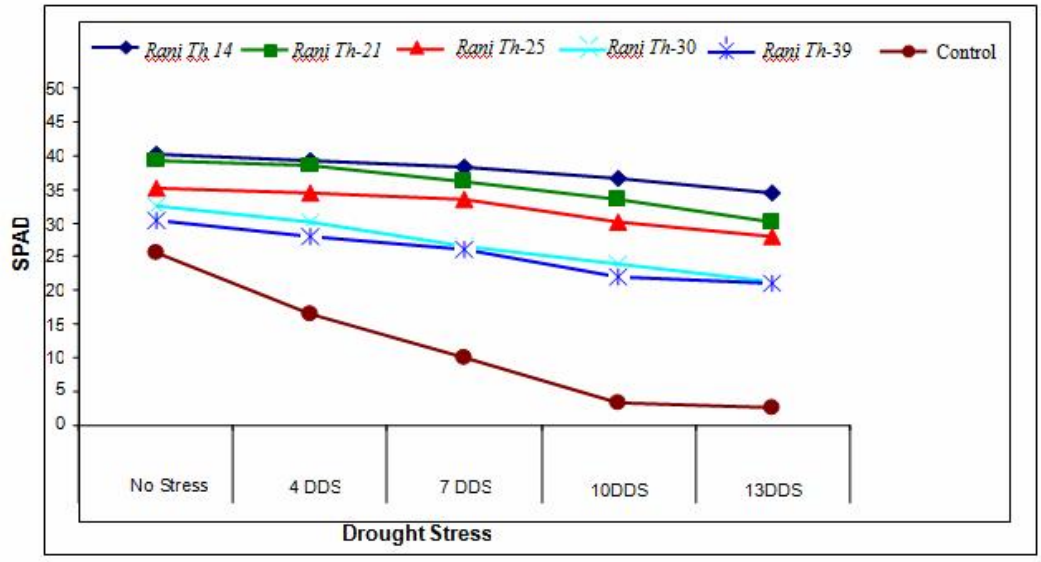

Figure 2 Effect of *Trichoderma* isolates on SPAD value of wheat plants under drought stress

Note: Treatments: Control (non-colonized seedlings) and *Trichoderma* isolates *Rani Th-14, Rani Th -21, Rani -Th 25, Rani Th -30* and *Rani Th -39* colonized seedlings with drought treatments viz., no stress (watering every alternate days) and drought by withholding water for 4, 7, 10 and 13 DDS(days drought stress)

An increase in the amount of hydrogen peroxide content was observed due to the action of drought stress. Concentration of H_2O_2 content increased significantly by 47 per cent in response to the time without water increased from 1 to 13 d in untreated wheat plant (Figure 3). However, this fluctuation was less dramatic in the plants treated with *Trichoderma*, wherein minimum per cent increase in H_2O_2 content was recorded in *Rani Th*-14 (9.8%) followed by *Rani Th*-30 (16.9%) and *Rani Th*-25 (23.4%) as compared to unstressed plants of respective treatments. Inoculation with drought tolerant *Trichoderma* isolates reduced the H_2O_2 content in rice as compared to control. This might be due to increase in expression of stress related proteins such as, glutathione S-transferase (GST), glutathione-dependent formaldehyde dehydrogenase (FALDH) and peroxidase. Similar effect was observed by the inoculation of *Trichoderma* isolate strain T22 in maize plant (Shoresh et al., 2008).They reported that under conditions of environmental stress, when ROS such as H_2O_2 are produced, these detoxifying proteins triggered by *Trichoderma* inoculation act as scavenging enzymes and play a central role in protecting the cell from oxidative damage.

Measurement of lipid peroxide content serves as a reliable indicator of oxidative damage during abiotic stresses (Mittler et al., 2002). In the present study, MDA content was significantly influenced by drought and *Trichoderma* isolates ($p \leq 0.05$) (Table 4). It was found to be 7.0 folds higher in untreated plants at 13 DDS as compared to control (No stress). A less significant change was found among the treated plants with increase of 5-5.6 folds at 13 DDS. Minimum per cent increase in MDA content was found in *Rani Th*-14 followed by *Rani Th*-25. One such common mechanism could be the control of damage caused by the ROS. These molecules that play a crucial signaling role during abiotic stresses at higher concentration cause cellular and molecular damages (Mittler et al., 2002). In stress, untreated plants bleached white indicating the generation of ROS while treated plants remained green. This suggests that *Trichoderma* either scavenge ROS or induces plants to more efficiently scavenge ROS, or prevents ROS production when exposed to drought stress. Most ROS are unstable and are quickly converted to H_2O_2, which is either reduced to water during the ascorbate- GSH cycle, converted to water and oxygen by catalase enzyme, or used as a

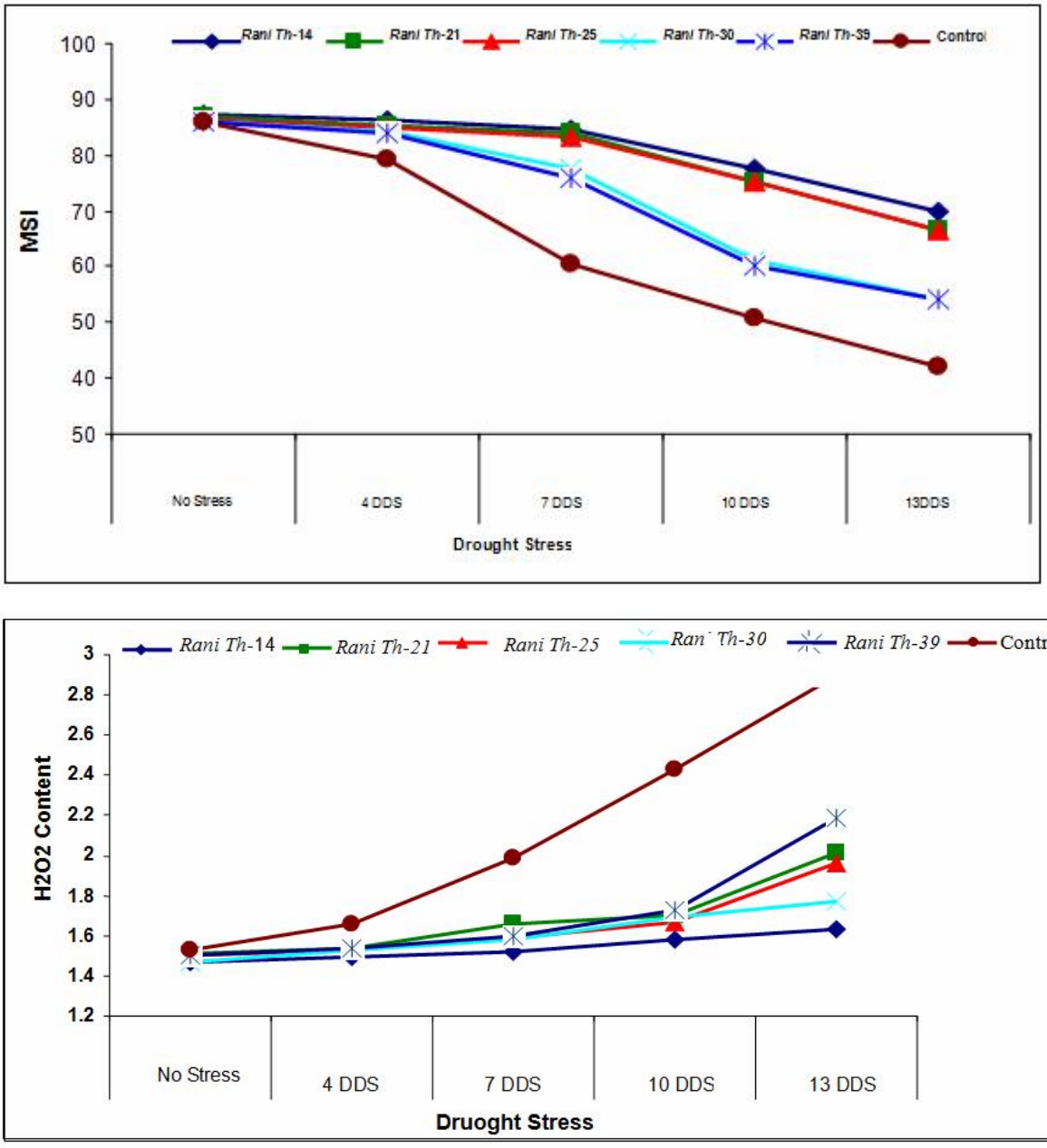

Figure 3 Effect of *Trichoderma* isolates on membrane stability index and hydrogen peroxide (H_2O_2) content (μ mol g^{-1} fresh weight) of wheat plants under drought stress

Note: The largest leaves were harvested before altering the water cycle and at 4, 7, 10 and 13 DDS. *Trichoderma* Treatments: Control (non-colonized seedlings) and *Trichoderma* isolates *Rani Th*-14, *Rani Th*-21, *Rani Th-25*, *Rani Th*-30 and *Rani Th*-39 colonized seedlings with drought treatments viz., no stress (watering every alternate days) and drought by withholding water for 4, 7, 10, and 13 DDS(days drought stress)

substrate of peroxidase enzymes (Foyer, 2003; Noctor et al., 1998). However, in the presence of transition metal ions, H_2O_2 is converted to hydroxyl radicals, which start chain reactions leading to the peroxidation of membrane lipids (Aust et al., 1985); that result in loss of membrane integrity and also damage to other macromolecules. On the other

hand, plant resistance to stress factor is associated with their antioxidant capacity, and the increased level of antioxidant constituents may prevent the stress damage (Khan and Panda, 2008). Additionaly, (Mastouri et al., 2010); reported that total lipid peroxides of tomato seedlings germinated under lowered water potentials or that of aged seed was found less when the seedlings were treatred with *Trichoderma* isolate T22 as compared to untreated control. The mechanisms where by *Trichoderma* spp. induce such changes are not known however, enhanced ROS level could act as a signal to regulate expression of some of the related genes. A transient increase in intracellular ROS has been detected 5 to 10 min after treating soybean cell culture with culture filtrate of *T. atroviride* (Navazio et al., 2007). Such signals, along with Ca++ signaling (Navazio et al., 2007); can induce plant ROS scavenging mechanisms (Moon et al., 2003); resulting in elevated protection against the oxidative damage.

An increase in the content of phenolics in drought conditions was of higher magnitude in *Trichoderma* treated wheat plants. Maximum per cent increase in phenolics content was found in *Rani Th-14* followed by *Rani Th*-30 (Table 4). The phenolic compounds, besides having antifungal, antibacterial and antiviral activities also possess antioxidant properties and thus act as scavengers of activated free radicles. Mastouri et al., (2010) also reported role of PAL mediated increased phenolics compounds viz., ferulic acid for drought tolerance in winter triticale through the action as efficient photoprotectant.The interaction between drought and treatment is presented in Table 5 which indicates non-significant effect of the interaction between drought and treatment for Photosynthetic rate, Chlorophyll content and chlorophyll fluorescens. However, effect of the interaction between treatment nad drought stress was observed on the exchanges in the total pool of MSI, H_2O_2, Proline content, MDA content and phenolic compounds.

Table 1 Colony forming units (Cfu's) of different *Trichoderma* isolates as affected by different moisture regimes at 14 DAI (days after inoculation)

Isolate code	**Cfu$^+$ on oven dried cow dung at 10^{-8} dilution at different moisture (%)**						**Mean**
	5	**10**	**20**	**40**	**70**	**90**	
Rani Th-1	0.0	0.2	1.7	4.3	4.0	1.1	**1.88**
Rani Th-2	0.0	0.1	2.4	5.2	4.1	1.6	**2.23**
Rani Th-3	0.0	0.3	1.8	4.1	3.7	0.1	**1.66**
Rani Th-4	0.0	0.8	2.2	5.4	5.1	2.1	**2.60**
Rani Th-5	0.0	0.1	3.1	5.5	5.1	2.2	**2.60**
Rani Th-6	0.0	0.1	2.1	4.5	3.6	0.1	**1.73**
Rani Th-7	0.0	0.2	2.5	5.2	4.2	1.2	**2.21**
Rani Th-8	0.0	0.1	2.1	5.3	4.0	0.1	**1.93**
Rani Th-9	0.0	0.1	3.1	4.9	4.1	1.0	**2.20**
Rani Th-10	0.0	1.6	4.1	5.7	4.1	1.1	**2.76**
Rani Th-11	0.0	1.7	4.1	5.9	4.3	0.3	**2.71**
Rani Th-12	0.0	1.1	2.4	4.9	4.2	0.7	**2.21**
Rani Th-13	0.0	0.1	1.8	4.8	4.1	1.1	**1.98**
Rani Th-14	1.0	2.7	5.4	7.8	7.2	4.3	**4.73**
Rani Th-15	0.0	0.2	1.1	4.0	3.1	0.1	**1.41**
Rani Th-16	0.0	0.7	2.8	5.0	3.2	0.1	**1.96**
Rani Th-17	0.0	1.1	4.0	5.4	4.7	1.3	**2.75**

Rani Th-18	0.0	1.2	3.8	5.2	4.7	1.4	**2.71**
Rani Th-19	0.0	1.0	1.0	3.9	3.1	0.1	**1.51**
Rani Th-20	0.0	1.5	4.9	6.7	5.9	2.1	**3.51**
Rani Th-21	0.7	2.1	5.1	7.4	7.1	4.2	**4.43**
Rani Th-22	0.0	0.1	2.0	4.2	4.2	1.1	**1.93**
Rani Th-23	0.0	0.1	0.3	2.3	3.1	0.1	**0.98**
Rani Th-24	0.0	0.0	0.8	2.9	3.7	0.5	**1.31**
Rani Th-25	0.0	1.9	4.8	7.2	6.1	3.5	**3.91**
Rani Th-26	0.0	0.1	2.0	3.4	3.3	0.7	**1.58**
Rani Th-27	0.0	0.1	1.8	3.1	2.1	0.1	**1.20**
Rani Th-28	0.0	0.1	2.4	4.1	3.7	1.1	**1.90**
Rani Th-29	0.0	0.1	2.2	3.6	3.7	0.6	**1.70**
Rani Th-30	0.0	2.0	5.3	7.3	5.6	3.3	**3.91**
Rani Th-31	0.0	0.1	3.0	4.8	2.2	0.1	**1.78**
Rani Th-32	0.0	0.2	0.2	2.6	2.9	0.1	**1.00**
Rani Th-33	0.0	0.1	2.0	3.1	3.2	1.1	**1.58**
Rani Th-34	0.0	0.1	2.7	4.7	4.1	1.6	**2.20**
Rani Th-35	0.0	0.1	0.2	2.5	1.5	0.1	**0.73**
Rani Th-36	0.0	0.1	0.2	2.0	2.1	0.1	**0.75**
Rani Th-37	0.0	0.1	1.1	3.2	3.1	0.1	**1.26**
Rani Th-38	0.0	0.1	3.2	5.9	5.1	1.5	**2.63**
Rani Th-39	0.0	1.6	5.4	7.2	7.0	3.7	**4.15**
Rani Th-40	0.0	0.1	0.1	2.6	2.7	0.1	**0.95**
Mean	**0.04**	**0.60**	**2.53**	**4.69**	**4.07**	**1.14**	**1.88**

	Isolate (A)	**Moisture (B)**	**A × B**
S.Em.±	0.060	0.012	0.027
CD at 5%	0.162	0.028	0.071

Table 2 Effect of seed biopriming with drought tolerant isolates of *Trichoderma harzianum* on wilting per cent of the wheat plants under different levels of drought stress

Days	**Per cent wilting**					
	Rani Th-14	*Rani Th-21*	*Rani Th-25*	*Rani Th-30*	*Rani Th-39*	*Control*
No stress	-	-	-	-	-	-
4 DDS*	-	<20	_	-	<20	40-60
7 DDS	-	45-60	<20	<20	45-60	81-100
10 DDS	<20	45-60	20-45	20-45	61-80	-
13 DDS	20-40	65-80	41-60	41-60	81-100	-

Note: *DDS= Days Drought Stress

Table 3 The effect of drought and *Trichoderma* colonization on Stomatal conductance (gs), Net Photosynthesis (Pn,) Chlorophyll content (Cc) and chlorophyll fluorescens (Fv/Fm)

	% REDUCTION			
Treatments	**Stomatal Conductance (gs)**	**Net Photosynthesis (Pn)**	**Chlorophyll Content (Cc)**	**Chlorophyll fluorescence (Fv/Fm)**
Drought effect				
0 d	-	-	-	-
4 d	35.6C	24.6D	25.2D	8.67D
7 d	56.7AB	51.3C	47.4C	23.10C
10 d	75.6B	73.8B	76.2B	37.38B
13 d	95.0A	94.9A	99.4A	56.31A
***Trichoderma* effect**				
Rani Th-14	19.1C	13.3C	13.9C	16.63D
Rani Th-21	34.7B	27.5B	26.5B	36.81B
Rani Th-25	25.6CB	17.5C	18.7C	19.84CD
Rani Th-30	25.6CB	18.3C	20.6BC	24.93C
Rani Th-39	34.3B	27.9B	27.8B	37.33B
Non colonized	67.7A	63.1A	64.0A	52.63A

Note: d PW =days post watering

Table 4 Effect of drought tolerant *Trichoderma harzianum* isolates on proline, MDA and total phenolics content of wheat plants under different levels of drought stress

Treatments	Proline content (μ mol g^{-1} fresh weight)					MDA content (μ mol g^{-1} fresh weight)					Total phenolics content (μg g^{-1} fresh weight)				
	No Stress	4 DDS	7 DDS	10 DDS	13 DDS	No Stress	4 DDS	7 DDS	10 DDS	13 DDS	No Stress	4 DDS	7 DDS	10 DDS	13 DDS
Rani Th-14	4.0G	4.9G	5.9FG	6.4E	8.1DEF	1.9G	2.3FG	2.6FG	3.1F	3.3EF	69.4GH	100.6FG	148.4DE	186.3CD	279.9A
Rani Th-21	4.0G	6.0FG	7.1DFG	9.0DE	17.0B	1.8G	2.9FG	3.9FG	4.2EF	7.6BC	61.6H	89.9FG	114.0F	148.3DE	196.7C
Rani Th-25	3.7G	5.1G	6.0EFG	7.5DEFG	9.6D	2.0G	2.6FG	3.0FG	3.7EF	4.1E	65.9GH	99.4FG	136.8EF	161.5D	242.8B
Rani Th-30	4.1G	4.9G	6.1EFG	7.5DEFG	9.6D	1.7G	2.4FG	2.9FG	3.9EF	4.2DE	66.8GH	97.3FG	137.3E	164.3D	257.7A
Rani Th-39	3.9G	5.2FG	7.0DEF	9.4D	15.5BC	1.4G	3.9EF	4.6DE	5.1D	7.8BC	55.7H	80.0GH	125.2EF	141.2DE	175.6CD
Control	3.8G	9.8D	14.0C	18.0B	27.9A	1.5G	5.0DE	7.1C	8.2B	9.7A	54.4H	68.8GH	85.6G	98.0FG	118.9EF
LSD(0.05)			2.87					0.940					23.12		

Table 5 Mean squares from analysis of variance of data for physiological and biochemical parameters of wheat grown under five levels of drought stress after seed biopriming with drought tolerant isolates of *Trichoderma*

Sources of variation	df	Net photosynthesis (μ mol CO2 m^{-2} s^{-1})	Stomatal conductance (mol H_2O $m^{-2}s^{-1}$)	Chlorophyll content (mg g^{-1} fr. wt.)	Chlorophyll flourescence (Fv/Fm)	Osmotic Potential (MPa)	Relative water content (%)
Drought (a)	4	158.03[3]	0.261[3]	9.44[3]	0.212[3]	4.08[3]	498.22[3]
Trichoderma (b)	5	125.55[3]	0.217[3]	7.05[3]	0.165[3]	3.66[3]	101.12[3]
a × b	20	10.95[ns]	0.019[ns]	1.16[ns]	0.037[ns]	0.063[3]	3.91[3]
Error	58	1.68	0.005	1.025	0.004	0.011	19.55
LSD (0.05)		2.677	0.061	0.257	0.054	0.359	7.301

Sources of variation	df	Membrane Stability Index (%)	Hydrogen peroxide content (μ mol g^{-1} fr. wt.)	Proline content (μ mol g^{-1} fr. wt.)	MDA Content (μ mol g^{-1} fr. wt.)	Total phenolics content (μ mol g^{-1} fr. wt.)
Drought (a)	4	2411.55[3]	3.03[3]	438.40[3]	51.25[3]	88802.60[3]
Trichoderma(b)	5	700.08[3]	2.22[3]	152.39[3]	19.89[3]	17365.76[3]
a × b	20	72.81[3]	0.072[3]	18.40[3]	3.19[3]	3437.52[3]
Error	58	27.73	0.023	2.11	0.127	153.67
LSD (0.05)		8.606	0.245	2.87	0.940	23.12

Note: a: No Stress (control), 4 DDS, 7 DDS, 10 DDS and 13 DDS; DDS = days drought stress; b: *Rani Th*-14, *Rani Th*-21, *Rani Th*-25, *Rani Th*-30 and *Rani Th*-39 and control; 3: Significant at 5% level of probability; ns = non-significant; fr. wt. = fresh weight

2 Conclusion

The results of the experiments suggests that seed biopriming with drought tolerant *Trichoderma harzianum* isolates increased the ability of wheat to grow successfully under drought stress conditions. Isolate *Rani Th*-14 was considered best in providing drought tolerance to wheat plant. Regardless of the mechanisms involved in *Trichoderma* conferred stress tolerance, it is clear that these fungi can have significant effects on the ecophysiology of plants. This may result in the rapid adaptation of plants, allowing them to establish and survive in high-stress habitats. The most important and consistent response, which enabled plants to tolerate drought was increased root vigour. Evidence presented in this research also indicates that drought stress resistance is alleviated through osmoregulation, amelioration of damage caused by ROS, and accumulation of stress induced metabolites. Other important factors in drought tolerance are the protective mechanisms involving, among others, phenolic compounds as one of the effective photoprotectors. These effects, along with increased root and shoot growth, all energy requiring, suggesting that both photosynthesis levels and efficiency are increased in the presence of drought tolerant *Trichoderma* isolates. The experiments confirmed that the drought tolerant isolates of *Trichoderma* through seed bio priming are critical in inducing tolerance to drought. The most important and consistent response, which enabled plants to tolerate drought was increased root vigour. The ability to perform the process of osmoregulation, the efficiency of the utilization of excitation energy by the photosynthetic apparatus and the functioning of protective mechanisms involving the level of total phenols, free

proline and H_2O_2 content in leaf tissues.

This research merits attention and could additionally open the avenue for the use of *Trichoderma* inoculation through seed biopriming in the plants for enhanced drought tolerance.

3 Materials and Methods

3.1 Experimental Site

The experiment was carried out at Biocontrol Laboratory of Plant Pathology Division, College of Forestry, Ranichauri, V.C.S.G UUHF.

3.2 *Trichoderma* collection and evaluation at different moisture regimes

A set of 40 *Trichoderma* harzianum isolates, maintained in the repository of Biological Control Laboratory, College of Forestry, Ranichauri, were used for the study. Those isolates were grown on 50g cow dung in 250 ml Erlenmeyer flask at different moisture regimes i.e., 5, 10, 20, 40, 70 and 90 per cent (based on oven dried cow dung) and evaluated for their growth performance (Zaidi et al., 2004). Five best isolates were selected for further studies.

3.3 Preparation of talc -based formulation of *Trichoderma*

Mass culture of each selected *Trichoderma harzianum* isolate was prepared separately on barnyard millet (*Echinocloa frumentacae*) grains. Grains were soaked in water for 12 h and filled in 250 ml Erlenmeyer flasks (@ 50 g/ flask). Those flasks were autoclaved at 15 *lbs psi* for 30 minutes. After cooling to room temperature, the flasks were inoculated with mycelial discs cut from three-day old culture of *Trichoderma* spp. and incubated at 28 ºC for 12 days. *Trichoderma* colonized barnyard millet grains were air dried in open shade and ground with Willy Mill to a fine powder. The powder was passed through 50 and 80 mesh size sieves, simultaneously to obtain a pure spore powder. The talc formulation was prepared by diluting this powder with talcum powder (350 mesh, 95% whiteness) and 1 % carboxy methyl cellulose (CMC), used as a sticker, to get desired concentration of biocontrol agents in the formulation. The final colony forming units of *Trichoderma* were adjusted to 5 X 10^6 Cfu/g of the formulation.

3.4 Seed collection and biopriming

Seeds of wheat (*Triticum aestivum* L.) variety UP-2572 were obtained from Crop Improvement Department, College of Forestry, Ranichauri, V.C.S.G UUHF. Prior to their use, seeds were surface sterilized with 1% sodium hypochlorite solution for 3 min, then rinsed with sterilized water and dried. Seeds were bioprimed separately with each drought tolerant isolates of *Trichoderma* spp @ 10g/kg of seeds. After presoaking of seeds in sterile distilled water, seeds were coated with powder formulation of *Trichoderma* spp. and mixed thoroughly to provide uniform coating. Seeds were then kept under warm and moist conditions at 25±2 ºC for 24 hrs until prior to radical emergence as this technique helps *Trichoderma* to increase in number by almost hundred times on spermosphere during incubation period as compared to normal seed treatment (Singh et al., 2004).

3.5 Maintaining drought stress in plants

Five best drought tolerant isolates of *Trichoderma harzianum* (*Rani Th-14, Rani Th-21, Rani Th-25, Rani Th-30 and Rani Th-39*) were selected on the basis of their growth at different moisture regimes. Along with control the selected isolates were tested in each drought treatment for their ability to enhance drought tolerance in wheat plants, wheat seeds were sown in plastic pots after filling them with soil prepared in the following manner. Bulk surface soil (0-15 cm) was collected from the Plant Pathology Research Block, Ranichauri (clay loam) having 3.2 mm water per cm depth of soil at field capacity. The soil was air dried, mixed thoroughly and passed through 2-mm sieve.Seeds were sown in plastic pots (2.5 kg capacity) filled with 2.0 kg autoclaved soil and saturated with water holding calibrated tensiometer . Five plants per pot were maintained for each treatment combinations including control. Moisture was maintained by applying 100 ml of water per pot every alternate day until plants attained the age of five weeks and at

this point drought treatments were given by altering the water cycle. Watering was stopped for subsequent days for each drought treatment which included 4, 7, 10 and 13 days drought stress (DDS), while control seedlings were continued to be watered every alternate day. Subsequent to drought treatment application, observations were recorded on wilting and biochemical and physiological responses of wheat. For recording observations on wilting the reading for the non-colonized seedlings at 4, 7, 10 and 13 dPW were divided by the readings for 0 d watered non-colonized seedlings at those same time points, and the readings for the colonized seedlings at 4,7, 10 and 13 d PW were divided by the readings for 0 d watered colonized seedlings at those same time points within each replication. These values were subtracted from 1 and multiplied by 100 to determine percentage reduction.

3.6 Growth parameters

Plants were uprooted carefully and washed with distilled water. The root length and shoot length of 35 days old seedlings were observed and measured manually before altering the water cycle.

3.7 Measurement of leaf gas exchange and greenness

Net photosynthesis and stomatal conductance were measured using a CO_2 gas analyzer (CID Inc. USA) on intact leaves under full sunlight. Leaf greenness was measured by estimating total chlorophyll content. Total Chlorophylls were extracted with 80 per cent acetone and estimated according to Arnon (1949). Estimation of nitrogen status of the crop was done using portable SPAD meter (Model- Opti Science, CMM-200, USA).

3.8 Chlorophyll fluorescence (Fv/Fm ratio)

Chlorophyll "a" fluorescence emitted by green plants reflects photosynthetic ability of PS-II. A handy plant efficiency analyzer (Handy PEA, Hansatech, UK) was used to monitor chlorophyll fluorescence (Fv/Fm) according to the equation;

$$Fv/Fm = (Fm-F0)/ Fm$$

3.9 Measurement of biochemical parameters

Assessment of biochemical (Proline, MDA, total phenolics, MSI and H_2O_2 content) responses of treated and untreated wheat plants against drought stress were carried out using fresh plant material that was immediately extracted and assayed according to the appropriate methods listed below:

4.0 Proline content

Proline content in the tissue was estimated by colorimetric method as described by Bates et al., 1973.

4.1 Malondialdehyde (MDA) content

Lipid peroxidation was measured by the amount of malondialdehyde (MDA), a product of unsaturated fatty acid peroxidation. MDA concentration (mg g^{-1} fresh weight) was estimated by the method of Kramer et al., 1997.

4.2 Total phenolics content

Total phenolics were estimated by the method of Swain et al., 1959 using Foline Ciocalteu reagent and the absorbance was measured at 725 nm against each blank. The content of phenolics was obtained from different concentration of catechol and expressed as mg g^{-1} fresh weight.

4. 3 Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide was measured spectrophotometrically after reaction with potassium iodide (KI). The reaction mixture consisted of 0.5 ml of 0.1 percent trichloroacetic acid (TCA) leaf extract supernatant, 0.5 ml 100 mM K-phosphate buffer and 2 ml reagent (1 M KI w/v in fresh double-distilled water H_2O). The blank probe consisted of 0.1 percent TCA in the absence of leaf extract. The reaction was developed for 1 h in darkness and absorbance

measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2 (Alexivea et al., 2001). The H_2O_2 content was expressed as mg g^{-1} fresh weight.

4.4 Membrane stability index (MSI)

Membrane stability index (MSI) of fresh leaves was determined as per the method suggested by Bailly et al., 1996. For this, two leaves of each treatment were randomly chosen per replicate and two leaf samples per plant were taken as follows. One sample from second leaf below the shoot apex and the second sample from third leaf above the base were taken to represent developing and mature leaves, respectively (Kaya et al., 2003). The conductivity of solution was measured using a conductivity bridge meter using the formula,

MSI = 1 - C1/C2

Where, C1= conductivity at 40 ºC; C2= conductivity at 100 ºC.

4.5 Data analysis method

Data presented are the averages of three replicates, obtained from two independent experiments carried out under similar conditions. The experiments were performed in a factorial completely randomized design. The data from the experiments were subjected to two-way ANOVA followed by separation of means at $P \leq 0.05$. The CD values were computed by multiplying the standard error of difference (SEd) with table t value at error degrees of freedom (Gomez et al., 1984).

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