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Abiotic stress tolerant, antagonistic *Trichoderma* spp. as an adaptation strategy for crop disease management under climate change conditions

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ABSTRACT

Climate change and climatic variability are major over-riding concerns for Indian agriculture impacting ultimate farm profitability and sustainability.Both biotic and abiotic stresses are influenced by these phenomena. In the current study 13Trichoderma isolates were screened in-vitroat CRIDA - Hyderabad during 2018-2019, for their biotic and abiotic stress tolerance levels.In-vitro antagonistic activity with phytopathogens Macrophominaphaseolina (Mp), Fusariumoxysporumf.sp. ricini (FoR), Rhizoctoniasolani (Rs) and Sclerotiumrolfsii (Sr) revealed that T6,T7,T9,T12 and T13 isolates were most effective against Mp, FoR and Rs with 97-100% inhibition, in Rs T4, T12 and T13 with 69-75% inhibition were found to be best.In plant growth promotion traits, T5 was found to be the highest producer of IAA with 37.9g/mL, T6 and T8 produced the highest amounts of HCN (39.2ppm). Similarly ammonia was produced highest in T5 and T13 while in siderophore production was highest in T10 isolate with 80 SU. Under drought conditions, T3, T12, T11 and T13 isolates were tolerant up to -1.8MPa and in salinity conditionsT6, T12 and T13 isolates were resistant up to 220 dSm^{-1}. T6, T12 and T13 isolates were found to sustain elevated temperatures up to 43°C. Most of the Trichoderma isolates were found to be effective antagonists and abiotic stress tolerant.

Key Words: Climate change, antagonistic activity, *Trichoderma* spp., abiotic stress, PGP traits

INTRODUCTION

Soil-borne plant pathogens are important yield reducing factors in crop production systems with very few options to manage them. *Macrophominaphaseolina,Sclerotium rolfsii, Rhizoctonia solani*and *Fusarium oxysporum f.sp., ricni*are some of the most potential phytopathogens causing diseases such as charcoal rot, root rot, foot rot, seedling blight, stem blight, damping-off, collar rot, stem rot and wilt (Abbas *et al*., 2019; Zhu *et al*., 2018; Ajayi-Oyetundeand Bradley2018; Prasad *et al*., 2019). Chemical fungicides are effective but cost-prohibitive. On the contrary, the biological control agents (BCA) are the most promising alternatives to the fungicides without hazardous impact on the environment (Javaid *et al*., 2020). Additionally, these function plant growth promoters and thereby help in overall crop health management (Alekhyaand Gopalakrishna 2017). *Trichoderma* genus has been the most popular and well-established BCA against fungal phytopathogens (Rabinal and Bhat 2020), it suppresses the pathogen growth through various mechanisms including antibiosis, competition for nutrients, mycoparasitism, production of cell wall degrading enzymes and induction of plant defense response (Ali, *et al*., 2020). Climate change and climatic variability is an additional dimension that contributes significantly through its impact on all components of global, national and local food systems. Climatic variability is leading to increased frequency and intensity of extreme weather events and its first impacts are already being felt. Climate change variables influence biophysical factors including plant pathogens and biocontrol agents and thus increasing the risk in emergence of new disease patterns (Desai *et al.*, 2021). *Macrophominaphaseolina* has emerged into a devastating fungal pathogen under drought stress triggering significant damage in the cowpea (Muchero and Ehlers *et al* 2011). High salinity was found to breakdown the resistance in the *Fusarium* resistant cultivars (Daami-Remadi, 2009). High temperature and relatively high humidity have been observed to favor the Bakanae disease caused by *Fusarium fujikuroi* (Singh *et al*., 2019). Charcoal rot incidence had a significant and positive correlation with increase in temperature in soybean (Teja *et al*., 2020). If left unaddressed, such risks could roll back decades

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of development progress and undermine prospects for future sustainable development and food security. Considering the changing climate scenarios and higher sensitivity of crops to infections caused by soil-borne pathogens, there is an urgent need to develop suitable microbe-mediated adaptation strategies which are eco-friendly. In this backdrop the current studies were taken up with 13 *Trichoderma* isolates (T1 to T13) to evaluate their antagonistic ability against four major soil-borne phytopathogens (*Macrophominaphaseolina* (Mp), *Fusariumoxysporum*f. sp. *ricini* (FoR), *Rhizoctoniasolani* (Rs) and *Sclerotiumrolfsii* (Sr); screening for their plant growth promoting traits and assessing their ability to withstand abiotic stresses.

MATERIALS AND METHODS

Organisms and culture conditions

Thirteen(13) *Trichoderma* isolates and four major soil borne phytopathogens *viz*.,FoR, Mp, Rs and Sr maintained in culture bank of ICAR-Central Research Institute for Dryland Agriculture, Hyderabad, India were used in this study. All the isolates were maintained on malt extract dextrose agar (MDA) with a composition of malt extract 20g, dextrose 5g, yeast extract 2g, peptone 2g, agar 20g and distilled water 1L.The study took place at the Plant Pathology Laboratory of the Central Research Institute for Dryland Agriculture (CRIDA) in Hyderabad during 2018-2019.

In-vitro antagonism against test phytopathogens

Dual culture assay: *Trichoderma* isolates were screened for their antagonistic activity by dual culture assay against FoR, Mp, Rs, and Sr (Dennis and Webster 1971a).*Trichoderma* isolates and the phytopathogen were inoculated opposite to each other in MDA Petri dishes and incubated for six days. Plates containing only pathogens served as control. Three replicates were maintained for each treatment and the experiment was repeated thrice.The radial growth of the phytopathogen was recorded in cm and percent inhibition was calculated as described by Whippsand Budge (1990).

Volatileproduction by *Trichoderma*: *Trichoderma* isolates were tested for their ability to produce volatiles against phytopathogens using the inverted plate technique, and percent inhibition was calculated as described by (Dennis and Webster 1971a).Six mm diameter mycelia plugs from a 7-day old culture of either pathogen or Trichoderma test isolate were centrally inoculated into MDA petridishes. After removing the lids from both plates, the bottom plate containing Trichoderma was inverted over the bottom plate inoculated with test pathogen. To make it leaf-proof, the entire assembly was tightly wrapped in parafilm. As a control, a plate inoculated with only the pathogen was used. The radial growth of the phytopathogen was measured in cm after six days of incubation at 28°Cand percent inhibition calculated as described by Whipps and Budge (1990).Three replicates were maintained for each treatment and the experiment was repeated thrice.

Screening for plant growth promoting traits

Trichoderma isolates (13) were screened for plant growth promoting traits such as production of Siderophores, Indole acetic acid, Ammonia andHydrogen cyanide.

Siderophore production: Estimation of siderophores was carriedout in CAS Blue-agar medium as described by (Schwyn and Neilands, 1987).*Quantitative estimation was carried by CAS shuttle assay.* Siderophore content in the aliquot was calculated by using following formula:

$$\%\, Siderophore\, units = Ar - \frac{As}{As} x\ 100$$

Where, Ar = absorbance of reference at 630 nm (CAS reagent) and As = absorbance of sample at 630 nm.

Screening for Indole Acetic Acid (IAA) production: Screening and estimation of IAA phytohormone production by *Trichoderma* isolates was carried out as mentioned by Tsavkelova *et al*. (2007). Modified Trichoderma liquid enzyme media with 0.1% bactopeptone, 0.03% urea, 0.2% KH_2PO_4, 1.4% $(NH_4)_2SO_4$, 0.03%$MgSO_4$ and 0.5% glucosewas amended with 0.1% L-tryptophan inoculated and incubated for 72 h at 28°C.The IAA was quantified based on the standard curve prepared with known amount of IAA and recorded as µg per mL of broth.

Detection of ammonia production: Ammonia production by the *Trichoderma* isolates in the culture medium was assayed following the method of Dye (1962). The accumulation of ammonia was detected by the addition of Nessler's reagent to each tube. A faint yellow colour indicated a small amount of ammonia (+), yellow colour indicated medium amount of ammonia (++) and deep yellow to brownish colour indicated high production of ammonia (+++).

Screening for Hydrogen Cyanide production: Malt dextrose medium amended with 0.44% of L-Glycine was used for detection and quantification of hydrogen cyanide following the method of Bakker and Schippers (1987). The total cyanides (in ppm) content was estimated as described by Haque and Bradburg(1999) and calculated using the following equation:

$$Total\ cyanides\ content\ (ppm) = 396\ \times A_{510}\ nm$$

Screening for Abiotic Stress tolerance

Drought tolerance: Drought tolerance studies on *Trichoderma* isolates were conducted in MD broth supplemented with polyethylene glycol (PEG)-6000 to induce osmoticum stress, as described by Basal et al (2020). An osmotic pressure of-1.2Mpa was created by approximately 32.6 percent of PEG-6000. To determine the tolerance levels, the total dry biomass obtained after inoculating the media with spore suspension ($1x10^6$spores mL^{-1}) and incubating at 28°C for 10 days was calculated.

Salinity tolerance: The tolerance of *Trichoderma* isolates to high salt concentrations was determined by calculating the total dry biomass obtained after amending the media with quantified amounts of NaCl (70.1gm NaCl per 1 L medium created a saline pressure of 120ds m^{-1}), inoculating with spore suspension ($1x10^6$spores mL^{-1}) and incubating at 28°C for 10 days.

High temperature tolerance: *Trichoderma* isolates thermotolerance was assessed by calculating the total dry biomass obtained after inoculating the media with spore suspension ($1x10^6$sporesmL^{-1}) and incubating for seven days at 35°C, 38°C, 40°C, and 41°C.

RESULTS AND DISCUSSION

In vitro antagonism against phytopathogens

*In-vitro*screening for antagonism against the test phytopathogens revealed that T4, T6, T7, T9, T10 and T13 inhibited growth of Rs by more than 90% after six days of incubation.T9, T10 and T13 isolates produced volatiles that inhibited growth of Rs by more than 40%. Lowest inhibition of Rs was noted in T1 isolate. T4 and T12 isolates inhibited growth of Sr by 71 and 76%, respectively. While T4 exhibited highest volatile production, the least volatile production was recorded in T5 and T11 isolates. T3, T4 and T9 inhibited FoR with more than 85% inhibition percentage whereas only 25% inhibition was recorded against Mp and FoR in case of T6, T7 and T11 isolates. The ability of the volatiles of *Trichoderma* isolates to inhibit MP ranged from 17-48%, maximum being by T6 and T11 isolates (Fig 1).

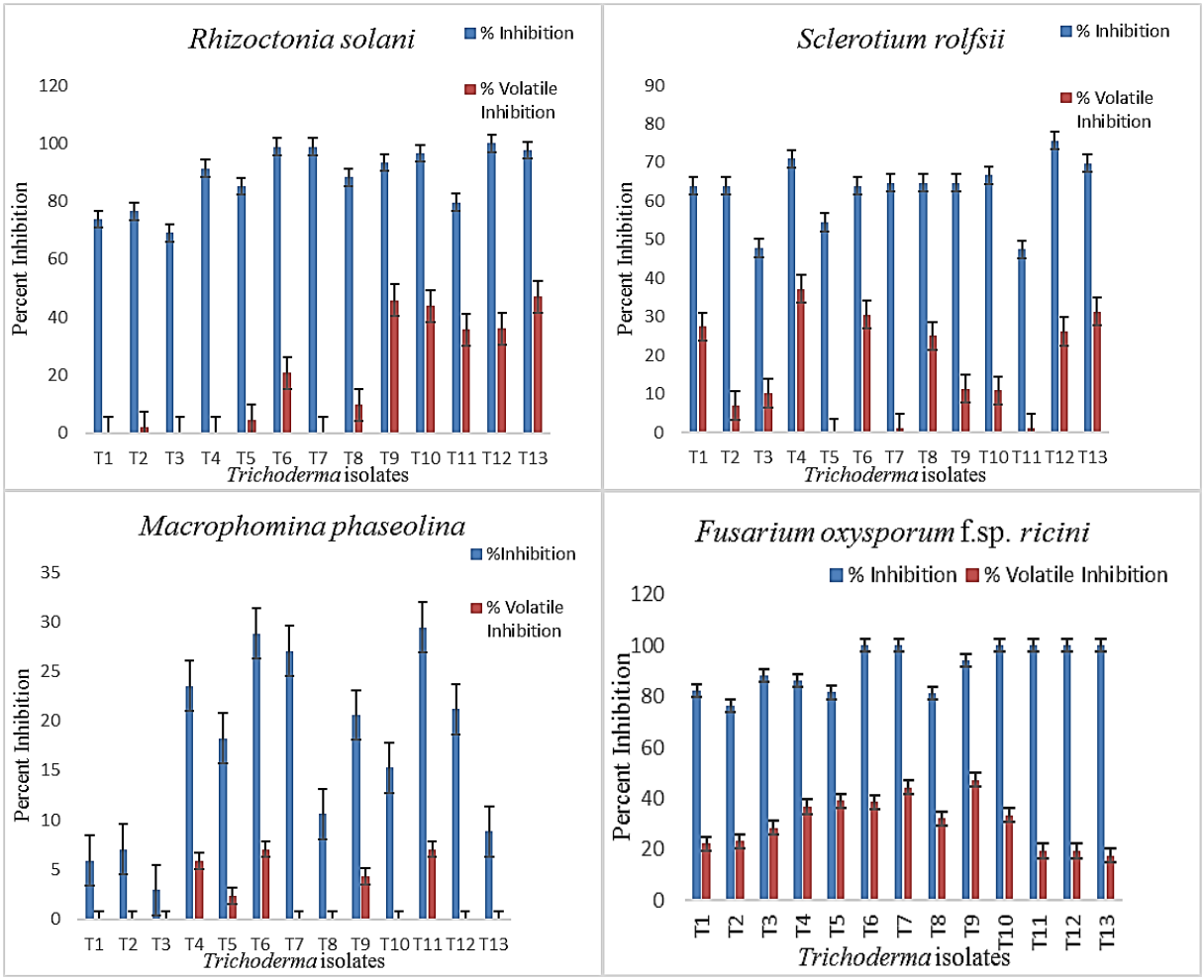

Fig 1: *In vitro* antagonistic activity of 13 isolates of *Trichoderma* against *Rhizoctoniasolani* (top left), *Sclerotiumrolfsii* (top right), *Macrophominaphaseolina* (bottom left) and *Fusariumoxysporum*f.sp. *ricini*(bottom right)

Screening for production of PGPR traits

Siderophore production: All the 13 isolates tested were positive for siderophore production (Fig 2).In the quantitative assay the production of siderophores was in the range of 27-80 units. T10 isolate produced a highest of 80SU, followed by 66 SU by T9 and lowest production was observed in T1 and T8 strainswith 27 SU. (Table 1).

Indole Acetic Acid (IAA) production: All the *Trichoderma* isolates produced IAA. In quantitative assay, the IAA producing ability of the isolates ranged between 5.7 and 37.9 µg/ml. and T5 isolate produced highest IAA (37.9 g/L, while T11 was found to be the lowes tproducer in broth medium (Table 1).

Fig 2: Siderophore production in CAS medium by *Trichoderma* isolates

Ammonia production: All the isolates were found to be positive for ammonia production, T5 and T13 were high producers whereas T8, T9 and T10 were found to be lowest producers (Table 1). Production of ammonia is well studied in bacterial isolates than in *Trichoderma* spp.considering the fact that these are low producers of ammonia compared to bacteria.

HCN production: All the *Trichoderma* isolates were found to produce HCN. In quantitative assaymaximum production of 39.2 ppm was observedin two isolates *i.e.,* T6 and T8 and lowest by T3 17.82 ppm (Table 1).

Table 1: Production of IAA, HCNSiderophoresand ammonia by 13 *Trichoderma* isolates

Trichoderma isolates	Mean IAA µg. mL-1	HCN (ppm)	Siderophore units (SU)	Ammonia
T1	6.29 ± 0.0	20.988	27	++
T2	8.62 ± 0.06	19.404	31	++
T3	6.45 ± 0.05	17.82	30	++
T4	25.74 ± 0.7	32.868	40	++
T5	**37.92 ± 0.13**	28.116	60	**+++**
T6	5.96 ± 0.06	**39.204**	49	++
T7	9.06 ± 0.12	37.62	37	++
T8	6.79 ± 0.01	**39.204**	27	+
T9	7.46 ± 0.0	29.304	66	+
T10	5.71 ± 0.13	22.176	**80**	+
T11	4.46 ± 0.06	24.552	46	++
T12	8.29 ± 0.03	38.016	50	++
T13	9.59 ± 0.02	26.532	52	**+++**

Screening for tolerance to abiotic stresses

Drought stress/ Osmoticum stress tolerance: All the isolates varied in their growth pattern and could tolerate osmoticum stress up to -1.5 MPa (Fig 3). At (-1.5 MPa) highest growth was observed by T12 isolate and lowest by T6. However, with one-unit increase in stress, there was a drastic reduction in the growth of all the isolates and the degree of reduction varied across isolates.Interestingly, T3 isolate showed less growth at -1.5 MPa, but sustained the stress even at -1.8 MPa showing relative more growth as compared to other isolates. T5 isolate showed least growth. T12 which showed maximum growth at -1.5 MPa also could resist -1.8 MPa stress and survive (Table 2).

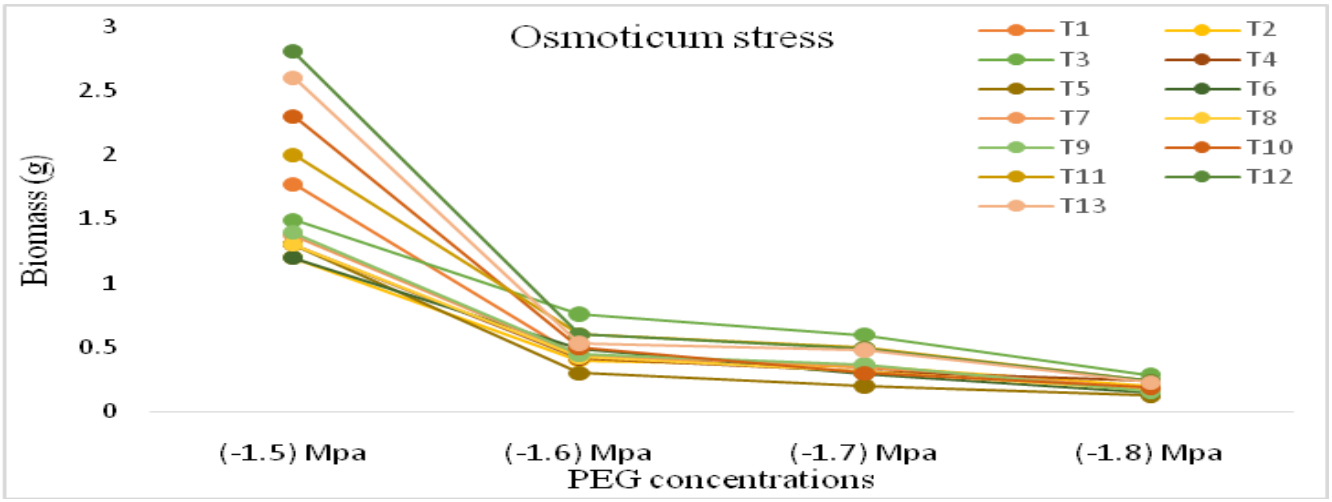

Fig 3: Variation in biomass production by *Trichoderma* isolates at with increasing osmoticum/ drought stress *in vitro.*

Table 2: Growth of *Trichoderma* isolates in terms of dry mass (g) with increasing concentrations of PEG inducing osmotic stress (-1.5Mpa to -1.8Mpa) in liquid media

Trichoderma isolates	Biomass(g) of *Trichoderma* isolates (± SD) under drought stress (Mpa)			
	(-1.5) Mpa	(-1.6) Mpa	(-1.7) Mpa	(-1.8) Mpa
T1	1.74±0.021	0.43±0.008	0.35±0.007	0.17±0.001
T2	1.2±0	0.4±6.798	0.35±6.798	0.19±0.000
T3	1.49±0.005	0.76±0.000	0.58±0.011	0.29±0.006
T4	1.28±0.011	0.41±0.011	0.32±0.01	0.24±0.003
T5	1.29±0.011	0.29±0.005	0.2±3.399	0.12±0.003
T6	1.16±0.057	0.5±0	0.29±0.005	0.15±0.000
T7	1.39±0.017	0.43±0.011	0.35±0.002	0.17±0.002
T8	1.23±0.057	0.43±0.015	0.30±0.005	0.16±0.000
T9	1.4±0.01	0.45±0.001	0.36±0.001	0.16±0.004
T10	2.29±0.011	0.50±0.006	0.29±0.005	0.18±0.001
T11	1.99±0.011	0.60±0.004	0.50±0.011	0.24±0.003
T12	2.76±0.057	0.59±0.008	0.49±0.005	0.23±0.000
T13	2.63±0.057	0.53±0.01	0.49±0.011	0.22±0.001

Salinity stress tolerance: At the lowest concentration of 20dSm^{-1} (0.2M) NaCl, all isolates showed growth and as the concentration increased to 80dSm^{-1}, there was considerable reduction in growth (Fig:4). With increase in salt concentration to 120 dSm^{-1}, T5 ceased to grow showing its highest sensitivity. At 140 dSm^{-1}, T4 ceased to grow whereas T1, T2 and T3 failed to grow at 160 dSm^{-1}. At highest concentration of 220 dSm^{-1}, T6, T12 and T13 isolates could grow showing their ability to withstand such high levels of stress.

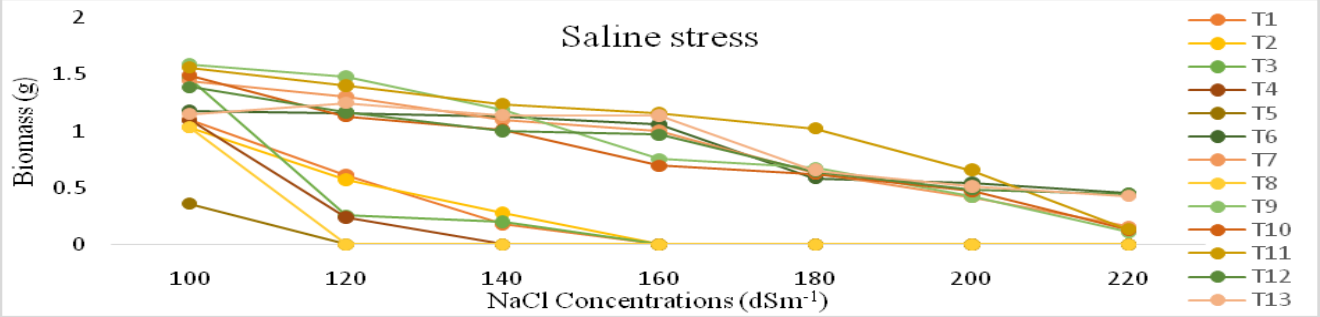

Fig 4: Growth of *Trichoderma* spp. in terms of dry mass (g) with increasing concentrations of sodium chloride (120-220 dSm^{-1}) in liquid media

Table 3: Growth of *Trichoderma* isolates in terms of biomass (g) with increasing temperatures inliquid media

Trichoderma isolates	Biomass(g) at ambient and high temperature (± SD)				
	28°C	38°C	40°C	41°C	42°C
T1	0.85 ± 0.01	0	0	0	0
T2	0.8 ± 0.01	0	0	0	0
T3	0.40 ± 0.02	0	0	0	0
T4	0.63 ± 0.03	0	0	0	0
T5	0.46 ± 0.03	0.049 ±0.00	0.0432 ± 0.0	0	0
T6	0.55 ± 0.04	0.054 ± 0.00	0.042 ± 0.00	0.04 ± 0.001	0.038 ± 0.00
T7	0.86 ± 0.01	0.05 ± 0.00	0.036± 0.00	0	0
T8	0.82 ± 0.02	0	0	0	0
T9	0.78 ± 0.01	0	0	0	0
T10	0.62 ± 0.02	0	0	0	0
T11	0.46 ± 0.01	0	0	0	0
T12	0.48 ± 0.01	0.042 ± 0.00	0.039 ± 0.00	0.04 ± 0.00	0.029 ± 0.00
T13	0.45 ± 0.03	0.043 ± 0.00	0.041 ± 0.00	0.036 ± 0.00	0.035 ± 0.00

High temperature tolerance: At the temperature of 28°C and 30°C *Trichoderma* isolates exhibited maximum growth as it being the optimum growth temperature. T5, T6, T7, T12 and T13 isolates could resist high temperatures of 38°C and 40°C, with the increase temperature to 40°C T5 and T7 isolates did not exhibit any growth. The highest tolerance levels was found to be at 42°C, where T6, T12 and T13 exhibited growth whereas other isolates didn't exhibit any signs of growth(Table 3). The growth, sporulation pattern and color of spores was also recorded for 13 isolates at different temperatures (Table 4)**.**

Table 4: Morphological variations of 13 *Trichoderma*isolates at different temperatures

Trichoderma isolates	Growth pattern						Sporulation pattern						Spore colour					
	28°C	30°C	35°C	40°C	42°C	43°C	28°C	30°C	35°C	40°C	42°C	43°C	28°C	30°C	35°C	40°C	42°C	43°C
T1	C	C	C	++	++	++	+++	+++	-	-	-	-	Dg	Dg	-	-	-	-
T2	C	C	C	-	-	-	+++	++	-	-	-	-	Dg	Dg	-	-	-	-
T3	C	C	F	-	-	-	+++	+++	-	-	-	-	G	G	-	-	-	-
T4	C	C	F	-	-	-	+++	++	-	-	-	-	Dg	Dg	-	-	-	-
T5	C	C	F	-	-	-	+++	++	-	-	-	-	Dg	Dg	-	-	-	-
T6	C	C	C	C	C	-	+++	+++	+++	++	-	-	Yg+ Dg	Yg+ Dg	Yg+ Dg	Dg	-	-
T7	C	C	F	-	-	-	+++	+++	-	-	-	-	Yg	Yg	-	-	-	-
T8	C	C	C	-	-	-	+++	+++	-	-	-	-	Dg	Dg	-	-	-	-
T9	C	C	F	-	-	-	+++	+++	-	-	-	-	Dg	Dg	-	-	-	-
T10	C	C	C	-	-	-	+++	+++	-	-	-	-	Dg	Dg	-	-	-	-
T11	C	C	C	-	-	-	+++	++	-	-	-	-	Dg	Dg	-	-	-	-
T12	C	C	C	C	C	-	+++	+++	+++	++	-	-	Yg+ Dg	Yg+ Dg	Yg+ Dg	Dg	-	-
T13	C	C	C	C	C	-	+++	+++	+++	++	-	-	Yg+ Dg	Yg+ Dg	Yg+ Dg	Dg	-	-

Trichoderma isolates have been shown to induce plant resistance under abiotic stresses, among the 13 isolates tested, T3, T6, T7, T10, T12 and T13 exhibited higher abiotic stress tolerance to either drought, salinity or high temperature. Nevertheless, *Trichoderma* by themselves are not immune to abiotic stress like moisture deficiency, higher temperature, etc., that tend to cause morphological, physiological, biochemical and molecular changes and adversely affect the beneficial consequences of these bioagents (Sowmya *et al*., 2016). Even though *Trichoderma* promote plant growth and induce resistance to biotic and abiotic stresses (Hermosa *et al*., 2013), their role as bio pesticide has primarily contributed to their commercial success as bio-agents. In this study, three isolates *i.e.,* T6, T12 and T3, could tolerate high temperature of 42°C and also possessed other desirable traits and hence could be candidate strains for future exploitation to promote them commercially to cope with the impacts of climate change.

The primary goal of this study was to find *Trichoderma* isolates with abiotic stress tolerance and antagonistic activity against known phytopathogens, as well as the ability to produce various PGPR traits. Proper deployment of isolates with one or more traits may have a synergistic effect on plant growth and disease management under climate change scenarios, through proper root colonization and beneficial effects in the rhizosphere.

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