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RESEARCH ARTICLE

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THERMOTOLERANT *TRICHODERMA*

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ABSTRACT

The study focuses on the morphological and physiological cell responses to high temperature stress in thermotolerant isolates of *Trichoderma viz*., *T. longirachiatum* 673 (TaDOR673) and *T. asperellum* 7316 (TaDOR7316). Heat shock of 52 °C led to changes in morphological characteristic *viz*., decrease in the size and volume of spores but the reduction was significantly less in comparison to susceptible *Trichoderma* isolates tested. Scanning and transmission electron microscopy (TEM) of fungal cultures heated at 52 °C demonstrated shrinkage of cytoplasm and reduced intracellular spaces and mitochondrial count. The observations have supported the hypothesis of reduced metabolism during stress conditions. TEM analysis also revealed increased accumulation of vacuoles indicating their role as scavengers of toxic metabolites generated during heat stress. The isolates showed different patterns of lytic enzyme production and we observed that the thermotolerant isolate *T. asperellum* TaDOR7316 was able to produce β-1, 4-glucanases even after heat stress and thus it can be further tested for biological control of plant pathogens under stress conditions.

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INTRODUCTION

Trichoderma species are the most commonly used biopesticides in today's agriculture (Verma *et al*., 2007). *Trichoderma* antagonizes phytopathogenic fungi and the antifungal properties are principally due to their ability to produce antibiotics (Vinale *et al*., 2008) and/or hydrolytic enzymes (Benitez *et al.,* 2004) and competition for space and nutrients (Elad, 2000). Even through *Trichoderma* species promote plant growth and induce resistance to biotic and abiotic stresses (Hermosa *et al*., 2012), their role as bio-pesticide has primarily contributed to their commercial success as bio-agents. 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. For example, soil hydrological conditions influence the growth and antagonistic properties of *Trichoderma* (Tronsmo and Dennis, 1978; Luard and Griffin, 1981; Magan, 1988) and soil temperature affect the radial extension of *Trichoderma* (Knudsen and Bin, 1990).

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Thermotolerant strains have been identified in bio-control agents *Bacillus*, *Pseudomonas* (Kumar *et al*., 2014) and *Trichoderma* (Poosapati *et al*., 2014). The biocontrol ability of thermotolerant isolates of *Trichoderma* were established earlier (Poosapati *et al*., 2014; Prasad *et al*., 2016) and in the current study these isolates were used to study the morphological and physiological changes associated with heat stress tolerance. The preliminary observations provide supportive information for understanding the mechanisms of heat stress tolerance and these in turn can be employed for the improvement of potential biocontrol agents.

MATERIALS AND METHODS

Strains

The thermotolerant strains of *Trichoderma viz*., *T. longibrachiatum* 673 (TaDOR673) and *T. asperellum* 7316 (TaDOR7316), which were identified previously and deposited in Microbial type culture collection (MTCC), IMTECH, Chandigarh, India (Poosapati *et al*., 2014), were used for this study. These thermotolerant strains were identified from a pool of *Trichoderma* isolates, isolated from soil samples collected from various regions of India. To confirm their identity, multi-locus sequencing (elongation factor1 alpha and RNA polymerase subunit B) was performed using the primer

sequences mentioned in TrichoKey (http://isth.info/) and the results confirmed the identity of these strains (Accession numbers provided in Table 1).

Table 1. List of accession numbers of thermo tolerant *Trichoderma* isolates

Isolate	Accession numbers	
Gene	TEF1α	RNA Polymerase subunit B
T. asperellum 7316, TaDOR7316	KM190858	KX389492
T. longibrachiatum 673, TaDOR673	KM190859	KX389493

Determination of spore volume

Conidia of *Trichoderma* were grown on PDA at 28°C. Aerial conidia were harvested from 7–10 day old culture and the conidial suspension was filtered through three layers of cheesecloth. The filtrate was preserved at 4 °C until use (Agosin *et al*., 1997). Spore concentration was measured as described by Norton and Harman (1985). The spores in the suspension were pelleted by centrifugation and washed repeatedly with sterile distilled water until the microscopic examination indicated the absence of debris from cell lysis. The spore suspension of thermo tolerant and susceptible isolates *Trichoderma* were subjected to a heat shock of 52 °C for 1 h, 2 h and 4 h and observed using a phase-contrast microscope (Carl-Zeiss, Jena, Germany) to distinguish the variation in spore sizes. The dimensions of the spores were measured through micrometry. The untreated samples were used as controls.

Electron microscopy

Conidiophore samples from treated (exposed to 52 °C for 4 h) and untreated isolates of thermotolerant and susceptible *Trichoderma* were processed for Scanning electron microscopy (SEM) as per Pham *et al.* (2005). Samples were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 24 h at 4 °C and post fixed 2 % in aqueous osmium tetroxide for 4 h. Fixed samples were dehydrated in a series of graded alcohols and dried to critical point with CPD unit. The processed samples were mounted over the stubs with double- sided carbon conductivity tape and a thin layer of gold was coated over the samples by using an automated sputter coater (Model – JEOL JFC – 1600) for 3 minutes and scanned under SEM (SEM- Model: JOEL – JSM 5600) at required magnifications as per the standard procedures at RUSKA Lab's College of Veterinary Science, SVVU, Rajendranagar, Hyderabad, India. To further understand the intracellular changes of conidia during heat stress, the spores of treated and control samples of thermotolerant isolate of *Trichoderma viz*., *T. asperellum* 7316 (TaDOR7316) was processed through Transmission Electron Microscopy (TEM). The samples fixed in glutaraldehyde were dehydrated in a series of graded alcohols, infiltrated and embedded in araldite 6005 resin or spur resin (Spurr 1969). Ultra thin (50-70 nm) sections were made with a glass knife on ultratome (Leica Ultra cut UCT-GA-D/E-1/00), and mounted on copper grids. The sections were stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate and viewed under TEM (Model: Hitachi, H-7500 from JAPAN) at required magnifications.

Determination of Lytic enzymes

Lytic enzymes like chitinases, glucanases and cellulases were determined in thermotolerant isolates of *Trichoderma* and the enzyme profiles were compared with other isolates of *Trichoderma* available at Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad (IIOR). Chitinase production was studied by inoculating the cultures in minimal medium supplied with 1% colloidal chitin as the sole source of carbon. After 7 days of culture the supernatants was extracted and used as the source of crude enzyme to determine the enzyme activity as explained by Xu-fen *et al*., 2007. The activities of other enzymes *viz*., β- 1, 3-glucanase and β- 1, 4-glucanase were estimated as explained by Diby *et al*., 2005. Plate assay was also performed to determine the activity of different lytic enzymes. Plates containing 2.4 % (w/v) chitin, 0.5 % (w/v) laminarin, 0.25 % (w/v) carboxymethylcellulose (CMC) were used to identify the activity of chitinases, glucanases and cellulases respectively. The cultures were inoculated onto the plates and incubated at room temperature for 4-6 days and then flooded with 1% congo red solution in water. The stain was removed after 30 min and plates were destained with 1 M NaCl for 15 min (Hagerman *et al*., 1985). Clear zones developing in the opaque agar around the colonies indicated the degradation of the substrate. Plates which were not flooded with any of the stains described above, served as the control.

Statistical analysis

Repeated measures Analysis of Variance (ANOVA) were used to analyze spore count and enzyme activity. The enzyme activity data were transformed with logarithmic transformation.

RESULTS

Morphological differences of thermotolerant *Trichoderma*

In the present investigation, two isolates of *Trichoderma*, *T. longibrachiatum* 673 and *T. asperellum* 7316, that were identified as thermotolerant strains at IIOR (Poosapati *et al.,* 2014), were used to study the morphological differences associated with heat stress in comparison to susceptible isolates of *Trichoderma*. The thermotolerant isolates were able to tolerate a heat shock of 52 °C for 4 h and were able to retain their morphological features after recovery from heat stress. The level of thermotolerance, however, was distinct between these isolates. The isolate TaDOR673 was highly tolerant to the heat shock condition tested, with a mean spore count (log c.f.u/ml) of 4.33 after the treatment whereas TaDOR7316 had a mean spore count of 1.16. The two isolates exhibited distinct morphology with TaDOR673 sparsely sporulating and with yellow pigmentation whereas TaDOR7316 was densely sporulating fungus (Poosapati *et al*., 2014). TaDOR673 was able to tolerate higher thermal stress even at hyphal stage when compared to TaDOR7316. Also, the biochemical analysis revealed that both these isolates accumulate a higher concentration of trehalose, a known compatible solute, during heat shock (Poosapati *et al*., 2014). The morphological differences of thermotolerant isolates and other *Trichoderma* isolates of IIOR repository were observed after recovery from heat stress and are tabulated in Table 2.

Table 2. Morphological variations associated with heat shock recovery process in high temperature tolerant strains of *Trichoderma*

Isolate	Morphological Features									
	Colony characters						Spore Dimensions			
	Color		Mycelial growth		Shape		*Length (µm)		*Width (µm)	
	W	R	W	R	W	R	W	R	W	R
T. viride 701	Green	Green	++	++	Round	Round	11.4	4.75	11.4	4.75
T. viride 85	Green	Green	+++	+++	Oblong	Round	5.34	10.45	3.8	10.45
T. viride 613	Green	Yellow pigmented	++	+	Spherical	Oblong	11.4	6.65	11.4	4.75
T. harzianum 736	Green	Greenish white	+++	++	Oblong	Oblong	6.65	10.45	4.75	4.75
T. longibrachiatum 673	Yellow pigmented	Yellow pigmented	+	+	Oblong	Round to Oblong	10.45	9.5	3.8	3.8
T. harzianum 763	Green	Yellow pigmented	++	+	Round	Oblong	7.98	8.55	7.98	4.75
T. viride 82	Green	Yellow pigmented	++	+	Oblong	Round to Oblong	6.65	5.7	3.8	4.75
T. asperellum 7316	Green	Green	+++	+++	Round	Round to Oblong	5.7	6.65	5.7	3.8
T. harzianum 712	Light green	Dull green	++	++	Round	Oblong	3.8	5.7	3.8	3.8
T. harzianum 693	Whitish green	Whitish green	++	++	Spherical	Spherical	5.7	5.7	5.7	5.7
T. harzianum 672	Dull whitish green	Whitish green	+	++	Round	Round	3.8	5.7	3.8	5.7
T. asperellum 294	Green	Fluorescent green pigmented	++	++	Oblong	Round to Oblong	5.7	5.7	3.8	3.8
T. viride 1	Green	Fluorescent green pigmented	++	++	Round	Round	3.8	5.7	3.8	3.8
T. koningii 1	Green	Fluorescent green pigmented	+	+	Round	Round to Oblong	5.7	5.7	5.7	3.8

W: Unstressed control; R: Isolates revived for a week after heat stress; *Values are mean of two replications

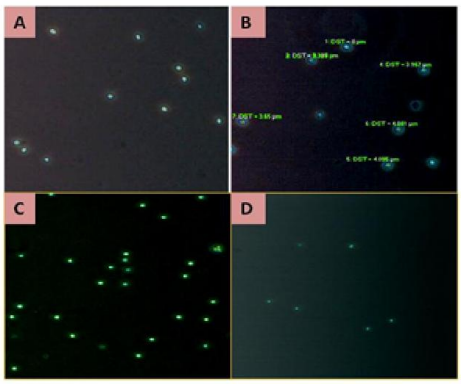

Fig. 1. Conidial morphology of thermotolerant and susceptible isolates of *Trichoderma* during heat stress. A. TaDOR7316 control; B. TaDOR7316 after heat stress; C. Tv5 control; D. Tv5 after heat stress.

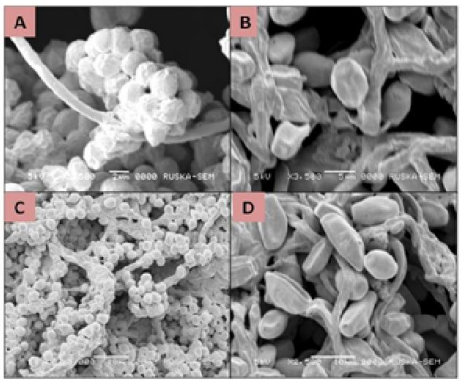

Fig. 2. Scanning electron microscopy of conidia of thermotolerant and susceptible isolates of *Trichoderma* during heat stress. A. TaDOR7316 control; B. TaDOR7316 after heat stress; C. Tv5 control; D. Tv5 after heat stress.

Table 3. Spore volumes of thermotolerant and susceptible strains of *Trichoderma* during heat stress at 52 °C

Isolate	Control		1 h heat shock		2 h heat shock		4 h heat shock	
	Spore diameter (µm)	Spore volume (μm^3) *	Spore diameter (µm)	Spore volume (μm^3) *	Spore diameter (µm)	Spore volume (μm^3) *	Spore diameter (µm)	Spore volume (μm^3) *
T. asperellum 7316	3.89	34.66	3.27	18.79	3.10	15.77	2.08	5.00
T. longibrachiatum 673	4.13	35.91	3.17	17.01	2.33	7.18	2.24	5.59
T. viride 5 (Susceptible)	3.21	17.54	2.21	4.52	1.82	4.23	1.37	1.11
T. harzianum 4d (Susceptible)	3.78	28.61	2.72	16.70	1.96	2.58	0.99	1.43
T. harzianum S12 (Susceptible)	3.67	27.80	2.47	13.08	2.02	2.55	0.87	0.99

The volumes were calculated assuming that the spores are an ellipsoid with volume V, where $V= \pi LW^2/ 6$, in which L represents the length and W the diameter

Conidial morphology after heat stress

The variation in spore sizes of thermotolerant isolates of *Trichoderma* were identified through Phase contrast microscopy (Fig. 1). It was observed that upon exposure to 52 °C for 4 h there was ≥10% reduction in spore diameter and spore volume in susceptible isolates *viz*., *Trichoderma viride* Tv5, *Trichoderma harzianum* Th4d and *Trichoderma harzianum* S12 (ThS12) compared to thermotolerant isolates of *Trichoderma* (Table 3). The results were consistent with changes observed through SEM and TEM analysis of conidia (Fig. 2 and Fig. 3). It was observed that there was shrinkage in cytoplasm and reduced intracellular spaces. TEM analysis also revealed increased accumulation of vacuoles after heat stress.

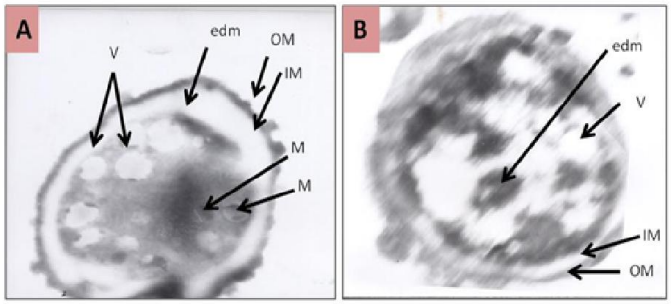

Fig. 3. Transmission electron microscopy of conidia of thermotolerant isolate of *Trichoderma, T. asperellum* 7316 under A. Control and B. Heat stress conditions. V-Vacuole; edm-electron dense bodies, IM- Inner cell membrane; OM-Outer cell membrane; M-Mitochondria

Determination of lytic enzymes

We observed that different isolates of *Trichoderma* uses different combination of lytic enzymes to antagonize the plant pathogens. *T. asperellum* 7316, *T. asperellum* 33, *T. asperellum* 564, *T. asperellum* S3 and *T. asperellum* 693 antagonize the fungal pathogens through the production of chitinases, glucanases and cellulases (Table 4 and Fig. 4). However other isolates tested lacked the production of one or the other of these enzymes signifying different modes of action of these isolates as biocontrol agents. TaDOR673 is a highly thermotolerant strain identified among all the isolates but it was observed that the isolate lacked the production of cellulases. Moreover upon exposure to heat stress at 52 °C for 4 h all the strains retained the activity of β-1, 4-glucanases and comparatively, TaDOR7316 retained better activity of lytic enzymes even after heat stress (Table 5).

Table 4. Determination of Chitinases, glucanases and cellulases in high temperature tolerant strains of *Trichoderma* through plate assay

Isolate	Zone of Clearance (cm)		
	Chitinases	Glucanases	Cellulases
T. asperellum 7316	1.6	2.0	1.3
T. asperellum 224	-	1.8	0.5
T. asperellum 33	1.0	1.8	1.1
T. asperellum 293	-	1.3	-
T. asperellum 294	-	1.4	-
T. asperellum 564	1.2	1.3	0.6
T. asperellum 79	-	1.5	-
T. asperellum 222	1.0	2.6	-
T. asperellum S3	0.7	1.4	0.4
T. harzianum 671	0.6	1.6	-
T. asperellum 693	0.5	1.5	1.2
T. harzianum 712	-	0.7	2
T. longibrachiatum 673	0.9	0.7	-

Values are mean of three replications.

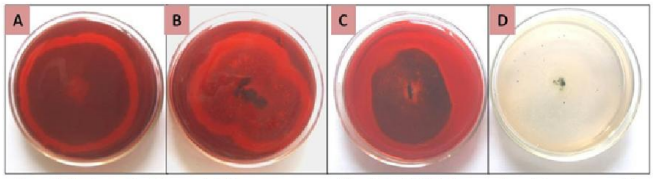

Fig. 4. Determination of lytic enzymes of TaDOR7316 through plate assay. The picture depicts the degradation of A- Chitin; B- Laminarin and C- CMC substrates by TaDOR7316 compared to D- Control. ZC indicates the zone of clearance.

Table 5. Production of β-1, 4-glucanases (U/ml) in liquid culture by high temperature tolerant isolates of *Trichoderma* measured at 500nm

Isolate	Control	Exposure to 52 °C for 4 h	Percent reduction of enzyme production (%)
T. asperellum 7316	3.19	2.44	23.5
T. harzianum 671	1.55	0.78	49.6
T. asperellum 33	2.05	0.84	59.0
T. asperellum 293	1.41	0.37	73.7
T. asperellum 294	1.12	0.82	26.7
T. asperellum 564	1.94	0.81	58.2
T. asperellum 79	1.73	0.52	69.9
T. asperellum 222	1.53	0.67	56.2
T. asperellum S3	1.92	0.82	57.2
T. asperellum 224	1.16	0.43	62.9
T. asperellum 693	2.02	0.85	57.9
T. harzianum 712	1.89	0.84	55.5
T. longibrachiatum 673	1.47	0.83	43.5
C.D at 0.05	0.206	0.266	-
CV%	6.84	19.00	-

DISCUSSION

Trichoderma species have been widely used as potential biological control agents in commercial agriculture over the past 2–3 decades. But the performance of *Trichoderma* under field conditions is highly limited by the soil hydrological conditions (Luard and Griffin 1981; Magan 1988). Hence we have tried to identify some thermotolerant isolates of *Trichoderma* (Poosapati *et al*., 2014) at IIOR that can be used as model organisms to understand the stress tolerance mechanisms of *Trichoderma.* The isolates, TaDOR7316 and TaDOR673 were able to survive after a heat shock of 52 °C. Thus these isolates were used to understand the morphological changes associated with heat stress in comparison to susceptible isolates of *Trichoderma*. At temperatures as high as 52 °C the concentration of viable spores has reduced but as expected the reduction was less in thermotolerant isolates of *Trichoderma* in comparison to other isolates of *Trichoderma* tested. SEM and TEM analysis also supported the observations and gave further insights into the intracellular changes associated with heat stress. In TaDOR7316, there was increased accumulation of vacuoles, reduced intracellular spaces and disintegration of mitochondria.

These observations probably indicate that, to encounter the heat stress, TaDOR7316 reduces the cellular metabolic process and removes the toxic metabolites through autophagy (Spear and Ng, 2003). Studies have shown that endocytic pathways, proteosome pathways and regulation of autophagy are induced during heat stress in filamentous biocontrol fungi (Wang *et al.,* 2014) to maintain cellular homeostasis. In support of this we observed an increased expression of an autophagy protein, Apg6 and two hypothetical proteins with conserved oligomeric-golgi domains (COG) during the stress conditions (Poosapati *et al*., Unpublished). The biocontrol potential of the thermotolerant isolates were attributed by the production of lytic enzymes. Even after heat stress the thermotolerant isolate, TaDOR7316 was able to produce β-1, 4-glucanases at a concentration of 2.44 U/mL. The isolate TaDOR7316 possessed better antagonist activity against *Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *carthami* in safflower (*Carthmus tinctorius* L) (Prasad *et al*., 2016). Thus the morphological differences observed during heat stress

provide insights into the probable mechanisms employed by thermotolerant isolates for stress tolerance. Moreover the ability of thermotolerant isolates to antagonize plant pathogens after heat stress widens their applicability as bioagents under stressed soil conditions.

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