

## Selection of *Trichoderma* spp. tolerant to abiotic stresses with antagonistic activities against date palm leaf spot diseases in Qatar

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### ABSTRACT

Biological control Agents (BCA) are a group of microorganisms that are used as biopesticides in diseases control. Their inconsistency in performance from the laboratory to the field and from a region to another is mainly due to the prevailing abiotic stresses. Therefore, selection of isolates with tolerance to abiotic stresses would benefit the development of a successful biopesticide.

In this study an attempt was made to isolate and identify morphologically and molecularly indigenous isolates of *Trichoderma* species. Then evaluate their stress tolerance and antagonistic effects against three main causal agents (*Alternaria alternata*, *Thielaviopsis sp.* and *Helmenthosorium sp.*) of leaf spot disease of date palm in Qatar. Six isolates of three *Trichoderma* species were isolated from soil samples. Screening these isolates on different pH and exposing them to the heat shock of (70°C and 90°C for 24h) showed the potential of all 6 Qatari isolates to grow well at pH 8 and to survive 70°C/24h. Whereas the early reports suggested for the acidic to neutral pH for growing the *Trichoderma* species and the potential tolerance to heat shock of 55°C for a few hours. The survival of heat shock was more pronounced in the isolates of *T. longibrachiatum* than those of *T. harzianum* and *T. viridi*. Two isolates of *T. longibrachiatum* were able to survive the 90°C heat shock for 24h. For bio safety reason we suggest avoiding using the *T. longibrachiatum* because this species is known as an opportunistic pathogen of immunocompromised mammals including humans. On the other hand *T. harzianum*, showed the ideal antagonistic affect *in vitro* and *in vivo* trials against the three causal agents of leaf spot disease in date palm. It can be recommended as a good candidate to develop a bio-pesticide against the three pathogens.

To conclude; this study showed the possibility of discovering and selecting isolates from Qatar that tolerate arid conditions and can be developed to a successful bio-pesticide. The identification of these *Trichoderma* isolates paved the way to develop a sustainable biological control agents for the arid regions.

**Keywords:** *Trichoderma*; Thermotolerance; pH tolerance; biocontrol; leaf spot disease, date palm

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## INTRODUCTION

Extensive academic and applied research were conducted in discovering and using biological control agents (BCAs) in plant protection. The outcomes of these research have led to the development of a number of biocontrol products on a commercial scale which were reviewed by Fravel (2005). *Trichoderma harzianum* was one of the first registered commercial BCA. with the United States Environmental Protection agency (EPA).

The genus *Trichoderma* is a fungus that is common in all type of soils and known for its potential in plant protection (Harman *et al.*, 2004). Using *Trichoderma* spp. as BCAs were studied in the past 80 years in controlling a wide range of plants diseases (Hjeljord and Tronsmo, 1998 and Verma *et al.*, 2007). They possessing different mechanisms of action against plant pathogens, including competition of nutrients, mycoparasitism and antibiosis as well as inducing resistance through activation of plant defense reaction (Harman 2006; Verma *et al.*, 2007; Mendez-Vilas 2010; Valencia *et al.*, 2011)

This genus like all soil microbes are known to be affected by abiotic stress such as high temperature, pH and salinity. The effect of temperature on the *Trichoderma* was reported by Widden and D. Hsu, (1987). The commercial success of BCA in arid area such as Qatar would depend on their ability to withstand and proliferate under adverse environments such as high temperatures and alkaline pH. This necessitate the identification of potential high temperature and high pH tolerant *Trichoderma* strains that could sustain and survive the conditions of arid regions or the fluctuating temperature rising due to global warming.

Native isolates from *Trichoderma* species were identified as BCA against leaf spot diseases of date palm in Qatar (El-Badawy and Kharbotly, 2014). They were isolated from the rhizosphere of date palms. The presence of these *Trichoderma sp.* under the harsh environmental conditions of Qatar reflects their potential to be used as BCA in arid land. The isolation and the selection of stress tolerant *Trichoderma* strains would increase their efficiency against plant pathogenic fungi and prolong their persistence in plant environment even under harsh environmental conditions.

Leaf spot diseases of date palm are the most common and widely spread diseases in the middle east and north Africa. In Qatar, leaf spot diseases affecting all date palm plantation. Our preliminary studies showed that *Alternaria alternate*, *Helmenthosorium sp.* and *Thialoviopsis sp.* fungi are the three main causal agent of these diseases. In Saudi Arabia, a few casual agents of the leaf spot diseases in date palm were reported including *Alternaria alternate* and *Helmenthosorium sp* (Al-sharidy and Molan, 2008; Ammar and El-Naggar, 2011). Application of a wide variety of fungicides and the removal of infected fronds are being used to control the diseases. Intensive use of fungicides can cause problems such as fungi resistance, environmental pollution and human and animal health hazards Such problems can be avoided by using BCAs against plant diseases (Cook and Baker, 1983)

Both the removal of infected fronds and the presence of black brown spots on the palm leaves affect negatively the date fruit production through the reduction of photosynthesis. The presence of the brown dry spots on the fruit reduce their quality dramatically. Date palms are also used in landscaping of metropolitan roads for shading and beauty. The view of dried or spotted leaves are sight disturbing. Using fungicides has

negative effects on the environment and humans in the metropolitan roads. A bio-pesticide is the ideal way to control such diseases in these areas.

The survival of BCAs is a challenging issue in arid agroecosystems due to the abiotic stresses. BCA inoculants for foliar application can be affected negatively by high temperature on plant canopy. In case of soil application, high temperature, pH, salinity and drought would influence the efficiency of BCA treatments. The variations in the efficiency of a BCA under laboratory and field conditions are mainly due to the abiotic stresses that present under field conditions. Such problems can be overcome by using isolates which are abiotic stress tolerant.

The present study was conducted to develop a BCA against leaf spot diseases of date palm through the isolation and identification of *Trichoderma* isolates. This followed by the selection of isolates that have the ability to withstand adverse environments such as high temperature, pH along with antagonistic activity to the causal agents of leaf spot diseases of date palm.

## MATERIALS AND METHODS

### Fungal isolates

Sixteen soil samples were collected from the rhizosphere of date palm plantation at the Agricultural Research Farm, Rwdet Al-Faras, Qatar. (25°82'N; 51°33'N). The date palm field contained Khalas cultivar older than 20 years old. The pH of all soil samples was determined. The soil samples were stored at -20°C for a few days until the isolation of the BCA (*Trichoderma* sp.). The isolation was conducted using PDA medium (Eddleman, 1998) supplemented with chloramphenicol by serial dilution plate technique as described by Johnson and Curl (1972). Samples were purified using single spore isolation technique according to Constantinescu (1988). The obtained *Trichoderma* isolates were stored in the culture collection at Qatar National Gene bank, Department of Agricultural Research, Doha, Qatar.

Three pathogenic fungi isolates (causal agent for leaf spot diseases on date palm) namely *Alternaria alternate* isolate QATMicF000032. and *Helmenthosorium* sp. isolate

QATMicF000033 and *Thialoviopsis sp.* isolate QATMicF000034 were obtained from the the culture collection at Qatar National Gene bank. These isolates were selected based on the results of the screening tests of their pathogenicity on date palms. They were grown on potato dextrose agar (PDA) and incubated at 28 °C for 4–6 days prior the antagonistic experiments.

### Morphological and Molecular Identification

Isolates were identified based on their phenotypic characters such as colony color, shape and growth as well as the microscopic morphology such as size and shape of phialdes and conidia (Kiffer and Morelet, 2000, Kumar *et al.*, 2011, Morton and Stroube 1955, and Muthumeenakshi *et al.*, 1994).

Morphological identification of *Trichoderma* isolates was confirmed by sequencing the ITS region of rDNA. DNA was extracted from 50 mg of fungal biomass from each isolates of *Trichoderma* growing on PDA media using Qiagen plant mini Kit. The amplification of ribosomal ITS region was carried out by polymerase chain reaction (PCR) using primers ITS1 (5' -TCCGTAGGTGAACCTGCGG-3') (White *et al.*, 1990). and ITS4-B (5' - CAGGAGACTTGTACACGGTCCAG-3'). (Gardes and Bruns. 1993) in a final volume of 20 µl containing 2µl (20 ng) of DNA template, 10µl of AmpliTaq Gold® 360 Master mix (Applied Biosystems), 1µl (10 pmol/ µl) of each forward and reverse primer in addition to 6 µl of nuclease free water. Amplification was carried out according to Kamhawy *et. al.*, 2011) The PCR products were visualized on 1.5% agarose gel using SYBR Green staining. They were purified using ExoSAP-IT then were sequenced by a Sanger's Dideoxy method (Sanger *et al.*, 1974). The Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer) were used for sequencing. Sequences obtained were analyzed together with sequences available in GenBank (NCBI) using BLAST (Basic Local Alignment Search Tool) program. <http://www.ncbi.nlm.nih.gov/blast/>.

### Evaluation of culture conditions for the Qatari isolates

#### Preparation of standard inoculums

Standard inoculum culture was prepared according to Kendrick and Ratledge (1996). A total of 10% (v/v) of the standard inoculum was used as initial inoculant in each replicate of the experiments. Three replicates for each treatment were incubated at 25 °C (Singh *et. al.*,

2014). The growth (biomass production) was measured as dry weight of mycelium (Madan and Thind. 1998).

**Media:** The effects of media on fungal growth were studied on PDA, TSM broth (*Trichoderma* selective medium) (Elad et al., 1981), and chapix. They were prepared according to the manufacturer's instructions. The isolates were cultivated on different media then growth diameters were measured in interval of 24 hours.

**pH:** The influence of initial medium pH on fungal growth in liquid TSM medium was investigated at pH 4.0, 6.0 and 8.0. Fifty ml of media were inoculated for each replicate then incubated at 25°C in an orbital shaker at 150 rpm for 7 days.

**Extreme heat stress tolerance:** Selection of heat stress tolerant isolates was performed by exposing 1.5 ml liquid culture of each replicate to 70 or 90 °C for 24 hrs. then cultured on PDA media plates. They were incubated at 25 °C then observe the presence or absence of the growth after 5 days.

#### **Evaluation of antagonistic activity of *Trichoderma*:**

The *Trichoderma* isolates were evaluated *in vitro* and *in vivo* for their potential to control the *Alternaria alternate*, *Helmenthosorium sp.* and *Thialoviopsis sp.*:

***In vitro* test:** dual culture with the pathogen:

The antagonistic potentialities of the *Trichoderma* isolates were determined against the three pathogens by dual culture on PDA media according to Morton and Stroube (1955). During 5 days of the incubation period, radial growth of pathogen was recorded and percentage inhibition calculated in relation with control by following formula:

$$L = (C - T) / C * 100$$

L= Percentage of inhibition

C= radius of the radial growth of the pathogen towards opposite side in control plate

T=radius of the radial growth of the pathogen towards the opponent antagonist in test plate.

***In vivo* test:**

*Trichoderma* isolates were evaluated on 2-years-old date palms in pots inside a greenhouse. Spore suspensions of pathogens and *Trichoderma* isolates were prepared from 7days old culture. The spore concentration was adjusted to  $2.5 \times 10^5$  spores /ml by using a hemocytometer. 100 ml of the *Trichoderma* spore suspension was sprayed on each palm. Palms were covered with plastic sheet for 48 hrs. then 100 ml of pathogen spore suspension was sprayed. Palms were covered again for another 72 hrs. Control palms were sprayed with

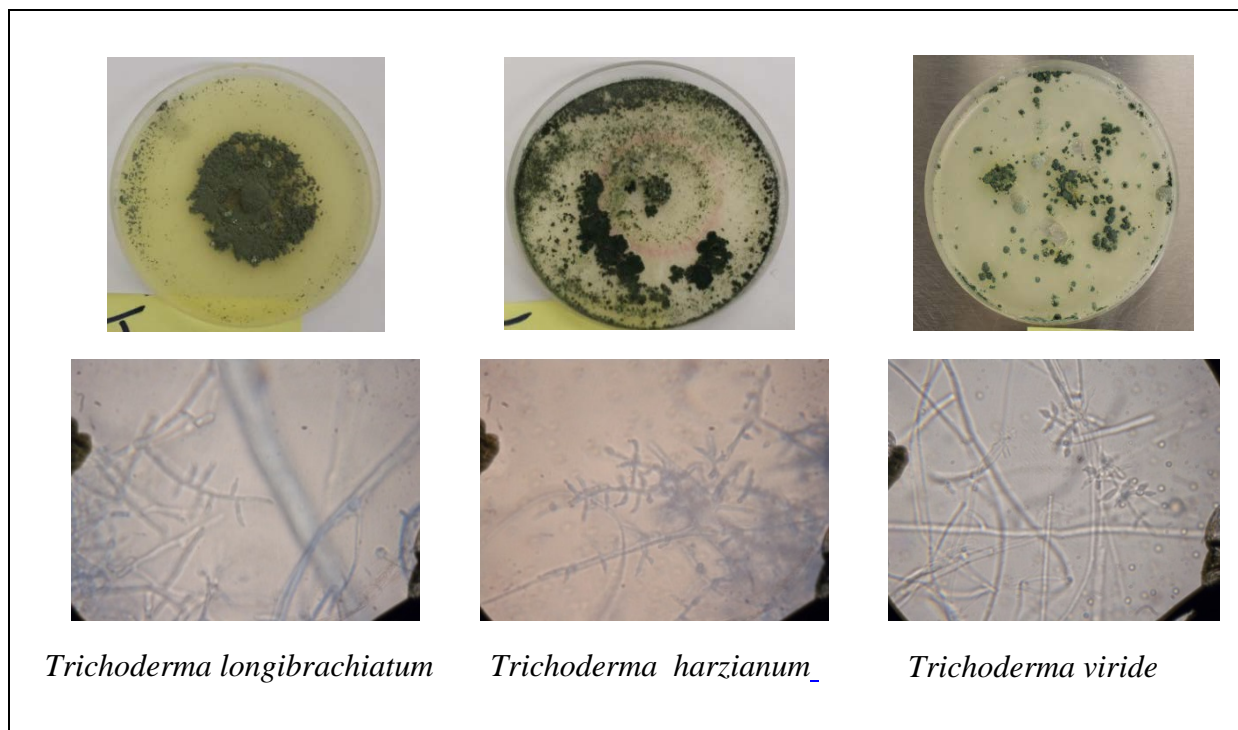
only the pathogens spore suspensions. Each treatment was performed in triplicate. The pots were watered once a week. The appearance of symptoms was observed every two days for 14 days after spraying. Disease severity was calculated using the following formula (James, 1971).

Disease severity = (Mean of plant tissue infected) / (Mean of total area of the leaflet) X 100

## RESULTS

### Isolation of *Trichoderma*

Six isolates of *Trichoderma* were isolated from 16 soil samples. Colonies that produced green color conidia were picked up and observed under the microscope after staining with lactophenol cotton blue stain. The initial microscopic analysis of the mycelium with spore revealed that the isolates were belong to genus *Trichoderma*. (Fig. 1). Isolates were then sub cultured and stored in PDA slants at -20°C till identification and evaluation.



**Fig. 1. Six isolates of *Trichoderma* were classified into three species, *Trichoderma longibrachiatum*, *Trichoderma harzianum* and *Trichoderma iride*.**



## Morphological and Molecular Identification

Bases on the morphological characters, the six isolates of *Trichoderma* were classified to three species. *T. longibrachiatum* (4 isolates), *T. harzianum* (one isolate) and *T. viridi* (one isolate) (Fig. 1). The color of the colonies changed from light green shade to dull green after the production of conidia. The growth characters of culture and sporulation patterns varied noticeably within and between the species as shown in Table 1.

**Table 1. Morphological and molecular identification of the six isolates of *Trichoderma***

Isolation Codes	T1	T2	T3	T4	T5	T8
<b>Qatar genebank accession no</b>	QATMicF000047	QATMicF000048	QATMicF000049	QATMicF000050	QATMicF000051	QATMicF000052
<b>Morphological characters</b>						
Colony growth diameter after 48 hours	9 cm .	9 cm	9 cm .	9 cm .	9 cm .	8 cm.
Colony color	Light green to yellowish green	Light green to yellowish green	Light green to yellowish green	yellow	yellow	Light green to dark green
Reverse colony color	Light yellow	Light yellow	Dark yellow	colorless	Light yellow	colorless
Culture smell	No smell	No smell	No smell	No smell	No smell	malt
Phialide shape	Oblong	Oblong	Oblong	Oblong	Oblong	Ampulliform
Conidial color	Yellowish green	Yellowish green	Yellowish green	colorless	colorless	Dark green
<b>Molecular identification</b>						
NCBI, Genbank						
Accession numbers.	KY054892	KY054893	KY054894	KY054895	KY054897	KY054896
NCBI, GenBank Similarity	99%	99%	99%	99%	99%	99%
Organism	<i>T. longibrachiatum</i>	<i>T. longibrachiatum</i>	<i>T. viridi</i>	<i>T. longibrachiatum</i>	<i>T. longibrachiatum</i>	<i>T. harzianum</i>
Accession no	<b>Z48935</b>	<b>Z48935</b>	<b>AJ230678</b>	<b>Z48935</b>	<b>Z48935</b>	<b>AJ224013</b>
<b>Definitive identification</b>	<i>T. longibrachiatum</i>	<i>T. longibrachiatum</i>	<i>T. viridi</i>	<i>T. longibrachiatum</i>	<i>T. longibrachiatum</i>	<i>T. harzianum</i>



The molecular identification based on the ITS rDNA sequences of the isolates confirmed the species identity. The sequences of the isolates were submitted to NCBI, Genbank with the submission ID numbers shown in Table 1.

### Evaluation of culture conditions for the Qatari isolates

#### Media:

All isolates showed significantly fast growth on PDA media compared with other two media, ranged from 4.13 to 4.6 cm/ 24 hrs. for isolates belong to *T. longibrachiatum* while reached 4.37 and 3.83 cm/ 24 hrs. for isolates belong to *T. viridi* and *T. harzianum* respectively. The slowest growth rate was observed on Chapix agar media. The TSM showed intermediate growth fell between the PDA and Chapix media (Table 2).

**Table 2. Effect of media and pH on growth of different *Trichoderma* isolates at 25°C**

BCA Isolates	Growth rate (cm/24 hrs) Media.	Dry weight (g) pH							
		PDA	TSM	Chapix	LSD	4:0	6:0	8:0	LSD
<i>T. longibrachiatum</i>	T1	4.37	2.17	1.10	0.319	0.096 <sup>a*</sup>	0.072	0.11 <sup>a</sup>	0.013
	T2	4.13	2.40	1.10	0.305	0.077	0.069	0.11	0.02
	T4	4.43	2.47	1.27	1.038	0.08 <sup>a</sup>	0.072 <sup>a</sup>	0.07 <sup>a</sup>	0.026
	T5	4.60	1.97	0.73	0.221	0.075 <sup>a</sup>	0.075 <sup>a</sup>	0.07 <sup>a</sup>	0.030
<i>T. viride</i>	T3	4.37	2.23	1.23	0.199	0.060	0.077	0.11	0.014
<i>T. harzianum</i>	T8	3.83	2.97	1.47	0.365	0.064 <sup>a</sup>	0.063 <sup>a</sup>	0.086	0.018

\*Averages indicated by the same letter in the same row have no significant differences at (P≤0.05%).

#### pH:

The effect of the pH on growth biomass measured as dry weight varied between species as well as between isolates from the same species. The pH had no significant effect on the two isolates T4 and T5 (*T. longibrachiatum*) while pH 6 significantly affected negatively the isolates T1 and T2. The growth in *T. viride* (T 3) was increased significantly as the pH increased. The isolate T8 (*T. harzianum*) produced dry biomass on media of pH 8 significantly higher than on the both media of pH 4 or 6. There were no significant

differences between the growth of this isolate on pH4 and 6. In general the pH 8 was the favorite pH for all isolates from the three species (Table 2). It was noticed that the favorite pH for all isolates is very close to the pH of the soil samples that ranged from 8.4 to 8.6

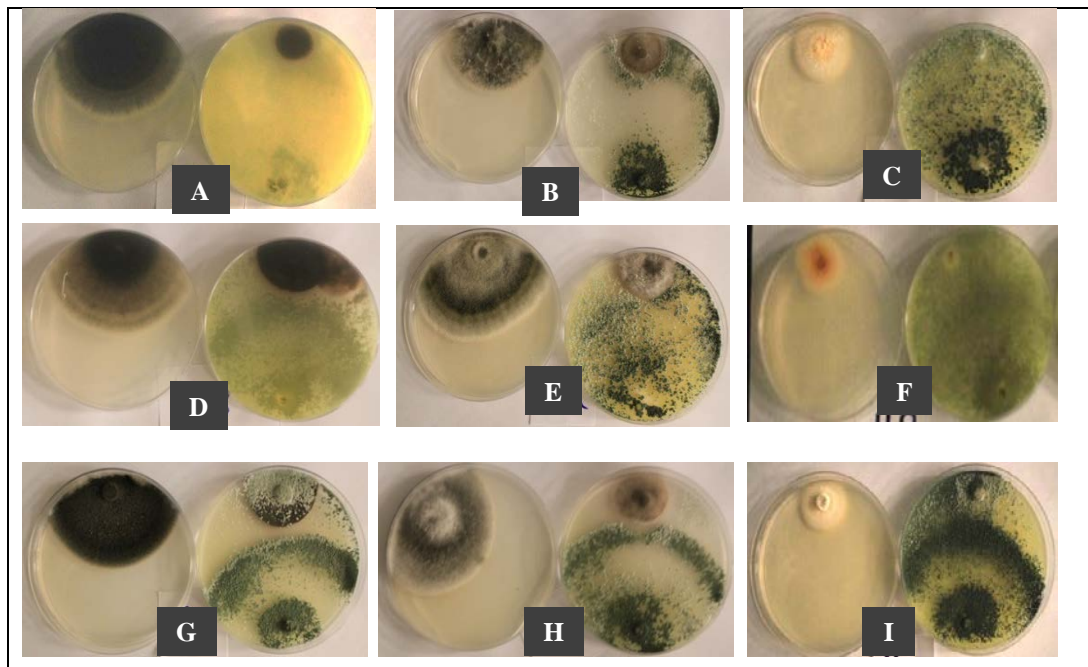
#### Extreme heat stress tolerance:

The results after exposing the isolates to either 70 or 90 °C for 24 hrs before culturing was remarkable. All the isolates exposed to 70 °C grew normally, meanwhile only isolates T2 and T5 (*T. longibrachiatum*) grew after exposed to 90 °C.

#### Evaluation of antagonistic activity of *Trichoderma*:

##### *In vitro* test:

Suppression of mycelial growth of the three pathogens (*Alternaria alternata*, *Thialoviopsis sp.* and *Helminthosporium sp.*) was observed in dual culture with all isolates. It ranged from 61.28 % to 79.36 % of *Alternaria alternata*, 68.6 % to 75.31 % of *Helminthosporium sp.* and 69.16 % to 90.22% of *Thialoviopsis sp.* depending on the *Trichoderma* isolate that was used (Fig 2 and table 3). The highest percentage of suppression was observed using isolate T8 (*T. harzianum*).



**Fig. 2. Inhibition effect of different species of *Trichoderma* on *Alternaria alternata*, *Helminthosporium sp.* and *Thialoviopsis sp.* respectively.**

**A, B, C: *T. longibrachiatum*, D, E, F: *T. viridi*. and G, H, I: *T. harzianum***

**Table 3** The effect of *Trichoderma* isolates on three casual agents of leaf sopt disease of date palm

BCA Species and Isolates	Pathogens						
	<i>Alternaria alternata</i>		<i>Helminthosporium sp.</i>		<i>Thialoviopsis sp</i>		
	<i>In vitro</i> % suppression	<i>In vivo</i> % Disease severity	<i>In vitro</i> % suppression	<i>In vivo</i> % Disease severity	<i>In vitro</i> % suppression	<i>In vivo</i> % Disease severity	
<i>T. longibrachiatum</i>	T1	66.70	19.57	69.84	25.00	75.05	13.4
	T2	62.32	18.07	70.32	22.22	76.28	13.95*
	T4	62.74	19.45	68.60	15.30	75	13.93 <sup>a</sup>
	T5	61.28	22.23	68.92	19.47	75.48	13.97 <sup>a</sup>
	T3	68.69	11.12	68.08	14.45	69.16	8.94
<i>T. viride</i>	T3	68.69	11.12	68.08	14.45	69.16	8.94
<i>T. harzianum</i>	T8	79.36	13.90	75.31	13.90	90.22	9.33
Control		0.0	50.00	0.0	30.45	0.0	25.00
LSD		0.0173	0.0914	0.01066	0.0616	0.00293	0.053

\*Averages indicated by the same letter in the same column have no significant differences at ( $P \leq 0.05\%$ ).

### ***In Vivo* test:**

The efficacy of *Trichoderma* as a BCA against *Alternaria alternata*, *Helminthosporium sp.* and *Thialoviopsis sp.* was evaluated based on the disease severity. The disease severity decreased significantly in palms treated with different *Trichoderma* species before applying pathogens spores.

The lowest disease severity of the three pathogens was observed in palms treated with T3 (*T. viridi*) and T8 (*T. harizianum*) (Table 3). All other isolates (T1, T2, T4 and T5) belongs to *T. longibrachiatum* showed significantly higher disease severity ranged from 18.07% to 22.23% with *Altenaria alternata*, 15.3% to 25% with *Helminthosporium sp* and 13.4 to 13.97% with *Thialoviopsis sp.* The disease severity on the control palms were significantly much higher than all other treatments scoring 50%, 30.45% and 25% for *Alternaria alternata*, *Helmenthosporium sp.* and *Thialoviopsis sp.* respectively.

### **DISCUSSION**

This study showed the possibility to select *Trichoderma* isolates as BCA for arid regions where soil has alkaline pH and the temperature is high during summer months. *Trichoderma* species were abundant in the date palm field of the experiment station. From 16 samples three species of *Trichoderma* were found. Also variations were observed between

isolates from the same species which reflects rich biodiversity and potentialities to select the suitable isolates as BCA for arid conditions.

The antagonistic effect of isolates of T3 (*T. viridi*) and T8 (*T. harzianum*) were more efficient in controlling leaf spot diseases of date palm than the four isolates of *T. longibrachiatum*. *T. harzianum* was reported as BCA against black scorch disease (*Ceratocystis radicola*) in date palm (Al-Naemi, *et al.*, 2016) These findings support the preference of using the isolates of *T. harizianum* as BAC to develop a bio-pesticides. Also the isolate of *T. viridi* showed potential for controlling the leaf spot disease of date palms. Isolates of *T. longibrachiatum* are less favorable to be used as BCA because of their low efficiency. Moreover *T. longibrachiatum* is known as an opportunistic pathogen of immunocompromised mammals including humans (Kredics *et al.*, 2003). So for the biosafety reason it is better not to use such species. Also for the same reason, it is important to have the correct identification of any unknown isolates of *Trichoderma*. In this study, the identification was based on the morphology and the growth characters of the isolates then was confirmed by the molecular analysis of the ITS region of rDNA.

Qatari isolates showed the preference for high pH (pH 8). Measuring the pH of the media after the experiment revealed no changes. This observation indicated that the *Trichoderma* isolates existing in an ecosystem can have characters suitable for their survival under that condition. They might differ from the same species of *Trichoderma* isolated from different ecosystem in their performance in growth and inhibition for pathogens. Earlier reports demonstrated that, *Trichoderma* preferred the acidic conditions more than alkaline (Bitton and boylan, 1985, and Limón *et al.*, 2004). Joshi *et al.*, (2010) demonstrated the effect of the ecosystem including the pH of the soil on the performance of 62 *Trichoderma* isolates from western Himalayas. Also, Jackson *et al.*, (1991) reported that *T. harzianum* isolates showed optimum mycelial growth between pH 4.8 to 6.8. Most probably the main reason is that these *Trichoderma* isolates were isolated from acidic soils. In most cases *Trichoderma* is used as bio-fungicide agents (Verma *et al.*, 2007). In case of applying it for soil fumigation, the soil pH will play a vital role on its success. That may necessitate the adjustment of the soil pH. Other option would be the selection of the *Trichoderma* that match the soil pH. In our case, the selected isolates can be used effectively under Qatari conditions for both air born and soil born diseases.

All six isolates from the three *Trichoderma* species in this study survive the extreme heat shock of 70 °C for 24 hrs. and only two belong *T. longibrachiatum* survive the 90 °C for

24 h. These temperatures are much higher than those reported by Poosapati *et al.*, (2014). In their study, they expose their isolates of *Trichoderma asperellum* to heat shock at 48°C, 50°C and 52°C for 1, 2 and 4 hrs., respectively. Screening large number of isolates from both species *T. viridi* and *T. harzianum* for heat shock followed by selection of isolates that can grow on higher incubation temperature might be possible as was reported for *T. asperellum* by Poosapati *et al.*, (2014). Moreover, The Qatari isolates were obtained from soil samples that were stored for a few days at -20 °C before isolation. Also they were stored after purification and subcultured in PDA slants at -20°C till evaluation. That showed their potential to survive the cold and heat of the arid regions during winter and summer month respectively. It also gave indications on the possibility of long storage of the biopesticide produced from them at wide range of temperature.

Thus, the study identified a potential thermotolerant and high pH tolerant isolates of *Trichoderma* that could be used as potential BCA under arid conditions. Such strains could survive the fluctuating of temperatures and sustain their existence the whole year round in the arid regions.

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