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Antifungal Potentiality and Physiological Characterization of *Trichoderma* isolates from Port Said Governorate

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A TOTAL of twenty *Trichoderma* isolates belonging to seven species were isolated from several locations at Port Said Governorate. Physiological characteristics of these isolates and their antagonistic effect against *Sclerotinia sclerotiorum*, the causative agent of white mold of *Phaseolus vulgaris* were evaluated. *Trichoderma polysporum* 2 showed the highest activity of total cellulase reached up to 2.95 filter paper unit ml^{-1}. *T. harzianum* 2 had the highest endoglucanase activity (5.47 IU ml^{-1}), whereas *T. polysporum* 1 had the highest chitinase activity (1.01 IU ml^{-1}). Siderophores production was assayed using $FeCl_3$, tetrazolium, Arnow's and spectrophotometric tests. Most of the isolates were able to produce hydroxamate and carboxylate types of siderophores. The ability of *Trichoderma* to produce indole acetic acid (IAA) and gibberellic acid (GA_3) were also investigated. *Trichoderma harzianum* 2 showed the highest concentration of IAA in broth medium reached up to 13.19µg/ml, whereas *T. polysporum* 1 showed the highest concentration of GA_3 by 586.51µg/ml. The antagonistic potentialities of *Trichoderma* isolates against *Sclerotinia sclerotiorum* were tested *in vitro* by using dual culture, base to base assay and the crude liquid extract effect of the isolates. Percentages of inhibition of radial growth of the pathogen by *T. polysporum* 1 in dual culture and crude extract methods were 45.97% and 87.78%, respectively. Also, the volatiles of *T. piluliferum* 2 showed a reduction of the radial growth by 66.85%. So, some *Trichoderma* isolates collected from Port Said soil showed high biocontrol activity against *S. sclerotiorum.*

Keywords: Biocontrol, Enzymes, Hormones, *Sclerotinia sclerotiorum,* Siderophores, *Trichoderma.*

Introduction

Trichoderma is anamorphic fungal genus, green-spored, belongs to the family of ascomycetes, free-living plant symbiont, found in many habitats such as root, soil and foliar environments (Shukla et al., 2016). Several species of *Trichoderma* are used as biofertilizers and biofungicides in agricultural soils to enhance crop growth and control a wide range of aerial and soil-borne fungal plant pathogens. The success of *Trichoderma* to survive in plant rhizosphere is due to their high reproductive capacity, ability to survive under unfavorable conditions, efficiency in utilizing nutrients, capacity to modify the rhizosphere and strong aggressiveness against plant pathogenic fungi (Benítez et al., 2004).

Trichoderma can suppress plant diseases by one or more many mechanisms, including competition for nutrients or space, mycoparasitism, production of antibiotics and/or hydrolytic enzymes such as chitinases and cellulases, enhancement of plant development, and induction of plant systemic resistant even at sites away from the point of application (Contreras-Cornejo et al., 2016). One factor that contributes to the beneficial biological activities by *Trichoderma* strains is the wide variety of secondary metabolites which include: i) Non-volatile with high molecular weight and ii) Volatile organic compounds with low molecular weight (Hermosa et al., 2014).

Many *Trichoderma* species can produce the auxin phytohormone indole-3-acetic acid (IAA),

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and its production has been suggested to promote root growth. IAA synthesis can help in plant tolerance to biotic and abiotic stresses (Nieto-Jacobo et al., 2017). Many studies have been focused on the positive role of *Trichoderma* spp. in enhancement of many regulators such as zeatin and gibberellins which have been used in breaking of seed dormancy in some plant species, stimulating of stem elongation and regulation of gene expression (Al-Askar et al., 2016).

Iron is essential trace element for all living organisms due to its role in the biosynthesis of chlorophyll and many physiological activities (Sujatha & Ammani, 2013). However, low availability of soil-iron to plant roots is generally noticed in alkali soils. Siderophores are small (low molecular weight < 1000 Daltons), extracellular compounds, which are generally produced by microbes under low-iron stress to chelate or bind residual iron that is subsequently brought back into the cell by specific uptake mechanisms (Winkelmann, 2007). Siderophores produced by biocontrol agents (BGA) and plant growth promoting microbes (PGPM) is one of the most important mechanisms for plant growth promotion and disease suppression (Vinale et al., 2014). Microbial siderophores are classified to catecholates, hydroxamates and α carboxylates, depending on chemical nature of their coordination sites with iron (Winkelmann, 2002).

Sclerotinia sclerotiorum (Lib.) de Bary is a facultative parasitic Ascomycete fungus, which can grow well even under unfavorable conditions causing white mold disease, it can survive for up to 8 years in soil in the form of sclerotia and it has a wide host range (de Figueirêdo et al., 2010). *S. sclerotiorum* considers a serious problem with a large number of beans and lately; this pathogen caused a considerable yield loss of *Phaseolus vulgaris, L*. in Ismailia, Egypt (Elsheshtawi et al., 2017). Beside it is very difficult to completely eradicate the sclerotia produced by this pathogen, an integrated disease management strategy includes biological control, and chemical fungicides, is desired to ensure efficient control of bean white rot disease (Clarkson et al., 2006). However, using chemical fungicides is expensive and causes a negative ecological impact due to toxic residues (El-Sharkawy & Alshora, 2020). Several species of *Trichoderma* have been reported as potential biocontrol agents of *S. sclerotiorum* (Bastakoti et al., 2017). Therefore, this study aimed to isolate *Trichoderma* spp. from different soil habitats at Port Said Governorate, to investigate their physiological characteristics and *in vitro* assay of their antifungal potentiality against the white mold pathogen (*S. sclerotiorum*).

Materials and Methods

Collection of soil samples and isolation

Seventeen soil samples were collected from different soil habitats at Port Said Governorate as demonstrated in Fig. 1. *Trichoderma* isolation from these soils was performed by the pour plate method using seven media. These media were RB-S-F medium (Davet & Rouxel, 2000), TSM modified (Williams et al., 2003), TME (Vargas et al., 2009), TSM (Attitalla et al., 2012), Davet's medium (Ommati et al., 2013), GRSM (Haddad et al., 2014) and Czapek Yeast Autolysate CYA (Visagie et al., 2014).

Chemical analysis of soil samples

Soil to water extract (1:5) for each soil sample was prepared and the electrical conductivity (EC), total dissolving salts (T.D.S), NaCl (%) and pH were measured (He et al., 2012).

Morphological identification of Trichoderma isolates

Trichoderma isolates were plated on 2% MEA at 25°C for 3-4 days, morphological characteristics were detected on basis of conidiophore branching pattern and size, conidium morphology and size, by using the identification key provided by Rifai (1969).

Assay of total cellulases and β-glucanase activity

The total cellulases and endo-1,4-β-glucanase (EC 3.2.1.4) production capacities of the isolates were carried out using Mandel's broth medium supplemented with Whatman filter paper 50mg, (1.0 × 6.0cm) or 1% (w/v) carboxy methyl cellulose, inoculated with fungal disk 5mm obtained from 7 days old culture of each isolate on PDA and incubated at 28°C on a rotary shaker (180rpm) for 10 days in FP and 7 days in CMC methods. After that, the supernatants were tested for cellulase activity assays using 3,5-dinitrosalicylic acid (DNS) reagent, absorbance was noticed at 550nm and compared to glucose standard (Jahangeer et al., 2005).

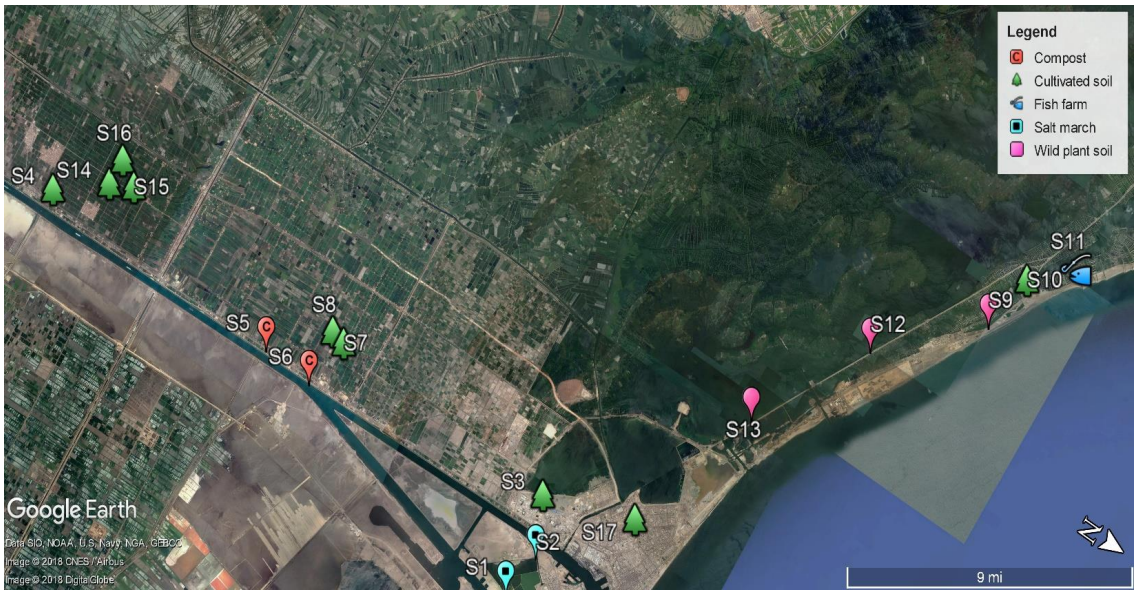

Fig. 1. Sampling sites at Port Said Governorate showing 9 cultivated, 3 wild plant, 2 compost, 2 salt marches and 1 fish farm soils.

Total cellulase activity was expressed in term of filter paper unit (FPU) per ml of culture filtrate. A filter paper unit (FPU) is defined as an amount of enzyme that produces one µ mole of glucose per minute per ml of culture filtrate under standard conditions (Eveleigh et al., 2009). Endo-1,4-β-glucanase activity was determined in terms of International Unit (IU) which is defined as an amount of enzyme that produces one µ mole of glucose per minute per ml of culture filtrate (Andrade et al., 2011).

Chitinase activity assay

Chitinase (EC 3.2.1.14) assay was applied using a basal broth medium supplemented with 1% (w/v) colloidal chitin, the isolates were inoculated in the medium and incubated on a rotary shaker at 26±2ºC, 100rpm for 10 days then the enzymatic activity was measured using p-dimethylaminobenzaldehyde (DMAB) reagent, the absorbance of the released reducing sugar was measured at 585nm in a spectrophotometer and the results were compared to N-acetylglucosamine standard (Jha & Modi, 2017). Chitinase unit is defined as the amount of enzyme, which produces 1µ mole of N-acetylglucosamine in 1ml of reaction mixture under standard assay condition (Jha et al., 2016).

Screening for siderophores production

For siderophores production 5mm plugin of each isolate was grown on MEB medium (2% Malt Extract, pH 5.6), incubated on orbital shaker at 120rpm, 28°C for 9 days then culture free supernatants were examined for extracellular siderophores production (Ghosh et al., 2015).

Detection of catecholate nature of siderophores

The catecholate nature of siderophores was detected following spectrophotometric assay. To each 1ml of supernatant, 3ml of freshly prepared 2% aqueous $FeCl_3$ solution was added, the formation of a wine colored complex ferric hydroxamate showing a peak at 495nm indicates presence of catecholate siderophores (Yeole et al., 2001). Arnow's Test, to 1ml of culture supernatant, 1ml of 0.5N HCl, 1ml of nitrite molybdate reagent, 1ml of 1N NaOH were added and the formation of red coloration indicates the presence of catecholate siderophores (Baakza et al., 2004).

Detection of hydroxamate nature of siderophores

The hydroxamate nature of siderophores was detected following spectrophotometric assay, to each 1ml of supernatant, 3ml of freshly prepared 2% aqueous $FeCl_3$ solution was added, the formation of orange to wine colored ferric hydroxamate showing a peak between 420- 450nm indicates the presence hydroxamate siderophores (Dave et al., 2006). Tetrazolium test, to a pinch of tetrazolium salt, 1-2 drops of 2N NaOH and 1ml of culture free supernatant were added. The presence of hydroxamate siderophores was confirmed with formation of a deep red coloration (Yeole et al., 2001).

Detection of Carboxylate nature of siderophores

To 1ml of culture supernatant, 1ml of 250µM $CuS0_4$ and 2ml of acetate buffer (pH 4) were added. The copper complex was observed for formation of absorption peak between 190-280nm (Dave et al., 2006).

Determination of indole acetic acid hormone (IAA)

Each isolate was cultured in potato dextrose broth (PDB) supplemented with 2mg/ml of DL-tryptophan and other without tryptophan, incubated in the dark at 25°C on rotary shaker at 120rpm for 14 days. Then 2ml of Salkowski's reagent was added to1 ml of culture supernatant and incubated in the dark for 1hr. The absorbance of the formed pink color was measured at 530nm using double beam visible UV spectrophotometer (ST-UV-1901PC) (Bharucha et al., 2013).

Determination of gibberellic acid (GA_3) hormone

Each isolate was inoculated in Czapek broth media and incubated on rotary shaker at 120rpm for 10 days. The extraction of GA_3 from culture supernatants were performed using liquid-liquid (ethyl acetate/$NaHCO_3$) extraction method (Siddikee et al., 2010). The concentration of GA_3 was quantified using phosphomolybdic acid reagent at 780nm in a spectrophotometer and results were compared to standard curve of GA_3 (Ramya et al., 2015).

In vitro antagonistic activity

Dual culture test was performed by inoculating the petri dishes containing PDA, with 5mm agar discs of each *Trichoderma* isolate and *S. sclerotiorum* on opposite sides (Zhang et al., 2016). The isolates were ranked into four classes: R1= *Trichoderma* overgrew completely to pathogen and covered the whole surface of the medium, R2= *Trichoderma* overgrew two-thirds of the surface of the medium, R3= *Trichoderma* and pathogen colonized each half of the surface and nobody seemed to dominate the other, R4= The pathogen colonized the 2/3 parts of the medium surface and resisted invasion by *Trichoderma* (Castillo et al., 2011).

To determine the antagonistic effect of crude liquid extract of the isolates against *S. sclerotiorum* PDA plates were inoculated with 500µL of the filtrate of each *Trichoderma* isolates, then a disc (5mm) with active mycelium of *S. sclerotiorum* was inoculated in the center of each plate (Mishra, 2010).

The antagonistic activities of volatile metabolites produced by the isolates were assayed using inverted plate method (Nagamani et al., 2017). The percentage of mycelial radial growth inhibition in mm calculated with the following formula:

$$\text{inhibition (\%)} = [(C-T)/C] \times 100$$

where C= Radial growth of the pathogen in the absence of antagonist and T = Radial growth of the pathogen in the presence of antagonist (Zhang et al., 2016).

Statistical analysis

The results were subjected to an analysis of variance using one-way ANOVA. The significant difference between means at $P< 0.05$ was evaluated by Tukey test with SPSS 19.

Results

Distribution and diversity of Trichoderma isolates

Data presented in Tables 1 and 2 show the isolation sites at Port Said Governorate and the distribution of *Trichoderma* in these sites. It can be seen that the richest habitats in *Trichoderma* were radish and wheat rhizospheres with 17 total *Trichoderma* colonies. The most common *Trichoderma* species was *T. harzianum* which represented 25% of the isolates and most of the recovered isolates were obtained from Czapek yeast extract agar (CYA) medium and *Trichoderma* selective medium (TSM) modified. It was appeared that non-saline soils contain more count of *Trichoderma* than salt marshes.

Morphological characterization of Trichoderma isolates

Microscopic observations of the pure cultures of *Trichoderma* isolates grown on 2% MEA clarified that, seven species were identified from the 20 isolates as represented in Table 3. The isolates belonged to *T. aureoviride* were characterized by horn-shaped phialides (7-14*2-2.5µm) and ovoid conidia (3-4.3*2-3µm), *T. harzianum* had skittle-shaped phialides (5-7*3-3.5µm) and sub- globose conidia (2.8-3.2*2.5-2.8µm), *T. koningii* isolates had nine-pine shaped phialides (7.5-12*2.5-3.5µm) and angular to oblong conidia (3-4.8*1.9-2.8µm), *T. piluliferum* had a Pyriform phialides (4.5-6.5*2.8-3.5µm) and globose conidia (2.5-3.5*2.5-3.5µm). *T. polysporum* had flask-shaped phialides (4.5-

6.5*2.8-3.5µm) and oblong conidia (2.8-3.7*1.8-2 µm), *T. pseudokoningii* possessed skittle-shaped phialides (5.5-8*2.7-3.5µm) and oblong conidia (3.4-4.6*2-2.2µm) and *T. viride* showed nine-pine shaped phialides (8-14*2.4-3µm) and globose to ovoid conidia (3-4.8*3.5-4µm).

Enzymes activity

Results in Fig. 2 indicate the activity of total cellulases, endo-1,4-β-glucanase and chitinase enzymes in the filtrates of different *Trichoderma* isolates. All isolates showed total cellulase activity with significant differences among them (Fig. 2A). The highest cellulases was produced by *T. polysporum* 2 which reached up to 2.95 FPU ml^{-1} and the lowest activity was by *T. koningii* 2 which reached up to 0.56 FPU ml^{-1} only. The highest endo-1,4-β-glucanase activity was manifested by *T. harzianum* 2 with a value up to 5.47 IU ml^{-1}, whereas the lowest activity was appeared by *T. pseudokoningii* 1 with a value up to 1.28 IU ml^{-1} only (Fig. 2B).

TABLE 1. Isolation sites at Port Said Governorate and their soil properties.

Site	Geographic location	Habitat	Soil properties				
			Visual appearance	pH	NaCl (%)	TDS (g/L)	Conductivity (ms/cm)
1	31°13’14.32”N/ 32°20’9.66”E	Salt march	Sandy	8.0±0	52.2±0.1	13.5±0	26.9±0.1
2	31°13’27.13”N/ 32°18’39.30”E	Salt march	Sandy	8.4±0.3	43.8±0.3	11.3±0.1	22.6±0.1
3	31°13’27.01”N/ 32°18’1.75”E	Radish	Clay	8.4±0	1.1±0	0.3±0	0.5±0
4	30°57’43.30”N/ 32°18’33.61”E	Rice	Clay	8.2±0	18.1±0.2	4.6±0.1	9.3±0.1
5	31° 4’31.04”N/ 32°18’21.64”E	Compost soil 1	Crumbly	7.8±0	39.7±0.2	10.2±0.1	20.4±0.1
6	31° 5’45.87”N/ 32°18’13.21”E	Compost soil 2	Crumbly	7.9±0	49.9±0.3	12.9±0	25.8±0.1
7	31° 5’42.86”N/ 32°18’10.96”E	Courgette	Sandy	8.4±0	2.7±0	0.1±0	0.7±0
8	31° 5’42.83”N/ 32°18’10.76”E	Eggplant	Sandy	8.4±0.1	1.8±0	0.5±0	0.9±0
9	31°20’54.33”N/ 32° 5’27.48”E	*Halocnemum strobilaceum*	Sandy	8.2±0	16.1±0.4	4.0±0	8.0±0.1
10	31°21’24.96”N/ 32° 4’5.58”E	Arum plant	Loamy	7.6±0.3	19.7±0.1	4.7±0.1	9.9±0
11	31°21’19.82”N/ 32° 3’24.10”E	Fish farm soil/ water	Clay	8.3±0	11.0±0.1	2.9±0	5.9±0
12	31°18’29.28”N/ 32° 8’4.10”E	*Zygophyllum*	Sandy	7.5±0	4.1±0.1	0.1±0	0.2±0
13	31°16’38.83”N/ 32°11’46.43”E	*Arthrocnemum*	Sandy	7.8±0	32.6±0.2	8.4±0.1	16.7±0.1
14	30°58’57.34”N/ 32°17’27.58”E	Wheat	Clay	7.8±0	4.6±0.2	0.1±0	0.2±0
15	30°58’48.71”N/ 32°17’27.80”E	Cotton	Clay	7.9±0.1	7.1±0.1	1.9±0	3.8±0
16	30°58’57.44”N/ 32°16’39.82”E	*Vicia faba*	Clay	8.1±0	5.0±0.1	0.1±0	0.3±0
17	31°15’28.91”N/ 32°16’34.93”E	*Chenopodium quinoa*	Clay	8.2±0	1.9±0	0.5±0	1.0±0

Values are means ±SE.

TABLE 2. Distribution of *Trichoderma* isolates among different sites at Port Said Governorate and different isolation media.

Site	Habitat	Isolates	Total colonies count	Isolation media	Frequency
3	Radish cultivated soil	*T. koningii 1*	17	TSM modified	20%
		T. harzianum1		TSM modified	25%
		T. aureoviride1		CYA	10%
		T. harzianum 2		TSM	25%
		T. viride1		TSM modified	15%
		T. koningii 2		TSM modified	20%
4	Rice cultivated soil	*T. piluliferum1*	4	Davet's Medium	10%
10	Arum plant cultivated soil	*T. pseudokoningii1*	3	TSM modified	10%
11	Saline water	*T. koningii 3*	5	RB-S-F	20%
13	*Arthrocnemum* wild plant soil	*T. koningii 4*	1	Davet's Medium	20%
14	Wheat cultivated soil	*T. piluliferum 2*	17	TME	10%
		T. harzianum 3		RB-S-F	25%
		T. harzianum4		RB-S-F	25%
		T. harzianum 5		RB-S-F	25%
		T. viride2		CYA	15%
		T.polysporum 1		CYA	10%
15	Cotton cultivated soil	*T. viride3*	3	Davet's Medium	15%
17	*Chenopodium quinoa* cultivated soil	*T. aureoviride 2*	25	CYA	10%
		T. pseudokoningii 2		CYA	10%
		T. polysporum 2		CYA	10%

TABLE 3. Morphological characteristics of *Trichoderma* isolates.

Isolates	Phialides		Conidia	
	Shape	Dimensions (L*W) (µm)	Shape	Dimensions (L*W) (µm)
T. aureoviride 1	Horne-shaped	(7.4±0.1) *(2.4±0.1)	Ovoid	(3.3±0.2) *(2.8±0.1)
T. aureovirid 2	Horne-shaped	(7.2±0.2) *(2.3±0.2)	Ovoid	(3.4±0.01) *(2.7±0.1)
T. harzianum 1	Skittle-shaped	(6.9±0.3) *(3.1±0.1)	Sub globose	(2.8±0.1) *(2.5±0.04)
T. harzianum 2	Skittle-shaped	(6.4±0.1) *(2.9±0.1)	Sub globose	(2.7±0.2) *(2.4±0.03)
T. harzianum 3	Skittle-shaped	(6.7±0.1) *(3±0.1)	Sub globose	(2.7±0.05) *(2.7±0.1)
T. harzianum 4	Skittle-shaped	(6.2±0.2) *(3±0.2)	Sub globose	(2.8±0.1) *(2.7±0.1)
T. harzianum 5	Skittle-shaped	(7±0.2) *(3.3±0.2)	Ovoid	(3.1±0.04) *(2.7±0.1)
T. koningii 1	Nine-pine shaped	(8.9±0.1) *(2.9±0.1)	Angular to oblong	(3.2±0.2) *(2.7±0.1)
T. koningii 2	Nine-pine shaped	(7.5±0.2) *(2.5±0.2)	Angular to oblong	(2.8±0.1) *(2.1±0.2)
T. koningii 3	Nine-pine shaped	(7.9±0.1) *(3.5±0.2)	Angular to oblong	(3.1±0.2) *(2.6±0.1)
T. koningii 4	Nine-pine shaped	(7.5±0.2) *(2.5±0.1)	Oblong	(3.4±0.1) *(2.4±0.01)
T. piluliferum 1	Pyriform	(5.8±0.1) *(2.8±0.2)	Globose	(3.5±0.04) *(3.4±0.04)
T. piluliferum 2	Pyriform	(6.2±0.2) *(2.8±0.1)	Globose	(2.5±0.04) *(2.5±0.03)
T. polysporum 1	Flask-shaped	(6.5±0.02) *(2.8±0.2)	Oblong	(3.7±0.04) *(2.1±0.2)
T. polysporum 2	Flask-shaped	(6.3±0.05) *(3.1±0.1)	Oblong	(3.7±0.1) *(2±0.02)
T. pseudokoningii 1	Skittle-shaped	(7.7±0.2) *(2.8±0.1)	Oblong	(3.9±0.2) *(2.1±0.04)
T. pseudokoningii 2	Skittle-shaped	(7.1±0.1) *(3.4±0.03)	Oblong	(4.2±0.1) *(2.2±0.03)
T. viride 1	Nine-pine shaped	(8.7±0.02) *(3±0.2)	globose	(3.5±0.03) *(3.3±0.1)
T. viride 2	Nine-pine shaped	(8.8±0.1) *(3±0.3)	Broadly ovoid	(4.1±0.2) *(3.5±0.1)
T. viride 3	Nine-pine shaped	(10.5±0.03) *(2.6±0.2)	Ovoid	(4.2±0.1) *(3.5±0.003)

Values are means ±SE.

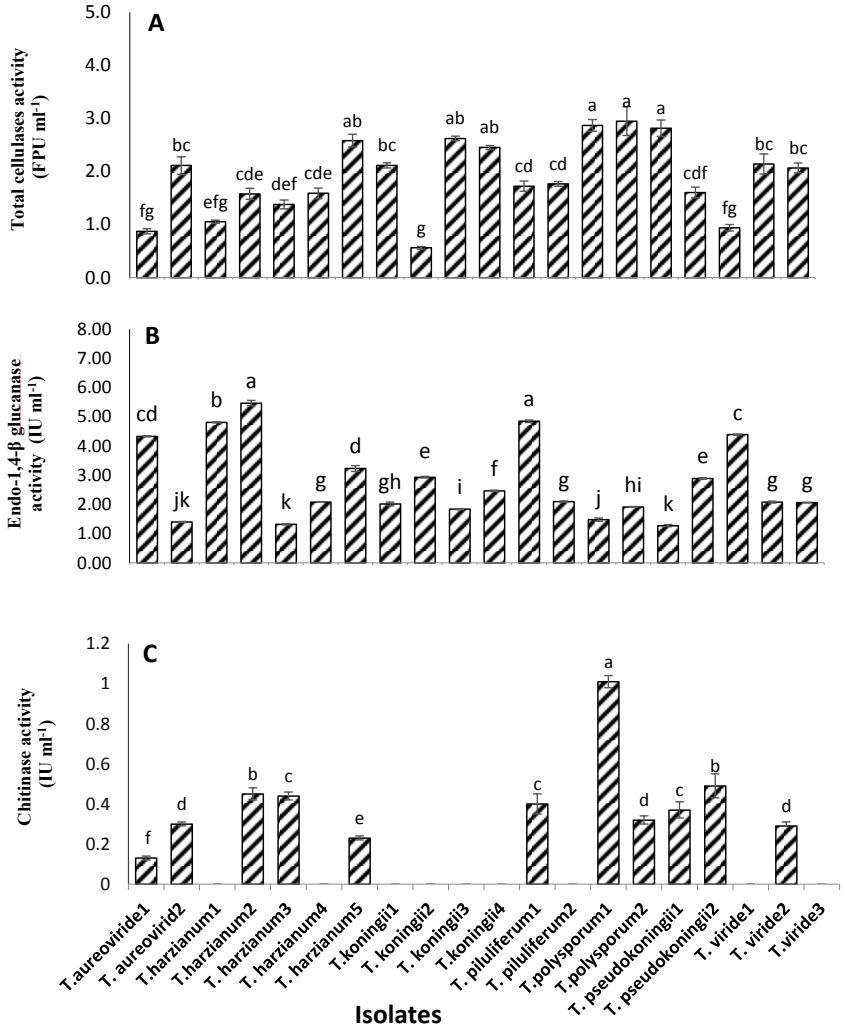

Fig. 2. ***In vitro*** **assays of enzymatic activities by** ***Trichoderma*** **isolates in broth media; (A)Total cellulases using the filter paper method, (B) Endo-1,4-β-glucanase using CMC method, (C) Chitinase using colloidal chitin [Values are mean ± SE, Values with different letter (s) are significantly different at P< 0.05].**

It can be seen from Fig. 2C that most of the isolates had chitinase activity, but with different degrees. The highest chitinase activity was appeared by *T. polysporum* 1 with a value up to 1.01 IU ml^{-1}. Conversely, *T. aureoviride* 1 showed the lowest activity of this enzyme with a value up to 0.13 IU ml^{-1} only.

Siderophores production

Results represented in Table 4 clarify that all the isolates showed siderophores productivity in broth media except *T. koningii* 2. The hydroxamate siderophores showed a peak between (420-450nm) after addition of aqueous $FeCl_3$ solution and deep red color in tetrazolium test, while carboxylate siderophores showed a peak between (190-280nm) after addition of $CuS0_4$ solution which indicates the formation of copper complex, thus the produced siderophores belong to hydroxamate and carboxylate categories, on the other hand no catecholate siderophores were detected.

Phytohormones production

Most of the isolates which represented in Fig. 3A showed productivity of IAA in broth media supplemented with DL-tryptophan after 14 days of incubation in dark conditions, the highest concentration was detected in *T. harzianum* 2 which reached up to 13.19µg per ml of culture filtrate and lowest concentration was by *T. polysporum* 2 with a concentration 1.62µg/ml with a significant difference between these two isolates at P< 0.05, while no IAA compound was detected in the tryptophan free medium.

TABLE 4. Screening for siderophores production by the isolates and determination of their types.

Isolates	$FeCl_3$ test	Catecholate siderophores		Hydroxamate siderophores		Carboxylate siderophores
		Spectrophotometer (495nm)	Arnow's test	Spectrophotometer (420-450nm)	Tetrazolium test	Spectrophotometer (190-280nm)
T. aureoviride 1	+	-	-	439 ±1.7	+	201.3±3.5
T. aureoviride 2	+	-	-	432.8±1.5	+	190.9±0.7
T. harzianum 1	+	-	-	-	-	215.6±2.4
T. harzianum 2	+	-	-	430.4±1.4	+	267.3±1.9
T. harzianum 3	+	-	-	445±0.6	+	230.6±2.1
T. harzianum 4	+	-	-	440±1.7	+	226.6±1.2
T. harzianum 5	+	-	-	448±0.6	+	201.1±2.1
T. koningii 1	+	-	-	430.4±1.8	+	190±0.009
T. koningii 2	-	-	-	-	-	-
T. koningii 3	+	-	-	-	-	199.4±1.1
T. koningii 4	+	-	-	433.6±1.6	+	200.7±3.3
T. piluliferum 1	+	-	-	-	-	215.6±2
T. piluliferum 2	+	-	-	435±0.8	+	202.7±0.7
T. polysporum 1	+	-	-	443.4±1.4	+	233.6±2.5
T. polysporum 2	+	-	-	-	-	196.9±1.2
T. pseudokoningii 1	+	-	-	440±0.8	+	-
T. pseudokoningii 2	+	-	-	440±1.5	+	201.3±2.2
T. viride 1	+	-	-	430.8±1.6	+	252±1.8
T. viride 2	+	-	-	433.8±2	+	251.4±1.4
T. viride 3	+	-	-	430.4±1.3	+	227.1±2

Values are means ±SE.

Results in Fig. 3B clarified that all the isolates had gibberellic acid metabolites in their broth extracts which showed molybdenum blue coloration after addition of phosphomolybdic acid reagent, the highest concentration was by *T. polysporum* 1 which reached up to 586.51µg per ml extract with a deep blue coloration and the lowest productivity was by *T. koningii* 1 with a concentration 27.6µg/ml only and faint blue coloration in comparison to control. Statistical analysis exhibited a significant difference between the isolates at $P< 0.05$.

Changes in antagonistic activities

All the isolates had antagonistic activities against *S. sclerotiorum* in *in vitro* assays. In the dual culture method, as it can be seen from (Fig. 4) most of the isolates belongs to class R1 which completely overgrow all the pathogen colonies, nearly covered all the surface of the media and no sclerotia formation was observed, whereas the highest recorded radial growth inhibition of the pathogenic fungi was by *T. polysporum* 1 which showed 45.97 % of inhibition, with a slightly significant difference between the isolates and the lowest radial growth inhibition was by *T. polysporum* 2 with 23.76% of inhibition after 96hrs.

The crude extracts of metabolites produced by nearly all of the isolates showed high antagonistic activities against the pathogen with a moderate significant difference between them at $P< 0.05$ after 72hrs, the highest radial growth inhibition was by *T. polysporum* 1 metabolites with a percentage up to 87.78%, while the lowest inhibition activity was by *T. harzianum* 2, *T. aureoviride* 1 metabolites with no significant difference between them and a percentages 4.44%, 4.26% inhibition successively, as represented in Fig. 5A.

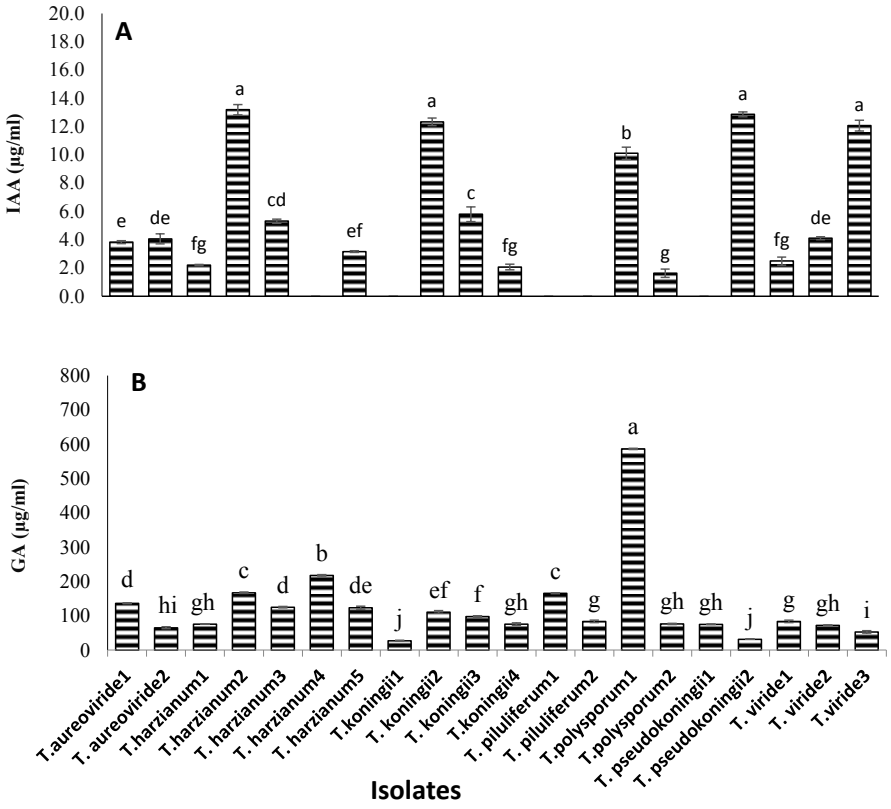

Fig. 3. ***In vitro*** **assays of phytohormones production by** ***Trichoderma*** **isolates in broth media; (A) Indole acetic acid (IAA) production, (B) Gibberellic acid (GA) production [Values are mean±SE, values with different letter (s) are significantly different at P< 0.05].**

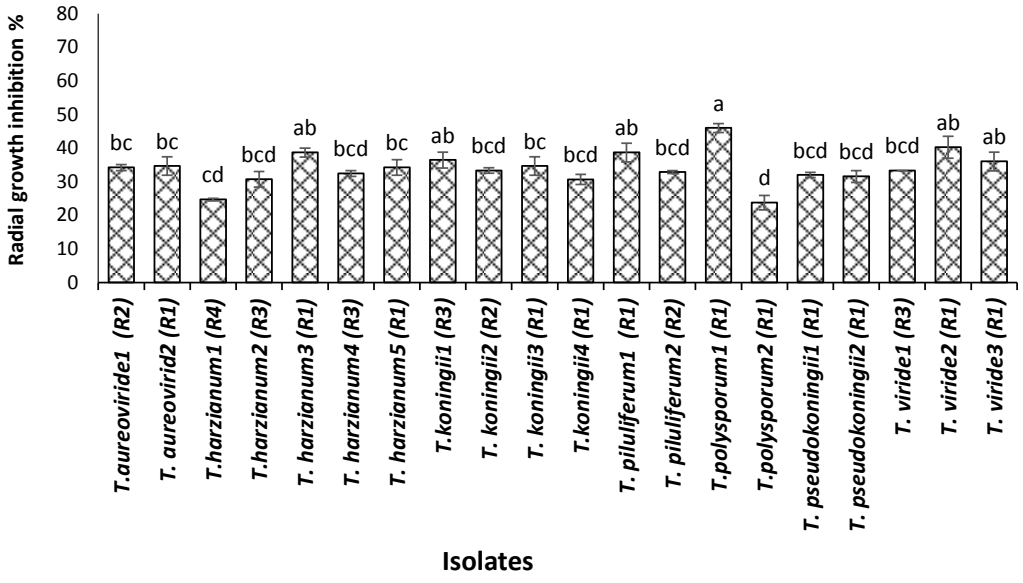

Fig. 4. ***In vitro*** **assays of antagonistic activities of** ***Trichoderma*** **isolates against** ***S. sclerotiorum*** **using dual culture method (96hrs) [Values are mean±SE, values with different letter (s) are significantly different at P< 0.05. Abbreviations indicate** ***Trichoderma*** **classes: R1= Isolates overgrew completely to pathogen and covered the whole surface of the medium, R2= Isolates overgrew two-thirds of the surface of the medium, R3= Isolates and pathogen colonized each half of the surface and nobody seemed to dominate the other, R4= The pathogen colonized the 2/3 parts of the medium surface and resisted invasion by** ***Trichoderma*****]**

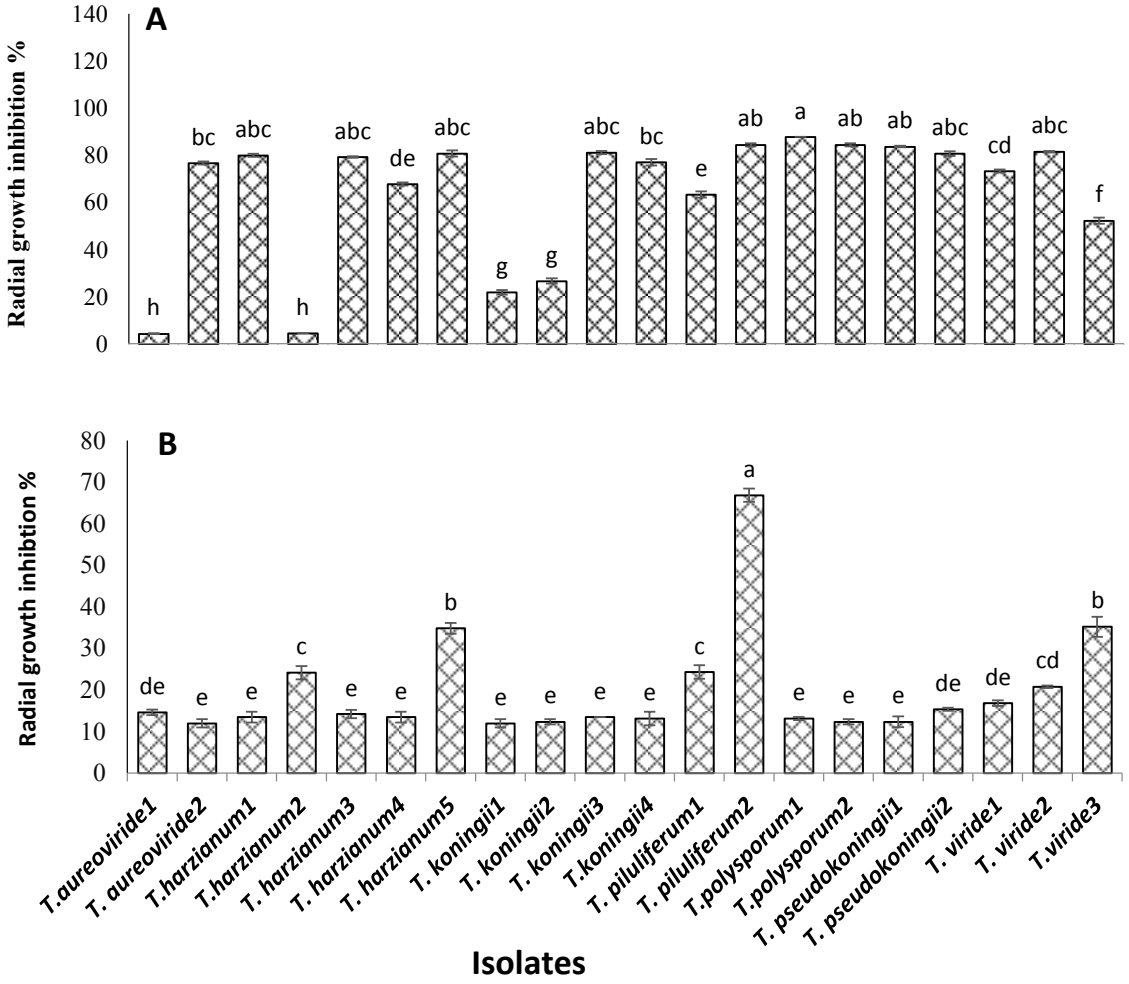

Fig. 5. ***In vitro*** **assays of antagonistic activities of** ***Trichoderma*** **isolates against** ***S. sclerotiorum*****; (A) Metabolic activity (72hrs), (B) Inverted plate method (72hrs) [Values are mean ± SE. Values with different letter (s) are significantly different at P< 0.05].**

The microbial volatile organic compounds produced by the isolates showed high antagonistic activities against the pathogen using an inverted plate assay, results in Fig. 5B clarified that volatiles by *T. piluliferum* 2 had the highest antagonistic activity with a percentage 66.85% radial growth inhibition with a significant difference at P< 0.05 in compared to other isolates, while *T. koningii* 1 and *T. aureovirid* 2 showed the lowest reduction with a percentage of 11.99% after 72hrs.

Discussion

Trichoderma spp. are well known as effective biocontrol agents, but there is a gap of knowledge about their distribution and activity at Port Said Governorate. In this study, we found that from the seventeen collected soil samples, cultivated soil habitats were the richest habitat in *Trichoderma*, followed by wild plant soil habitats but *Trichoderma* was absent in salt marshes. Most of the isolates were obtained from the rhizosphere of plants in clay soils due to high level of organic matter content and root exudates which provide a source of nutrient to the microorganisms followed by loamy and sandy soils. These results agree with results of Sadfi-Zouaoui et al. (2009) who found that from a total of 105 *Trichoderma* isolates, the number of recovered isolates was higher in rhizosphere (81.9%) than the other soil parts (18.1%). These results point out the preference of *Trichoderma* to rhizosphere layers.

All the isolates showed total cellulases and endo-1,4-β-glucanase activity in both filter paper and carboxy methyl cellulase assays. The highest

total cellulase activity was manifested by *T. polysporum* 2 which reached up to 2.95 FPU ml^{-1} and the highest endo-1,4-β-glucanase activity was elicited by *T. harzianum* 2 which reached up to 5.47 IU ml^{-1}. Our results support the general reputation that most of *Trichoderma* spp. have total cellulases and endo-1,4-β-glucanase activity in both solid state and fermentation broths (Pirzadah et al., 2014). In this respect, Pandey et al. (2015) found that *T. harzianum* had total cellulase, which reached up to 0.56 FPU ml^{-1} and endo-1,4-β-glucanase which reached up to 0.56 IU ml^{-1}.

Nearly most studied isolates showed chitinase activity in broth media with the highest value by *T. polysporum 1* which reached up to 1.01 IU ml^{-1}. This revealed the importance of our *Trichoderma* spp. which were isolated from Port Said Governorate as biocontrol agents and their promising usage as biological fungicides. The production of chitinolytic enzymes by this genus was also recorded in other studies (Agrawal & Kotasthane, 2012; Khatri et al., 2017).

Some *Trichoderma* spp. which had been used as biocontrol agents (BCAs) produce highly efficient siderophores that chelate iron. Siderophores can be beneficial to plants by solubilizing non- soluble forms of iron, make it available to be utilized by the plant and stop growth of pathogenic fungi by depriving them of iron sources (Vinale et al., 2014). Siderophores production of the obtained isolates was screened using $FeCl_3$ test showed that most of the isolates had siderophores productivity, belongs to hydroxamate and carboxylate siderophores, while no catecholate siderophores were recorded. These results matching with the results of Ghosh et al. (2017).

Many *Trichoderma* spp. are known as producers of the indole-3-acetic acid (IAA) phytohormone, and its production had been suggested to promote plant root growth (Nieto-Jacobo et al., 2017). In this study we found that most of these isolates showed productivity of IAA in media supplemented with DL-tryptophan. The highest IAA was detected in *T. harzianum* 2 while *T. koningii, T. harzianum4, T. piluliferum1, T. piluliferum 2* and *T. pseudokoningii1* showed no IAA production in the broth medium. These results are compatible with the finding of Yadav et al. (2011) who recorded 138.9µg IAA per ml by *T. harzianum* in 1000µg mL^{-1} DL-tryptophan medium and Kumar et al. (2017) who manifested 115µg IAA per ml by *T. viride* VKF3 in a medium supplemented with 0.5% DL-tryptophan. All the obtained isolates showed high gibberellic acid productivity in fermentation broth and the highest productivity was by *T. polysporum* 1. These results are consistent with the results of Deshmukh & Shinde (2016) who clarified the production of GA by *T. polysporum* isolated from the phyllosphere and endophytic region of *Cajanus cajan* L.

Several researches clarified the effectiveness of *Trichoderma* in biocontrol of root pathogens in both *in vitro* and *in vivo* studies (Thinggaard, 1989; Ahmed & El-Fiki, 2017; Alfiky & Eldenary, 2019). In our study nearly all the isolates appeared high antagonistic activity against *S. sclerotiorum* root pathogen, the causative agent of white mold of *Phaseolus vulgaris*. The highest antagonistic activity in dual culture method was by *T. polysporum* 1 which reached up to 45.97% of radial growth inhibition of the pathogen after 96hrs of incubation compared to control one. In this method where the pathogen and each *Trichoderma* isolate are placed in the same plate, the pathogen and the biocontrol agent began their radial growth and their colonies came in contact with each other after the incubation period, here both showed action over each other. In this study, the inhibitory effect of this isolate (*T. polysporum* 1) could be related to its high chitinase activity and siderophores production which result in lysis of fungal mycelium. Others attributed this inhibitory to the release of antibiotic or antibiotic like substances and competition for nutrients Bastakoti et al. (2017).

In our study, the crude extract of almost isolates showed high antagonistic activity against the used pathogen and the highest metabolic activity was noticed by *T. polysporum* 1 which reached up to 87.78 % inhibition of radial growth of the pathogen. These results are compatible with those of Yousef et al. (2017) who studied the effect of *Trichoderma* metabolites on radial growth of *S. sclerotiorum* and found that *T. harzianum* T8 and *T. koningii* T6 had a high antagonistic activity against the pathogen reached up to 80% inhibition activity. Also, the antagonistic activity of the MVOCs of different isolates against the pathogen was determined using the inverted plate method which revealed that *T. piluliferum* 2 metabolites had the highest antagonistic activity and inhibited radial growth of the pathogen by 66.85%. This antagonistic activity of *Trichoderma* MVOCs

against fungal plant pathogens has also been proved by Kottb et al. (2015).

Conclusion

Several bioactive *Trichoderma* isolates were recovered from Port Said habitats. Physiological characterization showed that most of the isolates had high enzymatic activity, phytohormones and siderophores production ability. *In vitro* antifungal activities of *Trichoderma* isolates showed that, both *T. polysporum 1* and *T. piluliferum 2* manifested high antagonistic activity against *S. sclerotiorum*. The next step is a pot experiment using the two promising *Trichoderma* isolates (*T. polysporum* 1 and *T. piluliferum* 2) against the white mold pathogen of *Phaseolus vulgaris* L.

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القدرة التضادية للفطريات والتوصيف الفسيولوجي لعزلات الترايكوديرما من محافظة بورسعيد

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تم عزل 20 عزلة لفطريات الترايكوديرما (*Trichoderma*) والتي تنتمي إلى سبعة أنواع مختلفة من عدة مواقع في محافظة بورسعيد وذلك لدراسة خصائصها الفسيولوجية وقدرتها التضادية لفطر اسكلورتينا اسكلوريشيورام *Sclerotinia sclerotiorum* والمسبب لمرض العفن الأبيض لنبات الفاصوليا وذلك فى المختبر بطريقتي العزل المزدوج والقاعدة إلى القاعدة. لقد أظهرت عزلة ترايكوديرما بولي سبورم (*T. polysporum 2*) أعلى نشاط لانزيم السليوليز وصل إلى 2.95 وحدة/مل. كما أظهرت ترايكوديرما هارزينم (*T. harzianum 2*) أعلى نشاط للاندوجلوكانيز وصل إلى 5.47 وحدة/ مل، بينما أظهرت ترايكوديرما بولي سبورم (*T.polysporum 1*) أعلى نشاط للكيتينيز والذي وصل إلى 1.01 وحدة/ مل. بالإضافة إلى ذلك، تم اختبار إنتاج مركبات السديروفورات باستخدام اختبارات كلوريد الحديد الثلاثي، التترازوليم، آرنوس والطيف الضوئي. واتضح أن معظم العزلات قادرة على إنتاج أنواع الهيدروكسيمات (hydroxamate) والكربوكسيلات (carboxylate) من مركبات السديروفورات. وقد سجلت ترايكوديرما هارزينم (*T. harzianum 2*) أعلى تركيز لهرمون أندول حمض الخليك في الوسط السائل. وصل إلى 13.19 ميكروجرام/ مل، في حين أظهرت ترايكوديرما بولي سبورم (*T. polysporum 1*) أعلى تركيز لهرمون حمض الجبريليك بمقدار 586.51 ميكروجرام/ مل. كما تم اختبار القدره التضاديه للعزلات ضد فطر الاسكلروتينا في المعمل وتم تسجيل النسبة المئوية للتثبيط في النمو الشعاعي للفطر الممرض وقد أظهرت نتائج تجربه العزل المزدوج التأثير المثبط بنسبة 45.97% في حالة ترايكوديرما بولي سبورم (*T polysporum 1*)، ونسبه 87.78% باستخدام مستخلص المواد الثانويه للفطر في حين أظهرت المواد المتطايرة المنبعثة من ترايكوديرما بيليفورم (*T piluliferum 2*) انخفاضا للنمو الشعاعي بنسبة 66.85%. وبذلك اثبتت بعض عزلات الترايكوديرما التي تم الحصول عليها من تربة بورسعيد فعاليتها في السيطرة على نمو فطر الاسكلروتينا.