

Full Length Research Paper

Internal transcribed spacers (ITS) based identification of *Trichoderma* isolates and biocontrol activity against *Macrophomina phaseolina*, *Aspergillus niger* and *Meloidogyne incognita*

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Ten *Trichoderma* isolates were isolated from different locations in Egypt. Amplification and sequencing of internal transcribed spacers (ITS) was employed to identify *Trichoderma* isolates that exhibited from 99 to 100% identity with three species of *Trichoderma*: *Trichoderma harzianum*, *Trichoderma asperellum* and *Trichoderma longibrachiatum*. The biocontrol activity of *Trichoderma* isolates against *Macrophomina phaseolina*, *Aspergillus niger* and *Meloidogyne incognita* was tested *in vitro* and under greenhouse conditions. The results show that the isolate Th2 (*T. harzianum*) gave the best antagonism against *M. phaseolina* and *A. niger* with inhibition rates of 72.85 and 64.28%, respectively. Moreover, the isolate Ta1 (*T. asperellum*) was the best efficient isolate in reduction of each second stage juveniles (J2), number of galls, egg masses and females per root system with 90.33, 90.59, 90.06 and 85.50%, respectively. Treatment with *Trichoderma* isolates improved tomato growth parameters (root length, plant height, roots and shoots fresh weight and shoots dry weight).

Key words: *Trichoderma*, internal transcribed spacers (ITS), antagonism, *Macrophomina phaseolina*, *Aspergillus niger*, *Meloidogyne incognita*.

INTRODUCTION

The chemical pesticides are the first choice for farmers to control plant pathogens in order to obtain abundant crop yield (Junaid et al., 2013). It was estimated that 12% of crop loss is due to plant pathogens (Sharma et al., 2012). The long term uses of pesticides can cause severe environmental and health problems in addition to their expensive costs for developing countries. *Trichoderma* is

a famous saprophytic fungus that can be isolated from any soil and can be used as efficient biocontrol agent due to its high ability to reproduce and colonize (Pandya et al., 2011; Pal and Gardener, 2006; El-Hassan et al., 2013; Rao et al., 2015). *Trichoderma* fight against many fungal phytopathogens such as *Phythium*, *Phytophthora*, *Macrophomina*, *Aspergillus*, *Rhizoctonia* and *Fusarium*

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Table 1. Isolation sites and identification of *Trichoderma* isolates based on ITS data

Isolate code	Source	BLASTn results identity (%)	<i>Trichoderma</i> spp.
Ta1	Qalubia governorate	99	<i>Trichoderma asperellum</i>
Ta2	Gharbia governorate	100	<i>Trichoderma asperellum</i>
Ta3	Behira governorate	100	<i>Trichoderma asperellum</i>
Ta4	Gharbia governorate	99	<i>Trichoderma asperellum</i>
Th1	Dakhalia governorate	100	<i>Trichoderma harzianum</i>
Th2	Ismailia governorate	99	<i>Trichoderma harzianum</i>
Th3	Ismailia governorate	99	<i>Trichoderma harzianum</i>
Th4	Sharkia governorate	99	<i>Trichoderma harzianum</i>
Th5	Agricultural Research Center, Giza, Egypt	99	<i>Trichoderma harzianum</i>
TI	Agricultural Research Center, Giza, Egypt	99	<i>Trichoderma longibrachiatum</i>

using many biocontrol weapons as mycoparasitism, production of antibiotics and competition of pathogens for food and space (Benítez et al., 2004; Rayatpanah et al., 2011; Krishnamurthy and Shashikala, 2006). Recently, the global attention rely on *Trichoderma* as safe alternative of pesticides and this led to increase in the number of *Trichoderma* biocontrol products (Woo et al., 2014).

Macrophomina phaseolina and *Aspergillus niger* are considered destructive pathogens of many important and economically crops causing huge loses of each quantity and quality of crop yield (Khaledi and Taheri, 2016; Njoki et al., 2017; Bhale et al., 2013). *M. phaseolina* is a dangerous fungus that can infect many of important crops as corn, cotton, sesame, soy bean and sunflower causing diseases such as soy bean charcoal rot (Khaledi and Taheri, 2016; Aly et al., 2007). In addition, *A. niger* is a common saprophytic fungus that can cause dangerous diseases as black-rot and collar rot in crops like onion and peanut leading to yield loss from 5 to 40% (Khokhar et al., 2012; Gajera et al., 2011; Bhale et al., 2013). Moreover, root knot nematode (*Meloidogyne incognita*) is one of virulent parasites that attack many crops and can lead to destructive damages in crop yield range from 40 to 50% (Bakr et al., 2011; Katooli et al., 2010; Sikora and Fernandez, 2005; Karssen and Moens, 2006; Maqbool and Kerry, 1997). The previous studies indicated a vital role for *Trichoderma* species in biocontrol of *M. phaseolina*, *Aspergillus* species and *M. incognita* (Al-Hazmi and Javeed, 2016; Khaledi and Taheri, 2016; Mendoza et al., 2015; Shoib et al., 2018; Gajera et al., 2011; Sharon et al., 2011). As the cell wall of nematodes and pathogenic fungi is mainly composed of chitin, *Trichoderma* have chitinase enzymes able to degrade the cell wall of these pathogens (Loc et al., 2011; Haran et al., 1995; Ike et al., 2006). Identification of *Trichoderma* spp. to be applied in the field of biological control is an important issue. It was reported that identification of *Trichoderma* based on morphological characters can give misleading results (Fahmi et al., 2016). Recently, molecular identification based on internal transcribed

spacers (ITS) amplification and sequencing is common and highly trusted (Savitha and Sriam, 2015; Fahmi et al., 2016; Oskiera et al., 2015; Jiang et al., 2016).

In this study, 10 isolates of *Trichoderma* spp. were isolated from soil rhizosphere of different locations in Egypt, characterized on molecular level and screened for their antagonistic ability against *M. phaseolina*, *A. niger* and root knot nematode.

MATERIALS AND METHODS

Isolation of *Trichoderma* isolates

Ten (10) isolates of *Trichoderma* spp. were isolated from soil rhizosphere of different sites in Egypt (Table 1) cultivated with different crops as described by Fahmi et al. (2016). Several soil samples were obtained from a 15 cm depth, placed in sterile bags and transferred to the laboratory for isolation process. Serial dilutions were made and 250 µL of 10³ dilution was distributed onto petri dishes containing potato dextrose agar medium. The plates were then incubated at 28°C for one week for isolation of single colonies.

Soil borne pathogens

Isolates of *M. phaseolina* and *A. niger* were kindly provided by Faculty of Science, Zagazig University, Egypt.

DNA extraction from *Trichoderma* isolates

DNA isolation of *Trichoderma* was performed as described by Al-Samarrai and Schmid (2000). The clear sharp bands were indicator for the quality of DNA.

Molecular identification of *Trichoderma* isolates

Polymerase chain reaction (PCR) was utilized to amplify the internal transcribed spacer regions of *Trichoderma* using ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5' - TCC TCC GCT TAT TGA TAT GC - 3') primers. PCR conditions were performed as described by Loc et al. (2011). PCR products were first purified using QIAquick PCR Purification Kit (QIAGEN Cat. No. 28104). Sequencing was performed using Big Dye Terminator v3.1 Cycle

Sequencing Kit in a total volume of 20 μ L using 3500 Genetic Analyzer, Applied Biosystems (Daejeon, Korea).

PCR amplification of chitinase gene

Amplification of chitinase gene was done using specific primers: CHI-F (5-ATG TTG GGC TTC CTC GGA-3) and CHI-R (5-TTC GGG ATG GTT GTC ATA CTG-3) as described by Loc et al. (2011).

Antagonism of *Trichoderma* isolates

The antagonism of *Trichoderma* isolates against *M. phaseolina* and *A. niger* was evaluated in dual culture plate technique by measuring the radial growth inhibition of pathogen(s) as described by Abdel-lateif et al. (2017).

Multiplication of *M. incognita*

Root-knot nematode, *M. incognita* was identified by observation of perineal patterns according to Hartman and Sasser (1985). Multiplication was done under greenhouse conditions at the experimental greenhouse, Department of Agricultural Botany, Faculty of Agriculture, Menoufia University, Egypt. Susceptible tomato seedlings Cv. GS (*Solanum lycopersicum* Mill) was transplanted in plastic pots 15 cm in diameter filled with sterilized clays and soil (1:2 v/v). Seedlings were then inoculated by one egg mass of *M. incognita*. Agricultural operations were carried as needed.

Preparation of *M. incognita* inoculum

Two months heavily galled tomato roots previously infested with *M. incognita* were used to prepare nematode inoculum. Massive infested roots removed from the pots and gently washed using tap water to separate the adherent soil particles. Then roots were cut into small pieces and macerated for two periods of 10 s each at high speed by using a blender. The macerated root solution then placed in a jar containing 0.5% of sodium hypochlorite (NaOCl) as described by Hussey and Barker (1973). The Jar was vigorously shaken for 3 min to release the eggs from the egg matrix with the help of NaOCl. The resulting solution then poured through serial sieves to separate the root tissue. Eggs were collected on the last sieve (20 μ m) and washed several times with tap water to eliminate NaOCl residuals. The eggs were then transferred to a flask containing tap water. The number of eggs/ ml was estimated by counting 4 samples of 1 ml using a counting dish under a stereomicroscope.

Preparation of *Trichoderma* inoculum

The *Trichoderma* isolates were cultured on potato dextrose agar (PDA) and incubated at 26°C for 10 days. The conidia were collected from the surfaces of plates by flooding with sterile distilled water and gently scraping the colony surface with a sterile scraper (Jansson et al., 1985).

Greenhouse experiment

Greenhouse experiment was carried out to evaluate the effect of 10 *Trichoderma* isolates on *M. incognita*. Four weeks old tomato seedlings (Cv. GS) were transplanted into plastic pots (15 cm

diameter) containing sterilized sand-clay soil (2:1 v/v). Each plant is inoculated with 3000 eggs of *M. incognita* and *Trichoderma* spore suspension (10^8 g⁻¹ of soil) around the young hairy roots and were mixed well within the pots. Control treatments included untreated seedlings and nematode treated seedlings without *Trichoderma*. Treatment with vydate (abroad spectrum nematicide) was used for comparison.

The treatments were replicated three times in a completely randomized block design under greenhouse conditions. Tomato plants were observed, watered and fertilized with a nutrient solution according to Epstein (1972).

Plant growth and nematode parameters

Two months after nematode inoculation, tomato plants were carefully uprooted. The roots were cautiously washed with running tap water. Plant growth parameters as root length (cm), plant height (cm), fresh weight of roots (g), fresh weight of shoots (g) and dry weight of shoots (g) were recorded. Number of galls/root system was counted directly, while number of second stage juveniles (J2S)/250 g soil was evaluated using serial sieves and modified Baermann technique as described by Goody (1957). Egg-masses were stained prior to counting by dipping the infected roots in 0.015% Phloxine-B solution for 20 min as described by Daykin and Hussey (1985). Number of females/root system was determined by cutting the root system to small pieces and submerging the roots in a beaker full of tap water for four days at room temperature until they became soft. Roots pieces were then washed through 500 and 250 μ m sieves to separate the females from the root debris and counted under a stereomicroscope.

Data and cluster analysis

BLASTn was used to compare *Trichoderma* sequencing results with known sequences on NCBI site (<http://www.ncbi.nlm.nih.gov/>). The alignment and phylogenetic analyses were conducted using MEGA version 6 (Tamura et al., 2013). The obtained data of antagonism was analyzed using costat 6.3 version program. Analysis of variance and comparison of means were done at the 5% level of significance according to the Duncan's multiple range test (1955).

RESULTS AND DISCUSSION

Isolation and molecular identification of *Trichoderma* isolates

Ten isolates of *Trichoderma* were isolated from rhizosphere soil samples collected from different sites in Egypt (Table 1). PCR based on ITS primers was used to amplify ITS region and gave one band about 600 bp. The PCR products were sequenced and the sequencing data was entered on NCBI site to search BLAST and compare these data with published ITS data. The amplified ITS regions of *Trichoderma* isolates were exhibited from 99 to 100% identity with three species of *Trichoderma*; five isolates were found to belong to *Trichoderma harzianum* (Th1, Th2, Th3 Th4 and Th5), four isolates classified as *Trichoderma asperellum* (Ta1, Ta2, Ta3 and Ta4), while one isolate (TI) was identified as *Trichoderma longibrachiatum* (Table 1).

The phylogeny analysis supported the ITS identification

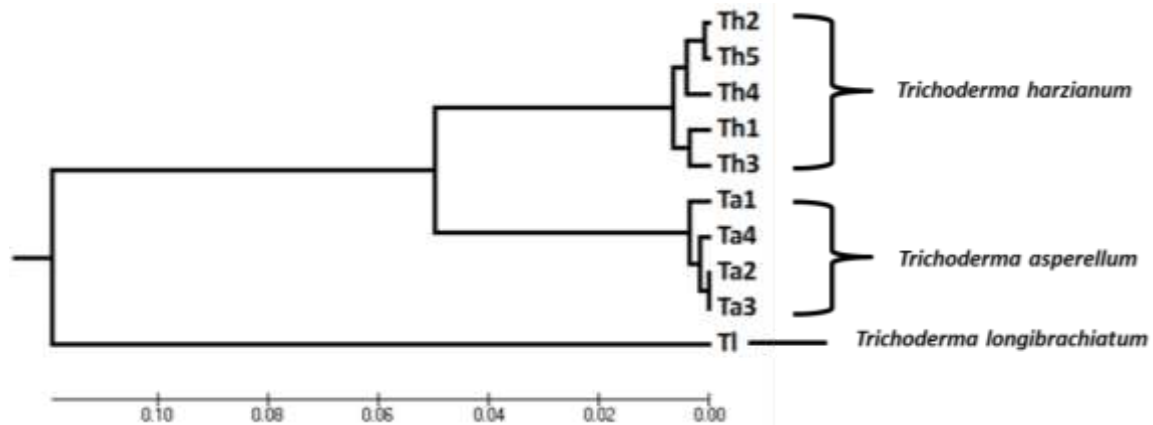


Figure 1. Phylogeny analysis of *Trichoderma* isolates based on ITS data using MEGA 6 (Tamura et al., 2013).

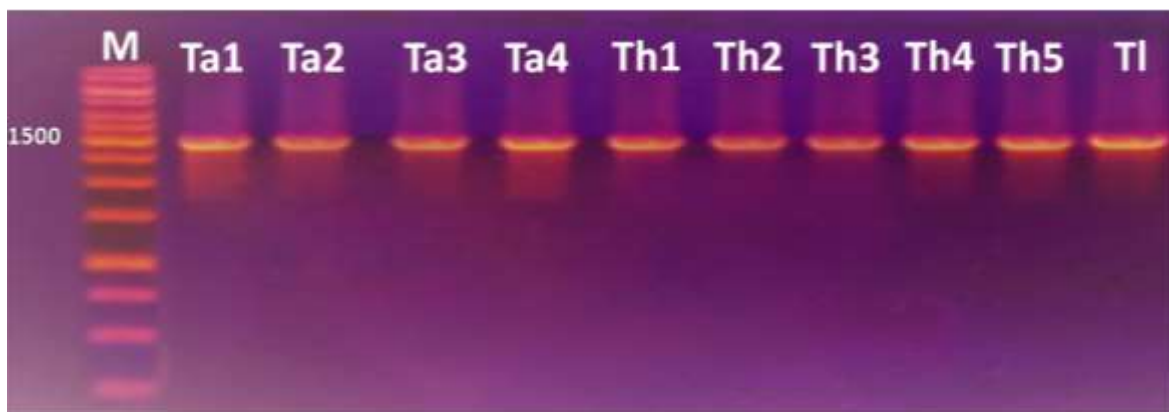


Figure 2. Amplification of chitinase gene in *Trichoderma* isolates based CHI-F and CHI-R primers, M; 1 kb plus DNA ladder.

and divided the isolates of *Trichoderma* into three clusters, the first cluster included the isolates of *T. harzianum* (Th1, Th2, Th3, Th4 and Th5). The second cluster included the isolates of *T. asperellum* (Ta1, Ta2, Ta3 and Ta4); finally, the third cluster contained the isolate TI of *T. longibrachiatum* (Figure 1).

These results confirm the efficiency of barcode DNA and are compatible with previous studies that employed ITS to identify *Trichoderma* spp. (Wu et al., 2017; Fahmi et al., 2016; Savitha and Sriram, 2015; Oskiera et al., 2015).

Chitinase gene of *Trichoderma*

However, chitin is key component of pathogen cell walls, *Trichoderma* secrete highly active chitinases that can destroy these cell walls and feed on them (Seidl-Seiboth et al., 2014; Hassan et al., 2015; Prasetyawan et al., 2018). Chitinase 42 is one of *Trichoderma* endochitinases that can degrade the β -1, 4-glycosidic

bonds between the N-acetyl glucosamine residues of chitin (Hassan et al., 2015). In this study, PCR based specific primers (CHI-F and CHI-R) was utilized to amplify and detect the chitinase 42 gene in *Trichoderma* isolates as shown in Figure 2. The PCR gave one band about 1500 bp homolog to that obtained by Loc et al. (2011) and confirmed the presence of this gene in all tested isolates. The amplification of chitinase gene confirms that these isolates are *Trichoderma* since the primers are specific for *Trichoderma* chitinases.

Evaluation of *Trichoderma* antagonism against *M. phaseolina* and *A. niger*

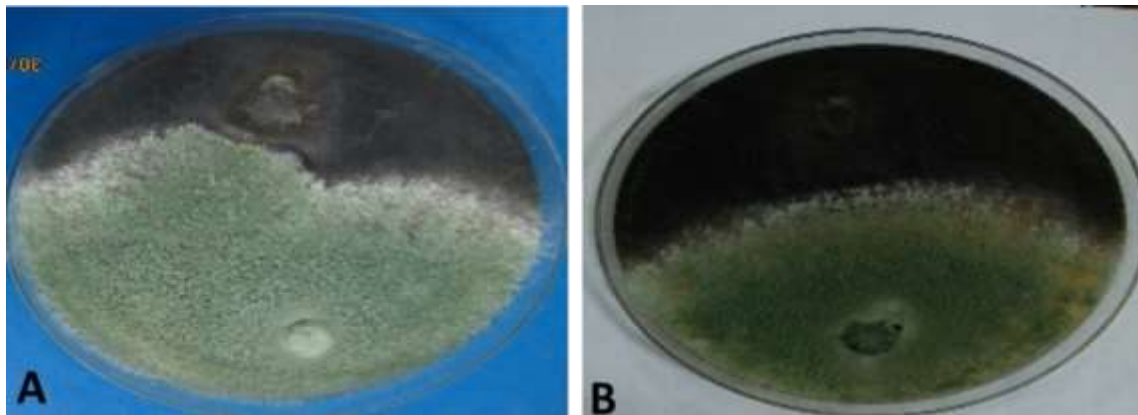
The antagonism of *Trichoderma* isolates against *M. phaseolina* and *A. niger* was evaluated *in vitro* by measuring the radial growth inhibition of the two pathogens (Table 2 and Figure 3).

The isolates Th2, Ta2, Th3 and TI were the most efficient isolates in suppression growth of *M. phaseolina*

Table 2. Radial growth inhibition of *Trichoderma* isolates against *M. phaseolina* and *A. niger* in dual culture plate technique

Isolates code	The radial growth inhibition of <i>M. phaseolina</i> (%)	The radial growth inhibition of <i>A. niger</i> (%)
Ta1	64.29 ^{bc}	57.14 ^d
Ta2	71.43 ^a	50.00 ^e
Ta3	50.00 ^d	42.85 ^f
Ta4	49.04 ^d	62.85 ^{bc}
Th1	54.29 ^c	54.28 ^{de}
Th2	72.85 ^a	64.28 ^b
Th3	67.14 ^b	58.57 ^{cd}
Th4	52.85 ^{cd}	57.14 ^d
Th5	25.71 ^e	71.43 ^a
Tl	65.69 ^b	31.43 ^g

Values followed by the same letter do not differ significantly ($P=0.05$) according to Duncan's multiple range test". Results are means of 3 replicates for each treatment.

**Figure 3.** Antagonism of *T. harzianum* against *M. phaseolina* (A) and *A. niger* (B).

with 72.85, 71.43, 67.14 and 65.69%, respectively. In addition, the isolate Th5 gave only 25.71% radial growth inhibition against *M. phaseolina*. For antagonism of *A. niger*, the isolates Th5 and Th2 were the most active isolates in suppression growth of *A. niger* with 71.43 and 64.28% respectively, while the isolate Tl exhibited the least radial growth inhibition against the same pathogen (31.43%). In general, the isolate Th2 of *T. harzianum* showed good antagonism against *M. phaseolina* and *A. niger* with inhibition rates 72.85 and 64.28%, respectively. Previous studies explained that *T. harzianum* is more vigorous in inhibition growth of pathogenic fungi than the other species of *Trichoderma* (Abdel-lateif et al., 2017; Shoaib et al., 2018; Al-Hazmi and Javeed, 2016). *Mycoparasitism* is one of *Trichoderma* biocontrol mechanisms that depend on secretion of lytic enzymes like chitinases and cellulases to destroy the cell walls of pathogens (Gajera et al., 2012). It was proved that chitin is major cell wall component of most pathogenic fungi and *Trichoderma* is excellent producer of chitinases

which can degrade the chitin and therefore killing them (Benítez et al., 2004; Agrawal and Kotasthane, 2012; Gajera et al., 2012).

Trichoderma genus is proved to be an excellent control agent in inhibition growth of *M. phaseolina*, *Aspergillus* spp. and root knot nematode under *in vitro* and green house conditions (Al-Hazmi and Javeed, 2016; Khaledi and Taheri, 2016; Khalili et al., 2015; Mendoza et al., 2015; Shoaib et al., 2018; Athira, 2017; Krishnamurthy and Shashikala, 2006). The variability in aggressiveness of the same *Trichoderma* isolates against *M. phaseolina* and *A. niger* may be due to the variation in pathogen cell wall structure and the defense ability of the pathogen.

The effect of *Trichoderma* isolates on nematode

The efficacy of *Trichoderma* isolates against *M. incognita* on tomato was evaluated in greenhouse experiments and the results were compared with those of vydate and

Table 3. The effect of *Trichoderma* isolates on nematode growth parameters

<i>Trichoderma</i> isolate	No. of J2/250 g soil	Reduction (%)	No. of galls	Reduction (%)	No. of egg masses	Reduction (%)	No. of females	Reduction (%)
Ta1	21.33 ^e	90.33	16 ^{gh}	90.59	16 ^g	90.06	19 ^f	85.50
Ta2	25.66 ^{de}	88.37	20 ^{efg}	88.24	21 ^f	86.96	25 ^e	80.92
Ta3	29.00 ^{cde}	86.86	22 ^{def}	87.06	22 ^f	86.34	27 ^{de}	79.39
Ta4	35 ^{bc}	84.14	24 ^{cde}	85.88	30 ^{de}	81.37	33 ^c	74.81
Th1	24.00 ^{de}	89.12	19 ^{fg}	88.82	20 ^f	87.58	21 ^f	83.97
Th2	30.66 ^{cd}	86.11	22 ^{def}	87.06	24 ^f	85.09	28 ^{de}	78.63
Th3	35.33 ^{bc}	83.99	25 ^{cd}	85.29	32 ^{cd}	80.12	35 ^{bc}	73.28
Th4	39.66 ^b	82.03	27 ^c	84.12	34 ^{bc}	78.88	36 ^{bc}	72.52
Th5	40.33 ^b	81.72	35 ^b	79.41	37 ^b	77.02	37 ^b	71.76
TI	34b ^c	84.59	23 ^{cdef}	86.47	28 ^e	82.61	29 ^d	77.86
vydate	21.00 ^e	90.48	14 ^h	91.76	14 ^g	91.30	12 ^g	90.84
Nematode alone	220.66 ^a	0.00	170 ^a	0.00	161 ^a	0.00	131 ^a	0.00

Values followed by the same letter do not differ significantly ($P=0.05$) according to Duncan's multiple range testⁱⁱ. Results are means of 3 replicates for each treatment.

Table 4. The effect of *Trichoderma* isolates on tomato plant growth parameters.

<i>Trichoderma</i> isolate	Plant height (cm)	Root length (cm)	Fresh root weight (g)	Fresh shoot weight (g)	Dry shoot weight (g)
Ta1	80.76 ^b	29.90 ^b	7.64 ^b	76.44 ^a	8.99 ^b
Ta2	78.63 ^b	26.80 ^{bc}	6.77 ^{bc}	64.85 ^b	8.24 ^{bcd}
Ta3	78.00 ^b	24.46 ^{cd}	6.75 ^{bc}	55.55 ^c	7.80 ^{cde}
Ta4	70.86 ^c	17.96 ^{fg}	5.64 ^{cd}	47.89 ^{cde}	6.58 ^{ef}
Th1	79.13 ^b	27.16 ^{bc}	6.89 ^b	70.83 ^{ab}	8.8 ^{bc}
Th2	72.96 ^c	23.00 ^{de}	6.70 ^{bc}	53.42 ^c	7.34 ^{def}
Th3	70.43 ^c	16.63 ^{gh}	5.59 ^{cd}	43.53 ^{de}	5.47 ^g
Th4	64.96 ^d	14.86 ^{gh}	5.54 ^{cd}	40.46 ^{ef}	5.20 ^{gh}
Th5	60.33 ^d	14.50 ^{gh}	4.62 ^d	39.97 ^{ef}	5.12 ^{gh}
TI	72.30 ^c	20.73 ^{ef}	6.52 ^{bc}	50.27 ^{cd}	6.79 ^{ef}
vydate	88.33 ^a	34.63 ^a	9.49 ^a	77.02 ^a	10.48 ^a
Nematode alone	43.7 ^f	10.66 ⁱ	2.26 ^e	22.57 ^g	3.61 ⁱ
Control	52.03 ^e	14.03 ^h	3.24 ^e	33.39 ^f	4.34 ^{hi}

Values followed by the same letter do not differ significantly ($P=0.05$) according to Duncan's multiple range testⁱⁱ. Results are means of 3 replicates for each treatment

control. The results show that treatment of tomato seedlings with *Trichoderma* decreased significantly root galling, egg masses, number of females and second stage juveniles (J2) per root system (Table 3), while improved tomato growth parameters (root length, plant height, roots and shoots fresh weight and shoots dry weight) as indicated in Table 4. The isolates of *T. asperellum* (Ta1, Ta2 and Ta3) and the isolates of *T. harzianum* (Th1 and Th2) exhibited high inhibition of nematode growth in levels comparable with those of vydate. The isolate of *T. asperellum* (Ta1) was the best efficient isolate in reduction of each second stage juveniles (J2), number of galls, egg masses and females

per root system with 90.33, 90.59, 90.06 and 85.50%, respectively and the effect of this isolate was similar to vydate. Wu et al. (2017) isolated new strain of *T. asperellum* with high antagonism against pathogens of cucumber wilt and corn stalk rot.

On the contrast, the isolate *T. harzianum* (Th5) was the least isolate in reduction of each second stage juveniles (J2), number of galls, egg masses and females per root system with 81.72, 75.41, 77.02 and 71.76%, respectively, as compared to vydate. It was shown that the growth of nematode was increased in absence of *Trichoderma*. These results highlight the significance of *Trichoderma* as excellent control agent in inhibition growth

of nematode (Spiegel et al., 2007; Chen and Dickson, 2004; Sharon et al., 2011). Interestingly, most of the isolates that exhibited high inhibition rate of nematode growth also improved each root length, plant height, roots fresh weight, shoots fresh, and dry weight of tomato plants (Table 4). These results are compatible with other studies showing the ability of *Trichoderma* in growth inhibition of nematode on various crops and enhancing the growth of host plants (Al-Hazmi and Javeed, 2016; Izuogu and Abiri, 2015; Sharon et al., 2011; Mascarin et al., 2012).

The variability among *Trichoderma* isolates in their aggressiveness against the tomato nematode may be due to the difference in their genetic structure, origin of isolates, pathogen cell wall structure and quantity of lytic enzymes secreted by *Trichoderma* (Al-Hazmi and Javeed, 2016; Abdel-lateif et al., 2017).

Conclusion

Identification of *Trichoderma* based on ITS markers exhibited high efficiency in discrimination among different *Trichoderma* spp. isolates. *Trichoderma* isolates showed variability in their aggressiveness against *M. phaseolina*, *A. niger* and *M. incognita*. In general, the isolate Th2 of *T. harzianum* showed the best antagonism against *M. phaseolina* and *A. niger*, while the isolate Ta1 of *T. asperellum* was the best efficient isolate in reduction of nematode growth.

In general, treatment of tomato with *Trichoderma* isolates improved their growth parameters (root length, plant height, roots and shoots fresh weight and shoots dry weight) as compared to untreated control. These results confirm the efficacy of *Trichoderma* as excellent biocontrol agent and also as plant growth promoting.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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