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Isolation and molecular characterization of *Trichoderma* isolates and assessment of their biocontrol efficiency against Rhizoctonia solani

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Hydrolytic enzymes producing from *Trichoderma* species have long been recognized as agents for controlling plant diseases caused by various phytopathogenic fungi. Fourteen *Trichoderma* isolates were isolated from rhizosphere of Wheat, Alfalfa and Faba bean plants using soil dilution plate method. The forteen *Trichoderma* isolates were showed positive test for cellulase production. Among of these isolates four *Trichoderma* isolates (FAYT1, FAYT10, FAYT11, and FAYT14) exhibited highest cellulolytic activities. The percentage inhibitory effect of all the fourteen *Trichoderma* isolates against growth of *Rhizoctonia solani* was calculated and ranged from 58.8% to 69.1%. Genetic distances for the four selected *Trichoderma* strains with high cellulase activity based on the 67 bands obtained from the results of RAPD revealed three groups, among which isolate FAYT1 and FAYT11 were closest to each other.

KEYWORDS:

Trichoderma species, *Rhizoctonia solani*, Enzyme activity, RAPD-PCR, Antagonistic.

1. INTRODUCTION

Fungal diseases are regarded as the most significant factor in major crops of vegetables, fruits and legumes causing yield losses. Diverse methods are being used to control pathogens in fungal plants. Members of the filamentous fungal genus *Trichoderma*

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are commonly found in specific plant soil and rhizosphere. Since the 1930s, the genus has been a common subject of basic and applied mycology research, mainly due to the fact that species *Trichoderma* is the most effective biocontrol agent against pathogenic plant fungi (Kacprzak et al., 2014). The *Trichoderma* genus and the efficient biocontrol strain are being developed as effective biological fungicides, and their biocontrol mechanism involves the role of secondary metabolites with prospective applications as new antibiotics (Agrawal and Kotasthane, 2012). They produce many antifungal enzymes, including celluloe-degrading enzymes, which are used economically as the basis of these kinds of proteins. Many reports suggest that chitinolytic enzymes produced by *Trichoderma* species are the most effective agents of different biological control of plant infections or diseases (Gajera et al., 2013). *Trichoderma* biocontrol mechanisms involve several events, such as antibiotics (Ghisalberti and Rowland, 1993), Mycoparasitism (Haran et al., 1996). *Trichoderma* species are attached to the host hyphae by coiling, hooking, and aspersorium-like bodies, and penetrate the host cell wall by secreting several lytic enzymes (Kubicek et al., 2001). All living organisms are made up of genes coded for specific proteins, which perform special functions. Genes play a major role in the biocontrol cycle by controlling certain signals and leading to the secretion of certain enzymes or proteins that enable the pathogens to degrade and hence are known as biocontrol genes. Increased gene expression helps in enhanced biocontrol activity which helps to promote plant growth and prevents the plant from attacking pathogen.This research aimed to isolation and molecular characterization of *Trichoderma* isolates and assessment of their biocontrol efficiency against *Rhizoctonia solani*.

2. MATERIALS AND METHODS

2.1. Collection of rhizosphere samples

Samples were collected from rhizosphere soil of different crops include Wheat, Faba bean, and Alfalfa grown in agricultural fields located in different area at Fayoum Governorate, Egypt.

2.2. Isolation of *Trichoderma* spp. from collected samples

Trichoderma isolates were isolated from rhizosphere samples according to soil dilution plate method described by Rahman et al. (2011).

2.3. Qualitative screening of *Trichoderma* isolates for their cellulase production

Fresh culture plugs of the *Trichoderma* isolates were placed in the middle of the plate contained culture medium amended with 1% of carboxymethyl cellulose (CMC). Plates were incubated at 28°C for 96 h and flooded with 0.3% Congo red for 20

min. cellulase-producing isolates can be discriminated and identified as they showed clear and prominent zones around the colonies.

2.4. *In vitro* evaluation of antagonism of *Trichoderma* spp. against *R. solani*

Trichoderma isolates were evaluated for their potential to antagonize the plant pathogenic fungus *R. solani in vitro* using dual culture technique according to the bioassay method described by Zhang and Wang (2012) on PDA medium.

2.5. Genomic DNA isolation from *Trichoderma* isolates

The four selected *Trichoderma* isolates were cultured in 100 ml Erlenmeyer flasks containing 20 ml PDA medium, after five days incubation, mycelium was collected. Genomic DNA was extracted as described by Doyle and Doyle (1987).

2.6. Random Amplified Polymorphic DNA–PCR fingerprinting of *Trichoderma spp.*

A total number of ten primers (OPA-1-OPA-10) obtained from OPA KIT (Operon Technologies Inc., Alameda, Calif., USA) were used in the present study to determine the genetic similarity between the four selected *Trichoderma* isolates. The PCR reaction mixture (25µl) contained 12.5µl Master Mix (one PCR^{TM}), 3 µl of primer, 2 µl of template DNA preparation and 7.5 µl of H_2O. PCR conditions were as follows; one cycle of initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation for 1 min at 94 °C, annealing primer for 1 min at 37°C and extension for 1 min at 72 °C, and one cycle for final extension step at 72 °C for 10 min using thermal cycler 2720 (Applied Biosystems, USA).

RAPD-PCR fingerprinting patterns were carried out with computer assisted analysis using RAPD software package, version 1.4 (Armstrong et al., 1994). Similarity of the band profiles was based on Excoffier matrix (Excoffier et al., 1992). The correlation coefficient was used to compare the number of the DNA patterns obtained. The clustering of the strains was determined by the unweight Pair Group Method using Arithmetic Average (UPGMA).

3. RESULTS AND DISCUSSION

3.1. Isolation and morphological identification of *Trichoderma* spp.

A total of 14 isolates of *Trichoderma* spp. were isolated from rhizospher soil of different cultivation crops (Wheat, Faba bean, Alfalfa and Cucumber). The *Trichoderma* isolates could be classified into three groups on the basis of culture and morphological characteristics. The conformation of species-level identification of *Trichoderma* isolates was carried out according to an interactive key http://nt.arsgrin.gov/taxadescriptions/keys/FrameKey.cfm?gen=Trichoderma.

These species of *Trichoderma* were identified as 9 isolates (*T. harzianum*), 3 isolates (*T. asperellum*) and 2 isolates (*T. aureoviride*). Colony appearance of the three different species grown on PDA for 5 days at 28^{o}C was shown in Fig. 1.

Morphological characterization was conventionally used in the identification of *Trichoderma* species, and it remains as a potential method to identify *Trichoderma* species (Anees et al., 2010). Besides macroscopic characteristics and growth rate, microscopic features of *Trichoderma* isolates are also important morphological keys in the identification of *Trichoderma* species. The microscopic features that are frequently studied include the shapes and sizes of conidia, the branching patterns of conidiophores, the shapes and sizes of phialides, and the production of chlamydospores (Anees et al., 2010; Gams and Bissett, 2002). However, information from morphological study alone is insufficient to precisely identify a *Trichoderma* species because *Trichoderma* species have relatively few morphological characters and limited variation that may cause overlapping and misidentification of the isolates (Anees et al., 2010).

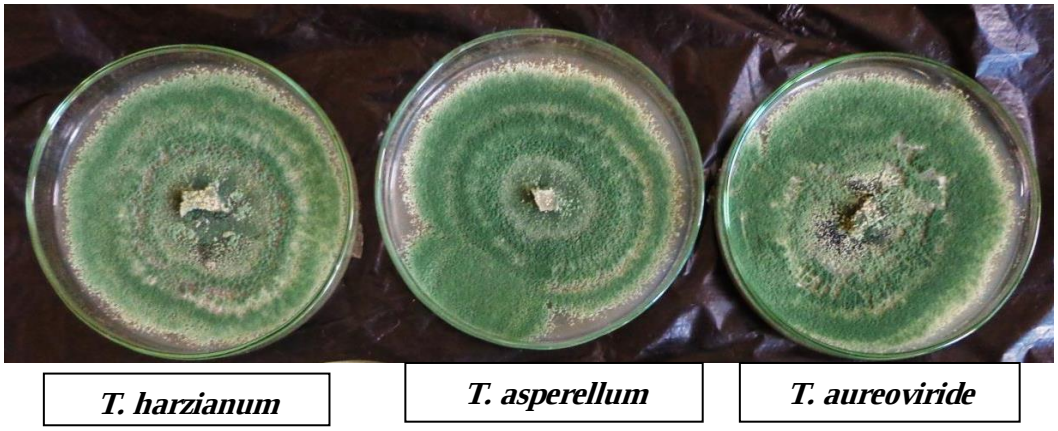

Fig. 1. Colony appearance of different species of *Trichoderma* isolates grown for 5 days at 28^{o}C on PDA plate.

3.2. Qualitative screening of *Trichoderma* isolates for their cellulase production

Forteen *Trichoderma* isolates were screened for their ability to produce cellulases. Results present in Fig. 2. showed both positive and negative cellulase producers for plates flooded Congo red. Clearing zones surrounding *Trichoderma* growing colonies after incubating for a suitable

period indicating their ability for cellulase production. The screening selection of the highly cellulolytic fungi isolate was based on the diameter size of clearing zone surrounding the colony of *Trichoderma* on the plate screening medium (Fig. 2). One the cellulose was degraded after 5 days of incubation, the red color disappeared leaving a place colored halo, which was indicative of the presence of cellulase relased into the culture medium. The cellulase production ability of fungi assessed by estimating zone around the colony formed due to ability of fungal isolates to hydrolyse cellulose. Results showed that isolate (1, 10, 11 and 14) has highest enzyme activity among total isolates. These isolates have been used for further studies into the enzyme production and their ability to degrade cellulose.

Fungi are the main cellulase-producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity (Lynd et al., 2005). Because the cellulase enzyme was a complex enzyme, not a single one, there were many genes to code cellulase proteins. Microorganisms of the genus *Trichoderma* are thought to be cellulose producers and crude enzymes produced by these microorganisms are commercially available for agricultural use. Many microorganisms have been classified as cellulolytic but, only few possess a complete cellulase complex capable of efficient depolymerization of crystalline cellulose. *Trichoderma, Penicillium, Aspergillus, Myrothecium, Fusarium* and *Chaetomium* are common soil fungus, which produces cellulolytic enzymes (Mandels et al., 1976).

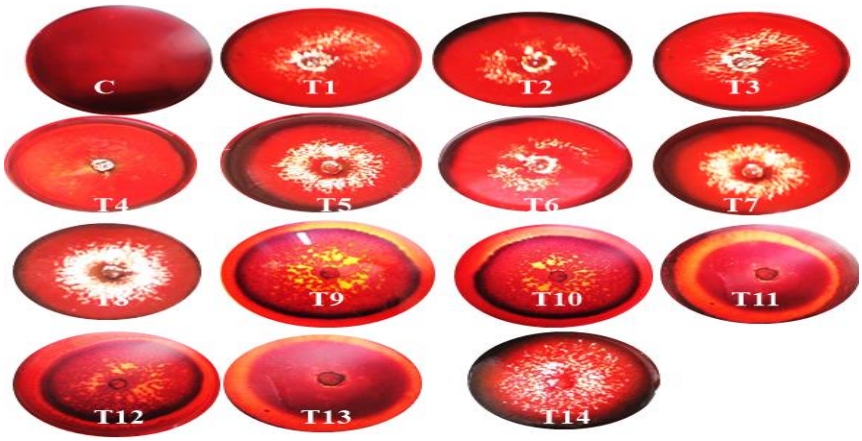

Fig. 2. Screening for cellulolytic *Trichoderma* isolates by covering the petri dishes with Congo red dye. A zone of clearance surrounding the colonies is indicative of carboxymethyl cellulose (CMC) hydrolysis by secreted CMCase.

3.3. Antagonistic efficacy of *Trichoderma* isolates against *R. solani in vitro*.

The *Trichoderma* species were evaluated *in vitro* for their potential antagonizes the plant pathogenic fungus *R. solani*. The results of antagonism between *Trichoderma* spp. and *R. solani*, showed in Fig.3. and Table 1. *Trichoderma* species were showed a significant reduction in mycelia growth of fungal colonies of *R. solani* face the *Trichoderma* spp. compared to the control. *Trichoderma* harzanium FAYT1 showed the highest inhibition (69.1%) of *R. solani* growth. On the other hand, *Trichoderma aureoviride* FAYT5 showed the lowest inhibition (Table 1). The antagonism was observed with the naked eye **(**Fig. 3**).** The *Trichoderma* isolates (FAYT1, FAYT3, FAYT5, FAYT8, FAYT9, FAYT10, FAYT11 and FAYT14) were overlapped with the pathogen *R. solani*. Wherase the *Trichoderma* isoaltes (FAYT2, FAYT4, FAYT6, FAYT7, FAYT12, and FAYT13) were able to inhibit *R. solani* growth before contact and did not coil over *R. solani* hypha. The data showed that the best antagonistic effect against the pathogen was obtained from *T. harzianum* isolates. The use of specific microorganisms that interfere with plant pathogens is a nature friendly, ecological approach to overcome problems caused by the chemical method of plant protection. Research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). According to the present results, colonies of *Trichoderma* always grew faster than pathogen fungi. Rapid growth of *Trichoderma* is an important advantage in competition with plant pathogenic fungi for space and nutrients (Deacon and Berry, 1992).

Many investigators reported that within the genus *Trichoderma*, species such as *T. asperellum*, *T. harzianum*, and *T. viride* have demonstrated efficient antagonistic activity against *R. solani* under laboratory conditions as well as under pots and field conditions (Wilson et al., 2008). *Trichoderma* isolates are well adapted to survival in crop soils, compatible with the indigenous soil microflora, and capable of colonizing the zone immediately adjacent to plants roots. Results of the present study agree with the finding of many researchers all over the world as well as in Egypt: several species belonging to the genus *Trichoderma* are capable of parasitizing fungal plant pathogens such as *R. solani*, producing antibiotics effective against soil borne pathogens and competing for infection sites against pathogens (Vinale et al., 2008).

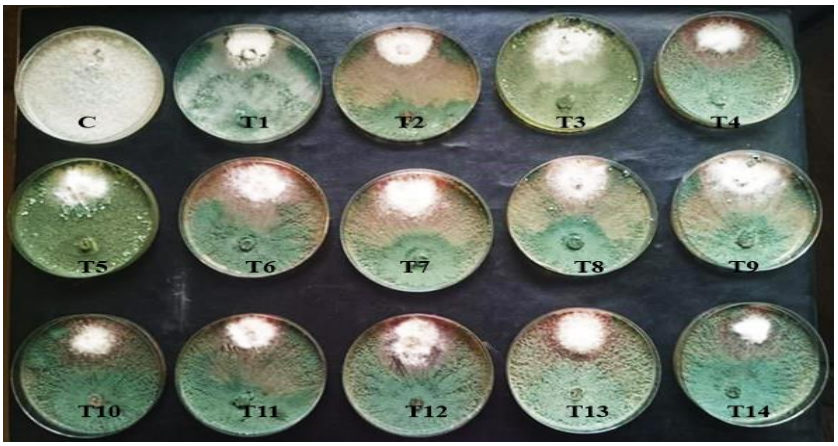

Fig. 3. Antagonistic activity of *Trichoderma* species against *Rhizoctonia solani* evaluated interaction on dual culture.

Table 1. Antagonistic effect of *Trichoderma* isolates against growth of *Rhizoctonia solani* on PDA.

Isolate code	*Trichoderma* species	Mean pathogen edge (cm)	inhibition %
C (*R. solani*)		6.8	00.00
FAYT1	*Trichoderma harzianum*	2.1	69.1
FAYT2	*Trichoderma harzianum*	2.45	63.97
FAYT3	*Trichoderma harzianum*	2.7	60.29
FAYT4	*Trichoderma harzianum*	2.58	62
FAYT5	*Trichoderma aureoviride*	2.8	58.8
FAYT6	*Trichoderma harzianum*	2.3	66.17
FAYT7	*Trichoderma asperullum*	2.2	67.6
FAYT8	*Trichoderma asperellum*	2.5	63.2
FAYT9	*Trichoderma harzianum*	2.56	62.35
FAYT10	*Trichoderma aureoviride*	2.12	68.8
FAYT11	*Trichoderma harzianum*	2.18	67.9
FAYT12	*Trichoderma harzianum*	2.56	62.35
FAYT13	*Trichoderma harzianum*	2.28	66.47
FAYT14	*Trichoderma asperullum*	2.12	68.8

3.4. RAPD-PCR for selected *Trichoderma* strains with high cellulase activities

Ten different (OPA 1 to OPA 10) random primers were tested with DNA samples of *Trichoderma* isolates. Out of 10 RAPD markers tested OPA

01, 02, 04, 05, 06 and 10 which were amplified in all isolates (Fig. 4). Classification of the four selected *Trichoderma* strains; FAYT1, FAYT10, FAYT11 and FAYT14 with high cellulase activity, at the molecular level were done using six different RAPD primers (OPA01, OPA02, OPA04, OPA05, OPA06 and OPA10). The results of RAPD were analyzed to construct dendrogram. The applicability of the method for determining genome similarities among *Trichoderma* strains was investigated by performing cluster analysis on the RAPD data. The number of scorable bands for corresponding primers ranged from 5 to 14 with an average of 11.2 bands. A total of 67 bands were scored against 4 isolates of the *Trichoderma* spp. The number of polymorphic amplicons per primer ranged from 45.5 % by primer OPA05 to 100% by primer OPA10. Genetic distances for the four selected *Trichoderma* strains with high cellulase activity based on the 67 bands obtained from the results of RAPD revealed three groups, among which isolate FAYT1 and FAYT11 were closest to each other (Fig. 5). The Dendrogram was constructed considering all bands generated by six primers and suggested two primary genetic clusters; the first cluster consisted of two sub-clusters that include *Trichoderma* isolates FAYT10 and FAYT14. The second cluster includes the other two *Trichoderma* isolates FAYT1and FAYT11 and the isolated (FAYT1 and FAYT11) were closely related in one linage, while the isolates (FAYT10and FAYT14) were highly distantly (Fig. 5). The polymerase chain reaction (PCR) technique has created new ways of revealing DNA polymorphisms among closely related genotypes with high sensitivity, via a fast and easy-to-perform protocol. Zimand et al. (1994) used RAPD markers obtained from 9 arbitrary primers to distinguish strains of *Trichoderma*. Ten of the strains identified as *T. harzianum* exhibited similarities, and it was possible to distinguish the isolate T-39, used commercially as a biocontrol agent for *Botrytis cinerea*. For efficient taxonomic identification of *Trichoderma* spp., Fujimori and Okuda, (1994) examined 74 strains by RAPD profiles and the results were consistent with the morphological, physiological and ecological data, which suggested that the technique can aid to eliminate strains duplicated in a program for microbial selection. Schlick et al. (1994) analyzed strains of *T. harzianum* and mutants induced by gamma radiation originated from a wild isolate, the analysis verified that with RAPD it was possible to differentiate all the mutant strains for at least one primer, concluding that the method was valuable for identification and fast differentiation of strains.

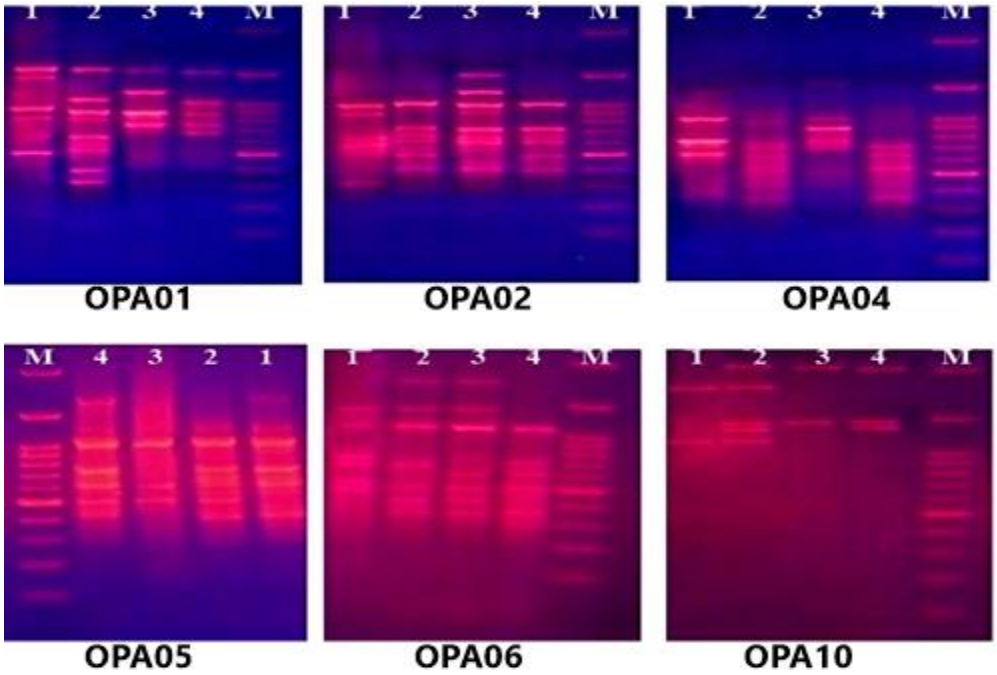

Fig.4. Banding patterns of *Trichoderma* spp. isolates obtained using RAPD primers OPA 01, 02, 04, 05, 06 and OPA 10; lane 1–4 = *Trichoderma* isolates (1 and 3 = *T. harzianum*; 2 = *T. asperellum*; and 4 = *T. aureoviride*) M – M: 100 bp DNA marker.

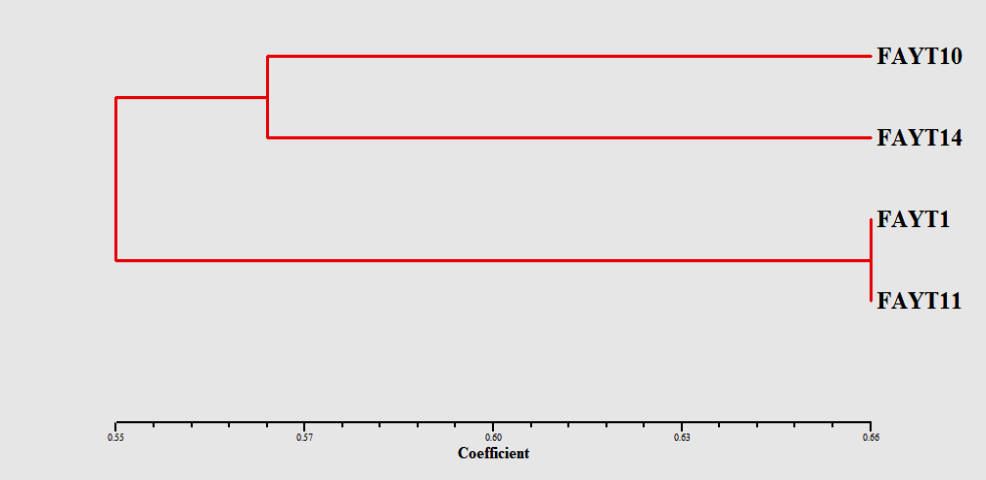

Fig. 5. Dendrogram showing genetic relatendes of *Trichoderma* isolates constructed considering all bands of 6 random primers (OPA01, OPA02, OPA04, OPA05, OPA06 and OPA10) using UPGMA and similarity matrices.

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عزل وتعريف جزيئي لعزلات فطر الترايكوديرما وتقييم نشاطها في المكافحة الحيوية ضد فطر الرايزوكتونيا سولاني

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تستخدم انزيمات التحلل المائي المنتجة من أنواع *Trichoderma* كعوامل للسيطرة على الأمراض النباتية التي تسببها الفطريات الممرضة للنبات. تم عزل أربعة عشر عزلة من *Trichoderma* من منطقة جذور نباتات الفول والبرسيم والقمح المنزرعة بمناطق مختلفة بمحافظة الفيوم. أظهرت عزلات *Trichoderma* الأربع عشرة اختبار إيجابي لإنتاج السليولاز. من بين هذه العزلات أربع عزلات (FAYT10 and FAYT14، FAYT11 ، و FAYT14) والتي أظهرت أعلى نشاط للتحلل. تم حساب التأثير المثبط بالنسبة المئوية لجميع عزلات *Trichoderma* الأربعة عشر ضد نمو *Rhizoctonia solani* وتراوح من ٥٨,٨٪ إلى ٦٩,١٪. تم تحساب درجة القرابة الوراثية بين السلالات الأربعة المختارة ذات نشاط السليولاز العالي بناء على ٦٧ حزمة تم الحصول عليها من نتائج RAPD والتي بينت ان العزلات الاربعة للترايكوديرما تقع في ثلاث مجموعات وكانت العزلتين FAYT1 و FAYT11 مرتبطتين ارتباطاً وثيقًا في مجموعة واحدة، في حين كانت العزلات (FAYT10 و FAYT14) في مجموعة منفصلة.

الكلمات الدالة: فطر الترايكوديرما– فطر الرايزوكتونيا سولاني– نشاط الانزيمات – تفاعل البلمرة المتسلسل العشوائي – التضاد٧.