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ISOLATION AND IDENTIFICATION OF *TRICHODERMA SPECIES* FROM SOILS FOR USING AGAINST SOME SOILBORNE FUNGAL PATHOGENS

[51]

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ABSTRACT

Hydrolytic enzyme producing *Trichoderma* species have long been recognized as an agent for controlling plant diseases caused by various phytopathogenic fungi. A study was done to characterize four isolates of *Trichoderma* isolated from the rhizosphere of different crop plants to characterize and identify certain biocontrol related enzymes (β-1,3-glucanase and chitinase). Morphological and molecular characterize action of antagonistic ability of *Trichoderma* species were studied. On the basis of morphological and culture characteristics, the Trichoderma isolates were identified as *T. harizanum* (1 isolate), *T. viride* (3 isolates). These isolates were tested against plant pathogens (*Rhizoctonia solani, Pythium* and *Fusarium oxysporum*).

The best isolate producing chitinase (overproduction) was *T. harizanum* from (160 to 0.64) mg/ml. On the other hand the best isolate gave over production β-1; 3-glucanase over production was *T. viride* from (2250 to 2.20 mg/ml)

Keywords: *Trichoderma species*, Biocontrol, Antagonism, Chitinase, β-1,3-glucanase

INTRODUCTION

The filamentous fungus *Trichoderma* is one of the most potent agents for the biocontrol of plant pathogens **(Cook and Vesth, 1991).** *Trichoderma* is among the most exploited fungal biocontrol in agriculture for the management of plant diseases caused by wide spectrum of pathogens **(Mathivanan et al 2000).** Production of extracellular proteolytic enzymes is one of the biocontrol mechanisms exerted by Trichoderma towards fungal pathogens. Several biocontrol agents alleviate the growth of pathogenic fungi by producing extracellular chitinase, which degrades the chitin polymers of fungal cell wall **(Mathivanan et al 2000).**

The antagonistic mechanism of *Trichoderma* spp. is due to inducing secretion of chitinolytic, proteolytic, and glycolytic enzymes and their controlling mechanism is still a hypothesis, in spite of numerous studies and reports. In time course of mycoparasitism, the cell wall-degrading enzymes act synergistically and exerted their action on pathogenic fungi. Thus, considerate the induction process of these enzymes is essential for selection of most significant *Trichoderma* spp. for biocontrol purpose.

Members of the fungal genus *Trichoderma* have been extensively studied, particularly due to their ability to secrete cellulose and chitinase degrading enzymes or to act as biocontrol agents . Most of the work has been carried out on strains of *T. harizanum* and *T.viride*. These strains have been extensively studied for their ability to produce extracellular β-1, 3-glucanase and chitins enzymes, which act synergistically in the conversion of cellulose and chitin to glucose **(Eveleigh, 1987).**

The filamentous fungus *Trichoderma* is well known for its ability to secrete distinct enzymes, to degrade the cell wall of other filamentous fungi **(Ridout et al 1985).** This property is related to the finding that certain *T. harizainum* isolates are considered as bio fungicide candidates **(Papavizas, 1985).** One of the main problems in the applica-

tion of this fungus as bio pesticide is that its chitinases and β-1, 3-glucanase are inductive enzymes **(Ulhoa and Peberdy, 1991)** and consequently its fungicidal effect slowly develops. Because of the low efficiency, it seems necessary to breed this organism by mutagenesis **(Sivan and Harman, 1991).**

MATERIALS AND METHODS

Isolation and identification of *Trichoderma* species

A total four isolates of *Trichoderma* spp*.* Were isolated from rhizosphere soil of different cultivated crops such as Grape from silt loam soil and eggplant from loam soil samples by using potato dextrose agar (PDA) medium. PDA media were prepared and autoclaved at 121^{o}C at15psi for 20 mints. Penicillin at 100,000 unitsL-1 and streptomycin at 0.2g were added to sterilized stock media just before pouring to inhibit the bacterial growth. The media were poured in 9cm diam. petri plates at 10 ml per plate. Samples were inoculated over plates by multiple tube dilution technique (MTDT) and the plates were incubated at 26˚C for 4-7 days. Inoculated plate shows a mixture of multiple fungi and bacteria cultures. The desired fungal colonies were then picked and purified by streaking or by single spore and incubated at 26˚C for 7-8 days. 1ml of distilled water was spread over the fully-grown culture of *Trichoderma* with a spade and then transferred to 99 ml of distilled water to make a suspension. A drop from the diluted fungal suspension was taken over the slide and observe *Trichoderma sp*. Was observed under the microscope at 10-40X magnification.

Pathogenicty

The three pathogens used in this study were obtained *Fusarium oxysporum, Rhizoctonia solani*, and *Pythium* sp from the ACGEB. All strains were grown routinely on potato dextrose agar (PDA) at 25°C and 0.1 % Triton X-100 was included in some instances to reduce colony growth. Glucose minimal medium (GMM) **(Cove 1966)** was used as a defined medium and soil extract agar (SEA) was used to reproduce soil conditions.

Identifiable phenotypes on PDA medium of *Trichoderma* SPP*.*

(*T. harzianum*) formed 1-2 concentric rings with green conidial production, (*T. viride1*) formed green conidia distributed throughout. An irregular yellow zone without conidia was present around the inoculum, (*T. viride* 2) formed white pustules were also found growing on the green mat of conidia and (*T. viride* 3) formed green conidia distributed throughout.

Enzyme determination

Enzyme source was prepared following the methods of **(Dutta and Cheterjee, 2004).** Seven-days old *T. harzianum* (both wild and mutant) cultured on PDA was used for inoculation of Czapek's Dox broth (basal medium). The later was supplemented with sucrose (3 % w/v), fresh mycelial mat and dry mycelial mat as the soil carbon sources. The growth medium in the flasks was passed through filter paper (Whatman No. 1) after 10 days of incubation. Collected culture filtrates (enzyme source) were preserved in refrigerator at 4 °C with 0.02% (W/V) sodium azide. β- 1, 3-Glucanase the dinitrosalicylic acid method of **(Miller, 1959)** was used to assay the β-1, 3-glucanase (laminarians) to study Chitins enzyme method of **(Ohtakara, 1988)** was followed to assay the enzymatic hydrolysis of colloidal chitin following the release of free N-acetyl-glucosamine (NAGA) from colloidal chitin. Colloidal chitin was prepared as the method of **Shimakara and Takiguchi (1988).**

Molecular characterization

The total genomic DNA was extracted from each isolate of *Trichoderma* using the DNeasy mini spin columns kit (qiagen).

Inter-simple sequence repeats (ISSR) technique.

The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94º C, 1 min at 45º C, and 2 min at 72º C. the reaction was finally stored at 72º C for 10 min. Amplified products were fractioned according to size (using 1 Kbp ladder marker) by electrophoresis in 1.5 % agarose gels in TBE buffer at 120 V for 1 h. The bands were visualized by ethidium bromide under UV florescence and photographed.

Table 1. List of the ISSR-PCR primer and their nucleotide sequences used in the study.

No.	Primer	sequence
1	HB08	5` GTGTGTGTGTGTGG 3`
2	HB10	5` GAGAGAGAGAGACC 3`
3	HB12	5` CACCACCACGC 3`
4	HB13	5` GTGTGTGTGTGTCC 3`
5	HB15	5` CACCACCACGC 3`

Table 2. Isolates of *Trichoderma spp*. Used in the present study and their origin.

Isolate code	Source of rhizosphere	species
T1	Grape	*Trichoderma harzianum*
T2	Grape	*Trichoderma viridi*
T3	Egg plant	*Trichoderma viridi*
T4	Egg plant	*Trichoderma viridi*

RESULTS AND DISCUSSIONS

Isolations and identification of *Trichoderma spp*.

The *Trichoderma* isolates were characterized by using distinctive morphological characters that includes rapid growth, green conidia, irregular yellow zone without conidia and white pustules.

A total of four isolates of *Trichoderma spp.* were isolated from rhizosphere soil of different cultivated crops such as Grape from silt loam soil and eggplant from loam soil samples by using potato dextrose agar (PDA) medium the *Trichoderma* isolates could be classified into two groups on the basis of culture and morphological characteristics descriped by **Gams and Bissett (1998).** These species of *Trichoderma* were identified as 4 isolates Trichoderma, 1 isolate (*T. harzianum*) and 3 isolates (*T. viridi*) **Table (2).**

Culture characteristics comprising growth rate, color and colony appearance were examined and the difference in morphological characteristics of *Trichoderma* spp. are summarized in **Table (3).** These characteristics were regarded as taxonomically useful characteristics for *Trichoderma* **(Samuels et al 2002).** Colony appearance of the different species grown on PDA for 5 days at 28 C. on PDA *Trichoderma harzianum* formed 1-2 concentric rings with green conidial production **(Table 3).** On PDA *Trichoderma viridi1* formed green conidia which distributed throughout the plate. An irregular yellow zone without conidia was present around the inoculum **(Table 3).** on PDA *Trichoderma viridi2* formed white pustules which were also found to grow on the green mat of conidia **(Table 3).** On PDA *Trichoderma viridi3* formed green conidia distributed throughout the plate **(Table 3).**

Table 3. morphological characteristics of Trichoderma spp. isolated from rhizosphere.

Cod #	Isolate	identifiable phenotypes on PDA medium	A Plant that has been isolated from rizosphere	Soil type
1	*Trichoderma* harzianum	formed 1-2 concentric rings with green conidial production	Grape	silt-loam
2	*T. viride. 1*	green conidia distributed throughout. An irregular yellow zone without conidia was present around the inoculum	Grape	silt-loam
3	*T. viride.2*	white pustules were also found growing on the green mat of conidia	Egg plant	loam
4	*T. viride.3*	green conidia distributed throughout.	Egg plant	loam

Antagonistic activity

The *Trichoderma* species were evaluated in vitro for their potential antagonizes the plant pathogenic fungi *Rhizoctonia solani , Pythium sp* and *Fusarium oxysporum* the result of antagonism between *Trichoderma* and three pathogens, was shown in **Table (4).** *Trichoderma* species showed significant reduction in mycelia growth of fungal colonies of *R.solani*, *Pythium* and *fusarium oxysporum* as compared to the control. The *Trichoderma harzianum* and *T.viridi 2* showed the highest inhibition . On the other hand, *Trichoderma viride 2* and *Trichoderma viridi 3* showed lowest inhibition. The data showed that the best antagonistic effect against three pathogens was obtained from *Trichoderma harzianum*. These results are in agreement with **Mathew and Gupta (1998)** who

found that the mycelial growth of *R.solani* was strongly inhibited in vitro by the antagonist *T. harizianum*.

Trichoderma harzianum and *T. viride* were reported by several workers as the best antagonists against several soil and root borne plant pathogen **(Poddar et al 2004). The** potentiality of *Trichoderma* spp. as biocontrol agent of phytopathogenic fungi in several crops is well known especially to *fusarium spp.* and *Rhizoctonia spp*. **(Poddar et al 2004 and Rogo et al 2007).**

Table 4. Antagonistic effect of *Trichoderma* species isolates against growth of phytopathogens (*Rhizoctonia solani, Pythium and Fusarium oxysporum*) on PDA in vitro.

strain	% growth of pathogens		
	Rhizoctonia solani	*Pythium sp*	*Fusarium oxysporum*
T.harizinum	78	75	75
T.viride1	92	89	85
T.viridi2	100	95	98
T.viridi3	98	95	100

Screening of the isolated *Trichoderma* spp. for enzyme activity

1- Production and assay of chitins and activity

The results present in **Table (5)** showed that all the four *Trichoderma* isolates were able to grow and produce chitins enzyme in the presence of chitin as the sole carbon source. The results indicates that *Trichoderma* isolates T1 and T2 exhibit highest activities of chitinase **(Table 5)** where the maximum amount of chitinase (from160 to 0.64 mg/ml) was ex/ml) was exerted by T1 isolate . On the other hand, the minimum amount of chitinase (from 142 to 0.64mg/ml) was exerted by T4.chitinase produced Chitinase produced by some *Trichoderma* species which is the key enzyme in the lysis of cell walls during mycoparasite action against phytopathogenic fungi.

Table 5. Assay of chitinase specific activity in *Trichoderma* culture filtrate.

Code	Chitinase activity (mg/ml) (mean± S.E)
	Chitinase
T1	160 ± 0.64
T2	142 ± 0.64
T3	125± 0.54
T4	121± 0.80

These enzymes may be important in the destruction of plant pathogens and could be used as the basis of screening for potential biocontrol agents (**DE La cruz et al 1992). El-Katatny et al (2000)** showed that *T.harzianum* Rifai (T24) was the most suitable isolates for the production of chitinase enzyme among 24 tested isolates.

Production and assay of cellulase and activity.

Results presented in **Table (6)** indicated that the maximum amount of β, 1-3 Glucanse ranged from 2250 to 2.20 mg/ml by T2 isolate. On the other hand, the minimum amount of β, 1-3 Glucanse from 1252 to 1.04 by T4 isolate. The role of β-1,3-glucanase in preventing fungal disease has been well studied, for example, glucanases have been shown to serve as bio-control agents in agricultural applications by protecting plants from fungal invasion. Bacteria secreted glucanase and other fungal cell wall-degrading enzymes are commonly considered as potential biocontrol agents against plant-pathogenic fungi .Therefore, degrading enzymes of the bacteria that produce these enzymes could be used as biological control agents. Many chitinolytic and glucanolytic microorganisms have potential for controlling fungal plant pathogens.

Table 6. Assay of β-1,3-glucanase specific activity in *Trichoderma* culture filter.

code	β-1,3-glucanase activity (mg/ml) (mean± S.E)
	B, 1-3 Glucanse
T1	2160 ± 2.99
T2	2250 ± 2.20
T3	1365 ± 1.15
T4	1252± 1.04

ISSR analysis

A total of five ISSR primers were used to screen four *Trichoderma* spp. genotypes **(Table 6).** A total of 58 bands were amplified among the four *Trichoderma* species genotypes, 21 were monomorphic, 6 species specific bands, and 23 were polymorphic distinct bands. The results showed that primers HB08 and HB15 were highly polymorphic (66% and 88% polymorphism) and produced wide molecular band length (151 to 1079 bp) and (149 to 1224 bp) respectively . Moreover, primer HB12 showed the least polymorphism (25%) and produced molecular band length that ranged from (333 to 1302). HB08, HB13 and HB15

gave the highest species- specific bands and HB10 and HB12 gave the least specific bands. Source of polymorphism in ISSR assay may be due to deletion, addition or substitution of base within the priming site sequence. High diversity is the reflection of adaptation to environment, which is beneficial to its propagation, conservation, the domestication of wild species, and the screen of specific locus **(Fig. 1)**.

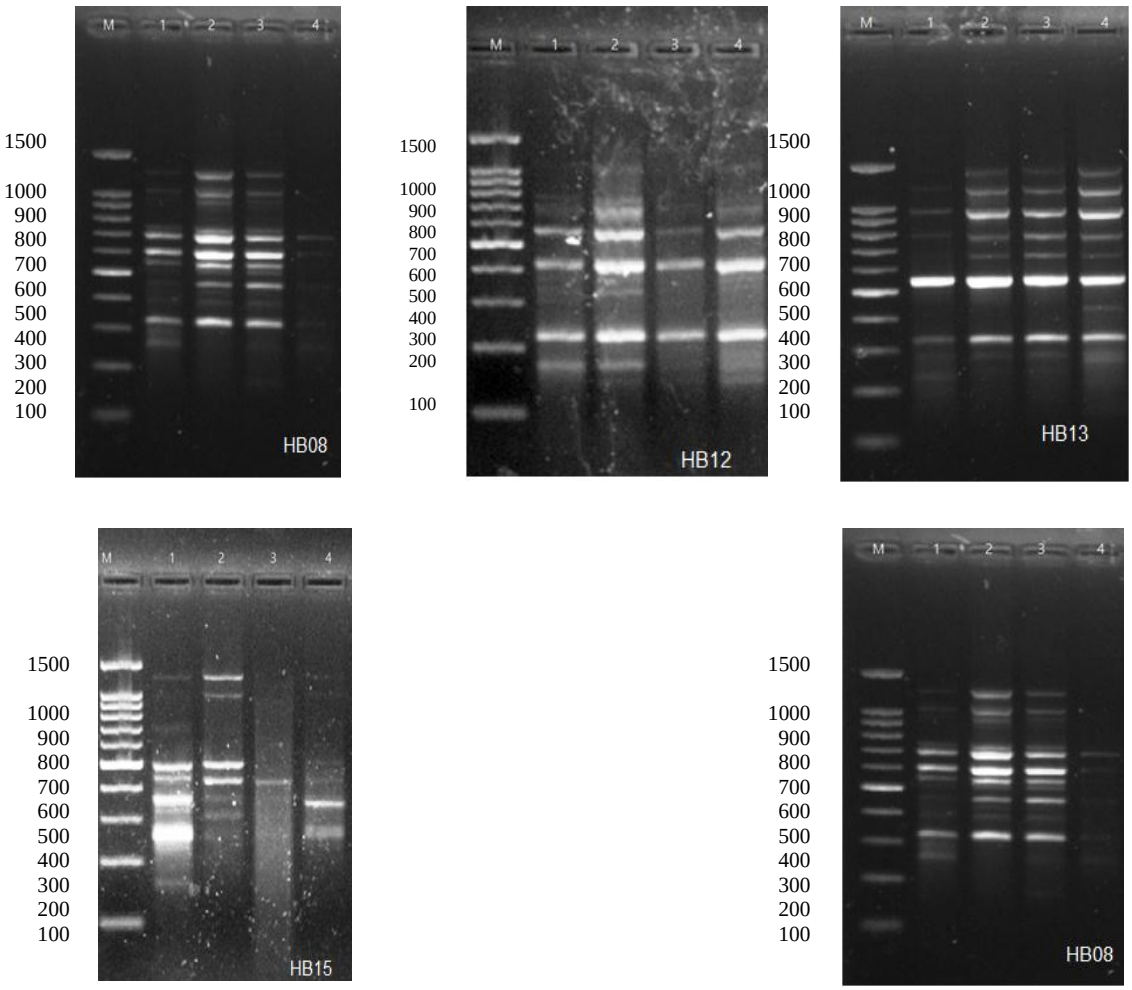

Fig. 1. PCR products with ISSR marks profiles, HB08, HB10, HB12, HB13, HB15and ISSR primers among the four Trichoderma spp. genotypes under different habitats. (M)= Marker, 1= (*T. harizinum*), 2= (*T. viride 1*), 3= (*T. viridi 2*), 4= (*T. viride 3*).

Table 7. HB08, HB10, HB12, HB13, HB15, ISSR primers among the four Trichoderma spp. genotypes under different habitats. (M)= Marker, 1= (*T. harizinum*), 2= (*T. viride1*), 3=(*T. viridi2*), 4=(*T.viride3*).

Polymorphism percentages	Total amplified bands	Unique bands	Polymorphic Bands	Monomorphic bands	Length Range(pb)	Primer sequence	Primer code
66%	12	2	6	4	151-1079	5` GTGTGTGTGTGTGG 3`	HB08
54%	11	0	6	5	222-1241	5` GAGAGAGAGAGACC 3`	HB10
25%	8	0	2	6	333-1302	5` CACCACCACGC 3`	HB12
50%	10	2	3	5	140-1084	5` GTGTGTGTGTGTCC 3`	HB13
88%	9	2	6	1	149- 1224	5` CACCACCACGC 3`	HB15

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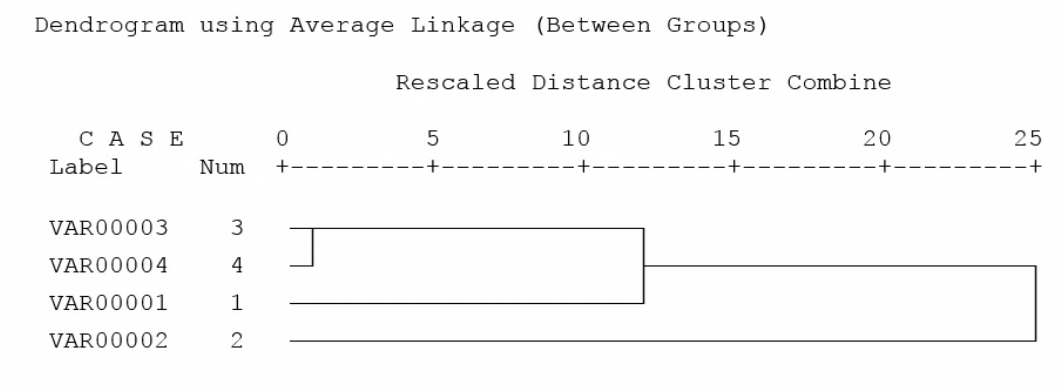
Dendrogram analysis

Similarity matrix was developed by SPSS computer package system, the closest relationship was scored between *T. viridi 2* (T3) and *T. viridi 4* (T4), while the least relationship scored between *T. virdie 3* (T2) and *T. viride 1*(T2).

Proximity Matrix

Case	Matrix File Input			
	VAR00001	VAR00002	VAR00003	VAR00004
VAR00001	.000	12.000	9.000	12.000
VAR00002	12.000	.000	9.000	16.000
VAR00003	9.000	9.000	.000	9.000
VAR00004	12.000	16.000	9.000	.000



Dendrogram using Average Linkage (Between Groups)

Rescaled Distance Cluster Combine

C A S E 0 5 10 15 20 25

Label Num +---------+---------+---------+---------+---------+

CONCLUSION

Trichoderma strains are known to produce extracellular lytic enzymes, including chitinase, cellulase, and protease which have a wide range of application in biological controlled, agriculture and other industries. Because of the important of these products, these present studies aimed to isolate and identification *Trichoderma spp*. to genetically improve extracellular lytic enzyme as chitinase and β-1,3-glucanase to use against plant pathogens such as *Rhizoctonia solani, Pythium sp* and *Fusarium oxysporum.*

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عزل وتعريف انواع التريكوديرما من التربه لاستخدامها ضد بعض المسببات الفطريه التي تنتقل عن طريق التربه

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الموجـــــز

تتميز العديد من انواع فطر التريكودرما *Trichoderma* بأهميتها الاقتصاديه، نظرا لمقدرتها علي انتاج مركبات تهم الانسان ذات قيمه اقتصاديه يمكن إستخدامها في المجالات الصناعيه والبيئيه والزراعيه المختلفه، حيث تتميز بعض سلالاتها بالقدره علي انتاج الإنزيمات المحلله لجدر الفطريات الممرضه للنبات، لذلك تستخدم في المكافحه الحيويه. تضمنت الدراسه عزل أربع عزلات محليه جمعت من منطقتين مختلفتين من محافظه البحيره– جمهوريه مصر العربيه، تتبع فطر الترايكوديرما *Trichoderma* وهي *Trichoderma* sp *T. harizinum* و *T. viridi* تم إجراء تجربه التضاد (antagonism) بين فطر الـ *Trichoderma* والفطريات الممرضه للنبات *Rhizoctonia solani*, *Pythium* and *Fusarium oxysporum* علي أطباق في المعمل واتضح ان السلالات T1, T2 هي السلالات الاكفأ في التضاد لفطريات *Rhizoctonia solani*, *Pythium* and *Fusarium oxysporum* علي أطباق في المعمل ونظراً لأهمية سلالات التريكوديرما موضوع الدراسه وامكانيه استخدامها في مجال المكافحه الحيويه فقد كانت هناك محاوله ناجحه لتعريف هذه السلالات علي المستوي البيوكيماوي والجزيئي باستخدام تقنيه تفاعل البلمره المتسلسل باستخدام خمسه من البادئات ISSR-PCR.

الكلمات الدالة: أنواع التريكوديرما، المكافحة الحيوية، مضاد الفطريات، شيتينيز، بادئات ISSR-PCR

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