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Antagonism Efficiency of *Trichoderma* against *Rhizactoni solani-15* and Molecular Diagnosed

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Abstract:

Six isolates of Trichoderma spp were used in this study, four isolates of T.harzianum were obtained from soil by direct plate and dilution methods, the isolates identified according to cultural and microscopic characters as well as T.harzianum (T.h) and T.viride UPM 29 available isolates, Trichoderma spp. isolates were screened in laboratory according to antagonism and antibiosis activity to choose the best one , antagonism experiment revealed a significant effect of T.harzianum (T.h), T.viride UPM 29 and T4 which were1.33 , 1.33 and 1.66 respectively , the broth culture filtrate of T.harzianum (T.h) and T.viride UPM 29 record the highest inhibition percentage 61.39% and 60.52% respectively, while T2 recorded the lowest inhibition percentage 32.8%. The identification of local isolates of T.harzianum (T.h), T1, T2, T3, T4 was confirmed with molecular diagnosis by using PCR technique, DNA of the five strains have been extracted, and the target region (ITS1 5.8S ITS2) of the rDNA has been amplified by using the universal primers ITS1– ITS4, The gel photo visualized five bands with molecular size 625 bp, the results compatible with microscopic and morphological identification.

Key words: Antagonism Efficiency of *Trichoderma*, *Rhizactoni solani-15*, Molecular Diagnosed

INTRODUCTION

The control of seed-borne diseases caused by fungi relies mainly on fungicide seed treatments (Ivic,2014). However the regular use of fungicides can lead to pollution of the environment, particularly if the residues persist in the soil or enter water way also the use of fungicides may lead to the appearance of new resistant strains of pathogens. (Den hond *et al*, 2003; Al-kaabi *et al*, 2010).Therefore the control of plant pathogen by developing new non-chemical strategy including microbial control has been considered as alternative methods. *Trichoderma* is one of the best bio-control agents because of the antagonistic activity which depends on multiple synergistic mechanisms include antibiosis, parasitism, inducing host-plant resistance, competition, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds as well as its properties in easily spread and multiply , The possibility of adaptation in different environments and soils also its resistance to a wide range of plant pathogens. (Al-Isawi and Jarjees, 2010). *Trichoderma* spp. comprise common soil Hyphomycetes found in all climate zones ranging from Antarctica to the tropics. The taxonomy of the genus *Trichoderma* has been and still is under some dispute, however, the basis being on the work of Rifai (1969) assigning the *Trichoderma* strains to nine species aggregates differentiated primarily by patterns of conidiophore branching and conidium morphology. Recent studies have used DNA fingerprinting, restriction fragment length polymorphism and amplification, and ITS sequencing to further clarify the taxonomy of *Trichoderma*.(Penttila *et al* , 2004) . The aim of this study was to evaluate the biological potential of some *Trichoderma spp.* isolates gainst seed borne pathogen *Rhizactoni solani-15* .

MATERIALS AND METHODS

Fungal isolates used in this study :

Plant pathogen

***Rhizctonia solani-15*:**

Isolated from infected chickpea seeds, the pathogenicity test performed previously in laboratory on cabbage seeds which proved highly pathogenic ability .

***Trichoderma* isolates:**

1. *T.viride* UPM29: obtained from UPM university / Malaysia.
2. *T.h* : Mutant isolate of *T. harzianum* obtained from Dr. Bassam Yahya, university of Mosul / college of Agricultural and Forestry .
3. Four isolates of *T.harzianum* (T1,T2,T3 and T4) isolated from okra , paprika , grape and eggplant soils from Shoraw/Kirkuk agricultural fields . The dilution and direct method were applied to isolate the bio-control agent from soils .The preliminary identification of *Trichoderma* isolates was done based on morphological observation and comparison with morphological identification keys from Ranasingh *et al* (2006) .

Evaluation of antagonistic activity of *Trichoderma* spp.

Dual culture technique :

The antagonistic effect of *Trichoderma* spp. isolates (*T.viride UPM29, T.h* (*T.harzianum*),T1,T2 , T3 and T4) against pathogenic fungus *R.solani-15 in vitro* was evaluated using the dual culture technique,each *Trichoderma* spp. isolate and *R.solani-15* were cultured , separately on PDA medium for 7 days at 25^0C . Disc (0.5 cm-diameter) from each bio-control fungus was inoculated on surface of PDA medium in side of petri dish . A disc (0.5 cm-diameter) of *R.solani-15* was inoculated at equal distance of the opposite side of petri dish .

Each replicates have three plates . Petri dishes were inoculated with pathogenic fungus only as control . The inoculated petri dishes were incubated at 25^0C for 7 days antagonistic degree was calculated according to Bell *et al* (1982).

Detection of antifungal activity by non-volatile metabolites .

Antibiosis activity :

To determine the effect of non-volatile metabolites on mycelia growth of pathogen poisoned food technique was used . Discs of mycelial agar plugs (0.5cm-diameter) obtained from edges of 7 days old culture of Trichoderma spp. were inoculated in to 100 ml sterilized potato dextrose broth (PDB) in 250 ml conical flasks and incubated at 25±2^0C for 10 days , with shaking the flasks each 2-3 days .The control conical flasks were inoculated with sterile PDA plugs .Three conical flasks for each treatments .After incubation the culture was filtrated through the Whitman No.1 filter paper and then through Millipore 0.22µ.Filtrates were amended in PDA to make 50% concentration in petri plates .Solidified agar plates were inoculated at the Centre with 0.5 cm diameter mycelial disc of pathogen and incubated at 25±2^0C for 5days . Plates without filtrate served as control.(Ibraheem ,2009).

Estimation of biomass :

The fungal mycelium was harvested after the end of incubation period , separated from PDB by filtration through dried and weighted a Whitman No.1 filter paper by using Buchner Funnel and vacuum , then dried at 70 C^0 overnight .The dry weight of the fungus was calculated by Subtracting the (weight of dried filter paper alone) from (dried weight of filter paper with mycelium)(AOAC ,1980) .

Molecular diagnosis of *Trichoderma harzianum* isolates :
Extraction of genomic DNA:
Genomic DNA of *T.harzianum* isolates were extracted by a commercial genomic DNA purification kit (BIO BASIC, Canada) according to the manufacturer's instructions .

Estimation of genomic DNA concentration and purity:
The DNA concentration of samples are measured using Nano drop by placing 1µl of the extracted DNA in the instrument to determine the concentration and purity is detected by noticing the ratio of O.D. 260/280 to detect the concentration of DNA samples with protein.The accepted 260/280 ratio for pure DNA, which is between 1.8 and 2 DNA quality, could be assessed by 1% agarose gel electrophoresis (Sambrook and Russell, 2001).

Gel Electrophoresis
After genomic DNA extraction, agarose gel electrophoresis is adopted to confirm the presence and integrity of the extracted DNA**.**

Preparation of agarose gel 1%:
1- The amount of 1 X TBE (50 ml) is taken in a beaker.
2- Agarose powder (0.5 gm) is added to the buffer.
3- The solution is heated to a boiling point, using a water bath or oven until all gel particles are dissolved.
4-Two µl of ethidium bromide (5 ng/ml) is added to the agarose solution.
5- The agarose is stirred in order to be mixed and to avoid bubbles.
6- The solution is left to cool down at 50 – 55C^{o}.

Agarose Gel
After fixing the comb in 1cm away from one edge of the gel tray, the agarose solution is poured into the gel tray. The agarose is allowed to solidify at room temperature for 30 minutes. The

fixed comb is carefully removed and the gel tray is placed in the gel tank. The tank is filled with1X TBE buffer until the buffer reached 3-5 mm over the surface of the gel, (Sambrook and Russell, 2001).

DNA Loading and Electrophoresis.

DNA (7µl) is mixed with 3µl of bromophenol blue dye. Samples are loaded carefully into the individual wells of the gel, and then electrical power is turned on at 5 volt/cm^2 for 1hour. The DNA is then moved from cathode (-) to anode (+) poles. The Ethidium Bromide stained bands in the gel are visualized using a UV transiluminator at 350 nm, and then photographed (Sambrook and Russell, 2001).

Polymerase Chain Reaction:

Table 1. Gene sequence with universal primers.

Primer	Nucleotide sequences (5' ⟶ 3')	Length	Reference
Forward ITS-1	**TCTGTAGGTGAACCTGCGG**	**19**	Shahid *et al* , 2013
Reverse ITS-4	**TCCTCCGCTTATTGATATGC**	**20**	

Table 2. The thermocycling conditions program

No. of cycles	Time	Temperature	Steps
1	4 min	95 C°	Initial denaturation
30	30 sec	95 C°	Denaturation
	30 sec	52C°	Annealing
	1 min	72 C°	Extension
1	7 min	72 C°	Final extension

On accomplishment of PCR, the product is electrophoresed in 2% agarose. The electrophoresis is performed at 70 Volts for approximately 90minutes. The gel is removed and then, the bands on the gel are visualized under UV Tran illuminator.

RESULTS AND DISCUSSION

Trichoderma isolation

The results of this study indicated that the dilution method is more efficient than the direct method in isolation . Three pure isolates of *T. harzianum* was obtained by dilution method and one isolate by direct method .

Dual culture technique:

Results from the dual culture test showed that all isolates of *Trichoderma spp.* inhibited the mycelial growth of the pathogen *R.solani -15*.Two isolates *T. viride* UPM29 *and T.h* (*T.harzianum*) showed statistically significant inhibition which reach 1.33 (Table.3) .The isolate T4 was the next best isolate in inhibiting the growth of the pathogen i.e 1.66. Followed by T1,T2 and T3 which cover 2/3 the petri dish plate .The difference in antagonistic activity among *Trichoderma spp*. isolates resulted from the interference of genetic and environmental factors , the results of testing three isolates of *T. harzianum* (T1,T2 and T24) indicated a variance in production of chitinase , ß-1,3 glucanase , T24 was the most productive isolate .(El-katatny,*et.al*,2003).Kredics,*et al*,2003 reported that the eighteen *Trichoderma* strains screened for their ability to degrade bacterial cells , the specificity spectrum and the intensity of degradation were highly variable .Antibiotics are often associated with bio-control activity , for example, the production of a pyrone-like antibiotic from *T. harzianum* exhibited bio-control activity against *Ganumannomyces graminis* (Ghisalberti ,*et al*., 1990).

Table 3 antagonistic activity of *Trichoderma* spp.
Dual culture technique

Trichoderma spp. isolates	Antagonistic activity
T.h (T. harzianum)	1.33 C
T. viride UPM29	1.33 C
T1	2 B
T2	2 B

T3	2 B
T4	1.66 * AB

* values are average of 3 replicates. Means with the same letter in each column are not significantly different in L.S.D. at ($P \leq .05$).

Effect of non-volatile metabolites (Antibiosis activity):

The cultural filtrate of all six isolates of *Trichoderma spp.* exhibited antibiotic potential against *R.solani-15* by inhibiting its mycelial growth (Fig1,2).The maximum inhibition percentage was observed by treated *R.solani-15* with culture filtrate of *T.h (T. harzianum)* and *T. viride* UPM29 (61.39 % and 60.52%) respectively while T2 recorded the minimum inhibition percentage (32.8%) .Sreedevi *et al* (2011) reported that the metabolites released from *T. harzianum* and *T. viride* were tested in culture medium against *Macrophomina phaseolina* the root rot pathogen , cell free metabolites of *T. harzianum* and *T. viride* inhibited the growth of pathogen in vitro and appeared to be fungicidal , the inhibition varied depending on the Trichoderma species producing metabolites ,*T. viride* inhibited pathogen growth up to 90% and *T. harzianum* up to 72% .The inhibitory effects of *T. harzianum* culture filtrates is due to its ability to produce various secondary metabolites such as Trichothecin , Gliotoxin , Viridin , Trichodermine and Pyrones (Ghisalbert *et al* 1990) or due to the presence of various defense enzymes such as Estrase,Lipase,Protease,B-glactosidase and Phospho -amidase (Aziz,*et al*,1993) . Raza *et al* (2013) indicated that the *Trichoderma* strains have been reported to produce different antimicrobial compounds like 6-pentyl-α-pyrone, with a strong, coconut-like aroma, was produced by *T. harzianum* in liquid culture .

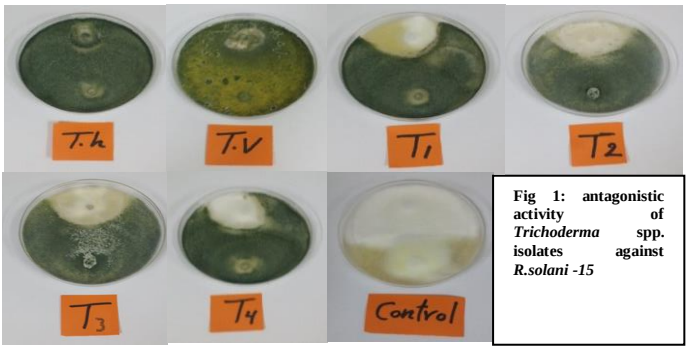

Fig 1: antagonistic activity of *Trichoderma* spp. isolates against *R.solani -15*

Estimation of biomass :

The maximum average of biomass was achieved by *T. harzianum* and *T. viride* UPM29 (0.40 and 0.39 gm/100 ml) respectively followed by T3 and T4 (0.34 and 0.36 gm/100 ml) respectively while T1 and T2 recorded minimum average of biomass which was 0.33 gm/100ml for each one (Fig 3) .The increased biomass refers to that *Trichoderma spp*. are highly successful colonizers of their habitats (PDB) which is reflected both by their efficient utilization of the substrate and their capacity in competition ,antagonism and secretion antibiotic metabolites and enzymes (Ibraheem , 2009) .

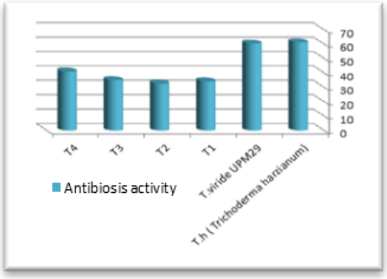

Fig 2: The difference in antibiosis activity among bio-control isolates *T.harzianum* (T.h) , *T.viride UPM29* , T1 , T2 , T3 and T4

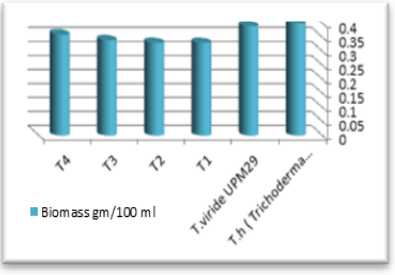

Fig 3 : The biomass of bio-control isolates *T.harzianum* (T.h) , *T.viride UPM29* , T1 , T2 , T3 and T4

Molecular diagnosis of *Trichoderma harzianum* isolates:

Extraction of genomic DNA:

DNA extraction is a crucial initial step for these molecular applications.

Extraction of high-quality genomic DNA for PCR amplification from filamentous fungi (*Trichoderma* spp.) is difficult because of the complex cell wall and the high concentrations of polysaccharides and other secondary metabolites that bind to or co-precipitate with nucleic acids. Traditional methods of DNA extraction, such as phenol/chloroform extraction, use toxic chemicals and are time consuming . These have in many instances been replaced by commercial DNA extraction kits that use fewer chemicals and are generally much more rapid. (Muller,*et al.*1998 ; Chen *et al.* 2010; Raihan *et al* . 2016) .Genomic DNA of *T. harzianum* isolates were extracted by a commercial genomic DNA purification kit (BIO BASIC , Canada) and then the extracted genomic DNA of *T. harzianum* isolates was analyzed using gel electrophoresis in 1%agarose. The results of gel electrophoresis showed sharp bands of chromosomal DNA (Fig.4) .The DNA concentration and purity are determined by using Nano drop 1000 spectrophotometer at 260/280nm. DNA concentration ranges between 24.3-117.4 ng/µl and purity ranging from 1.8 to 2 .The result of current study showed the important use of commercial kits such as BIO BASIC extraction kit as a rapid extraction method for the genomic DNA comparing with conventional DNA extraction methods such as alkaline lysis, boiling and salting out methods .The result of this study goes together with results of (Raihan *et al* , 2016) which referred that the purity of the DNA extracted from the *Trichoderma* was between 1.8 and 2.0 .

PCR amplification of ITS region:

The target regions of the rDNA ITS1, ITS2 regions and 5.8S gene were amplified symmetrically using primers ITS1(5-

TCCGTAGGTGA ACCTG CGG-3) and ITS 4 (5-TCCTCCGCTTATTGATATGC-3) , The purified DNA was amplified using Inter Transcribed spacer (ITS) primers, and the amplification products were separated on a 2% agarose gel, stained with ethidium bromide and visualized with UV light. Bands were found for 625 bp (Fig 5). This results matching 100% with the results of preliminary identification of *Trichoderma* isolates which based on morphological observation.

The finding of this study in agreement with findings of Mohammed and Eidan (2016) which referred that the PCR amplifications with primers ITS1 and ITS4 for the bio-control isolate of *Trichoderma harzianum* was 625 bp .

Also Sadfi-Zouaoui *et al* , 2009 indicated that the internal transcribed spacer (ITS) of the DNA region of 43 isolates showed extensive length polymorphism ,PCR amplifications with primers ITS1 and ITS4 for the ITS region generated bands ranging from 600 to 650 bp between strains. While Shahid *et al* , 2014 identified thirty isolates of *Trichoderma viride* which collected from rhizosphere soil of chickpea, pigeonpea and lentil crop , ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600bp products in all isolates. As well as Raihan *et al* (2016) used ITS1 and ITS4 primers during the PCR method to identifying the specific ITS1 region of rDNA of *Trichoderma* spp. , bands were found for the line T2 and T3 at 600 bp .

Shahid *et al* (2013) and Chakraborty *et al* (2010) indicated that the bands of ITS region of *Trichoderma* spp. within the range of 600-700 bp. While Hermosa et al , 2000 obtained a single product of approximately 560 to 600 bp from all the PCR amplifications with primers ITS1 and ITS4 for 17 biocontrol isolates of *Trichoderma* spp.

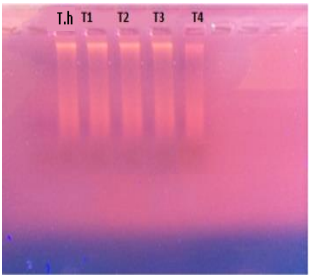

Fig 4 : Gel electrophoresis of extracted genomic DNA of *T. harzianum* isolates using 1% agarose gel at 7volt /cm for 30 minutes .

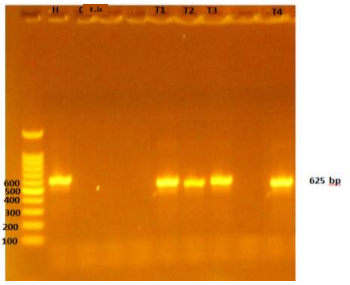

Fig 5 : Gel electrophoresis of PCR product of *T. harzianum* isolates using 2 % agarose gel. M: 100 bp DNA ladder, C: control negative , (T.h ,T1,T2,T3,T4) DNA bands of *T.harzianum* isolates .

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome. They also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU) , the 5.8S and the 28S large subunit (LSU) genes.(Iwen,*et.al*,2002;Hausner and Wang,2005; Chakraborty ,*et al*, 2010). Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies (Druzhinina,*et.al*,2005 ; Mancini , *et al* ,2005 ; Chakraborty *et al* , 2010) . Blaszczyk *et al* (2011) investigated the diversity of *Trichoderma* in Poland utilizing a combination of morphological and molecular/phylogenetic methods , 170 Trichoderma isolates were identified to the species level by the analysis of their ITS1, ITS2 rDNA sequences.

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