University of Sadat City
Environmental Studies and Research Institute
The4Th International Conference
Environmental for Studies and Research Smart Sustainable Environment

Journal of Environmental Studies and Researches (2017), 7(2-D): 946-954

EFFICIENCY OF LOCAL TRICHODERMA ISOLATES AGAINST ROOT ROT PATHOGENS, *PYTHIUM ULTIMUM AND RHIZOCTONIA SOLANI.*

M.A. Elsbag , Ashraf, M.M. Nofal, * M.A. El-Howeity **.

*Plant Pathology **Soil Microbiology

ABSTRACT:

Twenty three isolates of *Trichoderma spp*. were isolated from rhizospheric soil of different plants in different regions of Egypt. By using dual culture method, they were tested for antagonistic Efficiency against tested phytopathogenic fungi (*Rhizoctoniasoloni* and *pythiumultimum*)The results revealed that *Trichoderma atroviride* and *Trichoderma koningi i*causes completely overgrowth (100%) on tested pathogenic fungi. Analysis of filtrated of *Trichoderma atroviride* contain of antibiotics Trichorzins(13.0mg/ml) But in case of *Trichoderma koningii* contain high amount of protease enzymes (6.34 m/mg protein). The results of this study identify *Trichoderma atroviride* and *Trichoderma konigiv* have a significant antagonistic effect against root rot Phytopathogen fungi and as promising biological control agents for further test against root rot disease.

Key words: Antagonistic activity, *Trichorderma Spp*., root rot pathogens

INTRODUCTION:

Biological control of plant diseases is considered as one of the viable alternative methods to manage plant diseases (Barakat&Almasri, 2005 and Pal, 2006). Application of fungicides is not economical in the long term because they pollute the environment, leave harmful residues and can lead to the development of resistant strains of the pathogen with repeated use (Vinale*et al.,* 2008). However, use of biological control is safe, non-hazardous for human, farm animals and avoids environmental pollution (Barakat&Almasri, 2005; Abdel Kadir*et al.,* 2002). The application of biological controls using antagonistic microorganisms has proved to be successful for controlling various plant diseases in many countries (Janisiewiez*et al.,* 2009).

One of the most important biological control agents is the use of Trichoderma spp. that are most frequently isolated soil fungi and presenting plant root ecosystems (Whipps&Lumsden. 2004; Harman *et al.,* 2004). They colonize the root and rhizosphere of plant and suppress plant pathogens by different mechanisms, such as competition, mycoparasitism; antibiosis production and induced systemic resistance, improvement of the plant health by promote plant growth, and stimulation of root growth (Harman *et al.,* 2004; Mohidden*et al*., 2012).

Species of thegenus Trichoderma are well documented fungal biocontrol agents (Papavizas,1985;Elad,and.Kapat,1999; Howell,2002).The antagonistic action of *Trichoderma* species against phytopathogenic fungi might be due to either by these cretion of extracellular

hydrolytic enzymes(Chet, 1987; Di Pietro *et al.,*1993; Schirmbock*et al.,*1994)or by the production of antibiotics(DennisandWebster, 1971a; Dennis and Webster, 1971b; Claydon*et al.,* 1987; Howell,1998).

The objective of this study was to evaluate the potential of the bioagent *Trichoderma isolates* recovered from Egypt agricultural fields in controlling the soil borne phytopathogenic fungi *Pythium ultimum* and *Rhizoctonia solani*.

2. MATERIALS AND METHODS

2.1 Isolation and identification of test pathogen

Root showing symptoms of fungal infection were collected. Isolation of the pathogen was done from each of the distinct symptoms observed on roots. Infected root parts (1 to2 mm) were cut into small pieces by sterilized blade then surface sterilized with mercuric chloride (0.1%) for1min. The pieces were then washed twice with sterilized distilled water and dried by sterilized blotting paper. These pieces were placed on Petri dishes (90mmdiameter) containing 20mL potato dextrose agar medium and incubatedat28±2°C.Thefunginamely,*Rhizoctonia solani* and *Pythium ultimum* were isolated and identified with the aid of standard literature available (Ellis, 1971; Barnett, 1960).

2.2 Isolation of Trichoderma spp

Rhizospheric soils were collected from different locations in Egypt governments,.From the rhizosphere soil samples,*Trichoderma spp* were isolated by using PDA and *Trichoderma* selective medium (TSM) by dilution plate technique (Johnson, 1957). The isolated species were identified up to species level based on colony characters, growth, structure of mycelium, conidiophores,phialides and conidia (Kubicek and Harman,2002). All *Trichoderma spp* were purified by hyphaltip technique(Tuite,1996).The isolated *Trichoderma spp* were maintained throughout the study by periodical transfers on PDA and TSM slants under aseptic conditions to keep the culturefresh and viable. The identificaiotn of *Trichodermal* isolates were confirmed in Mycology center, Assiute university.

2.3. Dual culture experiment

Antagonistic efficiency of *Trichoderma spp*,were tested against the isolated pathogenicfungi by dual culture experiment (Morton and Stroube,1955).*Trichoderma spp* and test fungi were inoculated 6 cm apart. Three replicates were maintained for each treatment and incubated at 28 ± 2°C for 7 days. Mono culture plates of both served as control. Seven days after incubation, radial growth of test fungi and *Trichoderma spp* were measured. Colony diameter of test fungi in dual culture plate was observed and compared with control.Percentage of radial growth inhibition (%RGI) was calculated by using the formula:100×[C-T/C], Where C = growth in control and T = growth in treatment (Vincent,1947).

2.4Enzymes assays

2.4.1. Cellulase activity :

Suitable aliquot (100 µl) of the culture supernatant was incubated with 400 µl of 100mM sodium citrate buffer (pH 5.2) containing 1 % CMC (Collmer, *et al*., 1988).After incubation at 55ºC for 15 min., the glucose released was measured by the dinitrosalicylic acid (DNSA) method (Sadasivam, &Manickam,1992). A known volume of aliquot was taken in test tube and final volume of 1.0 ml adjusted with distilled water. To this, 0.5 ml DNSA reagent (1g DNSA + 200mg crystalline phenol + 50mg sodium sulphite in 100ml of 1% sodium hydroxide) was added and mixed properly. The content was heated in boiling water bath for 5 min. When the contents of the tubes were still warm, 1.0 ml of 40 % sodium potassium tartrate (Rochelle salt) solution was added and cooled. The final volume was made 5.0 ml with distilled water and read at 540nm using spectrophotometer. Reagent blank was also performed by addition of 1.0 ml of distilled water in place of enzyme aliquot and treated in the same way as above procedure. A known concentration of standard of glucose was calibrated by following the above procedure and the enzyme activity expressed as appropriate.

2.4.2. Poly galacturonase (PG) activity (EC 3.2.1.15):

The culture supernatants (100 µl) were incubated with 400 µl of 50mM sodium acetate buffer (pH 5.2) containing 0.25 % sodium polypectate (Collmer, *et al*., 1988). After incubation at 37ºC for 1 h, the galacturonic acid released was measured by the DNSA method (Sadasivam, &Manickam,1992).

2.4.3. Chitinase activity (EC 3.2.1.14):

Reaction mixture contained 200 µl of 0.5 % chitin in 10mM sodium acetate buffer (pH 5.2) and 100µl of culture supernatants (Boller,Mauch,1988) were incubated for 1 h at 50ºC . The formation of sugar N- acetylglucosamine was measured by Dimethylamino benzaldehyde (DMAB) method (Reissig, *et al*., (1955).Known aliquot of reaction mixture (0.5ml) was taken into test tube and 0.5 ml, 120mM potassium borate buffer (pH 8.9) was added. The tubes were vigorously boiled in water bath for 3 min. and cooled. Then, 3 ml DMAB reagent (5.0 g DMAB dissolved in 500 ml of glacial acetic acid containing 12.5 % v/v 10 N HCl, stored at 20ºC as a stock and prior to use, it was diluted with nine volume of glacial acetic acid) was added in each tubes and incubated at 38ºC for 20 min. Tubes were then cooled and absorbance was measured at 544nm in spectrophotometer. Standard N-acetylglucosamine was prepared in borate buffer and measured following the above procedure. The amount of N-acetylglucosamine was calculated and expressed as appropriate.

2.4.4. β-1, 3 glucanase activity (EC 3.2.1.39):

The reaction system contained 100 µl of 4 % laminar in in 50mM sodium acetate buffer (pH 5.2) and 100 µl of culture supernatants (Kauffman, *et al*.,1987).Reactions were carried out at 37ºC for 10 min. After incubation, the glucose released by enzyme b-1,3glucanase was measured by DNSA method (Sadasivam, &Manickam,1992).

Specific activity of cellulase, PG, chitinase and b-1,3glucanasewere expressed as Unit.mg^{-1} protein. However, Unit activity was defined as the amount of enzyme necessary to produce one µM of corresponding reducing sugar per min per ml of culture supernatants. Non enzymatic controls were also performed using boiled enzymes and were subtracted from the enzymatic values.

2.4.5. Protease activity (EC 3.4.21.4):

The reaction system contained 500 µl enzyme solution and 500 µl of 0.36 % casein and 2.0ml of 100mM acetate buffer (pH 3.6). Reactions were allowed to proceed for 1 h at 50ºC and stopped with 3 ml of 5 % trichloroacetic acid (Malik, Singh,1980).Blank was treated as zero time incubation. The reaction mixtures were then centrifuged at 5000 rpm for 10 min. to settle down precipitate and known volume of supernatants (500µl) were used for estimation of released free amino acids by ninhydrin method (Lee, Takahashi,1966).Specific activity of protease was expressed as Unit.mg^{-1} protein and one unit of protease activity was defined as the amount of protein necessary to produce µg free amino acids per min per ml of culture supernatant.

2.4.6. Xylanase activity

Xylanase activity was assayed by the method described by Bailey *et al*. [Bailey,*et al*. 1992,]. Oat spelt xylan (Sigma-Aldrich, St Louis, MO, USA) was used as the substrate. The amount of released sugar was assayed via the dinitrosalicylic acid (DNS) method using glucose or xylose as the standard [Miller *et al*., 1960,].

2.4.7. β-Glucosidase activities

β-Glucosidase activities were determined using 4-nitrophenyl-β-D-glucopyranoside with para-nitrophenol as the standard [Berghem and Petterson , 1974]. One unit (U) of enzyme activity was defined as the quantity of enzyme that liberated substrate at the rate of 1 µmol per minute.

2.5. Antibiotic

After induction, the medium was separated from mycelia by vacuum filtration, and the peptides were extracted by adding ethyl acetate (1:2 v/v, Dinâmica). After phase separation in a separating funnel, the a polar phase was collected, centrifuged and rotoevaporated. The residue formed was resuspended by washing with water and then with acetonitrile, and collected separately. The acetonitrile fraction was lyophilized and used as a peptaibol source.

The structures of the compounds mentioned above were elucidated by spectroscopic (UV, IR and NMR) and chemical methods.

3. Results

3.1. screening of *Trichoderma* native antagonists fungi against mycelial growth of *Rhizoctonia solani* and *pythium ultiumum*.

Twenty three nativeisolates of *Trichoderma spp*. were screened for their antagonism *invitro* against the *R. solni* and *pythium ultimum* by dual cultural technique. The result in Table (1) and Fig. (1). plate (1) showed thatisolates T2, T5, T11, T12, T13, T14, T18, T20, T21 and T22 showed highest inhibition in mycelial growth in case of *R. solani* where *Trichoerma* completely overgrow on *R.solani* (100% over growth). Also the results revealed that,*Trichodermal isolates* No., T2, T3, T4, T5, T11, T13, T20 and T22 showed completely overgrowth on *pythium ultimum* (100% overgrowth). However Isolate No T19 and T17 showed lowest % of inhibition (73.31) and 68.11against mycelial growth of *R.solani* and *pythium ultimum* respectively.

Fig. (1): Effect of *Trichoderma* antagonists on the mycelial growth of Rhizoctonia solani and *pythium ultimum* under *in vitro* conditions.

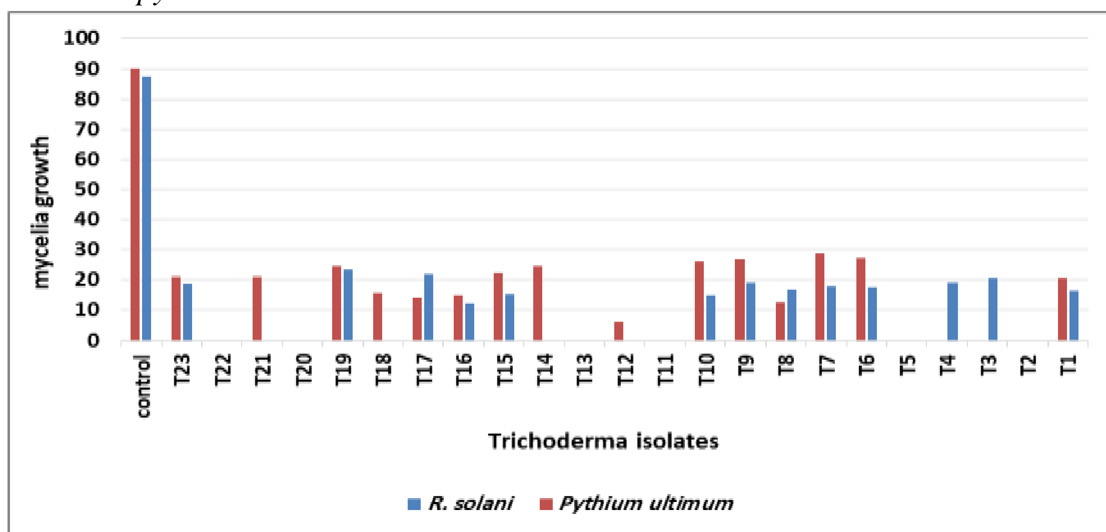

Table (1) Effect of *Trichoderma* antagonists on the mycelial growth of *Rhizoctonia solani* and *pythium ultimum* under *in vitro* conditions.

***Trichoderma* isolates**	**Tested phytopathogenic fungi**			
	Rhizoctonia solani		***Pythium ultimum***	
	Mycelial growth(mm)	**%inhibition**	**Mycelial growth(mm)**	**%inhibition**
T1	16.30	81.32	20.66	77.00
T2	0.00	100	0.00	100
T3	20.73	76.29	0.00	100
T4	19.33	77.89	0.00	100
T5	0.00	100	0.00	100
T6	17.70	79.73	27.33	69.67
T7	18.00	79.38	28.66	68.11
T8	17.00	80.53	12.66	86.00
T9	19.00	78.24	26.66	70.44
T10	15.00	82.02	26.00	71.11
T11	0.00	100	0.00	100
T12	0.00	100	6.00	93.33
T13	0.00	100	0.00	100
T14	0.00	100	24.33	73.00
T15	15.33	82.47	22.33	75.22
T16	12.33	85.91	15.00	83.33
T17	22.00	74.79	14.00	84.44
T18	0.00	100	15.66	82.56
T19	23.33	73.31	24.33	73.00
T20	0.00	100	0.00	100
T21	0.00	100	21.00	76.67
T22	0.00	100	0.00	100
T23	18.73	78.58	21.00	76.67
control	87.33	-	90.00	-

Mycelial growth and percent inhibition values are means of three replicates values within a column following by the same letter are not significantly different according to I SD test (p=0.05).

3.2. Identification of selected *Trichoderma* isolates

The ***Trichoderma*** isolates T2, T5 and T11cause completely over growth(100% inhibition) aganist tested phytopathogenic fungi and were selected for identification of their species. The identiftion were confirmed from, Mycological center, Assiut University, Egypt.

Isolate No.	AUMC No.	Identification
T2	10639	***Trichoderma atroviride*Karsten**
T5	10640	***Trichoderma koningii*Oud**
T11	10641	***Trichoderma atroviride*Karsten**

3.3. Analysis of the fungal filtrate of most active *Trichoderma* isolates

The filtrate of isolateT11 and T5obtained by filtration of these fungalculture by.using sterilized bacterial filter under sterilized conditions. The analysis of these fungal filtrate take place by Enzyme essays and identication of antibiotics which produced in filtrate. The assay method were made in research labs unit of Research National Center (Giza) and showed that T11 produce antibiotic Trichorizins13.0 ug/ml , T5 not produced this antibiotic. The enzyme which hydrolysis chitin in fungal pathogens (chitinase) produced by T11 (1.93), T5 (0.98) u.mg^{-1} protein.The results showed that the filtert of T5 produce high amount of protease enzyme 6.34 u/mg protein.

Table (2): Analysis of the fungal filtrate of most active of *Trichoderma* isolates.

No	Enzymes (U.mg^{-1} protein)	T11	T5
1	Chitinase	1.93	0.98
2	B, 1-3-exoglucanse	3.22	2.12
3	Protease	5.65	6.34
4	Cellulase	3.41	1.92
5	Poly galacturonase (PG)	7.56	3.42
6	β-glucosidase (U/mgprotein)	3.98	1.12
7	Xylanase (U/mg protein)	7.30	3.12
	Antibiotic		
1	Trichorzins PA (peptaibols) ug/mL	13	

4. Discussion:

Trichoderma koningii and *Trichoderma* atrovirde effectively inhibited the mycelial growth of tested phytopathogenic fungi *Rhizoctonia solani* and *pythium ultimum* our results similarly with results of Bhale*et. al* (2013) which showed that T. *koningii* over grow on *R.solni*.Dual culture of parhogens &*Trichoderma spp* rreveled that T. viride highly inhibited the mycelial growth over control (Faheem *etal.,* 2010).

Also shalini 2007 showed that *T. virde*&*T. aureoviride* inhibited the growth of *R.solani* (Shalini 2007). The species of *Trichoderma* significantly inhibited the mycelial growth of plant pathogenic fungi. (Rajhonda*etal.* 2011). The results showed that completely over growth of *Trichoderma koningii*& *Trichoderma autrovirde* on *R.solani*& *pythium ultimum*, Also showed that *T. autrovirde* produce antibiotic (Trichorzins). The *Trichoderma spp*. are generally considered to be aggressive competitors (samuels 1996). Our results agree with the results of (Dennis and Webster 1971a) which showed that. Many isolats of *Trichoderma spp*. Produce antibiotics these antibiotic inhibit growth of fungal pathogens.

Effective biological control agents inhibit the growth of the target organisms through their ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients (Harman, *et al*., 2004). Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens. Competition is effective when the pathogen conidia need exogenous nutrients for germination and germ-tube elongation (Elad, 2000).

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