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Original Research Article

In vitro Antagonistic Activity against *Fusarium* Species of Local *Trichoderma* spp. Isolates

Çiğdem Küçük*
Harran University, Science and Art Faculty, Department of Biology, Şanlıurfa, TURKEY

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ABSTRACT

***Trichoderma* spp. isolates were isolated from rhizosphere soils of cotton and maize. The antagonistic effects of local isolates of *Trichoderma* sp. against some *Fusarium* species such as *Fusarium solani, F.moniliforme, F.culmorum, F.verticillioides* and *F.chlamydosporum* were studied by using the dual culture technique. The isolates of *Trichoderma* spp. had a inhibitory effects on the growth of tested *Fusarium* species. Also, the influence of abiotic stress such as temperature, drought and NaCl on the growth of *Trichoderma* isolates was studied in this study. The most resistant isolates to abiotic stress were T8, T12 and T14, respectively. The specific activity of chitinase produced by local isolates was tested. The antagonist T8 isolate produced higher chitinase activity in culture supernatant.**

Keywords: Abiotic stress, Chitinase activity, Local isolate, *Trichoderma* spp.

INTRODUCTION

*Trichoderm*a species have been known to be able to attack plant pathogenic fungi to produce antibiotics and to act as biocontrol agents (Singh *et al.* 2013). *Trichoderma* species are opportunistic, avirulent plant symbionts and many phytopathogenic fungi are antagonists (Arjona-Girona *et al.* 2014; Srivastava *et al.* 2012). Kim and Knudse (2013) reported that *Trichoderma* protected agricultural crops against plant pathogens. The use of *Trichoderma* species have many advantages as biocontrol agents. Biocontrol of plant pathogens with *Trichoderma* isolates has proven to be a potential alternative to chemical control (Harman *et al.* 2004). Today, *Trichoderma* spp. are the most studied biocontrol agents and are commercially available as biofertilizer and biopesticide (Arjona-Girona *et al.* 2014). The soil application of *Trichoderma* conidial preparations has been demonstrated experimentally to increase the crop growth and increasing the plant's ability to resist *Fusarium* diseases (Ferrigo *et al.* 2014). *Trichoderma* species can suppress pathogens by competition and isolates produces growth factors that increased the rate of the plant growth (Harman *et al*. 2004, Howell 2003, Shoresh *et al*. 2010). Lytic enzymes released by *Trichoderma* isolates are very important in the biocontrol of root rot fungi such as *Rhizoctonia, Sclerotium, Phytium* and *Fusarium* species (Kim and Knudsen 2013; Mairzano *et al*. 2013; Singh *et al.* 2013; Srivastava *et al.* 2012).

Trichoderma is a fungus that exists in almost all soils and a wide range of habitats (Arjona-Girona *et al.* 2014; Srivastaus *et al.* 2012). *Trichoderma* spp. isolates are important as biocontrol agents against several soilborne pathogens including *Fusarium* species. *Fusarium* species are one of the yield limiting factors of crops in agriculture areas of the World (Kim and Knudsen 2013, Saravanakumar *et al*. 2017). The diseases caused by *Fusarium* species can effect at any stage of growth of the crop.

Hence, the present study carried to evaluate the effects to local *Trichoderma* isolates of abiotic stress factors such as temperature, salinity and drought, chitinase activity of *Trichoderma* sp. isolates and antagonistic activities of *Trichoderma* sp. local isolates against some *Fusarium* species.

MATERIALS AND METHODS

Fungal isolates

Local isolates of *Trichoderma* sp. were used in this study. *Trichoderma* isolates were isolated from rhizosphere soils of healthy cotton and maize during cropping season collected from the fields arround Harran Plain, Turkey. The soil was sieved (< 2 mm). The some physico-chemical analysis of soil samples are given in Table 1. The

* Corresponding author: ckucuk@harran.edu.tr

local strains of *Trichoderma* spp. were isolates using a *Trichoderma* selective agar (TSA) (Elad *et al.* 1981). The serial soil dilutions were spread on TSA. *Trichoderma* colonies were incubated for 10 day in light at 25 ºC (Elad *et al.* 1981). The isolates were identified to primarily on the macroscopic (pigmentation, growth rate, colour etc.) and microscopic morphology (spore morphology, formation etc.) according to the method by Gams *et al*. (1980) and Rifaii (1969). The microscopic examination was made by observing the slide after staining with lactophenol cotton blue. Isolates were preserved on Potato Dextrose Agar (PDA) slants at 4ºC.

Fusarium solani, F.moniliforme, F.culmorum, F.verticillioides and *F.chlamydosporum* were used as plant pathogenic fungi. Plant pathogenic fungi were observed from culture collection of Department of Biology, Harran University, Turkey.

Table 1. Physical and chemical properties of the soils isolated of *Trichoderma* spp. isolates.

Isolate no	Some soil properties					
	Organic matter (%)	N(%)	$CaCO_3$ (%)	EC (dS m^{-1} at 25 ºC)	pH	Texture grade
T1,T7,T16	1.46	0.090	26	0.45	8.35	clay
T2,T4, T5,T9,T12	1.37	0.084	39	0.78	8.21	clay
T3	1.57	0.069	19	0.71	8.34	clay
T6	2.16	0.084	29	0.76	8.2	clay
T8, T18	1.33	0.086	34	0.76	8.46	clay
T10	1.99	0.022	39	0.62	8.35	clay
T11	0.95	0.070	26	0.46	8.55	clay
T13	2.13	0.025	40	0.63	7.9	clay
T14	1.25	0.086	26	0.82	8.36	clay
T15	1.74	0.097	30	1.32	8.33	clay

Dual culture experiments

Competitive interactions between antagonistic *Trichoderma* spp. local isolates and plant pathogenic fungi were evaluated in dual culture experiments on petri dishes (90 mm diameter) containing 20 ml Potato Dextrose Agar (PDA). Two 5 mm diameter mycelial discs cut from 5 day old cultures of pathogenic fungi and *Trichoderma* sp. were placed at opposite sides, 30 mm apart in petri dishes and incubated in darkness at 30 ºC. Four replicates were prepared for each pairing.

Radial growth reduction was calculated in relation to growth of the control as follows;

% inhibition of mycelial growth = [(C-T)/C]x100

where C is the radial growth of pathogenic fungi in control plates; T is the radial growth of pathogen in presence of *Trichoderma* (Dennis and Webster 1971).

Determination of effects to growth of *Trichoderma* sp. of abiotic stress factors

The influence of temperature on the growth of *Trichoderma* spp. isolates was determined at 30, 45, 50 ºC on PDA for 5 days (Proosapati *et al*. 2014). The influence of different NaCl concentrations (0, 70, 150, 240, 300 and 350 mM) on the growth of *Trichoderma* spp. isolates was determined on PDA for 5 days (Mohammed *et al*. 2005).

Trichoderma spp. isolates were grown at increasing polyethylene glycol (PEG) levels (10, 20, 30, 35 and 40 %) in PDA containing petri dishes to tested drought tolerance. All these treatments were replicated there times for 5 days (Amalraj et al. 2010). The colony diameter of isolates were measured.

Enzymatic activity of isolates

The isolates were grown in synthetic medium (SM) containig (grams per liter of distilled water); glucose, 15; $MgSO_4.\ 7H_2O$, 0.2; KH_2PO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; Fe^{2+}, 0.002 and Zn^{2+}, 0.002 (Elad *et al.* 1982; El-

Katatny *et al*. 2001). Flasks containing 100 ml of liquid SM medium were inoculated with 1 ml of a conidial suspension ($1x10^8$ conidia/ml) of isolates. The level of conidia was determined in the solution using a haemocytometer. The glucose in the medium was substituted with chitin (2 mg/1). The cultures were incubated at 30 ºC for 5 days at 120 rpm. After incubation time, flaks were centrifuged at 15.000 xg at 4 ºC for 10 min (Harman *et al*. 1993). Chitinase activities of isolates were determined by following the released of 1 mol GLcNAc from chitin (Elad *et al.* 1982) Protein was determined by the method described by Bradford (1976) using Bovine serum albumin as the standard.

Statistical analysis

All the experiments were laid out in completely randomized design with three replications and the all data were analysed using of variance analysis (Yurtsever 1984).

RESULTS AND DISCUSSION

The dual culture method widely used in antagonistic assay (Arjona-Girona *et al.* 2014, Şehirli and Saydam 2016). Our results showed variations in the antagonistic activities of *Trichoderma* sp. isolates against the tested *Fusarium* species that inhibition percentage was maximum in *F.chlamydosporum* (92.6 %) with T1 and T5 isolates (Table 2). Ferrigo *et al.* (2014), Li *et al.* (2017) reported *T.asperellum, T.harzianum* as most effective growth inhibitors of *Fusarium* species under in vitro. *Trichoderma* isolates grew much faster on PDA than the tested *Fusarium* species under culture conditions. Effect of T8 and T13 against *F.moniliforme* was shown in Figure 1. The effects on pathogenic fungi of local isolates showed differences (Table 2). T3 and T4 isolates were effective against *F.verticilloides* (83.3 %). T16 isolate was inhibited the grown of *F.solani* at a rate of 84.4 %. The dual culture experiment as described by many researchers has been widely observed in antagonistic activity experiment (Altınok and Erdoğan 2015; Kim and Knudsen 2013; Küçük and Kıvanç 2004; Nakkeeran *et al.* 2005; Singh *et al*. 2013, Srivastava *et al.* 2012). T10 isolate was effective against *F.moniliforme* (82.8 %). In this study, *Trichoderma* isolates tested were determined antagonistic effect against the some *Fusarium* species. These differences; it can be caused by having different resistance to pathogens is throught to be derived from their produce different antifungal compounds of the isolates. In our study, although not single effective isolate, against of fungal plant pathogens tested, T3 and T8 isolates were found to be effective compared to other isolates against the tested *Fusarium* species. Arjona-Girona *et al.* (2014), Kim and Knudsen (2013) reported that there is no single isolate of *Trichoderma* isolates effective against plant pathogenic fungi.

Table 2. Inhibition rate (%) of growth of pathogenic fungi by *Trichoderma* spp. local isolates in dual culture.

Isolates	*F.solani*	*F.moniliforme*	*F.culmorum*	*F.verticilloides*	*F.chlamydosporum*
T1	71.1	65.7	60	56.7	92.6
T2	77.8	71.4	45	63.3	83.8
T3	64.4	65.7	67.5	83.3	88.2
T4	77.8	71.4	50	83.3	79.4
T5	77.8	57.1	63	33.3	92.6
T6	17.8	65.7	70	53.3	82.3
T7	77.8	68.5	40	50	82.3
T8	82.2	71.4	25	60	85.2
T9	77.8	65.7	37.5	56.7	91.1
T10	82.2	82.8	40	50	85.2
T11	80	57.1	50	53.3	80.8
T12	73.3	65.7	47.5	46.7	86.7
T13	77.8	71.4	52.5	50	83.8
T14	73.3	71.4	42.5	63.3	77.9
T15	73.3	62.5	35	56.7	79.4
T16	84.4	68.5	55	50	82.3
T17	60	60	35	60	85.2
T18	64.4	62.8	37.5	40	79.4

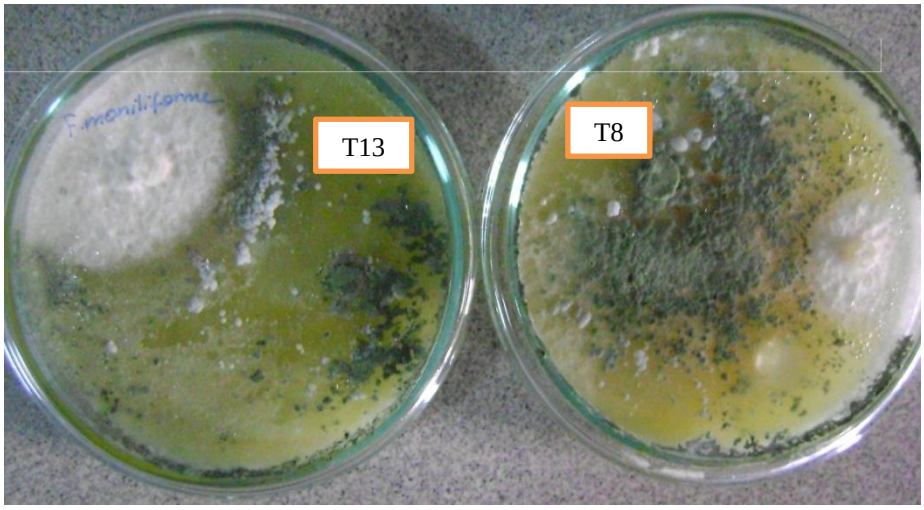

Figure 1. Effects of T13 and T8 against mycelial growth of *F.moniliforme* in dual culture assay.

Today, the majority of researchers has focused on the determination of psychrophilic and salt tolerant isolates of *Trichoderma* (Amalraj *et al*. 2010; Rawat *et al.* 2013). Also, the isolates of *Trichoderma* developed at temperatures above 35 ºC have been reported (Poosapi *et al*. 2014). In this study, all of the isolates showed good development at 30 ºC (Table 3). T1, T3, T4, T15 and T17 isolates were formed quickly the mycelia at 45 ºC (Table 3). Also, the spore formation of isolates with the increase of temperature was reduced.At 50 ºC, the growth of isolates decreased. Similarly, Yu et al. (2015) reported that mycelial growth of *Trichoderma* was highly sensitive to heat stress. T1, T3, T4, T15 and T17 isolates can grow effectively in hot climates. The results of the temperature tolerance of isolates were statistically analysed as given Table 3. Treatments, control and other, other mean squares are significant at 1 % level. Isolates best grown at 30 ºC. In this study, tolerance experiments on salt and drought were carried out at 30 ºC. The growth of *Trichoderma* sp. is affected by soil temperature, the water activity, moisture level of the soil (Kredics *et al.* 2003). Kredics *et al*. (2003) reported that the combinations or abiotic stress were negatively affected the growth of fungi. It has been determined that all isolates growth in medium containing 10 % PEG (Table 3). The growth of isolates were decreased at increasing PEG concentrations. This suggests that isolates are more sensitive to drought conditions (Table 3). T13, T10 and T8 isolates respectively have been observed as the most drought-resistant isolates. The isolates were showed different tolerance at different temperature and PEG treatments. There are differences in treatments ($p<0.01$). Similar observations have been reported (Kredics *et al*. 2003). The tolerances of the isolates to temperature and drought differed (Table 3).

Table 3. The medium mycelial growth (mm) of isolates at different temperature and drought levels.

Isolates	Temperature (°C)			PEG (%)			
	30	45	50	10	20	30	35
T1	86	90	41	90	32	15	15
T2	84	33	11	65	33	22	21
T3	82	90	26	70	40	12	10
T4	90	90	34	75	30	10	10
T5	83	44	30	90	35	16	15
T6	84	78	33	90	40	16	14
T7	82	62	41	90	21	15	15
T8	86	70	32	90	36	15	15
T9	84	61	48	80	40	22	20
T10	87	41	33	90	80	50	37
T11	90	83	31	90	65	16	14
T12	86	78	41	90	60	21	19
T13	87	41	33	90	75	70	68
T14	83	80	40	90	37	20	20
T15	90	90	39	90	30	13	12
T16	87	82	30	72	40	20	20
T17	86	88	39	90	30	18	15
T18	52	25	18	57	52	35	33

Variation source	degrees of freedom	Mean square	Variation source	degrees of freedom	Mean square
Replication	1	7.1	Replication	1	77903.1
treatment (tre)	3	22304.67**	treatment (tre)	3	42964.5
Control and others	1	188881.3**	Control and others	1	53613.01**
Others	2	24016.3**	Others	2	37640.2
Isolate (Iso)	17	669.6**	Isolate (Iso)	17	5548.2
Iso x tre	51	219.7	Iso x tre	68	615.1**
Error	72	1.19	Error	89	1.31

** significant %1 level

The mycelial growth of local isolates of *Trichoderma* sp. was examined in media containing different concentrations of NaCl (Table 4). At 70 mM NaCl, growths of T3, T4, T5, T6, T7, T8, T9, T10, T1, T13 and T14 isolates were not affected. One of the environmental factors the limiting antagonistic activity of *Trichoderma* species was determined as salinity (Rawat *et al*. 2013; Poosapati *et al.* 2014). It has been explained that the antifungal metabolites of the isolates reduce against salinity (Mohammed *et al.* 2005; Rawat *et al*. 2013). In 350 mM NaCl, the mycelial growths of T12 and T2 were inhibited at the highest rate (77.8 % and 72.2 %, respectively). As seen in Table 4, the growth of isolates affected at different rate in increased salt levels. T18 isolate has been most affected. The growth of T4 isolate was not affected in 150 mM NaCl. The most resistant isolates to abiotic stress were T8, T12 and T14, respectively and the most sensitive isolates were examined as T3, T18 and T11.

Table 4. Inhibition (%) of mycelial growth of *Trichoderma* sp. isolates in NaCl levels.

Isolates	NaCl (mM)				
	70	150	240	300	350
T1	7.08	10	15.6	66.7	61.1
T2	2.22	6.67	13.3	50	72.2
T3	-	3.3	8.8	18.9	20
T4	-	-	4.4	11.1	31.1
T5	-	1.2	2.2	8.9	35.6
T6	-	1.1	6.67	25.6	35.6
T7	-	1.2	1.2	4.4	16.7
T8	-	1.6	11.1	15.6	15.6
T9	-	17.8	23.3	42.2	51.1
T10	-	3.3	15.6	37.8	55.6
T11	-	4.44	4.44	8.9	33.3
T12	14.4	22.2	27.8	52.2	77.8
T13	-	8.9	12.2	13.3	27.8
T14	-	11.1	22.2	26.7	38.9
T15	2.2	4.4	13.3	21.2	28.9
T16	8.9	18.9	20	25.6	31.6
T17	11.1	22.2	14.4	16.7	40
T18	3.3	34.4	44.4	48.9	52.2

We have compared the activity of chitinase of *Trichoderma* isolates. The chitinase activities of isolates are seen in Table 5. The levels of production of chitinase showed differences among tested local isolates (Table 5). Similar results in different isolates of *Trichoderma* have been observed (El-Katatny *et al.* 2006; Elad *et al*. 1982, Harman *et al*. 1993, Küçük and Kıvanç 2004). When all of the isolates tested were compared, the highest enzyme production was observed in T8 (47 mU mg protein $^{-1}$). As shown in Table 5, the enzyme activity was found to different between isolates ($p<0.01$). The significance of the difference in values was determined through ANOVA at a significance level of 0.01. The lowest chitinase activity was obtained in T18 (6.2 mU mg protein $^{-1}$). Harman *et al.* (1993), determined that chitinase activity was highly dependent on the isolate. The most fungi have cell walls that contain chitin as a structural backbone and laminarin as a filling material. Chitinase activity produced by *Trichoderma* species is important for the degradation of cell walls of plant pathogenic fungi during mycoparasitic attraction (El-Katatny *et al*. 2006; Elad *et al*. 1982).

Table 5. Chitinase activities by *Trichoderma* sp. isolates and analysis of variance

Isolate	Specific activity (mU mg protein^{-1})	Isolate	Specific activity (mU mg protein^{-1})
T1	15 ± 0.06	T10	25 ± 0.04
T2	31 ± 0.00	T11	27 ± 0.03
T3	21 ± 0.02	T12	20 ± 0.06
T4	17 ± 0.06	T13	36 ± 0.05
T5	24 ± 0.03	T14	25 ± 0.03
T6	27 ± 0.07	T15	21 ± 0.07
T7	12 ± 0.01	T16	29 ± 0.02
T8	47 ± 0.08	T17	12 ± 0.02
T9	10 ± 0.01	T18	6.2 ± 0.01
Variation sources	Degrees of freedom		Mean square
Replication	1		4.69
Isolate	17		272.7**
Error	17		1.03

Values are the means ± SD of two measurements. Significant at the **0.01 level of probability

CONCLUSIONS

In this study, the effects of abiotic stress factors on *Trichoderma* isolates were observed and the antagonistic activities of local isolates were studied against some *Fusarium* species such as *F.solani, F.culmorum, F.moniliforme, F.verticilloides* and *F.chlamydosporum*. Also, local *Trichoderma* isolates were produced chitinase in liquid medium. *Trichoderma* isolates have a very tolerance of temperature, NaCl and PEG. Adaptation of *Trichoderma* isolates to environment with different stress factors seems to be an important mechanisms of evolution enabling the effective biocontrol activity against plant pathogens. The quality of the activity produced was specific to isolate. The chitinase activity produced by local isolates of *Trichoderma* sp. may be effective in biological control of *Fusarium* species.

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