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Research Article

In vitro efficiency of *Trichoderma harzianum* mutants in biocontrol of *Fusarium oxysporum* f. sp. *radicis-cucumerinum*

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Abstract: *Trichoderma* is very important as a bio control agent and probably a good alternative for chemical fungicides. *Fusarium oxysporum* is a plant pathogen that causes wilt in a wide range of plants. The use of gamma irradiation can be employed to increase *Trichoderma* efficiency against *F. oxysporum*. The induced mutation provides genetic changes in *Trichoderma* and in some of the mutated isolates the efficiency of bio control may be improved. In this study the efficiency of mutated *T. harzianum* CS5 against the *F. oxysporum* f.sp. *radicis-cucumerinum* was evaluated. Among 16 wild isolates of *Trichoderma*, CS5 isolate was chosen based on synchronous and nonsynchronous mutual cultivation with pathogen. The spore suspensions of *T. harzianum* CS5 were irradiated in a cobalt- 60 γ- irradiator at a dose rate of 0.23 Gy/Sec in Nuclear Agriculture Research School, (NSTRI, AEOI). Then antagonist screening of 76 mutants was investigated in the experiments of synchronous and nonsynchronous dual culture with pathogen. The results showed that only 17 mutants were able to control the pathogen better than wild type, and YFTM80 isolate had the most prevention. Using the ERIC-PCR marker, the distinction was investigated between the wild type and the 17 selected mutants. The results showed that the gamma ray is able to improve biocontrol efficiency of *Trichoderma* and the ERIC-PCR marker can differentiate among derived mutants, but it does not have the ability to distinguish mutants from wild type. In terms of antagonistic superiority, mutations might have occurred in antagonistic sites, which have led to improved antagonistic efficiency. Probably the ERIC-PCR marker has failed to replicate these areas.

Keywords: *Trichoderma,* Gama ray, Mutation, *Fusarium oxysporum* f. sp. *radicis-cucumerinum*

Introduction

One of the alternative methods to control pathogens is to use biological control agents instead of chemical pesticides. Biological control is a safe and suitable method for humans and the environment. The population of biocontrol agents is controlled by the presence of a pathogenic agent, which is one of the advantages of using biological control method. Trichoderma is one of the most suitable biocontrol agents. This fungus has the ability to control many plant pathogens

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(Waghunde *et al*., 2016). Owing to its potential, researchers have been looking into ways to enhance antagonistic levels of these fungi. One approach is inducing mutations whereby it is possible to create more effective strains. So mutation can help improve the biocontrol agents (Abbasi Iranog *et al*., 2014). Cucumber *Cucumis sativus* L. is an important agricultural product in Iranian families basket. The high volume of cucumber cultivation under greenhouse conditions has caused increase in pathogens and insect population and increased damage. Diseases such as cucumber mosaic, bacterial wilt, anthracnose, stem and root rot and pests such as aphids, white flies, beetles and mites can cause much damage to the plant. One of the most important diseases of this plant in the world is fusarium wilt. Farmers use different methods to cope with this disease, including agronomic, chemical and biological control. There are reports of resistance to fusarium wilt. Certain cultivars have been introduced as resistant cultivars (Molavi *et al.*, 2009; Shahriari *et al.*, 2011); however, due to the spread of pathogenic strains, there is not yet a suitable cultivar with resistance to all strains (Shahriari *et al.* 2011). One of the safest methods to control pests and diseases is biological control. The *Trichoderma* genus was identified by Persson over 200 years ago (Persson, 1794). *Trichoderma* is the non-sexual form of Hypocreales (Samuels and Seifert, 1987). This fungus is a soil inhibitor and is present in the rhizosphere of plants (Samuels, 1996). Some strains are saprophyte and some of them have antagonistic properties (Samuels, 1996; Elahinia, 2008). Trichoderma species, including *T. viridae*, *T. virens*, and mostly *T. harzianum*, have been known for their greater antagonistic effects against pathogenic fungi (Rahdar, 2012). Their efficacy is owing to high sporulation rate, good survival ability under adverse conditions, effective competition for food, and ability to ameliorate rhizosphere and high mycoparazitism (Rahdar, 2012; Moradi *et al.,* 2013). *Trichoderma* is also useful for plants as a plant symbiont, and has several evolved mechanisms that improve the plant resistance to plant pathogens (Harman, 2006). Moreover, Trichoderma controls the ascomycetes and basidiomycetes fungi, and acts better in acidic than in alkaline soils (Rahdar, 2012). Various *Trichoderma* species have been used to control Fusarium Pathogens in different plants (Fani *et al.,* 2013; Norouzi *et al.,* 2014; Khatib *et al.,* 2017).

In this research, mutation was used to improve the efficiency of Trichoderma fungus as a biocontrol agent against *Fusarium oxysporum* f. sp. *radicis-cucumerinum*. Afterwards, derived mutants were compared with wild isolates in respect to antagonistic properties; and finally some isolates have been introduced with higher antagonistic effect than the wild isolate. Genetic diversity of mutant isolates has also been investigated using the ERIC-PCR marker.

Materials and Methods

Isolation and identification of pathogenic fungi

Wilted cucumber plants from greenhouse of Chemistry Company in Yazd province were collected and washed under tap water, the infected crown and stem were cut and were disinfected with 70% ethanol for 30 seconds. Samples were cultured in a solid medium (containing vegetable extracts (V8®)) and incubated at 25 °C. The fungus was isolated and purified after 3 days from the culture medium. pathogenicity of isolated fungi was tested according to the Koch's principles. The fungus was reintroduced into healthy plants after isolation and re-purification, and then the pathogen was isolated from the inoculated plants after 10 days.

Preparing Trichoderma fungi

Sixteen isolates of *T. harzianum* were received from the Agricultural Research and Education Centre of Yazd province. Their code and collection area are shown in Table 1. The isolates were cultivated on a solid medium Trichoderma selective medium (TSM).

Table 1 The code and location of the gathering *Trichoderma harzianum* isolates.

Code	Gathering location	Geographical location	Code	Gathering location	Geographical location
CT1	Cucumber Rhizosphere	Taft	CY27	Cucumber Rhizosphere	Yazd
SB1	Eggplant rhizosphere	Bafgh	SB2	Eggplant rhizosphere	Bafgh
SA2	Cucumber Rhizosphere	Abarkooh	CB1	Cucumber Rhizosphere	Bafgh
CS5	Cucumber Rhizosphere	Sadoogh	CS2	Cucumber Rhizosphere	Sadoogh
CA1	Cucumber Rhizosphere	Abarkooh	CS12	Cucumber Rhizosphere	Sadoogh
CY16	Cucumber Rhizosphere	Yazd	CS6	Cucumber Rhizosphere	Sadoogh
CT16	Cucumber Rhizosphere	Taft	CY7	Cucumber Rhizosphere	Yazd
CS4	Cucumber Rhizosphere	Sadoogh	CY9	Cucumber Rhizosphere	Yazd

Experiment of Trichoderma antagonistic against the pathogen

In this research, synchronous and asynchronous mutual cultivation was applied. This experiment was conducted in two steps.

Synchronous mutual cultivation

In this experiment, Trichoderma and pathogen (*F. oxysporum* f.sp *radicis cucumerinum*) were cultivated simultaneously on PDA medium. On one side of the petri dish was placed a disc with 5 mm diameter from three-day culture of the pathogen and on the other side, at a distance of 6 cm, a disc with 5 mm diameter from the 3 days old culture of Trichoderma was placed. (Ashrafizadeh *et al.,* 2003). As control, instead of Trichoderma, a disc of pure PDA medium with 5 mm diameter was used. Petri dishes were then kept in incubator at 25 °C. After 3 days, the growth rate of *F. oxysporum* was measured and data were placed in the following formula and the inhibition percentage was calculated in comparison with the control.

$$IG = (C - T) / C \times 100$$

Where IG = Inhibition percentage of *F. oxysporum* growth, C = Colony diameter of the *F. oxysporum* synchronous experiment without Trichoderma and T = Colony diameter of the *F. oxysporum* in synchronous mutual cultivation with Trichoderma.

Asynchronous mutual cultivation

This experiment was conducted as synchronous mutual cultivation, with the difference that the Trichoderma was cultured 24 hours after *F. oxysporum*. The two interactive cultures of synchronous and asynchronous were compared in factorial experiment.

Selection of superior isolate

Based on the inhibition percentage in mutual cultivation of synchronous and asynchronous, and comparing the interaction between these two types of mutual cultivation, the best isolate (CS5) was selected for mutation.

Induction of mutation on the selected isolate

After selection of superior antagonist isolate of the *Trichoderma*, washed spore suspensions (containing 10^6 spores in 1 ml) were prepared from 7-day-old culture of that isolate in sterile saline solution (9 $g.l^{-1}$ NaCl in distilled water) and irradiated in a cobalt-60 γ- irradiator (Issledovatel Gamma-cell, PX-30) at a dose rate of 0.23 Gy/sec, in irradiation Lab. of Nuclear Agriculture Research School, Alborz. Optimum dose of γ-radiation for inducing mutation in *Trichoderma* was selected based on approximately 40-50% inhibition of spore germination (Moradi *et al*., 2013) and spore suspensions irradiated at 250 Gy (in triplicate). Dosimetry was performed with Ferick reference standard dosimeter system.

After irradiation process, the serial dilution of spore suspensions (10^{-1} to 10^{-4}) were performed and 100µl from 10^{-4} suspension was cultured on water agar medium plates (incubated at 28 °C). The germinated Trichoderma spores were isolated every six hours using binocular and transferred to new PDA medium plates under sterile conditions (up to 72 h). 500 irradiated colonies were collected separately and re-cultured on PDA medium three times to ensure stability of morphological traits and colony forms of mutants.

A total of 102 mutants were selected based on higher spore production ability compared with the un-irradiated isolate, and growth rate of mutants were measured on PDA medium at 28 °C for 72 h. Spore suspensions were taken from the mutants and used in subsequent cultures.

Antagonistic tests against the pathogen

The experiments of synchronous and asynchronous mutual cultivation on mutants were performed similarly to Trichoderma wild type.

Molecular identification of *F. oxysporum* and wild superior *Trichoderma*

To prepare the pathogen mycelium, the fungus was cultured on PDA medium for a week and DNA was extracted. To prepare mycelium of *Trichoderma* fungus, 10 ml of *Trichoderma* spore suspension was cultured in malt medium, and then placed in a shaker incubator at 150 rpm and 29 °C. After two days, mycelium was separated from the liquid medium**.** DNA extraction was performed from mycelial tissue using modified CTAB method (Moradi *et al.*, 2010). The molecular identification of pathogen was done by amplification of tef region from fungal genome using EF1/ EF2 primers (Fig. 1). To amplify the genomic DNA with the EF1/EF2 primers, the following sequences were used respectively. 5′-ATGGGTAAGGA(A/G)GACAAGAC-3′ and 5′-GGA(G/A)GTACCAGT (G/ A)ATCATGTT-3′ (Karlsson *et al.*, 2016).

The PCR was carried out in ThermoCycler (BIORAD® T100TM, Germany) as follows: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles (94 °C for 50 seconds, annealing of primers at 60 °C for 50 seconds and final expansion at 72 °C for one minute), and then final expansion at 72 °C for 5 min. To increase the validity of the results, positive and negative controls were used for all PCR reactions. Electrophoresis was performed with agarose gel 1.5% for one hour. Separated bands of both pathogen and *Trichoderma* were purified and sequenced by the South Korean Macrogene Company (Posted by Pishgam Biotech Company). The obtained sequences for Tef areas were first reviewed with the BioEdit software, and then sequences were presented in the Gene Bank database (www.ncbi.nlm.nih.gov/BLAST). The sequences related to pathogen were compared based on the highest similarity to the sequences found in the Gene Bank and in the FUSARIUMID program (http://isolate.fusariumdb.org/) and sequences related to *Trichoderma* were compared on the basis of the highest percentage of similarities to the sequences in the Gene Bank and the TrichoKEY program on the Trichoderma site (http://www.isth.info/tools/molkey/index.php)
At the end, the sequences were registered on the NCBI site (Raja *et al.*, 2017).

Determination of genetic variation of mutants using ERIC-PCR marker

This marker is one of the Rep-PCR markers that are used to determine the genetic variation of fungi. In this study, we used ERIC 1R and ERIC 2I primers with the sequences of 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and 5′-AAGTAAGTGACTGGGGTGAGCG-3′ respectively (Dorneles *et al.,* 2014).

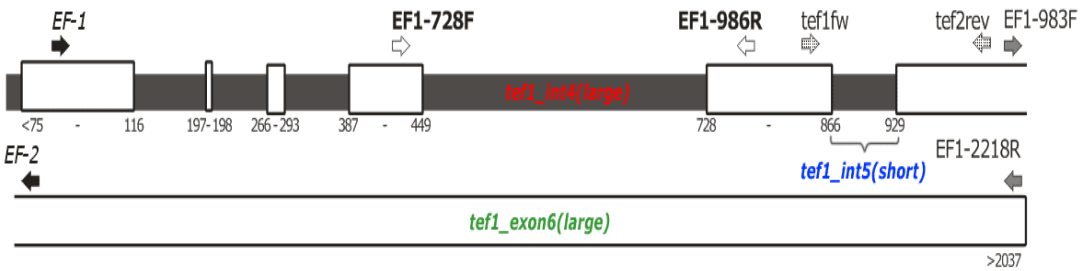

Figure 1 The studied sequences include the intron 4 tef1 (large) (about 300 bp) and the intron 5 tef 1 (small) (about 90 bp).

The PCR reaction was performed in the Thermocycler machine (Primus®, The UK) with the following conditions: Initial denaturation at 95 °C for 7 min and then 30 cycles: denaturation at 90 °C for 30 s, annealing at 52 °C for one minute, extension at 65 °C for 8 minutes, and final extension at 65 °C for 16 minutes. To increase the validity of the results, positive and negative control was considered in all PCR experiments. Electrophoresis was performed with agarose 1.5% for 90 min.

Statistical analysis of data

All statistical analyzes of Mutual cultivation were conducted as factorial (b × a) in a completely randomized design with three replications using SAS 9 software. The Arcsin $\sqrt{\%}$ formula was used to normalize the data. Grouping of the treatments was performed using LSD test at the level of $p < 0.01$. After detection of bands and giving 1 and 0 to the presence or absence of bands in each isolate in Excel software, analysis of obtained data from ERIC1R and ERIC2I primers was performed with NTSYS 2.02e software.

Results

Identification of the pathogenic fungus

After isolation and purification of the pathogenic fungus, its pathogenicity was confirmed by Koch's principles (Fig. 2). Based on the symptoms observed in inoculated plants and ontogenic and morphologic characteristics of isolated fungi from infected parts, this pathogen was detected as *F. oxysporum* f.sp. *radicis cucumerinum*.

The pathogen's molecular identification was also performed with the amplification of tef in the pathogenic fungus, using EF1/EF2 primers and a band of about 800 bp was detected (Fig. 3A). The results obtained from the comparison of amplified band sequences is shown in (Fig. 3B)

In the pathogenic fungus with reference sequences using the BioEdit software and the NCBI database and the Fusarium ID program showed 99% similarity. The total length of the sequenced region was approximately 700 bp. The sequences were registered on the GenBank of NCBI site with accession numbers of MN610445.

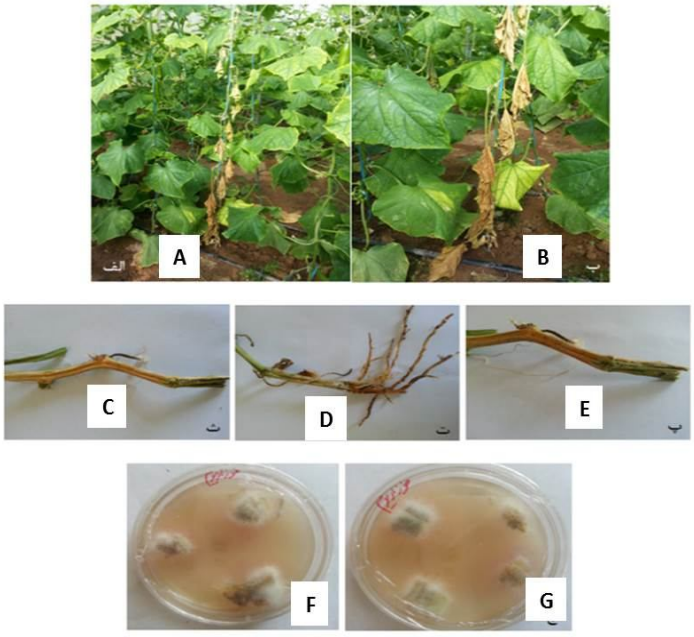

Figure 2 A and B: Cucumber bush infected with *Fusarium oxysporum*, next to the healthy cucumber bush. C, D, and E: root and shoot of infected cucumber. F and G: Pathogenic fungus 3 days after cultivation of infected cucumber samples on PDA medium.

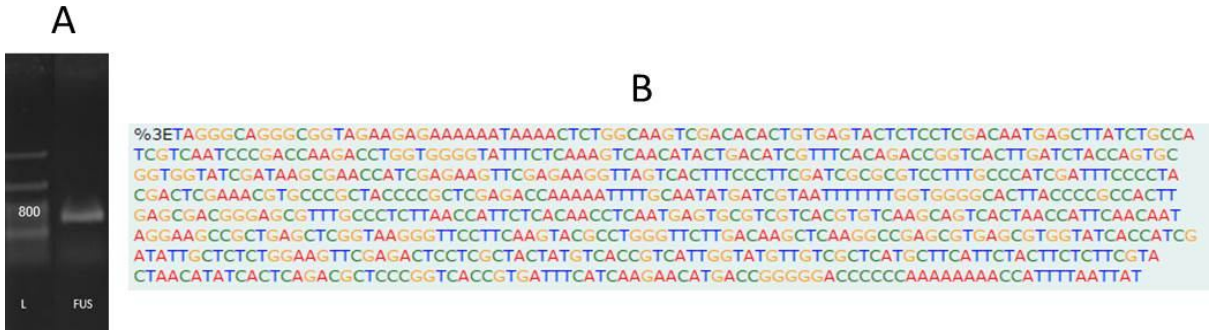

Figure 3 A: The amplified 800bp band of tef area in *Fusarium oxysporum* Using EF1/EF2 Primers, and B: The sequence of band.

Mutual cultivation of Trichoderma wild isolates against the pathogen

Analysis of variance showed that each one of synchronous and non-synchronous mutual cultivation and interactive effect of them was significant at 1% level (Table 2).

Comparison of Fusarium growth inhibition with Trichoderma isolates showed that all isolates of Trichoderma in both synchronous and non-simultaneous mutual cultivations inhibited the growth of Fusarium significantly (Fig. 4 and Fig. 5). Trichoderma isolates were divided into six groups in terms of mean inhibitory growth of Fusarium. The mean inhibition of Fusarium growth was between 40% and 70%. In the first group, two isolates belonged to the rhizosphere of cucumber in Sadough (CS5 and CS6). In the second group, an isolate belonged to the rhizosphere of the eggplant in Bafgh (SB1). In the third group, the isolate was from the rhizosphere of the eggplant in Bafgh (SB2), the fourth group consisted of five isolates from the rhizosphere of cucumber and Taft, Abarkuh, Sadoug and Yazd (CA1, CS12, CT1, CY7 and CS2). The fifth group consisted of six isolates belonging to rhizosphere of cucumber from Bafgh, Yazd, Taft, Sadoug and Abarkouh (CY16, CY27, CS4, CT16, CB2 and CA2). The sixth group included an isolate from the rhizosphere of cucumber from Yazd city (CY9) (Fig. 5). The non-significant difference in the mean of isolates between synchronous and non-simultaneous mutual cultivation showed good performance of isolates in both tests (Table 3). According to the results obtained from mutual cultivation, it was revealed that CS5 and CS6 isolates had the highest competitive ability with 68.59 and 68.23% inhibition respectively.

Induction of mutation in superior Trichoderma isolate

After mutation on CS5 isolate by Gamma gamy, and cultivation of mutants on the PDA medium, some mutants were destroyed in the first culture that indicates destruction of the heredity material as a result of mutation on the important genes by the Gamma gamy. Out of 102 primary isolates, 76 isolates could grow in PDA medium after three subcultures and were used for antagonistic tests.

Mutual cultivation of mutants against the pathogen

The results of analysis of variance showed that each of synchronous and asynchronous mutual cultivation alone or in opposition to each other were significantly different at P = 1% (Fig. 6).

Comparison of *Fusarium* growth inhibitory by *Trichoderma* mutant isolates indicated that all of the isolates controlled *Fusarium* growth at both synchronous and asynchronous mutual cultivation significantly (Table 4). The mean of the inhibition effect of mutant strains of *Trichoderma* was between 63.66- 53.83%. The mutant strains of Trichoderma were classified into 39 groups in terms of the mean of Fusarium growth inhibition. The wild isolate and the YFTM36 isolate were grouped together. In general, the average of

inhibitory effect of 16 mutants was increased in comparison with the wild type. These results showed that most of the isolates had better results in synchronous mutual cultivation (Table 5).

The results of mutual cultivation showed that the isolate of YFTM80 with 53.83% growth inhibition, had higher competition for food gain than wild type and other mutated isolates.

Table 2 Analysis of variance of synchronous and non-synchronous mutual cultivation.

Sources	MS	df
Synchronous mutual cultivation	401.35**	15
Asynchronous Mutual cultivation	4797.80**	15
Synchronous mutual cultivation * Asynchronous Mutual cultivation	294.88**	15
Error	108.80	64
CV	20.09	

** Indicates significance at 1%.

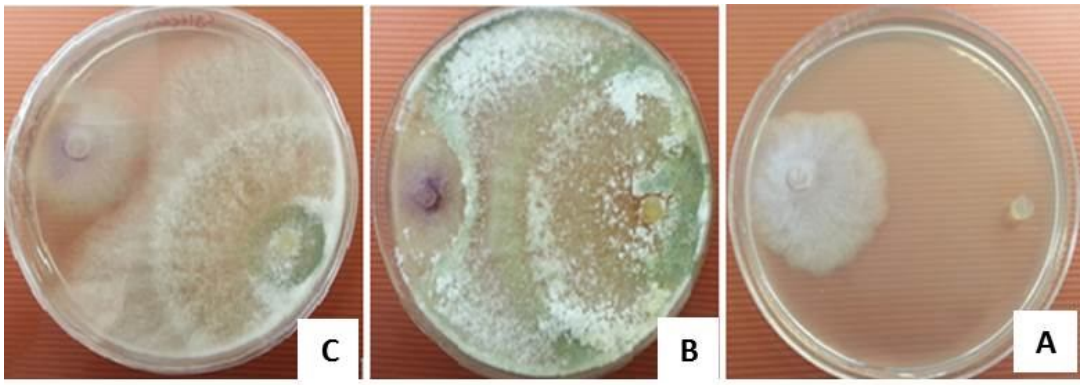

Figure 4 Mutual cultivation: A: control, B: synchronous mutual cultivation and C: Asynchronous Mutual cultivation (Trichoderma on the right and Fusarium on the left side of the petri dish).

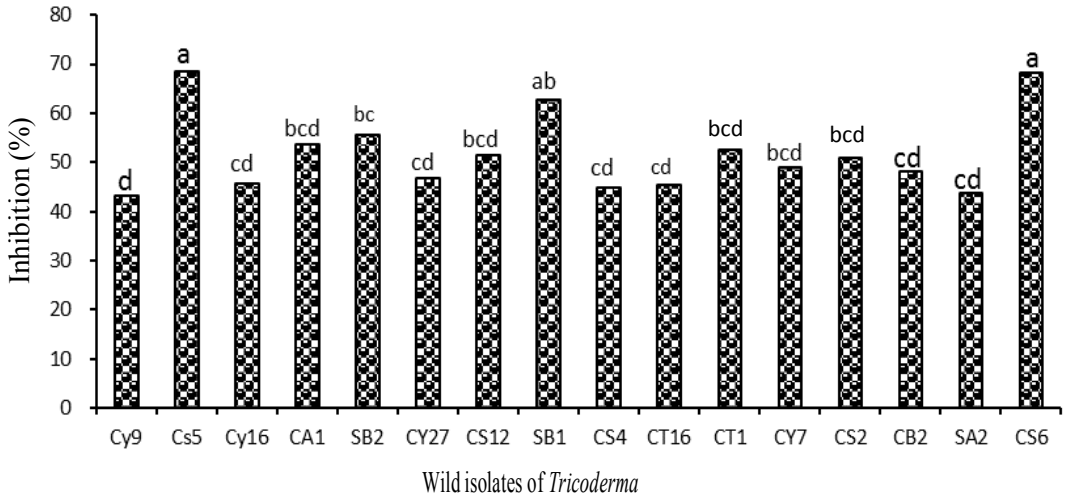

Figure 5 The percentage of *Fusarium* growth inhibition by *Trichoderma* isolates. Means with similar letters based on LSD test did not show any significant difference at 1% level.

Table 3 Comparison of the mean of inhibition percentages of *Fusarium* growth in synchronous and asynchronous cultivation of isolates.

Isolate	Synchronous mutual cultivation	Asynchronous mutual cultivation	Significance
Cy9	48.59	38.03	ns
Cs5	64.10	73.07	ns
Cy16	52.34	39.11	ns
CA1	54.29	52.85	ns
SB2	68.80	42.50	**
CY27	58.25	35.35	**
CS12	44.02	58.83	ns
SB1	81.48	43.91	**
CS4	50.61	38.98	ns
CT16	55.22	35.36	ns
CT1	64.98	40.27	ns
CY7	56.83	41.22	ns
CS2	64.79	37.11	*
CB2	59.94	36.22	ns
SA2	51.02	36.22	ns
CS6	68.22	68.22	ns

* and ** indicate significant differences at 5 and 1% probability level, respectively. Ns: Non-significant differences.

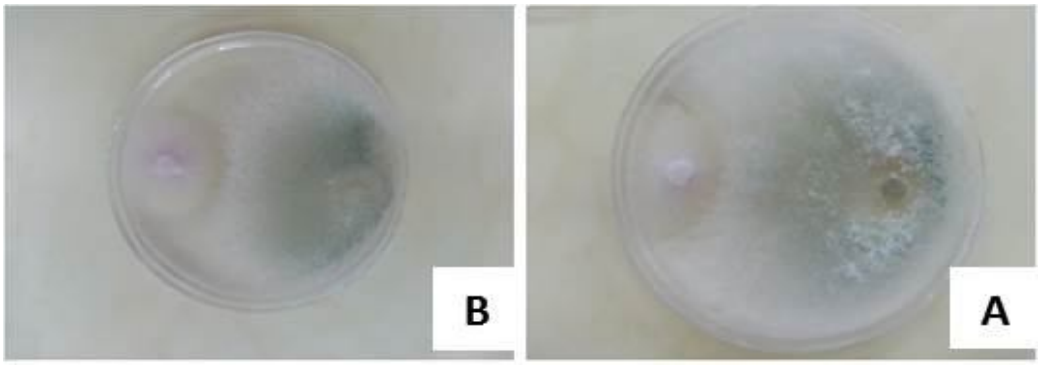

Figure 6 Mutual cultivation of mutant *Trichoderma* isolates on PDA medium after 3 days: A: Synchronous mutual cultivation and B: Asynchronous mutual cultivation (*Trichoderma* on the right and *Fusarium* on the left).

Molecular identification of the superior isolate of Trichoderma

The amplification of tef region in the superior isolate was done successfully using EF1/EF2 primers and a band of about 800 pairs was replicated (Fig. 7A). Comparison of the result obtained from the isolate sequence (Fig. 7B), using the BioEdit software and the NCBI database and the TRICHOKEY program, showed that the superior isolate is valid as *T. harzianum* with a similarity of 99% and 100% with reference sequences. The total length of the sequenced region was approximately 900 bp. The sequences were registered on the GenBank of NCBI site with accession numbers of MN610446.

Genetic variation of mutants with ERIC-PCR marker

The amplification with ERIC1R and ERIC2I primers was successfully achieved for the mutants. Ten bands (with length 200 bp-2000 bp) were amplified (Fig. 8).

After determining the number and weight of bands in different isolates, analysis was performed with NTSYS 2.02e software. The obtained dendrogram showed that this marker is able to differentiate mutant isolates (Fig. 9).

Table 4 Comparison mean of inhibitory effect of *Trichoderma* mutated isolates against *Fusarium* growth.

Mutated Trichoderma isolates	Percentage of inhibition	Group
YFTM80	53.83	a
YFTM46	53.18	ab
YFTM95	51.91	a-c
YFTM35-YFTM83	51.32-51.62	a-d
YFTM47	50.19	a-e
YFTM62	48.36	a-f
YFTM21	47.84	a-g
YFTM86	47.41	a-h
YFTM69-YFTM29-YFTM70-YFTM66	45.93-47	a-i
YFTM17	45.66	b-j
YFTM74-YFTM42	45.10-45.13	b-k
YFTM36-CS5	44.71-44.75	c-l
YFTM15-YFTM3-YFTM67	44.24-44.48	c-m
YFTM102-YFTM43-YFTM55	33.05-44.20	d-n
YFTM60	43.61	d-o
YFTM5	43.06	e-o
YFTM28	42.58	e-p
YFTM57	42.47	f-q
YFTM87-YFTM73-YFTM88-YFTM59	41.87-41.93	f-r
YFTM48-YFTM53	41.33-41.45	f-s
YFTM11-YFTM31	40.38-40.70	f-t
YFTM101-YFTM14-YFTM49-YFTM4-YFTM44-YFTM12	39.90-40.14	f-u
YFTM37-YFTM96-YFTM16	39.36-39.40	g-u
YFTM45-YFTM6	39.28	h-u
YFTM61	37.77	i-u
YFTM1	7.22-37.50	j-v
YFTM26-YFTM39-YFTM52-YFTM64	36.42-36.66	k-v
YFTM32-YFTM18	36.12	l-v
YFTM25	35.84	n-v
YFTM65	35.84	o-v
YFTM24-YFTM2	35.56-35.58	o-w
YFTM10-YFTM71-YFTM9	34.70-34.79	p-w
YFTM8	34.41	q-w
YFTM22-YFTM63-YFTM40	34.06-34.12	r-x
YFTM13	33.41	s-x
YFTM34-YFTM51-YFTM33	32.65-33.19	t-x
YFTM30-YFTM7	30.08-30.90	v-x
YFTM85	27.75	wx
YFTM38	26.69	x

Means followed by similar letters based on LSD test did not show any significant difference at 1% level.

Table 5 Comparison of the mean of inhibition percentages of Fusarium growth in synchronous and asynchronous cultivation of mutated Trichoderma isolates.

Isolate	Synchronous mutual cultivation	Asynchronous mutual cultivation	Sig.	Isolate	Synchronous mutual cultivation	Asynchronous mutual cultivation	Sig.
CS5	55.07	34.35	**	YFTM42	62.82	27.38	**
YFTM1	49.89	25.64	**	YFTM43	61.54	26.58	**
YFTM2	39.52	31.59	ns	YFTM44	50.85	29.95	**
YFTM3	50.95	37.54	*	YFTM45	52.34	26.45	**
YFTM4	59.91	21.09	**	YFTM46	77.27	29.08	**
YFTM5	57.85	28.26	**	YFTM47	78.46	21.91	**
YFTM6	49.68	29.04	**	YFTM48	59.91	22.99	**
YFTM7	46.81	13.35	**	YFTM49	52.99	28.24	**
YFTM8	53.86	14.96	**	YFTM51	44.08	22.15	**
YFTM9	55.34	14.06	**	YFTM52	58.10	16.49	**
YFTM10	46.60	22.97	**	YFTM53	64.91	17.75	**
YFTM11	49.54	32.61	**	YFTM55	59.01	29.08	**
YFTM12	52.52	28.24	**	YFTM57	59.37	25.57	**
YFTM13	42.09	24.73	**	YFTM59	58.09	25.65	**
YFTM14	52.25	29.15	**	YFTM60	56.39	30.83	**
YFTM15	50.95	39.02	*	YFTM61	46.11	32.45	*
YFTM16	53.31	26.48	**	YFTM62	66.71	30.02	**
YFTM17	64.86	26.45	**	YFTM63	48.73	19.44	**
YFTM18	42.04	30.81	ns	YFTM64	49.65	24.78	**
YFTM21	48.35	47.33	ns	YFTM65	48.59	23.08	**
YFTM22	43.44	24.80	**	YFTM66	54.75	37.11	**
YFTM24	50.85	20.31	**	YFTM67	61.54	27.42	**
YFTM25	48.50	23.75	**	YFTM69	61.42	32.58	**
YFTM26	61.00	14.00	**	YFTM70	65.09	27.36	**
YFTM28	55.18	29.99	**	YFTM71	47.51	22.02	**
YFTM29	58.43	35.27	**	YFTM73	53.80	29.98	**
YFTM30	49.89	11.90	**	YFTM74	55.92	34.35	**
YFTM31	56.13	25.70	**	YFTM80	66.98	40.68	**
YFTM32	47.65	25.66	**	YFTM83	51.28	51.35	ns
YFTM33	43.11	22.19	**	YFTM85	22.01	33.49	*
YFTM34	57.85	8.53	**	YFTM86	64.86	29.96	**
YFTM35	72.39	30.85	**	YFTM87	54.77	29.10	**
YFTM36	63.88	25.62	**	YFTM88	56.52	27.25	**
YFTM37	53.04	28.23	**	YFTM95	71.24	32.58	**
YFTM38	44.20	9.19	**	YFTM96	53.44	26.56	**
YFTM39	43.02	31.72	ns	YFTM101	51.36	30.04	**
YFTM40	39.90	28.22	*	YFTM102	54.96	33.45	**
YFTM41	47.56	26.56	**				

* and ** indicate significant differences at 5 and 1% probability level, respectively. Ns: Non-significant differences.

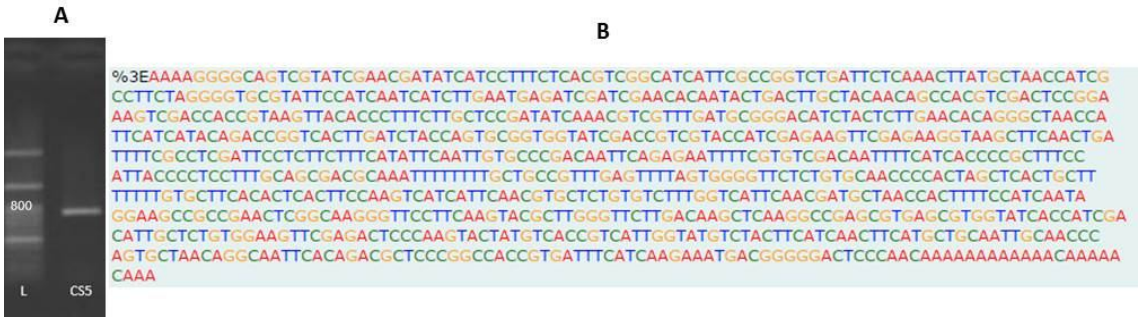

Figure 7 A: The banding pattern generated by amplification of the tef region of the mutated *Trichoderma,* YFTM80 isolate, using EF1/EF2 primers and B: The sequence of band in the Trichoderma YFTM 80 isolate.

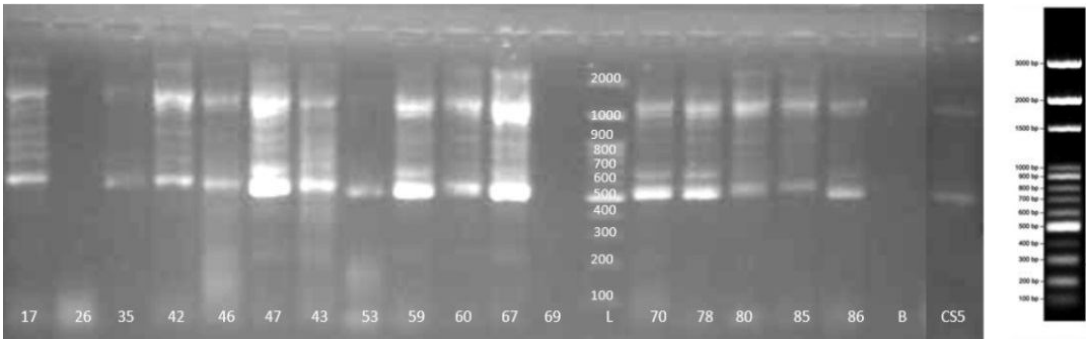

Figure 8 Bond pattern resulting from amplification of ERIC1R and ERIC2I primer pairs in mutated isolates of *Trichoderma*. CS5: superior isolate, L: size marker, and other lanes indicated with number are mutants derived from CS5.

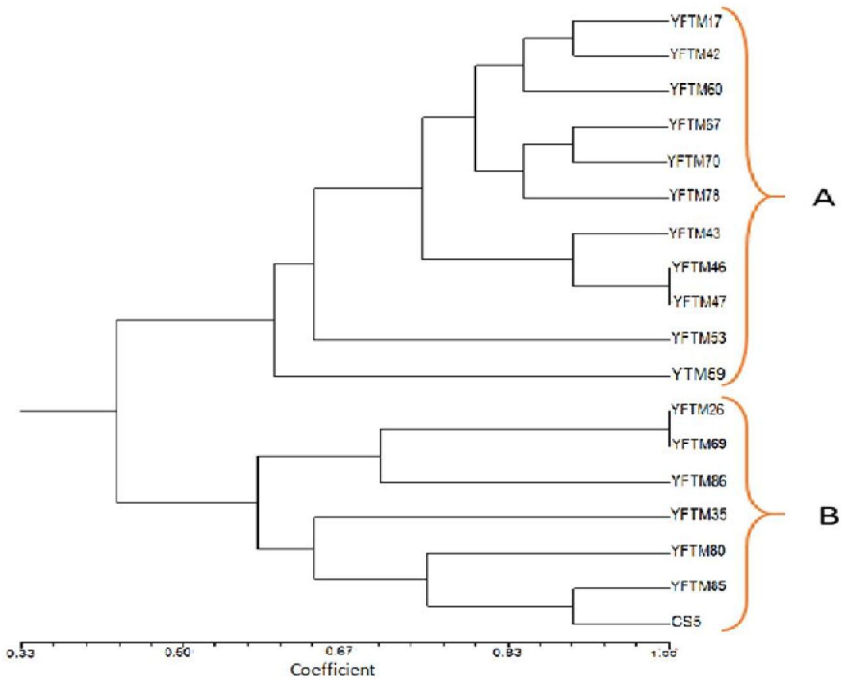

Figure 9 Dendrogram of *Trichoderma harzianum* CS5 and its mutants.

Regarding the dendrogram, the isolates were divided into two distinct clades. The first clade (A) consists of 11 mutated isolates and the second clade (B) consists of 6 mutated isolates and the wild type. The results showed that the isolates of YFTM46 and YFTM47 are the same and the isolates of YFTM26 and YFTM69 are the same and do not differ. Regarding antagonist effect of isolates against Fusarium, the isolate YFTM80 proved as a superior isolate and YFTM85 isolate as the poorest isolate, however, along with CS5(the wild type), they were placed in the same clade, indicating that the marker is not able to separate the mutated isolates from the wild type.

Discussion

The fungal pathogen, *F. oxysporum,* has two different forms on the cucumber plant, which can be distinguished according to the symptoms on the host. The symptoms of *F. oxysporum* f.sp. *cucumerinum* can be only identified on the root and crown of the plant and these areas are dried. Whereas whole infected plant dries up in case of *F. oxysporum* f.sp. *radicis cucumerinum* fungus and as result, the pathogen was recognized as *F. oxysporum* f.sp. *radicis cucumerinum*. The results of the mutual cultivation test indicated the competitive ability in obtaining food and overgrowing Trichoderma fungi. The isolate No. 96 of Trichoderma used by Ashrafizadeh *et al* (2003), with 68.58% inhibitory effect in synchronous mutual cultivation and 59.39% in asynchronous mutual cultivation, prevented the development of Fusarium melon disease, and compared to other isolates had a higher nutritional competency. Trichoderma isolates studied by Behboodi *et al*. (2003) and Okhovat *et al*. (1996) prevented the growth of the studied pathogens. The isolate of *T. harzianum* in the study of Heydari *et al*. (2004) with 82.65% inhibition in synchronous mutual cultivation and 42.53% in asynchronous mutual cultivation prevented the growth of Pathogen. Isolates No. 30 and No. 85 of *T. harzianum* and *T. koningii* were also selected as the best isolates in the reduction of radial growth of the agent of *verticillium* wilt of pistachio in the study of Jamdar *et al*. (2013). All of these studies show that *Trichoderma* fungi can inhibit the growth of plant pathogens. In this study, the two isolates CS5 and CS6 had 68.88 and 68.22% prevention of Fusarium growth respectively. These isolates had the best preventive performance against Fusarium compared to the rest of the isolates. Irradiation with ionizing radiation can be associated with fungistatic and/or fungicidal effects in the filamentous fungi. There are reports of decreased mycelial growth and reduced sporulation of pathogenic fungi upon exposure to ultraviolet light (Mostafavi, 2009) and 400-900 Gry doses of gamma ray (Moradi *et al*., 2013). The use of induced mutations and physical mutagens (ionizing radiation such as: gamma rays) is an applicable approach to increase the bio control potential in BCAs (Mohammadi *et al*., 2014).

The tested γ-radiation induced mutants had greater potential of bio-control activity than their parental wild type strain (*T. viride*). Moradi *et al*. (2013) considered the 250 Gry dose as the optimum dose, which caused 47.3% reduction in spore germination and highest induced mutation rate in *T. harzianum*. Out of 102 isolates, eight isolates were eliminated in the first subculture and 18 isolates were removed during more subcultures due to deformation of conidia or mycelia or lack of sporulation and, 76 isolated were selected for future analysis. The results of mutual cultivation showed that the isolate YFTM80 with 59.85% prevention had higher competition for food than wild type and other mutant isolates. Abbasi Iranog *et al.* (2014), after investigation of some mutants of *T. harzianum* as a bio control of several plant pathogens in reciprocal cultivation, identified six mutants as superior strains. Shahbazi *et al.* (2012) introduced better bio control *T. viride* mutant than wild type against *R. solani* in their study.

The results of this research and other studies showed the ability of Gamma ray to enhance the effectiveness of Trichoderma fungus as a biocontrol agent. With the help of this beam

and making random mutations, it is possible to change parts of the genome responsible for Trichoderma's biocontrol mechanisms, and this can lead to increase efficiency of this fungus. In other words, this marker does not have the ability to amplify the regions that related to Trichoderma's biocontrol mechanisms in the genome, and this marker cannot distinguish between superior and poor isolate in terms of antagonistic ability. For this purpose, the level of proteins in mutated isolates should be investigated or genes that are relevant to biocontrol mechanisms should be investigated. In a study by Abbasi *et al*. (2012), it was found that the ERIC marker is capable of distinguishing between mutant isolates and their wild type, which is in contrast to the results of this study. In general, this marker has the ability of differentiation between different isolates, and this has been proven in various studies (Khodayari *et al*., 2008; Abbasi *et al*., 2012; Poozeshi Miab *et al.*, 2014).

The present study revealed that gamma mutation by optimal dose 250 Gray causes changes in genome and induces mutants with enhanced antagonistic activity compared with wild type against *F. oxysporum* in vitro and in vivo experiments. Mutagenesis is an excellent tool for developing *Trichoderma* mutants with enhanced secretion of enzymes yields as compared to the parent strains (Seidl and Seiboth, 2010; Singh *et al*., 2010; Singh *et al*., 2016). Mutation induction by gamma irradiation has been shown to increase; capability of Trichoderma species to produce enzymes like chitinase and antibiotics; better colonization of tomato roots than wild type and to be a superior biocontrol agent against Fusarium wilt of tomato (Mohamed and Haggag, 2006). Other researchers have proved that mutation induces enhancement of biocontrol ability on soil borne diseases such as *Fusarium oxysporum, Sclerotium rolfsii, Sclerotium sclerotiorum* (Mukherjee *et al*., 1997; Haggag and Mohamed, 2002; Haggag, 2008). Present findings are in agreement with the above- mentioned results and a superior biocontrol candidate is introduced against Fusarium wilt disease of cucumber. Gamma-mutagenesis by improvement of antagonistic properties of biocontrol agents can be used as a strategy to combat against soil borne plant pathogens such as the agent of Fusarium wilt disease of cucumber.

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کارایی جهش یافته‌های *Trichoderma harzianum* در بیوکنترل ***Fusarium oxysporum f.sp. radicis-cucumerinum*** در محیط درون شیشه‌ای

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چکیده: قارچ تریکودرما یک عامل بیوکنترل بسیار با اهمیت است که می‌تواند جایگزین مناسبی برای سموم شیمیایی باشد. *Fusarium oxysporum* یک عامل بیمارگر گیاهی است که باعث پژمردگی فوزاریومی در دامنه وسیعی از گیاهان می‌شود. کاربرد پرتوتابی با پرتو گاما می‌تواند راهی برای ارتقاء کارایی قارچ تریکودرما علیه *F. oxysporum* باشد. جهش القایی امکان تغییرات در ماده ژنتیکی تریکودرما را فراهم می‌کند و در برخی از جدایه‌ها جهش در جهت بهبود توانایی بیوکنترل می‌تواند باشد. در این پژوهش، کارایی جهش‌یافته‌های قارچ *Trichoderma harzianum* CS5 در مقابل قارچ *F. oxysporum* f. sp. *radicis-cucumerinum* مورد بررسی قرار گرفت. از بین ۱۶ جدایه وحشی تریکودرما در آزمایش‌های کشت متقابل هم‌زمان و غیرهم‌زمان با بیمارگر، جدایه CS5 انتخاب شد. سوسپانسیون اسپور جدایه *T. harzianum* CS5 با پرتو گاما توسط چشمه کبالت ۶۰ با نرخ دز ۰/۲۳ گری بر ثانیه در پژوهشکده کشاورزی هسته‌ای، پرتوتابی شد. سپس میزان آنتاگونیستی ۷۶ جدایه جهش‌یافته با کمک آزمایش‌های کشت متقابل هم‌زمان و غیرهم‌زمان با بیمارگر بررسی شد. با استفاده از نشانگر ERIC-PCR، امکان تفکیک بین تیپ وحشی و ۱۷ جدایه جهش یافته منتخب بررسی شد. نتایج نشان داد که تنها ۱۷ جدایه جهش یافته توانستند بهتر از تیپ وحشی، قارچ بیمارگر را کنترل کنند و جدایه YFTM80 بیش‌ترین ممانعت‌کنندگی را داشت. نتایج این پژوهش نشان داد که پرتوگاما می‌تواند در بهبود خاصیت آنتاگونیستی قارچ تریکودرما نقش داشته باشد. نتایج نشان داد که نشانگر ERIC-PCR می‌تواند جهش‌یافته‌ها را از هم تفکیک کند اما توانایی تفکیک جهش‌یافته‌ها و تیپ وحشی از لحاظ برتری در آنتاگونیسم را ندارد. به احتمال زیاد جهش‌ها در مکان‌های مرتبط با خاصیت آنتاگونیستی اتفاق افتاده که منجر به بهبود کارایی جهش‌یافته‌ها شده است. احتمالا نشانگر ERIC-PCR نتوانسته این نواحی را تکثیر کند.

واژگان کلیدی: تریکودرما، جهش، پرتوگاما، *Fusarium oxysporum* f. sp. *radicis-cucumerinum*