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Effective Biological Control of Carnation *Fusarium* Wilt Using a New Combination of *Trichoderma* Mutant Isolates

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ABSTRACT

Fusarium **infection in carnation is the most important limiting factor for carnation production. We isolated 38 fungal isolates from infected carnation plants collected from Mahallat, Iran, and 15** ***Fusarium*** **species were identified morphologically. Isolates of J14, k5, and k72 were the most pathogenic isolates in pathogenicity test on White Liberty cultivar. Molecular identification of J14, k5, and k72 isolates was done based on** ***Elongation Factor 1-alpha*** **(EF1-α) gene. To investigate the effect of** ***Trichoderma*** **and some of its mutants on reducing the** ***Fusarium*** **infection, six wild type isolates were examined. The species of** ***T. harzianum, T. virense,*** **and** ***T. ghanens*** **showed more inhabitation potential and were chosen for induced mutation via gamma irradiation at 250 Gy. The number of 270 mutants were screened morphologically and 60 mutants were screened using dual culture against J14, k5, and k72. Morphological and molecular identification of J14, k5, and k72 isolates recognized them as** ***F. oxysporum.*** **Three mutants, i.e. ThM7(67.17%), TgM1 (59.45%), and TvM17(57.55%) showed the highest efficacy and were selected. Evaluation test of efficacy in greenhouse by mixture of** ***T. harzianum, T. virense*** **and** ***T. ghanens*** **(TW) and mixture of mutant isolates ThM7, TgM1, and TvM17 (TM) showed that biological method had higher ability to control** ***Fusarium*** **infection on carnation plants in greenhouse condition, and mutation had no adverse effects on plants. The results of this experiment proved that the use of mutation in the** ***Trichoderma*** **genome with the use of gamma radiation could be an effective way to achieve isolates with better performance in this bio-control agent.**

Keywords: Dianthus caryophyllus, Fusarium oxysporum, Gamma radiation, Induced mutation.

INTRODUCTION

Malallat City in Markazi Province is one of the important centers for producing flowers and ornamental plants in Iran. About 87,710 tons of flowers and ornamental plants worth $ 20 million were exported from Iran to other countries in 2018. Based on data published by the Ministry of Jihad Agriculture in 2017, Iran's income from carnation exports was about $ 8 million (Shahbazi *et al.*, 2021). Carnation (*Dianthus caryophyllus* L.) belongs to the family *Cariofilaceae* and is one of the four central flowers of Iran's export. All over the world, vascular wilt on carnation caused by *Fusarium oxysporum* Schelchtend. Fr. f. sp. *dianthi* (Prill. & Delacr.) W.C. Snyder & H.N. Hans, is the most important fungal disease. *Fusarium oxysporum* f. sp. *dianthi* (Fod) was consistently isolated from wilted carnation plants in commercial stocks in many countries worldwide and Iran (Carver *et al*., 1996; Fattahi *et al.*, 2014). Symptoms observed on carnation include severe

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vascular browning, and root and crown rot. Fattahi *et al.* (2014) reported *Fusarium* species as the major cause of the wilt and root and crown rot disease in carnation. *Fusarium* species are associated with different parts of growing carnations isolated in Markazi Province.

Trichoderma spices are known as Biological Control Agents (BCAs) of *Fusarium oxysporum f. sp, dianthi* causing carnation wilt (Shanmugam and Sharma, 2008; Sharma and Nirupma, 2008). Among bio-control agents evaluated *in vitro* for inhibiting the mycelial growth of *Fod*, *T. viride*, *T. harzianum*, and *T. virens* inhibited the mycelial growth of the pathogen (Shanmugam and Sharma, 2008). Twenty-eight isolates of *Trichoderma* were screened *in vitro* for antagonistic ability against *Fod*, and *T. atroviride*, *T. pseudokoningii*, and *T. harzianum* were reported as antagonistic isolates (Sharma and Nirupma, 2008). The growth inhibitory effects of *Trichoderma*, including three isolates of *T. harzianum* obtained from Karaj (Th1), Shahrekord (Th2), and Hamedan (Th3), and two isolates, namely, *T. virens* (Tv), and *T. atroviride* (Ta) from Hamedan were studied *in vitro* on the *Fod* isolated from Mahallat Carnation. The most significant growth inhibition in the dual culture test occurred with the Th3 (*T. harzianum*), and the lowest inhibition was observed by *T. atroviride* (Kermajany *et al.*, 2017). One of the strategies to increase the efficiency of bio-control agents is to increase their antagonistic potency by inducing mutations in the genome and screening biocontrol agents with higher efficiency (Abbasi *et al.*, 2016). Several studies have been conducted to increase the *Trichoderma* antagonistic abilities by an induced mutation on the genome using gamma rays (Ghasemi *et al.*, 2019; Lava *et al.*, 2021; Abbasi *et al.*, 2014; Soufi *et al.*, 2021; Orojnia *et al.*, 2021).

Due to the outbreak of *Fusarium* damage on carnation in recent years and the lack of effective chemical fungicides available to flower producers in Mahallat, in this study, we aimed to find effective mutant isolates of *Trichoderma* to control this disease.

MATERIALS AND METHODS

Isolation and Identification of Pathogenic Fungi

The wilt pathogen (*Fusarium* spp.) was isolated and collected from the diseased root and crown tissues of carnation plants during the survey in Mahallat, in 2019 (Figure 1). The infected plant tissues were surface sterilized with ethanol 80% and transferred on the potato dextrose agar medium (Peeled potato; 250 g, Dextrose; 20 g, Distilled water1,000 mL at pH= 5.6±0.2) in Petri plate, which were incubated at room temperature (25±2°C) and observed

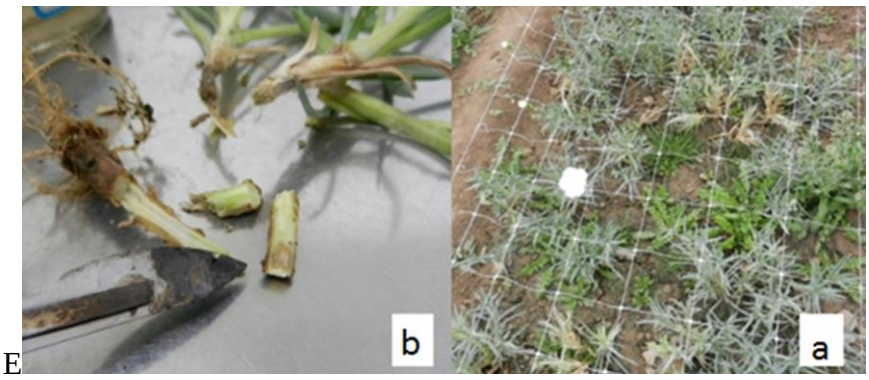

Figure 1. Isolation of pathogenic fungi from carnation plants: (a) Sampling of infected carnation plants from greenhouses in Mahallat, (b) Preparation of micro-samples of roots and crowns tissue and transfer to the culture medium.

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periodically for the growth of pure colonies. The single pure colonies were transferred to PDA slants for the maintenance of the culture. The *Fusarium* species isolated from carnation was identified based on the morphological and cultural characters by Leslie and Summerell's (2006) identification key. To identify *Fusarium* species morphologically, Carnation Leaf Agar (CLA) medium was used. Morphological identification of 38 isolates was performed (Table 1) based on characteristics such as macroconidia formation, presence or absence of microconidia and/ if their shape, location at the end of the conidiophore (single, chain and false heads), and type of phialids (non-monophialid or monophialid). The presence of chlamydospores on CLA culture media, as well as the specifications of propagules on the PDA culture medium, including the colour of the aerial hyphae and the average growth of the propagules, were recorded using Leslie and Summerell (2006).

Pathogenicity Test

A 10^6 spores mL^{-1} suspension of *Fusarium* species was prepared in sterile distilled water with a hemocytometer. Fifty mL of suspension was used for injection into the White Liberty cultivar of carnation plants (susceptible cultivar to all *Fod* isolates) for pathogenicity test. The sterilized 1 mL syringe was used to inject the suspension into several crown points of the carnation at a rate of one mL of the mentioned spore suspension (Figure 2). To ensure the special form of the isolated *F. oxysporum*, other plant seedlings were also inoculated by the same method [cucumber INCI F1 (ENZA-ZADEN ™), tomato Falat ™ and gladioli Oscar ™ cultivar white]. Inoculated plants were maintained in the germinator at 25°C for six weeks. The plants' symptoms were recorded once every three days (10 days after inoculation). The percentage of infected plants and symptoms presence was calculated using the Sharma and Nirupma (2008) formula. After pathogenicity testing for *Fusarium* isolates, because the three isolates J14, K5, and k72 had the highest pathogenicity, only these three isolates were used for subsequent examinations. Also, molecular identification of these three isolates was done.

Molecular Identification of F. oxysporum Isolates

Genomic DNA of *Fusarium* isolates was extracted using the extraction kit instructions of Pars Toos Company. Polymerase Chain Reaction (PCR) amplified a 700 bp region of TEF-1α using ef1 (5-ATGGGTAAGGA(A/G)GACAAGAC-3) as the forward primer and ef2 (5-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3) as a reverse primer (Geiser *et al*., 2004). The PCR was performed in Thermo Cycler (BIORAD® T100™, Germany) as the following program: Initial denaturation at 94°C for 5 minutes and 30 cycles including: 1 minute at 94°C, 50 seconds at 55°C, and 3 minutes at 72°C and a final extension at 72°C for 10 minutes. Amplified PCR products were separated on an agarose gel (1.5% w/v) in 1X TAE buffer at 100 V. The 100 bp DNA marker (MBI Fermentas, Lithuania) was used to estimate DNA band size. Also, a negative control was used to test the absence of contamination.

For DNA Sequencing, the band of 700 bp of DNA was separated from the gel and, after purification, was sequenced using the method of South Korean Macrogene Company (http://dna.macrogen.com, Korea). The sequences for TEF-1α areas were screened in FinchTV v.1.4.0 (https://digitalworldbiology.com/FinchTV) for possible machine errors, manually edited when needed, and subjected to BLAST searches (BLASTn) in GenBank (Altschul *et al.*, 1997). Further editing and preparation of an exact sequence were performed using forward and reverse sequences by BioEdit Sequence Aliment editor software (Hall, 1999). The obtained sequences were

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Table 1. Morphological identification and Pathogenicity potential of *Fusarium* isolated from Mahallat on carnation plants.

Isolate code	Pathogenicity	Morphologic identification	Sampling region	Geographical latitude and longitude of the sampling place
K5	Sever wilting and vascular necrosis	*F. oxysporum*	Crown	33.892175 50.499721
K6	non pathogen	*F. circinatum*	Root	33.892175 50.499721
K7	non pathogen	*F. circinatum*	Root	33.892175 50.499721
K8	non pathogen	*F. circinatum*	Root	33.892175 50.499721
K9	non pathogen	*F. circinatum*	Root	33.892175 50.499721
K10	non pathogen	*F. culmorum*	Soil (Rhizosphere)	33.892175 50.499721
K11	non pathogen	*F. oxysporum*	Root	33.892175 50.499721
K12	moderate wilting and vascular necrosis	*F. oxysporum*	Crown	33.892175 50.499721
K13	non pathogen	*F. oxysporum*	Root	33.892175 50.499721
K14	non pathogen	*F. oxysporum*	Root	33.892175 50.499721
K15	non pathogen	*F. pseudonygamai*	Root	33.892175 50.499721
K18	non pathogen	*F. nygamai*	Root	33.892175 50.499721
K72	Sever wilting and vascular necrosis	*F. oxysporum*	Root	33.892175 50.499721
J101	non pathogen	*F. thapsinum*	Soil (Rhizosphere)	33.884902 50.490541
J102	non pathogen	*F. denticulatum*	Soil (Rhizosphere)	33.884902 50.490541
J103	non pathogen	*F. acutatum*	Soil (Rhizosphere)	33.884902 50.490541
J104	non pathogen	*F. acutatum*	Soil (Rhizosphere)	33.884902 50.490541
J105	non pathogen	*F. acutatum*	Soil (Rhizosphere)	33.884902 50.490541
J106	non pathogen	*F. pseudocircinatu*	Root	33.892175 50.491129
J109	non pathogen	*F. pseudocircinatu*	Root	33.892175 50.491129
J11	low wilting	*F. oxysporum*	Crown	33.892175 50.491129
J12	non pathogen	*F. culmorum*	Soil (Rhizosphere)	33.892175 50.491129
J14	Sever wilting and vascular necrosis	*F. oxysporum*	Root	33.892175 50.491129
J15	non pathogen	*F. culmorum*	Soil (Rhizosphere)	33.892175 50.491129
J17	non pathogen	*F. circinatum*	Crown	33.892175 50.491129
J18	non pathogen	*F. beomiforme*	Root	33.892175 50.491129

Table1 Continued …

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Continued of Table1. Morphological identification and Pathogenicity potential of *Fusarium* isolated from Mahallat on carnation plants.

Isolate code	Pathogenicity	Morphologic identification	Sampling region	Geographical latitude and longitude of the sampling place
J120	non pathogen	*F. sterilihyphosum*	Root	33.892175 50.491129
J125	non pathogen	*F. sterilihyphosum*	Root	33.892175 50.491129
F103	moderate wilting and moderate root rot	*F.solani*	Soil (Rhizosphere)	33.892175 50.491129
F104	non pathogen	*F.solani*	Crown	33.892175 50.491129
F104	non pathogen	*F.solani*	Root	33.892175 50.491129
F112	non pathogen	*F. phyllophilum*	Crown	33.892175 50.491129
F115	non pathogen	*F. phyllophilum*	Crown	33.892175 50.491129
F101	moderate root rot	*F. proliferatum*	Soil (Rhizosphere)	33.892175 50.491129
F105	moderate root rot	*F. proliferatum*	Soil (Rhizosphere)	33.892175 50.491129
F114	non pathogen	*F. brachygibbosum*	Root	33.892175 50.491129
F115	non pathogen	*F. brachygibbosum*	Root	33.892175 50.491129
F110	moderate root rot	*F.solani*	Soil (Rhizosphere)	33.892175 50.491129

presented in the Gene Bank database (www.ncbi.nlm.nih.gov/BLAST). Then, these sequences were registered on the GenBank of NCBI site (https://www.ncbi.nlm.nih.gov/WebSub/).

Phylogenetic Analysis

The newly obtained Elongation Factor 1-alpha (EF1-α) sequences of *Fusarium* spp included j14, K5, and K72, together with the other sequences of the *Fusarium* genus already used by Lombard *et al.* (2019), Van Hove *et al.* (2011), Tirado-Ramirez *et al.* (2021), Crous *et al.* (2021), O'Donnell *et al.* (2018), and Jacobs-Venter (2019) were selected and obtained from https://www.ncbi.nlm.nih.gov/nucleotide for phylogenetic analyses. The dataset was updated by investigations in the database to obtain correct sequences. The sequence of TEF-1α from *Nectria cinnabarina* with accession number HM484527.2 *(*Hirooka *et al*., 2011) was considered an outgroup. All sequences were aligned using the Q-INS-i algorithm of MAFFT version 7 (http://mafft.cbrc.jp/ alignment/server/) (Katoh and Standley, 2013). The online version of Gblocks 0.91b (Castresana, 2000) was applied to remove ambiguous parts of the alignment, with all three options (including allowing smaller final blocks, allowing gap positions within the last blocks, and allowing less strict flanking position) for a fewer stringent selection (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The most suitable substitution model for the dataset was chosen using the Akaike Information Criterion (AIC) using PAUP*/MrModeltest v2.2 (Nylander, 2004). An asymmetrical model including a gamma distribution for rates (GTR+G) was picked out for EF1-α analysis. Bayesian Inference (BI) was carried out using MrBayes v3.1.2 (Ronquist

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and Huelsenbeck, 2003), choosing a random beginning tree and running the chains for 4 million for EF1-α. After casting off burn-in samples, the residual samples were reserved for additional analyses. The Markov Chain Monte Carlo (MCMC) method within a Bayesian framework was utilized to assess the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) by the 50% majority rule. The obtained phylogenetic tree was visualized via the online browser of icytree developed by Vaughan (2017) (https://icytree.org/).

Preparation of Antagonist and Induced Mutation

Trichoderma species isolates including [T. harzianum (NAS-101), T. auroviridae (NAS-105), T. lixii (NAS120), T. virens (NAS-115), T. ghanens (NAS-108, and T. atroviride (NAS-112)] investigated in this study are given in Table 1. They are maintained in the culture collection of Nuclear Agriculture Research Institute collection (NSTRI, AEOI) Alborz, Iran. Based on molecular identification using the ITS1 and ITS4 gene sequencing, the designation was proved as described in Table 2, and registered with the accession number at GenBank (NCBI) for all Trichoderma species isolates (Table 2).

Six isolates of *Trichoderma* species were screened against *F. oxysporum* (J14, k 5, and k72) by the dual culture method (Dennis and Webster, 1971). A nine mm mycelial disc of *F. oxysporum* (J14, k5, and k72) and each isolate of *Trichoderma spp*. were placed opposite to each other near the periphery of the Petri plate and incubated at room temperature (25±2°C). After 48 h of incubation, the mycelial growth of the pathogen and inhibition zone was measured in all the Petri plates, including the control plates. The Growth Inhibition (GI) of mycelial was calculated using the formula suggested by Pandey *et al.* (2000). *Trichoderma* species isolates were selected (based on ability to inhibit the growth of pathogenic fungi *F. oxysporum* (J14, k 5 and k72)), and to create genetic diversity in *Trichoderma* species genome and to achieve higher efficacy, mutations were induced in their genomes using gamma-ray irradiation.

The induced mutation on the selected *Trichoderma* species [*T. harzianum (NAS-101), T. virens (NAS-115),* and *T. ghanens (NAS-108)*] was performed by Ghasemi *et al.* (2020) method at 250 Gy doses of gamma-ray in a Cobalt 60 gamma resource with a dose rate of about 0.083 Gy s^{-1} using a Gammacell 220 irradiator (MDS Nordion, Ottawa, Canada) that was installed at the Radiation Applications Research School, Nuclear Science and Technology Research Institute, AEOI, Tehran, Iran. The irradiation was done at 25±1°C, with relative humidity of 45-55% for all samples, and the absorbed dose was checked by a Red-Perspex dosimeter (Harwell Dosimeters, UK). All treatments were carried out in triplicates. Mutant isolates were sub-cultured ten times to ensure the immutability of morphological characteristics on the Mandelś optimized medium (Lava *et al.*, 2021). Mutant isolates (270 mutants) were screened (based on spore production, mycelial growth rate, and spore germination percentage) because these indicators were effective in increasing efficiency in biological control (data not presented in this paper). Sixty selected mutant isolates were screened using dual culture with pathogenic fungi (against *F. oxysporum* J14, k5, and k72) to identify the best mutant isolates with more significant inhibitory potential than their parent isolates (Sahampoor *et al.* 2020).

Evaluation of the Greenhouse Efficiency of *Trichoderma*

Since the main purpose of this study was to find a more efficient combination for effective bio-control of *Fusarium* infestation of carnation in the greenhouse, spore suspension of the selected mutants *(ThM7,*

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Table 2. Morphological and antagonistic characteristics of *Trichoderma* isolates against *Fod* isolates. [a]

Isolate code	*Trichoderma* species	Gene Bank Accession number	GI (%) (k72)	GI (%) (k5)	GI (%) (J14)	Growth rate (mm d^{-1})	Conidia size (µm)
NAS120	*T. lixii*	MW719878.1	45.31 ^{gh} ±3.11	52.13 $^{d-h}$ ±2.41	43.21^{gh} ±2.01	2.18 ^{c}	2.14*2.09
NAS-105	*T. aureoviride*	MW719569.1	10.40^{j} ±1.32	2.42^{m} ±1.91	12.40^{j} ±1.22	1.28 ^{d}	2.06*2.17
NAS-112	*T. atroviride*	MW719255.1	44.83^{gh} ±5.30	41.99^{k} ±3.33	40.25^{gh}±3.22	2.03 ^{c}	2.21*2.07
NAS-101	*T. harzianum*	MW718882.1	60.66^{ab}±1.88	57.22 cd ±4.58	58.67^{ab}±1.08	3.74 ^{b}	2.44*2.14
ThM7	*T. harzianum*	-	68.27^{a}±0.98	59.14 ^{bc} ±4.12	67.17^{a}±1.09	4.91 ^{a}	2.44*2.14
NAS-108	*T. ghanense*	MW719590.1	55.65 $^{b-d}$ ±6.79	53.35 $^{d-g}$ ±4.73	54.55^{cd}±4.47	4.32 ^{b}	2.73*2.36
TgM1	*T. ghanense*	-	70.15^{a} ±1.08	55.76 $^{b-d}$ ±2.49	59.45^{bc}±3.05	5.32^{a}	2.73*2.36
NAS-115	*T. virens*	MW719876.1	50.28^{ef} ±3.10	54.30^{c-g} ±2.77	47.17^{ef}±1.15	2.18 ^{c}	2.45*2.13
TvM17	*T. virens*	-	54.44 $^{b-d}$ ±2.14	59.61^{a} ±3.13	57.55^{cd}±3.74	3.25 ^{bc}	2.45*2.13

[a] In the columns, means with different letters are significantly different at p=0.05. Means were compared using Least Significant Difference (LSD) Test using SPSS V.13 system.

TgM1, and *TvM17)*, as TM treatment, and high antagonist parental isolates (*T. harzianum*, *T. virense* and, *T. ghanens*), as TW treatment, were used for greenhouse evaluations at a concentration of 10^8 spores/mL of sterile distilled water. Fungi-free soil was obtained by twice treating the soil with steam (121°C) for 30 minutes. Disinfected soil was treated by an equal mixture of three isolates of the virulent pathogen (*F. oxysporum* J14, k5, and k72). To evaluate the efficiency of biological treatments, the Prados-Ligero method was used (Prados-Ligero *et al.*, 2007). The efficiency of biological treatments was compared to commercial chemical compound (CARBENDAZIM^{TM} 60% WP concentration 2 mL L^{-1} based on the manufacturer's recommendation). The experiment was conducted in a completely randomized design with four replications in the greenhouse.

RESULTS

Isolation and Identification of Pathogenic Fungi

Thirty-eight *Fusarium* fungal isolates were isolated from the collected diseased plant and soil samples (Figure 1) and 15 different species of *Fusarium* were identified morphologically. The pathogenicity potential, morphological identification, sampling region, and isolate codes are listed in Table 1.

Pathogenicity Test

In pathogenicity tests, all the three isolates of *F. oxysporum* (J14, k5, and k72) could cause severe disease symptoms on the Liberty White cultivar in the greenhouse condition. Necrosis and browning of the vessels in crown region subsequent wilting were observed in *F. oxysporum* (J14), and moderate browning and wilting were observed in plants inoculated with *F. oxysporum* (J11) with low pathogenicity (Figure 2).

The identical isolates were re-identified by re-isolation from infected tissues and purification on a PDA culture medium. Confirmation of the pathogenicity of each isolate (J14, k5, and k72) was done based on the pathogenicity test. The host specificity test for *F. oxysporum* (J14, k5, and k72) on other plant seedlings (cucumber, tomato, and gladioli) showed no symptoms based on Sharma and Nirupma disease index (2008) in any treatments. The non-pathogenicity on the other plants proved that J14, k5, and k72 belonged to F. *oxysporum f. sp, dianthi.*

Previous studies of Prados-Ligero *et al.* (2007) have shown that the reaction of *Fusarium*-susceptible carnation cultivars is visible in the form of browning of vessels in the crown region and vascular necrosis, and should not be limited to the symptoms of yellowing or wilting on the aerial parts of the plants. Therefore, in this study, vascular tissue was cut at the crown region (Figure 2). Selection of the virulent pathogen isolates was performed based on the degree of browning and necrosis of the vessel in the crown area (Prados-Ligero *et al.*, 2007).

Morphological Identification of *F. oxysporum* Isolates

Based on Leslie and Summerell identification key (Leslie and Summerell, 2006), the k5 (Figure 3) and J14 (Figure 4) and k72 (Figure 5) isolates were identified as *Fusarium oxysporum*. These isolates, on the PDA culture medium, produced white aerial mycelium gradually forming pale pigments (k72) or reddish-pink (k5 and J14). The underside of the colony turned reddish-pink and, in some cases, lacked color. Microconidia were false heads and formed short, simple, and sometimes branched monophialids. Macroconidia on the CLA culture medium were produced abundantly. They were slightly curved, sickle-shaped, and often had 2 to 3 septa, and they were primarily short and had a thin wall. The

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Figure 2. Symptoms incidence of some *F. oxysporum* isolates on Liberty White cultivar of carnation in pathogenicity test: (a) Control (un-inoculated), (b) J11 (*F. oxysporum* isolate with low pathogenicity), (c) J14 (*F. oxysporum* isolate with high pathogenicity caused severe wilting and vascular necrosis

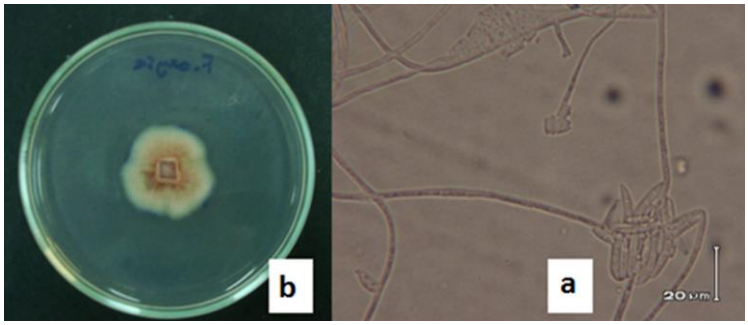

Figure 3. Macroscopic and microscopic features of **k5** (*F. oxysporum*) isolate: (a) Sickle-shaped macroconidia and macroconidia (bar: 20µm), (b) Colony on PDA culture medium.

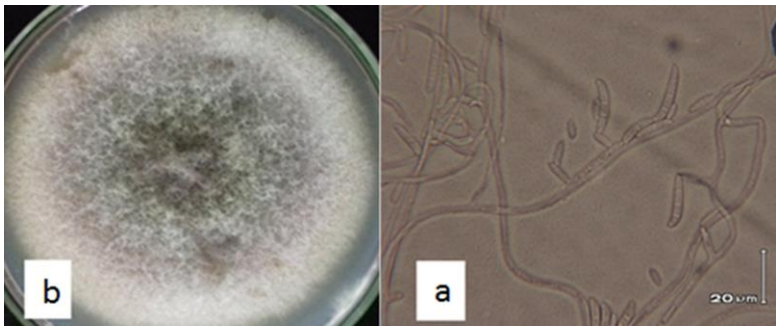

Figure 4. Macroscopic and microscopic features of **J14** (*F. oxysporum*) isolate: (a) Sickle-shaped microconidia and macroconidia on phialides (bar: 20µm), (b) Colony on PDA culture medium.

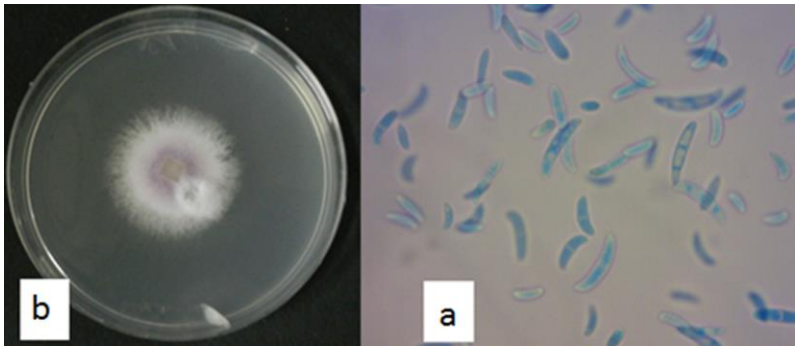

Figure 5. Macroscopic and microscopic features of **k72** (*F. oxysporum*) isolate: (a) Microconidia and macroconidia (bar: 20µm), (b) Colony on PDA culture medium.

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basal cell of the macroconidia was narrow and leg-shaped. Chlamydospores formed abundantly on the CLA culture medium after 14 to 20 days and were often spherical.

Molecular Identification of Fusarium Isolates

Molecular identification of *Fusarium* isolates J14, k5, and k72 were done based on Translation-*Elongation Factor-1alpha* gene (TEF-1α). The fragment of 700 bp was amplified using the Primers ef1/ef2 in the polymerase chain reaction. The presence of the 700 bp band in the J14, k5, and k72 isolates and its absence in the negative control indicates the correct amplification of the *TEF-1α* gene (Figure 6).

The sequences of *TEF-1α* gene related to J14, k5, and k72 isolates were registered on the GenBank of NCBI site with accession numbers of OM218668, OM218669, and OM218670, respectively. Molecular identification of *Fusarium* isolates supported their morphological identification. Bayesian phylogenetic tree was constructed based on TEF-1α alignment of J14, k5, k72, *F. oxysporum*, *F. keratoplasticum*, *F. albidum*, *F. caucasicum*, *F. musae*, *F. solani* and *Nectria cinnabarina* as outgroup (Figure 7).

As shown in Figure 6, the isolates of J14, k5, and k72 were grouped with MH485044.1_*Fusarium_oxysporum* (as type

Figure 6. Amplification of conserved region of *TEF-1α* gene. Left to right: Control, 100 bp DNA ladder, k5, k72, and j14 Fusarium isolates.

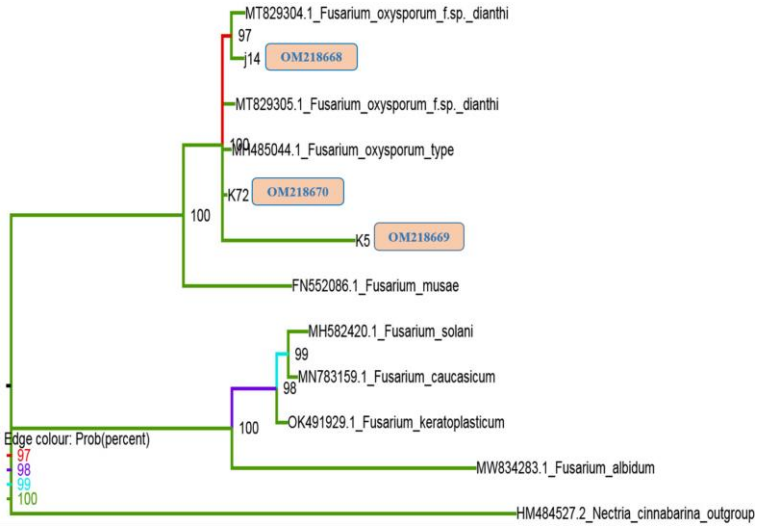

Figure 7. Bayesian 50% majority rule consensus tree deduced using EF1-α sequences of the *Fusarium* spp. Under the GT+G model. The newly generated sequences are in blue color in red frameworks.

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species) and two isolates of *F. oxysporum* f.sp. *dianthi*. So, isolates of *F. oxysporum* were easily distinguished from other species.

Screening of Antagonist (Wild Type and Mutants)

Analysis of GI means data showed that the activity of wild type species of Trichoderma [T. harzianum (NAS-101), T. auroviridae (NAS-105), T. lixii (NAS120), T. virens (NAS-115), T. ghanens (NAS-108), and T. atroviride (NAS-112)] in preventing the growth of pathogenic fungi (J14, k5, and k72) was statistically significant at the level of 5% (Table 2). The development of k72 isolate inhibited between 10-60% with wild type isolates, and T. harzianum (NAS-101) had the highest inhibition efficacy (Figure 8 and Table 2).

According to the results of dual culture experiments against k5, the highest inhibition was observed in *T. harzianum (NAS-101)* with 57%, and the weakest antagonist was *T. auroviridae (NAS-105)* (Figure 9 and Table 2).

Analysis of the mean inhibition of growth

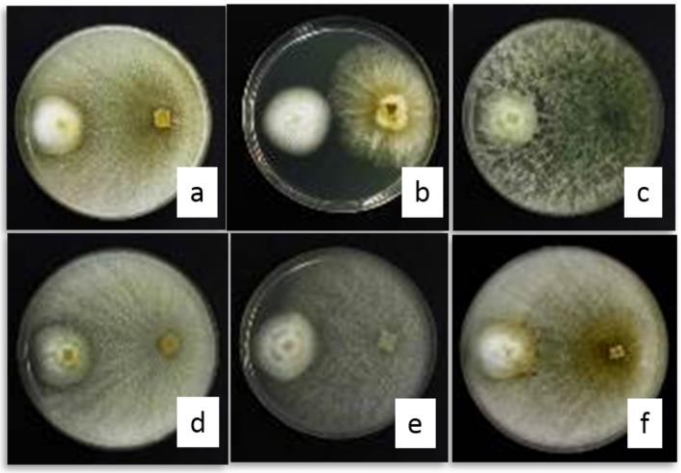

Figure 8. Antagonistic assay of *Trichoderma spp.* against k72 via dual culturing on PDA: (a) *T. harzianum*, (b) *T. aureoviride*, (c) *T. ghanense*, (d) *T. lixii*, (e) *T. atroviride*, and (f) *T. virens*).

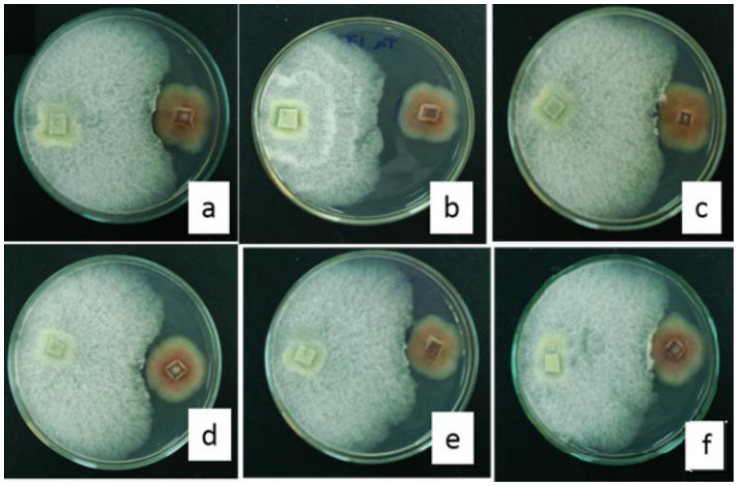

Figure 9. Antagonistic assay of *Trichoderma spp.* against *Fusarium oxysporum* (k5) via Dual culturing on PDA: (a) *T. harzianum*, (b) *T. aureoviride*, (c) *T. ghanense*, (d) *T. lixii*, (e) *T. atroviride and* (f) *T. virens*).

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showed that *T. harzianum (NAS-101)* had the highest inhibition efficacy with 58% (Table 2), but *T. auroviridae (NAS-105)* was the weakest bio-control isolate (Figure 10 and Table 2).

In all evaluations, *T. auroviridae* had the lowest ability to inhibit pathogens' growth, and *T. lixii*, and *T. atroviride* species were also significantly weaker in all assessments; therefore, they were not used for further studies. The wild types [*T. harzianum (NAS-101)* and *T. virens (NAS-115),* and *T. ghanens (NAS-108)*] were chosen for irradiation with 250 Gy gamma rays (Abbasi *et al.* 2016; Ghasemi *et al.* 2020) to induce mutation and increase antagonistic potency.

Induction of gamma-ray mutations is a random method for genetic diversity induction, so, a percentage of the population of radiation-induced mutants undergoes damaging mutations that cause them to lose their ability to produce spores, lose their conidial germination rate, or reduce their mycelial growth rate or malformation in structure. These damaging mutants were removed during the selection process (Abbasi *et al.* 2016; Ghasemi *et al.* 2020). Between 270 mutants generated from single-spore colonies, 60 mutants belonging to *T. harzianum (NAS-101), T. virens (NAS-115),* and *T. ghanens (NAS-108)* were selected (morphological screening data not shown in this paper). The mutants were sub-cultured ten times on PDA to ensure the stability of growth and morphological characteristics of the mutants. Since J14 isolate (*F. oxysporum*) had higher pathogenicity in pathogenicity evaluations (Figure 2), selection between 20 mutants was based on the ability to inhibit the growth of J14 in dual culture. *ThM7* showed 67% growth inhabitation effect (10% more than the parental isolate *NAS-101*), *TgM1* had 59% (5% more than parental isolate *NAS-108*), and *TvM17* was 10% more efficient than its parental isolate *NAS-115* (57%). As shown in Table 2, all of these selected mutants (*ThM7*, *TgM1*, and *TvM17*) were more efficient than their parental isolates in dual culture assay against k5 and k72.

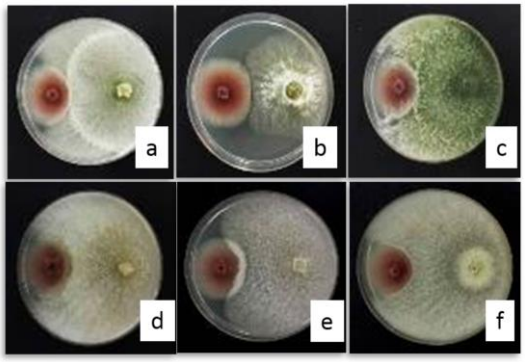

Figure 10. Antagonistic assay of *Trichoderma spp.* against *Fusarium oxysporum* (J14) via Dual culturing on PDA: (a) *T. harzianum,* (b) *T. aureoviride,* (c) *T. ghanense,* (d) *T. lixii,* (e) *T. atroviride* and (f) *T. virens).*

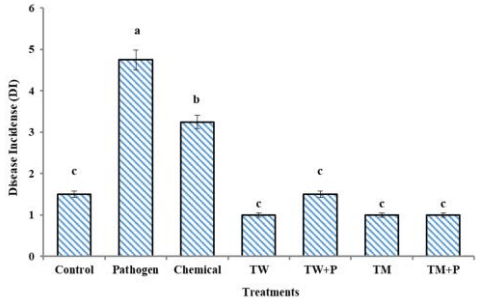

Figure 11. The effects of different treatments of TW (equal mix of *NAS-101*, *NAS-115*, and *NAS-108)* and their selected mutants TM (equal mix of *ThM7, TgM1,* and *TvM17*) on disease incidence in carnation plants, in the presence and absence of the pathogen (*F.oxysporum).*

Evaluation of the Greenhouse Efficiency of Trichoderma

Superior mutants (*ThM7, TgM1,* and *TvM17*) selected from the dual culture test (Table 2) were used to evaluate greenhouse performance. The plants treated with (TW+P) and (TM+P) did not statistically differ from un-inoculated control plants. Although (P): an equal mixture of three isolates of F. oxysporum's virulent pathogen (J14, k5, and k72) had the highest disease incidence, the chemical fungicide CARBENDAZIM TM 60%WP (chemical) was not as effective as biocontrol treatments (Figure 11).

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The pathogens treatment showed the maximum symptoms on the White Liberty cultivar and proved that this carnation cultivar is susceptible and the pathogen propagule was active (Figure 11). There was no active *Fusarium* propagule on the soil suspension test of the control plants. Thus the low degrees of chlorosis on the control plants maybe appeared because of the susceptibility of the White Liberty to irrigation and temperature. The evaluation test of efficacy in greenhouse showed that TW+P and TM+P (biological method) had statistically significant higher ability to control *Fusarium* infection on carnation plants in greenhouse condition than chemical treatment with fungicide CARBENDAZIM ™ 60%WP.

As shown in Figure 11, the *Fusarium* symptoms (chlorosis/ wilting or necrosis) were not observed in plants treated with mutant *Trichoderma* isolates (TM and TM+P). However, in plants treated with TW+P wilting symptoms appeared, but the amount of wilting was not statistically significantly different than the control plants. There was no a statistically significant difference between the efficiency of mutant isolates mix (TM) and parent isolates mix (TW) in infected soil, but in terms of quality and freshness of flowering branches, induction of flowering branches production in TM and TM+P treatment was significantly higher than the TW and TW+P (data not shown). To more accurately compare the efficacy of *Trichoderma* mutants with the wild type, evaluations are ongoing.

DISCUSSION

Carnation (the white Liberty cultivar studied in this study) has the largest area under cultivation as an ornamental plant in Mahallat, Iran. *Fusarium* wilt is one of the limiting factors in carnation cultivation. Its damage in the Mahallat was reported about 30-40%, with the peak damage in June to the end of October (Bani Jamali and Bayat, 2007). Fattahi *et al.* (2013) reported that the major cause of the wilt and root and crown rot disease in carnation was *Fusarium* species associated with different parts of the growing carnations, and they isolated them as *F. oxysporum, F. proliferatum, F. solani, F. equiseti* in Markazi Province. In another study, the causal agents of carnation inoculated by *Fusarium* from Mahallat were reported as *F. brachygibosum, F. solani, F. proliferatum,* and *F. oxysporum* (Shahbazi *et al.,* 2021). In this study, we isolated and identified the 38 *Fusarium* isolates from infected root and crown of carnation in Mahallat (Table 1). We identified 5 Fusarium spices including *F.oxysporum, F. beomiforme, F. denticulatum, F. solani,* and *F. foetens.* Our results showed that *F.oxysporum* could cause symptoms such as wilting, crown rot, and brownization of the vessels on the White Liberty cultivar in the greenhouse test.

The experimental results demonstrated that the J14 isolate belonging to *F. oxysporum* is more virulent than the others. It seems that the leading cause of vascular wilt symptoms in carnation is the presence of pathogenic isolates of *F. oxysporum* (J14, k5, and k72), but with the onset of drought stress and increasing plant age, the symptoms of crown rot increase. Therefore, by synergizing with each other, these *F. oxysporum* isolates (J14, k5, and k72), were responsible for the occurrence of the vascular wilt disease occurrence in carnation plants. The pathogenicity test of J14, k5, and k72 on other plant seedlings (cucumber, tomato, and gladioli) proved that J14, k5, and k72 belonged to *F. oxysporum* f. sp. *dianthi.* The molecular identification based on the *TEF-1α* gene supported the morphological identification. Shahbazi *et al.* (2021) also performed morphological and molecular identification of *Fusarium* spp. associated with carnation *Dianthus caryophyllus* in Mahallat.

Trichoderma is very important as a bio-control agent and probably a good alternative for chemical fungicides (Harman *et al*., 2004; Verma *et al.,* 2007). Many spices of *Trichoderma* spp. have always been considered potent biocontrol agents against *Fusarium* pathogens (Mohammad *et al.* 2006; Akrami *et al.,* 2012; Sahampoor *et al*., 2020; Lava *et al*., 2020). *T. polysporum* was the best promising antifungal agents in preventing the occurrence of *Fusarium* wilt in chickpea, with 56.9% inhibition (Moutassem *et al.,* 2020). Ommati *et al*.(2012) conducted a study to

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assess the biocontrol efficacy of some native *Trichoderma* isolates against *Fusarium oxysporum*, the causal agent of potato wilt disease, under laboratory and greenhouse conditions. Among the fourteen isolates of *Trichoderma*, *T. asperellum* (T2) and *T. atroviride* (T3) were almost more efficient than the others in inhibiting the mycelial growth of the pathogen, in comparison to the control. Best disease control was observed in potted plants treated with *F. oxysporum* + *T. asperellum* (T2), showing 2.5% disease incidence in contrast to *Fusarium* infested control, in which disease incidence was 73% (Ommati *et al*., 2012). In our study, three isolates, namely, *(T. harzianum (NAS-101), T. virens (NAS-115)*, and *T. ghanens (NAS-108)* had more efficacy to inhibit the mycelial growth of J14, k5, and k72 isolates (belonging to *F. oxysporum)* in dual culture.

The induced mutation provides genetic changes in *Trichoderma* and in some of the mutated isolates, the efficiency of biocontrol may be improved (Mohammad *et al.* 2006, Abbasi *et al.*, 2016; Ghasemi *et al.*, 2020; Orojnia *et al.*, 2021; Soufi *et al.*, 2021; Lava *et al.* 2020, Sahampoor *et al.*, 2020). We used 250 Gy gamma irradiation to increase *Trichoderma* efficiency against *F. oxysporum*. In our evaluation test of efficacy in the greenhouse, we used an equal mixture of *(T. harzianum (NAS-101), T. virens (NAS-115)*, and *T. ghanens (NAS-108)* (TW), and an equal mixture of three selected mutant isolates *ThM7, TgM1.* and *TvM17* (TM), and found that TW and TM treatment had higher ability to control *Fod* infection on carnation plants than the chemical compounds.

In greenhouse evaluations, the use of mutation induction in the *Trichoderma* genome with gamma radiation can be an effective way to achieve genetic diversity and isolates with better performance as bio-control agents. Comparing treatments of the mixture of mutants (TM) with the control, it can be concluded that induction of mutations in the *Trichoderma* genome does not lead to undesirable changes, but screening based on dual culture test increases the effectiveness of *Trichoderma* in controlling *Fod*. It seems that irradiation of *Trichoderma* isolates by gamma rays can increase its antagonistic potential and helps achieving an efficient isolate to inhibit the causative agent of *Fusarium* wilt in carnation. Further field evaluations using TM are the next step of this study.

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کنترل زیستی موثر پژمردگی فوزاریوم میخک با استفاده از ترکیب جدید جدایه های جهش یافته تریکودرما

ف. ذاکر تولایی، س. شهبازی، و ض. درودی

چکیده

آلودگی فوزاریومی مهمترین عامل محدود کننده تولید میخک است. در پژوهش حاضر ۳۸ جدایه فوزاریومی از گیاهان میخک آلوده جمع آوری شده از محلات جدا شد و ۱۵ گونه فوزاریوم از نظر مورفولوژیکی شناسایی شد. جدایه‌های J14، k5 و k72 پرآزارترین جدایه‌ها؛ در آزمون بیماری‌زایی انجام شده بر روی رقم لیبرتی وایت میخک بودند. شناسایی مولکولی جدایه‌های J14، k5و k72 نیز بر اساس ژن (EF1-α)انجام شد. اثر شش جدایه والد تریکودرما ((*T. harzianum* (NAS-101)، *T. auroviridae* (NAS-105)، *T. lixii* (NAS120)، *T. virens* (NAS-115)، *T. ghanens* (NAS- 108 و *T. atroviride* (NAS-112)) بر روی جدایه‌های J14، k5 و k72 بررسی شدند و سه والد کارآمدتر NAS-101، NAS-115 و NAS-108 علیه رشد فوزاریوم در کشت متقابل برای جهش القایی از طریق پرتوتابی با پرتو گاما با دز ۲۵۰ گری مورد استفاده قرار گرفت. تعداد ۲۷۰ موتانت از نظر مورفولوژیکی غربالگری شده و ٦۰ موتانت با استفاده از کشت دوگانه علیه J14، k5 و k72 غربالگری شدند. بر اساس شناسایی مورفولوژیکی و مولکولی، جدایه‌های J14، k5 و k72 به عنوان *F. oxysporum* شناسایی شدند. سه موتانت (ThM7 ٦۷/۱۷%، ٥۹/٤٥TgM1% و ٥۷/٥٥TvM17%) بالاترین اثربخشی را نشان دادند. آزمایش ارزیابی کارایی در گلخانه با مخلوط سه جدایه والد TW و مخلوط سه جدایه جهش یافته TM نشان داد که روش بیوکنترل توانایی بیشتری درکنترل بروز آلودگی فوزاریوم در شرایط گلخانه‌ای روی میخک دارد و القای جهش اثر ناطلوبی بر گیاهان ندارد. نتایج این پژوهش نشان می‌دهد که استفاده از جهش در ژنوم تریکودرما با استفاده از تابش گاما می‌تواند راهی موثر برای دستیابی به جدایه‌هایی با عملکرد بهتر در این عامل کنترل زیستی باشد.

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