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Research Article

Biological control of sesame charcoal rot by gamma-irradiated mutants of *Trichoderma aureoviride* and their RAPD-PCR fingerprinting

Elham Soufi[1], Naser Safaie[1*], Samira Shahbazi[2] and Shideh Mojerlou[3]

1. Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.
2. Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy Organization of Iran (AEOI), Alborz, Iran.
3. Department of Horticulture and Plant Protection, Faculty of Agriculture, Shahrood University of Technology, Shahrood, Iran.

Abstract: The potential of *Trichoderma* spp. for biocontrol of phytopathogenic fungi has been well documented. In this study, the wild-type isolate of *Trichoderma aureoviride* (Tv59) was treated with the target dose of 250 Gy as the optimum dose. Twenty-six mutants were preliminarily screened based on better growth rates. *In vitro,* antagonistic evaluation of the 26 mutants and wild-type was performed against two *Macrophomina phaseolina* isolates (F33 and H7). Six mutants (Tv2(4), Tv20(6), Tv25(6), Tv3(3), Tv4(5), and Tv3(4)) showed remarkable inhibitory activity and were selected for further examination in greenhouse trials. Greenhouse assessment of the selected mutants against *M. phaseolina* isolates revealed Tv20(6) and Tv25(6) as the most highly effective treatments screened for the measured indices. Moreover, the total genomic DNA of the wild-type isolate and its 26 corresponding mutants were analyzed to determine their genetic variability through the RAPD technique. Five RAPD primers generated different banding patterns and yielded a total of 178 amplified fragments, 172 amplicons (96.62%) were polymorphic. While the dendrogram obtained by UPGMA cluster analysis of combined RAPD fingerprints differentiated the wild-type from its mutants at approximately 40% similarity level, the mutants were categorized into two clusters. Based on Jaccard similarity coefficients, eight mutants (Tv25(6), Tv1(5), Tv14(5), Tv20(6), Tv3(4), Tv40(6), Tv33(6), and Tv14(6)) showed the lowest genetic similarities with the parental isolate. The possibility of improvement in biocontrol effectiveness of *T. aureoviride* through random mutagenesis and detection and differentiation of genetic changes induced by gamma rays using RAPD analysis was successfully proved in the present study.

Keywords: charcoal rot, gamma irradiation, *Macrophomina phaseolina*, RAPD-PCR analysis, *Trichoderma aureoviride*

Introduction

Macrophomina phaseolina (Tassi) Goidanich is a critical worldwide fungal pathogen causing stem canker disease in various plant species, commonly referred to as charcoal rot (Singh *et al*., 2012; Kaur *et al*., 2012). The pathogen is a seed- and soilborne and can infect plants at all stages of growth (Iqbal *et al*., 2010). The disease occurs under warm, dry growing conditions with considerable economic losses to the oilseed crops, including sesame *Sesamum indicum* L. (Abdel-Kader *et al*., 2010; Rayatpanah *et al*.,

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2012). Various disease management practices have been adopted against the pathogen, but different control measures have proven ineffective and impractical when the disease has emerged (Anis *et al*., 2010). Its broad host range (over 500 plant families), ability to survive up to 15 years in the soil as a saprophyte, inefficient control offered by resistant cultivars due to the variation among pathogen populations in varied agro-ecological regions, along with the emergence of resistant strains to fungicides and public concerns regarding the health and environmental effects of chemical compounds as a result of the application of fungicides, have given rise to scientific endeavors for alternative solutions to control this pathogen (Kaur *et al*., 2012; Iqbal and Mukhtar, 2014; Barari and Forutan, 2016; Khaledi and Taheri, 2016). Hence, biological control seems to be an alternative and nature-friendly manner towards disease control and reducing the application of chemicals in agriculture (Palizi *et al.*, 2009; Singh, 2014; Karimi *et al.*, 2016; Golafrouz *et al*., 2020). Regarding *M. phaseolina*, numerous studies have reported that some biocontrol agents (BCAs), particularly *Trichoderma* spp. could efficiently suppress the pathogen (Sreedevi *et al*., 2011; Singh *et al*., 2012; Mendoza *et al*., 2015; Shahid and Khan, 2016; Cherkupally *et al*., 2016; Shoaib *et al*., 2018).

Species of the genus *Trichoderma* are filamentous and soilborne fungi that have been known as the most extensively studied BCAs against plant pathogens (Ubalua and Oti, 2007). The common mechanisms of action in *Trichoderma* species are competition for nutrient resources and space, antibiosis, rhizosphere modification, mycoparasitism, promotion in plant growth, and stimulation of defense mechanisms that act in synergy leading to disease control (Benítez *et al*., 2004). *Trichoderma* species can colonize different habitats due to their efficient use of nutrients, their capacity to secrete various antibiotic compounds and enzymes, and their toleration to diverse environmental conditions (Schuster and Schmoll, 2010). Despite all the characteristics mentioned above that have made *Trichoderma* an omnipresent genus, large-scale application of these BCAs remains comparatively infrequent (Rey *et al*., 2001; Benítez *et al*., 2004). Because it has been documented that many antagonists are not capable of confirming their activity in field-scale experiments or when they are examined in various environmental conditions, outcomes will be unpredictable (Vannacci and Gullino, 2000). For this reason, significant progress has been achieved in improving the efficacy of *Trichoderma* species through genetic techniques, e.g., mutation, protoplast fusion, and genetic modification (Rey *et al*., 2001; Haggag and Mohamed, 2007).

Mutagenesis of the microorganisms, particularly BCAs, has been undertaken to enhance their survival, longevity, activity, antifungal potential, and antagonistic ability against a vast spectrum of phytopathogens (Mohamed *et al*., 2010). Accordingly, many researchers have uncovered a way to induce mutation through physical mutagens to obtain mutants with enhanced antagonistic activity or improved antifungal metabolite production ability (Mohamadi *et al*., 2014; Abbasi *et al*., 2014; Bagheri *et al*., 2018; Goharzad *et al*., 2019; Sahampoor *et al*., 2020; Orojnia *et al*., 2021; Lava *et al*., 2021). Besides, it has been feasible to obtain an enormous extent of genetic variability in many organisms including biocontrol agents (Jones and Kortenkamp, 2000; Taheri *et al*., 2014; Wang *et al*., 2017; Elkenawy *et al*., 2017). Such genetic variability can be recognized by assessing variation in the DNA sequence using molecular markers (Chauhan and Rajiv, 2010; Haratian *et al.*, 2013; Govindaraj *et al*., 2015; Rezaee *et al*., 2018; Shahbazi *et al*., 2021). Random amplified polymorphic DNA (RAPD) procedure introduced as a result of the independent efforts of Williams *et al*. (1990) and Welsh and McClelland (1990) relies on the PCR-assisted amplification of random segments of DNA using single arbitrary short primers (Kumari and Thakur, 2014). Although low reproducibility has been described as the major limitation and criticism of the RAPD technique (Atienzar and Jha, 2006); this methodology has been employed most frequently for examining genetic relationships and genetic

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diversity analysis in numerous fungal genera, including *Trichoderma* (Góes *et al*., 2002; Dodd *et al*., 2004; Gupta *et al*., 2010; Kumar and Sharma, 2011; Skoneczny *et al*., 2015; Rai *et al*., 2016; Carvalho *et al*., 2018).

In the current study, gamma irradiation was used to develop stable mutants of *T. aureoviride* (wild-type Tv59) and their biocontrol activities against *M. phaseolina*, the causative agent of charcoal rot of sesame, were evaluated *in vitro* and under greenhouse conditions. We also assessed the possibility of detecting genetic variations of wild-type and derivative mutants after the influence of gamma rays using the RAPD technique.

Materials and Methods

Fungal isolates

The *Trichoderma* wild type isolate (Tv59) was received from the plant pathology collection of Nuclear Agriculture Research School–Nuclear Science and Technology Institute (NSTRI), purified (Ghaneie *et al*., 2012) and subjected to molecular identification using the ITS1 and ITS2 gene sequencing analysis (Haratian *et al*., 2013) and successfully identified as *T. aureoviride* and registered with the accession number MW719569.1 at GenBank (NCBI) (Soufi *et al*., 2021). The two isolates of *M. phaseolina* (H7 and F33) were obtained from the Culture Collection of Tarbiat Modares University, Tehran, Iran.

Optimization of gamma radiation dose for induced mutagenesis

T. aureoviride spore suspensions (10^6 spore/ml) in a sterile saline solution (9 g/l NaCl in deionized water) were made from slant cultures after seven days of incubation and exposed to different doses of gamma radiation (0, 50, 150, 200, 250, 300, 350, 400 and 450 Gy (in triplicate)) provided from a cobalt-60 gamma cell with the activity of 2500 Curie and the dose rate of 0.23 Gy/s at Agricultural, Medical, and Industrial Research School, Nuclear Science and Technology Research, Alborz, Iran. Dosage validation was carried out using the well-known aqueous chemical dosimeter, Fricke dosimeter (Matthews, 1982). The concentration of irradiated suspensions was adjusted as 10^2 spore/ml, then spore suspensions were grown on PDA, stored at 28 °C for 12 h, and the quantity of germinated spores was verified. The optimal dose for induced mutagenesis was approximately defined based on 40-50% inhibition of spore germination on PDA (Ahari Mostafavi *et al*., 2010). The stability of mutants (including 260 isolates) was assessed through successive sub-culturing for ten generations on PDA medium at 25°, 28°, and 30 °C and colony characteristics of the mutants (radial growth rate, growth pattern on PDA, and colony color) were monitored and compared with their parental isolate. These experiments were set in three replications for each isolate.

In vitro antagonistic assays

Dual culture test

The antagonistic potentiality of *T. aureoviride* isolates (mutants and wild-type) against two isolates of *M. phaseolina* was examined through dual culture assay as illustrated by Dennis and Webster (1971a). Nine-mm mycelial discs were removed marginally from four-day-old cultures of the antagonistic and pathogenic isolates and placed on PDA dishes in the opposite direction at equidistance from the side of the dishes and incubated at 27 °C for four days. Three replicate plates were made for each treatment. Control plates contained sterile agar plugs instead of the antagonist. Radial growth measurements were recorded at 24-hour intervals. The growth inhibition percentage of the pathogen was assessed using the formula GI % = (dc-dt)/dc × 100, where GI is the percentage of growth inhibition, dc is the average radial growth in control, and dt represents the average radial growth of the pathogen in treatments.

Effect of volatile metabolites

The impact of volatile substances produced by *T. aureoviride* isolates on the tested pathogen was evaluated according to the technique clarified by Dennis and Webster (1971b). The PDA plates were inoculated centrally by placing nine-mm mycelial discs taken from four-day-old cultures of the *M. phaseolina* isolates and the antagonist. The lids of plates inoculated with the antagonists

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were replaced by the bottom portion of PDA plates inoculated with the tested pathogen. Then the matched plates were fixed and sealed together by Parafilm tape and incubated at 27 °C for two to three days. The plates inoculated with the pathogenic isolates alone served as control. Each treatment had three replications. Growth measurements were taken daily, and GI% was calculated, as described above.

Inhibitory impact of *T. aureoviride* culture filtrate

The growth inhibition of the examined pathogen through non-volatile substances of *T. aureoviride* isolates was assessed following the methodology of Dennis and Webster (1971a). The inoculum was prepared from four-day-old cultures of *T. aureoviride* isolates and transferred into 250 ml Erlenmeyer flasks containing 100 ml Potato Dextrose Broth (PDB) and stored at 25 °C with slow shaking (75 rpm) for seven days. Then, the cultures were filtered through Whatman filter paper No.1 and re-filtered through a Millipore membrane filter (0.22 µm, Syringe®) and preserved at 4 °C for two days. The filter-sterilized culture filtrate (FCF) of *T. aureoviride* isolates was amended in PDA to make the final concentration 10% in Petri dishes, and mycelial plugs of the pathogen (9-mm diameter) were put in the midpoint of dishes and stored for two days at 27 °C. Control treatment did not contain the FCF of the antagonist. Similarly, daily measurements were made, and GI% was calculated using the previously specified formula.

Statistical analysis

In all *in vitro* assessments, each experiment was accomplished through a completely randomized design (CRD), including three replicates, and data analysis was conducted by one-way ANOVA and Duncan's multiple range test ($p \leq 0.01$) embedded in the MSTAT-C statistical software (Michigan State University, version 1.42).

Greenhouse assessments of the selected *T. aureoviride* isolates

The efficacy of *T. aureoviride* isolates and Carboxin Thiram fungicide was studied to control charcoal rot and promote plant growth indices in greenhouse experiments. The greenhouse trials were performed two times under identical conditions in a Randomized Complete Block Design (RCBD), including four replicates. Inoculums of the selected antagonistic and pathogenic fungi were provided by growing on millet and wheat grains (in 250 ml flasks), respectively, as follows: the grains were kept immersed in distilled water, autoclaved for two subsequent days (121 °C for 45 min), and inoculated with five mycelial discs (9 mm in diameter) from four-day-old fungal colonies. Flasks were incubated at 27 °C in the darkness for 21 days. After that, colonized grains were transferred into paper pockets, dried, and ground. For both pathogenic and antagonistic fungi, an equal quantity of inoculum (i.e., 5 g) was added to 1 kg of the mixture of sterilized sandy soil, perlite, and peat moss (1: 1: 1) in each plastic pot (17 cm in diameter). In the case of the treatments in which both antagonistic and pathogenic fungi were used, the inoculum of *M. phaseolina* was added to the pots seven days before planting the seeds. Then the inoculum of the antagonist was embedded into the upper side of the pots at the time of planting the seeds. Before planting, the seeds (cv. Dashtestan II) were superficially disinfected by soaking in 2% sodium hypochlorite for one minute, rinsed three times with sterile distilled water, and left for drying overnight (except for the fungicide treatment in which the seeds were disinfected in 2:1000 Carboxin Thiram). The pots were kept at 35 °C and allowed to grow for 70 days. Observations were recorded for root and foliage wet weights, root and foliage dry weights, mortality rate, wilting percentage, and the length of stem lesions for 18 treatments, including 1: control (no pathogen, no antagonist and no fungicide); 2: the pathogen (the combination of two isolates of *M. phaseolina*); 3: Tv4(5); 4: Tv2(4); 5: Tv20(6); 6: Tv3(3); 7: Tv25(6); 8: Tv3(4); 9: Tv59 (the wild-type isolate); 10: Tv4(5) + pathogen; 11: Tv2(4) + pathogen; 12: Tv20(6) + pathogen; 13: Tv3(3) + pathogen; 14: Tv25(6) + pathogen; 15: Tv3(4) + pathogen; 16: Tv59 + pathogen; 17: Carboxin Thiram, and 18: Carboxin Thiram + pathogen.

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Root and foliage wet and dry weights were calculated using the formula below:

$$X\% = \frac{I - U}{U} \times 100$$

Where X is the percentage of increased or decreased wet and dry weight, I, represents the wet or dry weight of the treatments, and U denotes the wet or dry weight of the healthy control. Finally, the various sets of data were analyzed by the two-way ANOVA technique, and the means were compared by Duncan's multiple range test ($p \leq 0.01$) using the MSTATC statistical software (Michigan State University, version 1.42).

DNA extraction

The nine-mm mycelial plugs from four-day-old colonies of the *T. aureoviride* isolates were transferred into conical flasks containing 50 ml of PDB and incubated for four days on an orbital shaker incubator (120 rpm) at 25 °C (Torkamani *et al.*, 2014). The mycelial biomass was collected; the genomic DNA extraction of isolates was performed using Safaie *et al*. (2005) protocol. Quantity and quality of extracted DNA were assessed using spectrophotometric measurement (Eppendorf Biophotometer, Germany) and gel electrophoresis (agarose gels of 0.8%), respectively.

RAPD analysis

A set of 10 decamer primers of arbitrary sequence (Sinaclon, Tehran) was screened to investigate genetic variations among *T. aureoviride* isolates by RAPD-PCR. Five primers viz, OPA09 (5'-GGGTAACGCC-3'), OPA10 (5'-GTGATCGCAG-3'), OPA11 (5'-CAATCGCCGT-3'), OPA14 (5'-TCTGTGCTGC-3'), and OPA16 (5'-AGCCAGCGAA-3') were selected based on generating the reproducible polymorphic bands (Moore *et al*., 2001; Sharma *et al*., 2005). Amplifications were conducted within a thermocycler (Eppendorf AG, Germany) at least twice in 20 µl of a reaction mixture containing 1 µl template DNA (20 ng), 1 µl primer (10 pmol (Sinaclon, Tehran)), 0.4 µl of dNTP mix (10 mM), 0.5 µl Taq polymerase (5 unit. μl^{-1} (Sinaclon, Tehran)), 0.8 µl $MgCl_2$ (50 mM), and 2 µl PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH = 9.0). The PCR programs began with an initial step of denaturation for 3 min at 92 °C, 35 cycles at 92 °C for 1 min, 1 min at annealing temperature (33 °C), followed by a 2 min extension at 72 °C, and a final step of extension for 5 min at 72 °C. In each PCR experiment, a reaction missing template DNA was included as a negative control. PCR amplified fragments were visualized using 1.4% agarose gel electrophoresis by Gel Documentation System (Vilbert, Lourmat, Marne La Vallee, France) following ethidium bromide staining (0.5 µg/ml). To determine the fingerprinting patterns, the presence or absence of reproducible bands generated with each primer was scored as 1 and 0, respectively. Genetic similarities were measured by Jaccard's similarity coefficient, and cluster analysis was conducted by the UPGMA method provided in NTSYS-pc software (Applied Biostatistics Inc., USA, version 2.1).

Results

Determining the optimal gamma radiation dose for mutagenesis

Spore suspension of *T. aureoviride* wild-type isolate was radiated with the target doses of 0 to 450 Gy. According to the findings, a decrease in the spore germination (%) of *T. aureoviride* was detected after an increase in gamma radiation doses up to 450 Gy indicating an inverse relationship between the applied gamma radiation doses and the spore germination (%) of *T. aureoviride* (Shahbazi *et al*., 2014). The lethal dose based on the spore germination rate was 450 Gy, whereas the dose of 250 Gy showed an inhibitory effect on germination of *T. aureoviride* spores up to 43.4%. Since 40-50% inhibition of spore germination on PDA was regarded as the main criterion underlying the selection of the optimal dose (Ahari Mostafavi *et al*., 2010), 250 Gy dose was applied for mutation induction. Then, dilutions were conducted and plated on PDA, and single spores were individually transferred to fresh PDA plates. After successive subculture of the emerged

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colonies for ten times at various temperatures (25°, 28° and 30 °C) and comparison of their colony characteristics (radial growth rate, growth pattern on PDA and colony color) with their wild-type isolate, 260 mutants of *T. aureoviride* were chosen as stable mutants. All mutants and the wild type grew at 25° to 28 °C. They showed the fastest growth rate at 28 °C. Twenty-six mutants displayed statistically more mycelial linear growth rates on PDA than the parental isolate and were chosen for further experiments (*in vitro* assays). The colony color in all irradiated and non-irradiated isolates was green. Except for Tv10(6), Tv6(5), Tv11(5), Tv33(6), Tv14(6), and Tv14(5) (with petaloid colony), all other mutants had a smooth colony, a growth pattern similar to the non-irradiated isolates.

In vitro antagonistic assays

Dual culture test

Dual culture results demonstrated significant differences in growth inhibition (GI) of the pathogen within 96 hours of incubation ($p \leq 0.01$, Table 1). Substantial mycelial growth reduction of the *M. phaseolina* isolate F33 on PDA plates was observed in presence of the mutants Tv2(4) (51.67%), Tv20(6) (51.33%), and Tv3(4) (48.33%), while the lowest GI value was found with the mutant Tv2(3) (33%). In the case of isolate H7, four mutants (Tv4(3), Tv25(5), Tv6(3), and Tv5(3)) showed the lowest growth inhibitory activity, while the highest GI value was produced by Tv25(6) (60%) (Fig. 1a). In addition, of the 26 mutants, 19.23% of mutants were capable of reducing the colony growth of the pathogen to more than 50% within 96 hours of incubation.

Table 1 The mycelial growth percentage of *Macrophomina phaseolina* isolates (F33 and H7) affected by *Trichoderma aureoviride* mutants and wild-type (Tv59) in dual culture test within 96 hours of incubation.

Treatments	24 h		48 h		72 h		96 h	
	F33	H7	F33	H7	F33	H7	F33	H7
Control	100^{A}	100^{A}	100^{A}	100^{A}	100^{A}	100^{A}	100^{A}	100^{A}
Tv2(4)	52^{F-H}	47.29^{F-I}	51.33^{CD}	72^{G-J}	52.47^{DE}	51.33^{D-G}	48.33^{F}	50^{F-I}
Tv14(6)	61^{C-H}	79^{CD}	39.47^{D}	79.67^{D-H}	60^{B-E}	58.67^{B-E}	58.67^{B-E}	56.33^{B-H}
Tv5(4)	61.16^{C-H}	45.74^{F-I}	39.33^{D}	88.67^{A-E}	64^{B-D}	64^{BC}	61.67^{B-D}	60.33^{B-E}
Tv6(5)	61.91^{C-G}	89.15^{A-C}	39.49^{D}	83.67^{B-G}	58^{C-E}	62^{B-D}	57.33^{B-F}	60^{B-E}
Tv11(5)	56.55^{C-H}	27.13^{JK}	39.33^{D}	55^{LM}	62.67^{B-D}	44^{F-H}	59^{B-D}	52.33^{D-I}
Tv1(5)	64.73^{B-F}	66.98^{DE}	39.33^{D}	74^{F-J}	67.33^{BC}	52.67^{C-F}	64^{BC}	53.33^{C-I}
Tv3(3)	61.16^{C-H}	50.39^{FG}	39.33^{D}	69^{H-K}	60.33^{B-E}	57.67^{B-E}	57.33^{B-F}	55^{B-I}
Tv28(6)	66.95^{B-E}	48.84^{F-H}	39.33^{D}	67.33^{H-L}	63^{B-D}	59.33^{B-E}	60^{B-D}	56.33^{B-H}
Tv4(5)	62.20^{C-G}	95.35^{AB}	37^{D}	89^{A-E}	61^{B-E}	64^{BC}	58^{B-F}	59.67^{B-F}
Tv32(5)	61.02^{C-H}	46.98^{F-I}	39.33^{D}	76^{E-I}	64.33^{B-D}	61.33^{B-D}	60.67^{B-D}	56.67^{B-G}
Tv25(5)	67^{B-E}	56.59^{EF}	69.67^{A-D}	79.67^{D-H}	65.33^{B-D}	65.33^{B}	62^{B-D}	63^{BC}
Tv40(6)	70^{BC}	48.84^{F-H}	70^{A-D}	61^{J-M}	60^{B-E}	45^{F-H}	56.67^{B-F}	45.67^{IJ}
Tv3(4)	52.67^{E-H}	25.58^{K}	58^{B-D}	57.67^{K-M}	52.67^{DE}	40^{GH}	51.67^{D-F}	50.33^{E-I}
Tv20(6)	53.90^{D-H}	44.19^{F-I}	58.33^{B-D}	69^{H-K}	49.67^{E}	48^{E-H}	48.67^{EF}	46.67^{H-J}
Tv59	69.67^{BC}	34.88^{H-K}	69.67^{A-D}	64^{I-M}	63.67^{B-D}	57^{B-E}	59.67^{B-D}	63^{BC}
Tv10(6)	48.19^{GH}	84.5^{BC}	81.67^{A-C}	80.67^{C-H}	67.33^{BC}	61^{B-D}	63.33^{BC}	60.67^{B-D}
Tv2(3)	47^{H}	41.24^{G-I}	84.33^{AB}	64.67^{I-M}	71.67^{B}	48.33^{E-H}	67^{B}	48.67^{G-J}
Tv5(3)	60^{C-H}	41.86^{G-I}	83.67^{A-C}	74^{F-J}	62^{B-E}	64.16^{BC}	58.33^{B-F}	63^{BC}
Tv6(3)	67.67^{B-D}	83.72^{BC}	87.67^{AB}	95.33^{AB}	66.33^{BC}	65.33^{B}	62.67^{BC}	63^{BC}
Tv14(5)	64^{B-F}	78.91^{CD}	80^{A-C}	76.67^{E-I}	56.33^{C-E}	53.67^{B-F}	54.33^{C-F}	51.67^{D-I}
Tv31(5)	60.33^{C-H}	84.5^{BC}	78.67^{A-C}	86.67^{A-F}	60^{B-E}	62^{B-D}	58.67^{B-E}	59.67^{B-F}
Tv9(6)	70^{BC}	33.33^{I-K}	88.33^{AB}	62.67^{I-M}	67^{BC}	55.67^{B-F}	63.67^{BC}	53^{C-I}
Tv23(6)	77.33^{B}	90.70^{A-C}	88.67^{AB}	91^{A-D}	68.33^{BC}	63.33^{B-D}	63.33^{BC}	60.33^{B-E}
Tv25(6)	68^{B-D}	40.46^{G-J}	85^{AB}	52.33^{M}	60.67^{B-E}	37^{H}	56.33^{C-F}	40^{J}
Tv33(6)	77^{B}	34.11^{I-K}	91^{AB}	69.33^{H-K}	67.33^{BC}	44.33^{F-H}	64^{BC}	47^{G-J}
Tv2(5)	60.67^{C-H}	41^{G-I}	77.33^{A-C}	73.67^{F-J}	58^{C-E}	59^{B-E}	54.67^{C-F}	56.33^{B-H}
Tv4(3)	93.67^{A}	82.5^{BC}	88^{AB}	94.33^{A-C}	68^{BC}	64.67^{BC}	63.33^{BC}	63.67^{B}

The results are indicated as mean values. Significance differences are specified using superscripts carrying different letters in a column ($p \leq 0.01$, Duncan's multiple range test).

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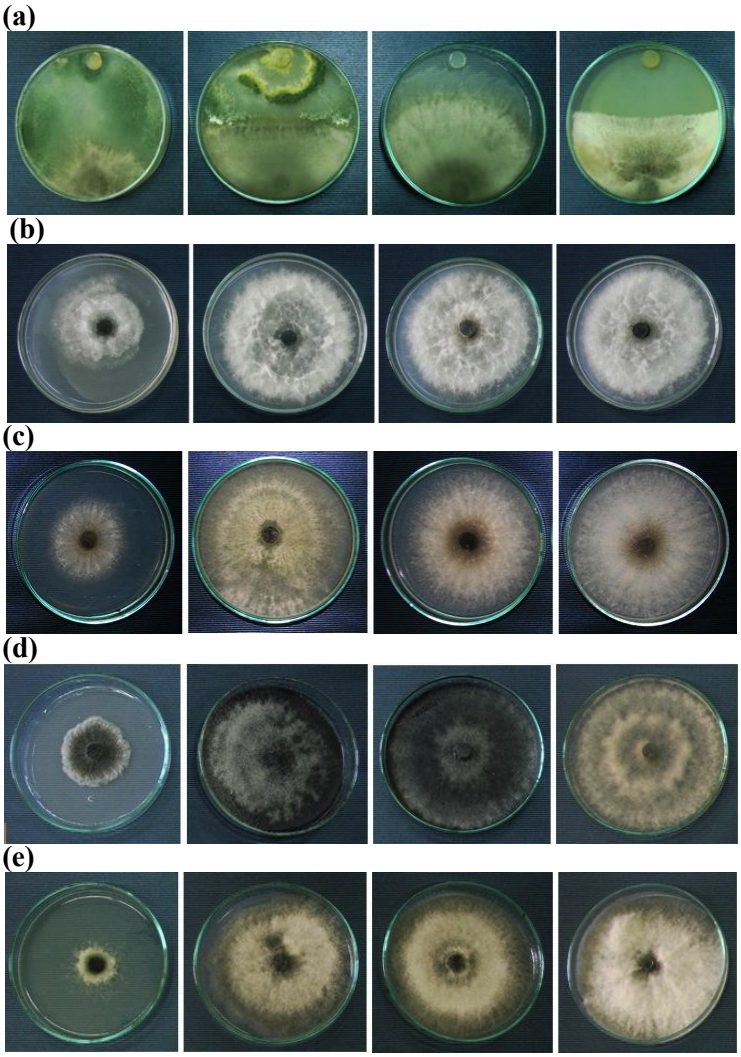

Figure 1 (a) Effect of *Trichoderma aureoviride* isolates on the colony growth of *Macrophomina phaseolina* (H7) in dual culture test. From left, Tv25(6), Tv59 (wild-type), control, and Tv4(3). (b) Effect of *Trichoderma aureoviride* isolates on the colony growth of *Macrophomina phaseolina* (F33) in volatile metabolite production test. From left, Tv11(5), Tv59 (wild-type), control, and Tv23(6). (c) Effect of *Trichoderma aureoviride* isolates on the colony growth of *Macrophomina phaseolina* (H7) in volatile metabolite production test. From left, Tv4(5), Tv59 (wild-type), control, and Tv2(3). (d) Effect of *Trichoderma aureoviride* isolates on the colony growth of *Macrophomina phaseolina* (F33) in culture filtrate test. From left, Tv3(3), Tv59 (wild-type), control, and Tv9(6). (e) Effect of *Trichoderma aureoviride* isolates on the colony growth of *Macrophomina phaseolina* (H7) in culture filtrate test. From left, Tv25(6), Tv59 (wild-type), control, and Tv31(5).

Effect of volatile metabolites

The impact of volatile inhibitors of *T. aureoviride* isolates on radial growth was evaluated after 24, 48 and, 72 hours of incubation (Table 2, $p \leq 0.01$). After 24 hours of incubation, the mutant Tv9(6) resulted in the lowest percentage of growth reduction of F33 isolate (9.4%), while the maximum growth inhibition was observed in presence of the mutant Tv14(5) (47.43%), which was followed by Tv40(6) (46.59%). After 48 hours of incubation, all mutants had GI values less than 50% indicating a weak activity in suppressing radial growth of the isolate F33. In addition, the percentage of mycelial growth of the pathogen in the case of four mutants (Tv5(4), Tv4(3), Tv10(6), and Tv23(6)) was similar to that of wild-type (Fig.

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1b). For isolate H7, the percentage of mycelial growth varied from 102% (Tv33(6)) to 50.33% (Tv5(4)) as compared to 4.33% of wild-type after 48 hours of incubation. Also, in the case of eight mutants (Tv33(6), Tv25(5), Tv10(6), Tv14(5), Tv2(5), Tv1(5), Tv28(6), and Tv5(3)), no significant antagonistic response was detected. On the third day of incubation, the GI rate of the isolate H7 versus ten mutants (Tv14(5), Tv32(5), Tv2(3), Tv33(6), Tv4(3), Tv28(6), Tv2(5), Tv1(5), Tv5(3) and Tv6(5)) decreased to 0%, whereas the highest GI was recorded from the mutant Tv4(5) (35.33%) (Fig. 1c). Furthermore, of the 26 mutants, only 2.30% of mutants held the capacity to reduce the colony growth of the pathogen to more than 50%.

Impact of non-volatile compounds of *T. aureoviride* isolates

As presented in Table 3, after 24 hours of incubation, the mutants Tv3(3) and Tv9(6) resulted in the highest (87.67%) and lowest (3%) GI of the isolate F33 as compared to 38.33% of wild-type. After 48 hours of incubation, the most effective antagonistic response was related to the mutant Tv3(3) (72.33%) (Fig. 1d), while in the case of seven mutants (Tv5(4), Tv31(5), Tv9(6), Tv6(5), Tv25(5), Tv28(6), and Tv6(3)), the rate of GI dropped to 0%. Regarding the isolate H7, the mutant Tv5(4) resulted in the lowest percentage of pathogen growth reduction (3.07%) after 24 hours, whereas the most inhibitory effect was obtained with the mutant Tv3(3) (83.78%), which was followed by Tv14(6) (80.21%). On the second day of incubation, four mutants (Tv25(6), Tv3(3), Tv5(3), and Tv14(6)) revealed strong inhibitory activity, whereas two mutants (Tv31(5) and Tv5(4)) had no significant differences compared with control (Fig. 1e). Moreover, of the 26 mutants, 23.07% caused more than 50% mycelial growth reduction of the pathogen

Table 2 The mycelial growth percentage of *Macrophomina phaseolina* isolates (F33 and H7) affected by *Trichoderma aureoviride* mutants and wild-type (Tv59) in volatile metabolite production test within 72 hours of incubation.

Treatments	24 h		48 h		72 h	
	F33	H7	F33	H7	F33	H7
Tv14(5)	52.57^{K}	95.33^{AB}	86.99^{A-D}	98.33^{A}		100^{A}
Tv40(6)	53.41^{K}	92.33^{AB}	91.22^{A-C}	91.67^{AB}		95.33^{AB}
Tv59	67.88^{F-J}	87.67^{A-C}	95.12^{AB}	95.67^{A}		100^{A}
Control	100^{A}	100^{A}	100^{A}	100^{A}		100^{A}
Tv25(6)	57.72^{JK}	88.33^{A-C}	88.86^{A-D}	95^{AB}		96.67^{AB}
Tv6(3)	60^{I-K}	70^{B-D}	85.28^{B-D}	69.67^{C-E}		92^{A-C}
Tv32(5)	62.12^{H-K}	87.33^{A-C}	92.68^{A-C}	88.67^{A-C}		100^{A}
Tv6(5)	56.97^{JK}	92.67^{AB}	91.38^{A-C}	94.67^{AB}		100^{A}
Tv20(6)	67.42^{F-J}	92^{AB}	92.68^{A-C}	88^{A-C}		98.33^{AB}
Tv4(5)	67.57^{F-J}	64.67^{C-E}	92.68^{A-C}	61^{DE}		64.67^{C}
Tv2(3)	68.33^{F-J}	98.33^{A}	88.29^{A-D}	86^{A-C}		100^{A}
Tv3(4)	70^{E-J}	93.67^{AB}	92.68^{A-C}	85^{A-C}		93.33^{AB}
Tv33(6)	88.48^{A-C}	90^{AB}	91.87^{A-C}	102^{A}		100^{A}
Tv9(6)	90.60^{AB}	41.67^{F}	92.92^{A-C}	57.33^{DE}		81.67^{A-C}
Tv5(4)	78.18^{B-G}	40.67^{F}	94.30^{AB}	50.33^{E}		71.67^{A-C}
Tv2(4)	87.72^{BC}	58.33^{D-F}	92.68^{A-C}	53^{E}		69.33^{BC}
Tv25(5)	80.30^{B-G}	87.33^{A-C}	92.68^{A-C}	100^{A}		92.67^{AB}
Tv31(5)	80.75^{B-F}	43.67^{EF}	91.95^{A-C}	60.33^{DE}		99.33^{A}
Tv4(3)	67.42^{F-J}	79.67^{A-D}	93.74^{AB}	74^{B-D}		100^{A}
Tv10(6)	67.12^{G-J}	89.33^{A-C}	95.28^{AB}	98.67^{A}		99.33^{A}
Tv3(3)	69.70^{E-J}	97.33^{A}	86.34^{B-D}	94^{AB}		98.33^{AB}
Tv23(6)	73.48^{D-H}	79^{A-D}	95.12^{AB}	88.33^{A-C}		89.33^{A-C}
Tv11(5)	70.30^{E-J}	78^{A-D}	80.16^{CD}	90^{A-C}		93.33^{AB}
Tv14(6)	82.57^{B-E}	87.67^{A-C}	91.87^{A-C}	93.67^{AB}		95.33^{AB}
Tv2(5)	75.45^{C-H}	92.67^{AB}	78.15^{D}	99^{A}		100^{A}
Tv1(5)	83.71^{B-D}	85.33^{A-C}	90.41^{A-D}	97.67^{A}		100^{A}
Tv28(6)	77.42^{C-G}	85.33^{A-C}	88.21^{A-D}	97.67^{A}		100^{A}
Tv5(3)	71.21^{D-I}	101^{A}	90.89^{A-C}	100^{A}		100^{A}

The results are indicated as mean values. Significance differences are specified using superscripts carrying different letters in a column ($p \leq 0.01$, Duncan's multiple range test).

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Table 3 The mycelial growth percentage of *Macrophomina phaseolina* isolates (F33 and H7) affected by *Trichoderma aureoviride* mutants and wild-type (Tv59) in culture filtrate test within 48 hours of incubation.

Treatments	24 h		48 h	
	F33	H7	F33	H7
Control	100^{A}	100^{A}	100^{A}	100^{A}
Tv4(5)	69^{D-G}	79.82^{B-D}	89.67^{BC}	92.60^{AB}
Tv5(4)	73^{CD}	96.93^{AB}	100^{A}	100^{A}
Tv1(5)	55.67^{HI}	69.22^{D-F}	86^{C}	74.63^{DE}
Tv10(6)	48.33^{IJ}	75.35^{C-E}	78.33^{DE}	78.53^{CD}
Tv59	61.67^{E-H}	74.20^{C-E}	90.33^{BC}	85.93^{BC}
Tv31(5)	84^{B}	91.83^{A-C}	100^{A}	100^{A}
Tv2(3)	61^{F-H}	68.32^{D-F}	87^{C}	77.76^{C-E}
Tv2(5)	47.33^{IJ}	54.28^{F-H}	70.67^{FG}	63^{FG}
Tv14(5)	40.67^{JK}	26.30^{I-K}	65.33^{GH}	50.98^{H}
Tv32(5)	48^{IJ}	59.38^{E-G}	73^{EF}	73.17^{D-F}
Tv2(4)	70.33^{D-F}	44.95^{G-I}	95.67^{AB}	66.99^{E-G}
Tv20(6)	54^{HI}	44.70^{G-I}	79^{DE}	59.92^{GH}
Tv40(6)	59.67^{GH}	71.90^{D-F}	84.67^{CD}	88.7^{B}
Tv33(6)	48^{IJ}	64.37^{D-F}	69.67^{FG}	60.24^{GH}
Tv14(6)	73.67^{CD}	19.79^{K}	96.67^{A}	34.96^{I}
Tv9(6)	97^{A}	54.15^{F-H}	100^{A}	68.41^{D-G}
Tv11(5)	81.33^{BC}	40.23^{H-J}	97.67^{A}	51^{H}
Tv6(5)	71.67^{C-E}	62.58^{D-G}	100^{A}	76^{C-E}
Tv25(5)	73.67^{CD}	67.56^{D-F}	100^{A}	71.80^{D-F}
Tv4(3)	75.67^{B-D}	62.58^{D-G}	99.67^{A}	70.20^{D-G}
Tv28(6)	68.67^{D-G}	60.66^{D-G}	100^{A}	76.67^{C-E}
Tv5(3)	36.67^{K}	28.73^{I-K}	62^{H}	34.75^{I}
Tv25(6)	23.33^{L}	22.35^{JK}	60.33^{H}	32.19^{I}
Tv3(3)	12.33^{M}	16.22^{K}	27.67^{I}	39.75^{I}
Tv23(6)	36.33^{K}	38.95^{H-J}	84.33^{CD}	67.80^{D-G}
Tv6(3)	73.67^{CD}	76.50^{C-E}	100^{A}	70.16^{D-G}
Tv3(4)	62^{E-H}	40.23^{H-J}	79^{DE}	51^{H}

The results are indicated as mean values. Significance differences are specified using superscripts carrying different letters in a column ($p \leq 0.01$, Duncan's multiple range test).

Greenhouse assessments of the selected *T. aureoviride* isolates

Out of 26 mutants, six mutants (Tv2(4), Tv20(6), Tv25(6), Tv3(3), Tv4(5), and Tv3(4)) were selected based on *in vitro* assays as potentially superior mutants (along with the wild-type (Tv59)) for further evaluation of their biocontrol activity in greenhouse experiments.

Root wet weight

Analysis of the results revealed highly remarkable differences among the treatments at the 1% significance level in Duncan's multiple range test; thus, the treatments were separated into five groups (Fig. 2a). In this regard, the mutants Tv20(6) and Tv2(4) demonstrated substantial increases in the wet weights of roots up to 2.83 and 2.39 times higher than that observed in control. In contrast, a significant decline in root wet weight was noticed in the pathogen treatment (38%), followed by the Carboxin Thiram + pathogen treatment (34%) compared to control. In addition, in the wild-type and Carboxin Thiram treatments, the root wet weight increased by 1.07 and 1.02 times over the control.

Root dry weight

As evident from Fig. 2b, the mutants including Tv20(6), Tv2(4), and Tv25(6) resulted in marked increases in the root dry weight up to 2.77, 2.48, and 1.92 times higher than control, respectively. On the contrary, the least efficiency was found with the pathogen treatment followed by Carboxin Thiram + pathogen and Tv4(5) + pathogen, which reduced the root dry weight by 71.4%, 63.8%, and 44.42% in comparison to

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control, respectively. In addition, in the wild-type and Carboxin Thiram treatments, the root dry weight increased by 57.91% and 50.54% compared to control (Fig. 2b).

Foliage wet weight

The results revealed that the treatment Tv2(4) resulted in the maximum increase (2.5-fold) in foliage wet weight as compared to control, which was followed by Tv20(6). Moreover, the wild-type and Carboxin Thiram treatments enhanced the wet weight of foliage by 79.29% and 81.84% over control, respectively. On the other hand, the Carboxin Thiram + pathogen and pathogen treatments led to the loss in the wet weight of foliage by 32.69% and 32.09% compared to control, respectively (Fig. 2c).

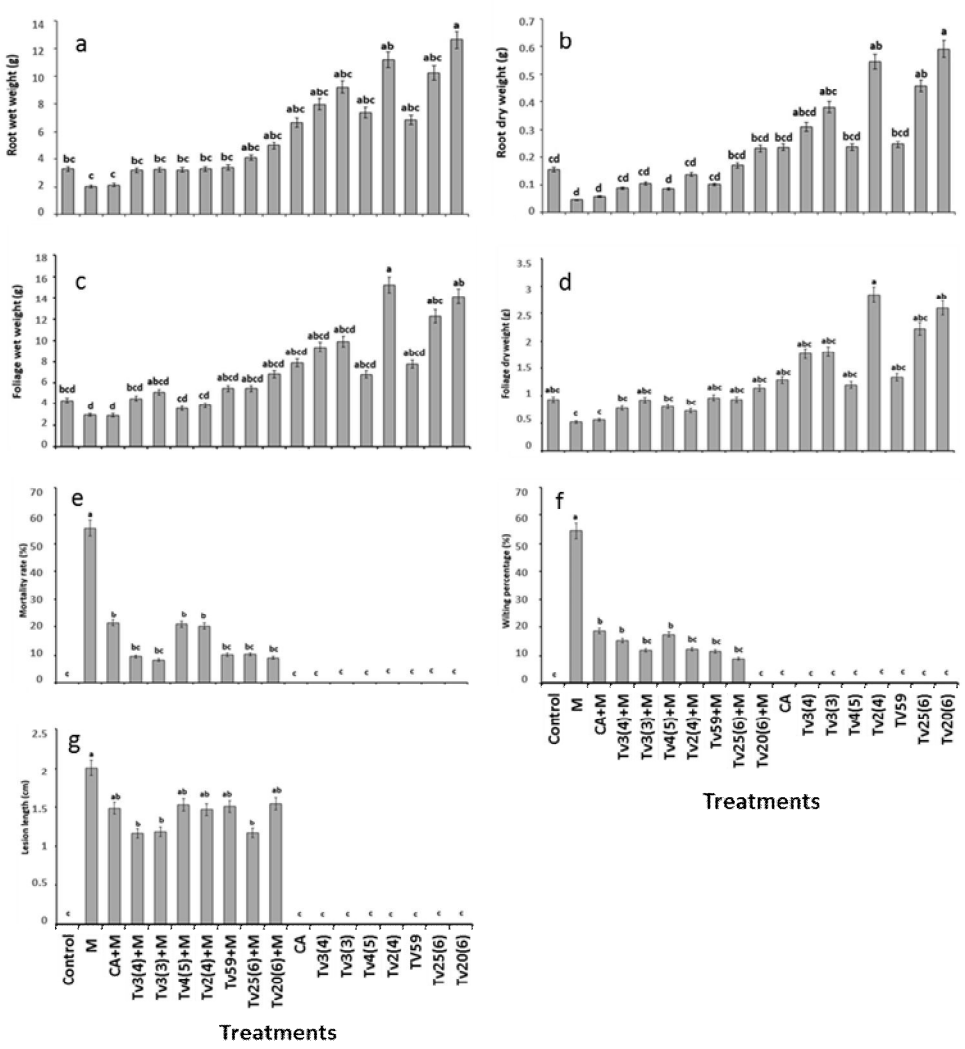

Figure 2 Effect of different treatments (wild-type, selected mutants of *Trichoderma aureoviride*, and Carboxin Thiram) separately and in combination with the *Macrophomina phaseolina* isolates on (a) root wet weight (g), (b) root dry weight (g), (c) foliage wet weight (g), (d) foliage dry weight (g), (e) mortality rate (%), (f) the wilting percentage (%), and (g) the length of stem lesion (cm) of sesame plants 70 days after planting in greenhouse conditions. M: The pathogen treatment and CA: The Carboxin Thiram treatment. Means labeled with identical letters show no significant difference (Duncan's test, $p \leq 0.01$).

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Foliage dry weight

Based on the results, as reflected in Fig. 2d, the treatments were split into five groups ($p \leq 0.01$). The maximum increase in the dry weight of foliage over control (2.06-fold) was achieved in the treatment Tv2(4), which was followed by Tv20(6). Also, the treatments of wild-type and Carboxin Thiram enhanced the dry weight of foliage by 44.26% and 38.63% compared to control, respectively. In contrast, the pathogen and Carboxin Thiram + pathogen treatments led to the loss in the dry weight of foliage by 44.39% and 40.45% compared with control, respectively.

Mortality rate

As in previous experiments, significant differences were observed among the treatments regarding plant survival and reducing the number of dead plants (Fig. 2e). According to the findings, the highest mortality rate was obtained with the pathogen treatment (55.47%). In contrast, the highest percentage of the survived plants was obtained from the Tv3(3) + pathogen treatment followed by the Tv20(6) + pathogen, which led to significant reductions in mortality rates of 8.36% and 9.13%, respectively.

Wilting percentage

The analysis of the recorded percentage of wilted plants showed that the best wilting control was related to the treatment Tv20(6) + pathogen followed by Tv25(6) + pathogen, which reduced the wilting percentage to 0% and 8.98%, respectively. In contrast, in the pathogen treatment, the wilting rate increased to 54.44% (Fig. 2f).

The length of stem lesions

The results revealed that the highest mean measured for lesion length was 2 cm, recorded for the pathogen treatment (Fig. 2g). On the contrary, no significant differences were detected among the treatments of Tv3(4) + pathogen, Tv3(3) + pathogen, and Tv25(6) + pathogen as for the lesion length, and the lesions in all these three treatments were significantly smaller than those of other inoculated treatments.

Based on the above-mentioned results, as expected, the pathogen treatment was determined as the least effective treatment evaluated for the measured indices, and Tv4(5) + pathogen treatment was recognized as one of the least efficient combined treatments. On the other hand, the Tv20(6) treatment was not only one of the most effective treatments for enhancing the root and foliage wet and dry weights but when it was applied in combination with the pathogen also resulted in significant rises in the root wet and dry weights, and the wet weight of foliage in comparison with control. Moreover, for mortality rate and wilting percentage, the positive effects of this treatment were observed. The only negative result regarding this treatment refers to the lesion length. This treatment was identified as one of the least efficient treatments and was similar to the pathogen. Although in the case of root and foliage wet and dry weights, the Tv2(4) was one of the most effective treatments, when it was applied in combination with the pathogen, the result was not so good. Another promising combined treatment, Tv25(6) + pathogen, showed significant increases in the root wet and dry weights and the wet weight of foliage compared to control.

Furthermore, for mortality rate, wilting percentage, and lesion length, this treatment was recorded as one of the most effective treatments. In sum, it can be concluded that the mutant Tv20(6) was the most effective treatment in the greenhouse experiments, since individually or in combination with the pathogen, it was able to improve the sesame growth indices and inhibit the pathogen. Regarding the efficiency of the mutant Tv25(6), whether individually or in combination with the pathogen, it can be regarded as the second most effective treatment in the greenhouse experiments.

RAPD Analysis

Five primers used in fingerprinting analysis successfully amplified clear and distinct

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banding patterns from all the 27 isolates. The number of amplified fragments was variable depending on the respective primers and the different isolates. Among the 27 tested isolates, 40, 37, 30, 36, and 35 fragments were generated by OPA09, OPA10, OPA11, OPA14, and OPA16 primers, respectively. PCR products ranged in size from less than 500 to approximately 4000 bp in OPA09, OPA10, OPA16, whereas in OPA11 and OPA14, they ranged from more than 250 to 6000 bp and from less than 500 to more than 8000 bp, respectively. All primers revealed a substantial proportion of polymorphism among the tested isolates. In this context, the primers OPA10 and OPA16 generated the highest polymorphic bands (100%). In contrast, the lowest polymorphism (93.33%) was observed with the OPA11 primer, followed by 94.44% and 95% of polymorphic bands obtained from the OPA14 and OPA09 primers, respectively.

Moreover, the mutants showed apparent changes in RAPD banding pattern, including presence/absence of bands, increase or decrease of band intensity compared with wild-type isolate. For instance, in the genetic profile obtained by the OPA09 primer, three bands of ~ 1400 bp, 800 bp, and 600 bp only appeared in the wild-type (Fig. 3a). Also, three bands with variable intensities and molecular weights of ~ 1800 bp, 1000 bp, 900 bp, and five additional bands of less than 500 bp were shared by the wild-type and some of the derived mutants (Fig. 3a). The OPA11 primer indicated that the bands with varied intensities and molecular weights around 700 and 650 bp (Fig. 3b), present in some mutants, were missed in the wild-type isolate. On the contrary, some mutants showed banding patterns similar to wild-type isolate (i.e. five bands of ~ 3000 bp, 1900 bp, 1000 bp, 900 bp, and 800 bp were observed in some mutants and wild type isolate (Fig. 3b)).

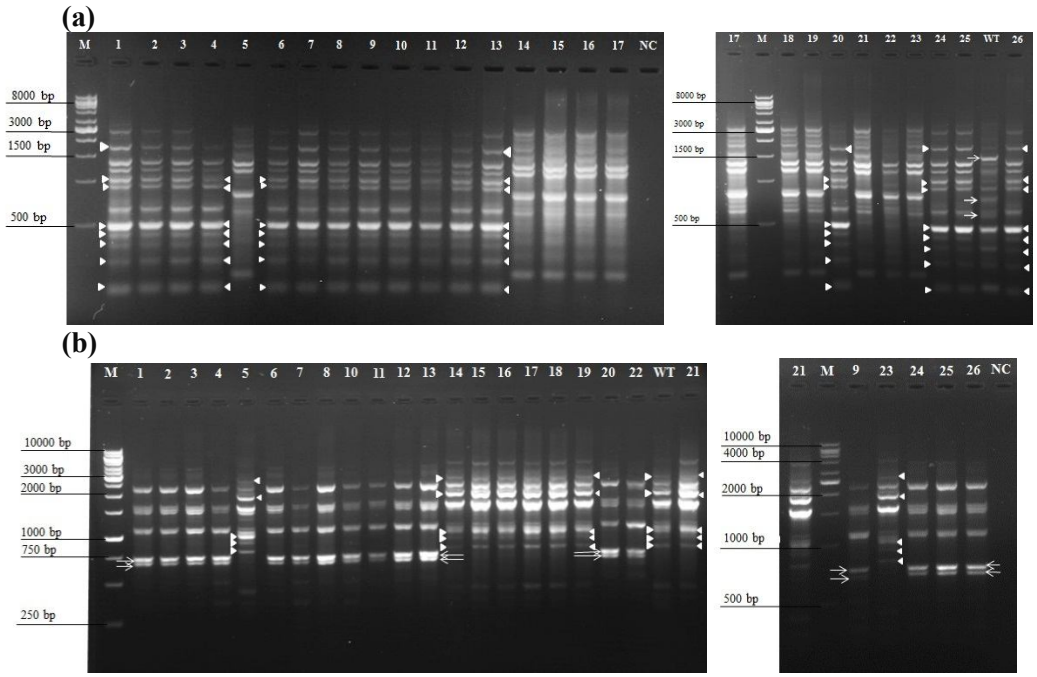

Figure 3 RAPD-PCR profile of the wild-type isolate of *Trichoderma aureoviride* and its 26 derivative mutants using (a) OPA09 and (b) OPA11 primers. Lanes 1-26 correspond to the mutants Tv10(6), Tv28(6), Tv2(3), Tv2(5), Tv1(5), Tv25(5), Tv5(4), Tv9(6), Tv6(5), Tv4(3), Tv31(5), Tv32(5), Tv6(3), Tv20(6), Tv11(5), Tv33(6), Tv40(6), Tv14(6), Tv3(4), Tv3(3), Tv14(5), Tv25(6), Tv2(4), Tv5(3), Tv4(5), and Tv23(6), respectively; Lane WT: Tv59 (wild-type isolate); Lane M: DNA molecular size marker (1 kb ladder); Lane NC: Negative Control. White arrows and arrowheads indicate polymorphisms.

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Cluster analysis of the RAPD banding profiles of the mutants and wild-type of *T. aureoviride*

To facilitate a more comprehensive understanding of the usefulness of RAPD methodology, cluster analysis of combined data obtained by all primers was carried out as a tool to distinguish between wild-type isolate of *T. aureoviride* and its mutants. The dendrogram revealed the differentiation of mutants from wild-type isolate at approximately 40% similarity level, and three clusters were observed among 27 isolates (Fig. 4). The wild-type isolate fell into the second cluster, and its 26 corresponding mutants were grouped in two separate clusters. These clusters got further split into two sub-clusters. Cluster I consisted of 38.46% of mutants, and the rest of the mutants belonged to cluster III (Fig. 4). The Jaccard's coefficients of similarity were in the range from 0.13 to 0.99. The overall genetic distance from wild-type was higher among the mutants belonging to cluster I than that observed in mutants which formed cluster III. The smallest genetic similarity for the mutants and wild-type isolate was recorded as 14.50% from the mutant Tv25(6), followed by 19.25%, 20.45%, 21.80%, 22.72%, 22.83%, 22.90%, and 23.66% genetic similarities, which were observed for seven mutants Tv1(5), Tv14(5), Tv20(6), Tv3(4), Tv40(6), Tv33(6), and Tv14(6) with the wild-type, respectively. Further, the least genetic distance (0.0136) was estimated between the mutants Tv5(3) and Tv4(5).

Finally, the present research investigated the extent of data compatibility in the antagonistic assays and those obtained from RAPD fingerprinting. According to the Jaccard similarity coefficients, eight mutants (Tv25(6), Tv1(5), Tv14(5), Tv20(6), Tv3(4), Tv40(6), Tv33(6), and Tv14(6)) showed the lowest genetic similarities with the wild-type isolate. As already mentioned, the mutants Tv25(6) and Tv20(6) were identified as the most highly effective treatments screened both in laboratory and greenhouse experiments. The mutant Tv3(4) was one of the selected mutants in the greenhouse studies. Also, the enhanced antagonistic capabilities of the mutants Tv40(6) (in dual culture test against the isolate H7 of *M. phaseolina*) and Tv14(6) (in culture filtrate test against the isolate H7 of *M. phaseolina*) were significant when compared to the wild-type isolate. On the other hand, in the *in vitro* assays, no significant inhibitory effects were observed for the mutants Tv1(5), Tv14(5), and Tv33(6). Further, the level of antagonistic activity of the mutants Tv3(3) and Tv4(5) based on their efficacy in the *in vitro* experiments was not consistent with their molecular grouping. It is clear from the results that there is variability in data consistency from *in vitro* assays with those observed from RAPD fingerprinting. Although the most superior mutants were successfully distinguished and remained in a distinct cluster with a far genetic distance from their wild-type isolate, there is not adequate evidence to deduce that the data compatibility is absolute.

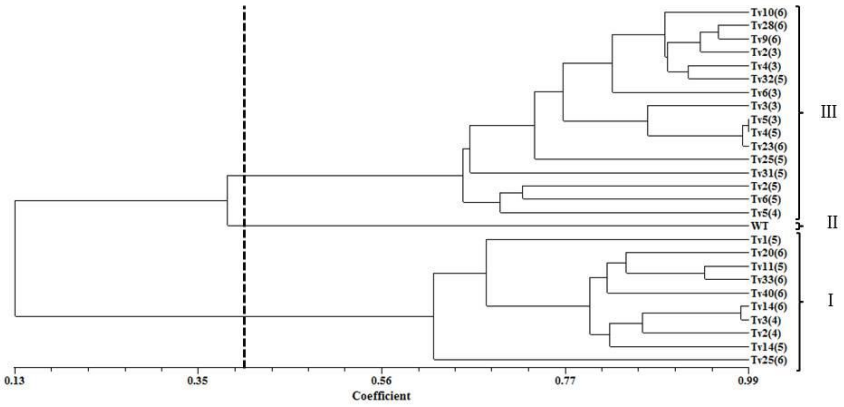

Figure 4 Dendrogram derived from UPGMA cluster analysis of combined RAPD primers depicting the genetic relationships between the mutants and the wild-type isolate of *Trichoderma aureoviride*.

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Discussion

The development of mutants through random mutagenesis is classified among the commonly used practices in the strain improvement program of BCAs (Patil and Lunge, 2012; Ribeiro *et al*., 2013). Despite all other advanced approaches, this technique still prevails as an attractive tool causing stable changes in the genome of host organisms (Yi *et al*., 2016). In the current study, a decrease in the spore germination (%) was detected after increasing gamma radiation doses up to 450 Gy. This result is in line with earlier studies that have indicated a negative dose-dependent relationship between the increasing applied levels of gamma radiation and the viability of irradiated spores or growth of *Trichoderma* species (Afify *et al*., 2013; Naseripour *et al*., 2014; Goharzad *et al*., 2020). Some factors such as the applied doses of radiation, the susceptibility of fungi to gamma radiation, and the density of spore or mycelium in the inoculum subjected to gamma rays have been associated with the loss in spore germination caused by gamma irradiation (Shathele, 2009; Aquino, 2011). Besides, the irradiation dose of 250 Gy did not negatively affect the growth rate and sporulation of *T. aureoviride*. After preliminary screening, 26 mutants were selected based on their higher radial growth rates. According to Haggag and Mohamed (2002), showing the greater efficacy due to gamma radiation might be attributed to the higher activity of constitutive metabolites in the irradiated mutants.

Considering *in vitro* experimental results, with the development of superior mutants whose enhanced biocontrol activities were remarkable against the isolates of *M. phaseolina*, it was proved that exposure to gamma radiation could improve the antagonistic ability of *T. aureoviride*. Further, differential antagonistic activity was observed for the evaluated isolates in various *in vitro* experiments. For instance, the mutants Tv2(4), Tv20(6), and Tv3(4) (in dual culture test against the isolate F33), Tv2(5), and Tv4(5) (in volatile metabolite production test against the isolates F33 and H7), and Tv3(3) (in culture filtrate test against the isolates F33 and H7), which were distinct due to their prominently superior abilities in one certain *in vitro* test did not unavoidably possess the same antagonistic efficacy in other assays. Numerous studies have documented that various *Trichoderma* spp. show differential antagonistic activity, which in turn, it can be an indication of semi-specificity in *Trichoderma*-host interactions (Aly *et al*., 2007; Gajera *et al*., 2012; Kumar *et al*., 2012; Nath *et al*., 2014). Finally, in the volatile metabolite production test, two mutants (Tv33(6) and Tv5(3)) which were expected to exhibit inhibitory effects, proved to have stimulatory effects on the radial growth of *M. phaseolina* isolate H7. These results confirm the findings from previous studies where a stimulatory effect of different *Trichoderma* species has been reported on the radial growth rate of *M. phaseolina* (Khan and Gupta, 1998; Aly *et al*., 2001; Mendoza *et al*., 2015). The BCAs reported in these studies had been commonly isolated from the soil under natural environmental conditions, whereas the isolates evaluated in the present study were the gamma-irradiated mutants.

According to the findings from *in vitro* studies, six mutants were picked to further assess their antagonistic abilities in greenhouse experiments. Greenhouse evaluation of the selected *T. aureoviride* mutants against *M. phaseolina* isolates revealed Tv20(6) and Tv25(6) as the most highly effective treatments for the examined parameters. In agreement with our findings, physical mutagenesis as a tool has been effectively employed throughout several studies to improve the biocontrol ability of *Trichoderma* species towards various plant pathogens, such as hyperhydrolytic mutants of *T. harzianum* with enhanced biocontrol activity against *R. solani* (Rey *et al*., 2001), mutants of *T. harzianum* with protease overproduction capacity and efficient biocontrol potential against various pathogens (Szekeres *et al*., 2004), mutants of *Trichoderma* species with improved production of antifungal metabolites and biological control proficiency against onion

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white rot disease (Haggag and Mohamed, 2002), salt-tolerant mutants of *T. harzianum* for control of *F. oxysporum* (Mohamed and Haggag, 2006), and mutants of *T. harzinum* with improved biocontrol ability against several soilborne phytopathogens (Abbasi *et al*., 2016).

Molecular genetics procedures have been applied to recognize mutation induction caused by physical or chemical mutagenic agents and reveal the feature of induced mutations (Li *et al*., 2016). RAPD analysis used in this study to identify genetic variations between *T. aureoviride* mutants and their wild-type isolates allowed the detection of changes induced by gamma rays at the molecular level. Five primers generated 178 amplified fragments, 172 of which (96.62%) were polymorphic. The discriminatory capacity of the RAPD technique was successfully confirmed when cluster analysis of the combined genetic profiles obtained by all primers enabled the differentiation of the wild-type isolate from its mutant derivatives at the similarity level of 40%. Only two investigations have described the applicability of the RAPD method for differentiation of the irradiation-induced mutants of *T. harzianum* from their wild-type isolate (Schlick *et al*., 1994; Abbasi *et al*., 2016).

Further, in some cases, specific bands only appeared in mutants, while some of the bands in wild type were absent in some specific or even all mutants. Previous studies have suggested that the disappearance of bands in the mutants may be ascribable to DNA damages (single or double-strand DNA breaks, modified bases, oxidized bases, and DNA bulky adducts), DNA-protein cross-links, point mutations, and rearrangements of chromosomes induced by gamma rays, whereas the appearance of new bands has been assignable to mutation rather than DNA damage (Najafi *et al*., 2011; Dhillon *et al*., 2014; Gaafar *et al*., 2017). Besides, in the present study, an absolute correlation was not observed between the *in vitro* antagonistic capability of *T. aureoviride* isolates and their RAPD polymorphisms. Though some earlier studies were similarly unable to detect a correlation between the ability of *Trichoderma* spp. to antagonize various fungal pathogens and their genetic variability resolved by RAPD markers (Góes *et al*., 2002; Sharma *et al*., 2009; El-Komy *et al*., 2015; Rai *et al*., 2016), Abbasi *et al*. (2016) reported a well-coherency between data from the *in vitro* antagonism of gamma-irradiated mutants of *T. harzianum* and those obtained from their molecular grouping based on RAPD and rep-PCR markers.

In conclusion, the proposal that mutation induction caused by ionizing radiation can be employed as a technique for enhancement of biocontrol capabilities of *T. aureoviride* against *M. phaseolina* was confirmed by the results obtained from *in vitro* and greenhouse experiments. Meanwhile, the RAPD fingerprinting technique was demonstrated to be a simple, rapid, and efficient procedure for detecting and distinguishing genetic variations induced by gamma rays in *T. aureoviride* mutants. Data in this work may have significant implications for the superior mutants' effective control of charcoal rot of sesame. However, using these mutants for charcoal rot control requires a complete understanding of the plant, physicochemical, biological, and agro-climatic features, which could influence the establishment and antagonism of delivered BCAs in different forms in the natural condition (Sharfuddin and Mohanka, 2012). In this respect, there is a necessity for further research on the biological control aspects of these promising mutants in field experiments. Also, further investigations are required to recognize the mechanisms employed by the potentially interesting mutants through enzymatic assays and sequencing of differential bands observed in the superior mutants showing the lowest genetic similarities with their wild-type isolate.

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The authors declare that they have no conflict of interest.

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کنترل بیولوژیک پوسیدگی ذغالی کنجد با استفاده از جهش یافته‌های *Trichoderma aureoviride* حاصل از پرتودهی با اشعه گاما و انگشت‌نگاری با نشانگر RAPD

الهام صوفی[1]، ناصر صفایی[1*]، سمیرا شهبازی[2] و شیده موجرلو[3]

۱- گروه بیماری‌شناسی گیاهی، دانشکده کشاورزی، دانشگاه تربیت مدرس، تهران، ایران.
۲- پژوهشکده کشاورزی هسته‌ای، پژوهشگاه علوم و فنون هسته‌ای، سازمان انرژی اتمی ایران، البرز، ایران.
۳- گروه باغبانی و گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه صنعتی شاهرود، ایران.
پست الکترونیکی نویسنده مسئول مکاتبه: nsafaie@modares.ac.ir
دریافت: ۱۰ خرداد ۱۴۰۰؛ پذیرش: ۱۹ آبان ۱۴۰۰

چکیده: پتانسیل گونه‌های *Trichoderma* برای کنترل بیولوژیکی قارچ‌های بیمارگر گیاهی به‌خوبی بررسی شده است. در این مطالعه، جدایه مادری *T. aureoviride* (Tv59) با دز هدف ۲۵۰ گری به‌عنوان دز بهینه مورد پرتودهی قرار گرفت. بیست و شش جدایه جهش‌یافته بر مبنای رشد بهتر غربال شدند. ارزیابی آنتاگونیستی درون شیشه‌ای ۲۶ جدایه جهش‌یافته و جدایه مادری علیه دو جدایه *Macrophomina phaseolina* (F33 و H7) انجام گرفت. شش جدایه جهش‌یافته ((Tv2(4)، Tv20(6)، Tv25(6)، Tv3(3)، Tv4(5) و Tv3(4)) فعالیت بازدارندگی قابل‌توجهی نشان دادند و برای بررسی بیش‌تر در آزمایشات گلخانه‌ای انتخاب شدند. ارزیابی گلخانه‌ای جدایه‌های جهش‌یافته منتخب نشان داد، جهش‌یافته‌های Tv20(6) و Tv25(6) مؤثرترین تیمارها برای شاخص‌های اندازه‌گیری شده می‌باشند. علاوه براین، DNA ژنومی جدایه مادری و ۲۶ جدایه جهش‌یافته به‌منظور تعیین تغییرات ژنتیکی آن‌ها از طریق تکنیک RAPD مورد تجزیه‌وتحلیل قرار گرفت. پنج آغازگر RAPD الگوهای باندی متفاوتی ایجاد کردند و درمجموع ۱۷۸ باند تکثیر شد، که از این تعداد ۱۷۲ باند (۹۶.۶۲٪) چندشکل بودند. درحالی‌که دندروگرام به‌دست آمده با استفاده از روش تجزیه‌وتحلیل خوشه‌ای UPGMA جدایه مادری را از جهش‌یافته‌هایش درسطح تشابه تقریبی ۴۰ درصد متمایز نمود، جدایه‌های جهش یافته به دو خوشه دسته‌بندی شدند. براساس ضرایب تشابه جاکارد، هشت جهش‌یافته ((Tv25(6)، Tv1(5)، Tv14(5)، Tv20(6)، Tv3(4)، Tv40(6)، Tv33(6) و Tv14(6)) کم‌ترین شباهت ژنتیکی را با جدایه مادری نشان دادند. امکان بهبود کارایی کنترل بیولوژیکی جدایه *T. aureoviride* از طریق جهش‌زایی تصادفی و تشخیص و تمایز تغییرات ژنتیکی ایجاد شده ناشی از اشعه گاما با استفاده از تکنیک RAPD با موفقیت در مطالعه حاضر اثبات شد.

واژگان کلیدی: پوسیدگی ذغالی، پرتوتابی گاما، *Macrophomina phaseolina*، تکنیک RAPD و *Trichoderma aureoviridae*

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