Research Article

Biocontrol Activities of Gamma Induced Mutants of *Trichoderma harzianum* against some Soilborne Fungal Pathogens and their DNA Fingerprinting

Sakineh Abbasi [1], Naser Safaie [*1] , Masoud Shams-bakhsh [1], Samira Shahbazi [2]

[1]Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University of Tehran, Tehran, Iran
[2]Nuclear Science and Technology Research Institute, Atomic Energy Organization of Iran, Karaj, Iran

[*]*Corresponding author*: Naser Safaie, Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University of Tehran, Iran.
Tel: +98-21-48292346, Fax: +98-21-48292200, E-mail: nsafaie@modares.ac.ir

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Background: Random induced mutation by gamma radiation is one of the genetic manipulation strategies to improve the antagonistic ability of biocontrol agents.
Objectives: This study aimed to induce mutants with more sporulation, colonization rate leading to enhanced antagonistic ability (*in vitro* assay) comparing to wild type (WT) and the assessment of genetic differences (*in situ* evaluation) using molecular markers. The superior mutants could be appropriate biocontrol agents against soil borne fungal diseases.
Materials and Methods: In this research sampling and isolation of *Trichoderma* isolates were performed from soils with low incidence of soil borne disease. *T. harzianum* 65 was selected and irradiation was conducted with gammacell at optimal dose 250 Gray/s. Mutants (115) were obtained from the WT. The antagonistic abilities of twenty-four mutants were evaluated using dual culture and culture filtrate tests.
Results: The results of *in vitro* assays revealed that *Th15*, *Th11* and *Th1* mutants exhibited stronger growth inhibition (GI) and colonization rate on *Macrophomina phaseolina* and *Rhizoctonia solani* AG4 compared to the wild type. *Th15* and *Th11* mutants exhibited stronger GI and colonization rate on *Sclerotinia sclerotiorum* in dual culture and culture filtrate tests and *Th1* and *Th11* mutants exhibited stronger GI on *Fusarium grminearum* in culture filtrate test.
The DNA fingerprinting was carried out using RAPD and rep-PCR markers. Two (*Th9* and *Th17*) out of the 24 mutants categorized distantly from the rest based on different polymorphism obtained by molecular markers. However, *Th9* was different in GI% from *Th17*. RAPD analysis separated WT from mutants, *Th9* from *Th17* and also phenotypically superior mutants from other mutants. Meanwhile, rep-PCR analysis categorized WT isolate and mutants according to their antagonistic properties.
Conclusions: The latter marker (rep-PCR) appeared to be reproducible and simple to distinguish mutants from a single isolate of *T. harzianum*. Mutants (3 isolates) were phenotypically and genotypically distinct from WT. These mutants demonstrated a pronounced biocontrol activities against soilborne fungal phytopathogens.
Keywords: Enhancement of antagonistic properties; Random Mutagenesis; RAPD and rep-PCR

1. Background

Advances in molecular aspects of antagonists have paved the way of creating improved biological control agents (iBCA). Induction of random mutations by physical mutagens such as UV, X, gamma radiation and chemical mutagens such as ethylmethane sulfonate have been used as useful tools to manipulate antagonists genetically (1, 2). Several studies have shown that gamma-ray radiation can cause genetic diversity of filamentous fungi and induce positive (3, 4 and 5) or negative mutants (6) of specific genes. *Trichoderma* species are filamentous fungi with teleomorphs belonging to the Hypocreales order of the Ascomycota. A number of potential biocontrol agents within the genus of *Trichoderma* have been reported that can act against soilborne plant pathogens, including *T. harzianum* (7). Several researchers have studied the enhancement of some metabolic functions such as

secretion of extracellular cell wall-degrading enzymes and antibiotic production of mycoparasite *Trichoderma* isolates after a treatment by physical mutagens (8, 9, 10, 5 and 11).

In situ identification of superior biocontrol isolate of *Trichoderma* was achieved by using Random Amplified Polymorphic DNA (RAPD) (12), Sequence Characterized Ampilfied Regions (SCAR) markers (13) and real-time PCR (14). Rep-PCR is the genomic fingerprinting method that is based on the use of DNA primers corresponding to naturally occurring interspersed repetitive elements in bacteria, such as the REP, ERIC and BOX elements and has been used to evaluate genetic diversity and distinguish strains in bacteria (15). This method has also been used for evaluation of genetic diversity of fungi including *Fusarium oxysporum* (16), *Verticillium chlamydosporium* (6) *Leptosphaeria maculans* (20), *Macrophomina phaseolina* (19), *Rhizoctonia solani* (20, 21) and *Tilletia* spp. (24); so far this method has not been applied for evaluation of genetic diversity in biocontrol isolates of *Trichoderma* species.

2.Objectives

Random gamma radiation with optimal dose (a dose at which near 50% of spores was abled to germinate) was applied on *T. harzianum*. Phenotypic alterations (antagonistic activities against some soil borne fungal pathogens) and genotype of gamma induced mutants were compared with WT via RAPD and rep-PCR (23, 24).

3. Materials and Methods

3.1. Culture of Microorganisms

T. harzianum was isolated from soil rhizosphere of healthy plants (*Beta vulgaris*) adjacent to or between two wilted plants in Khuzestan province, using dilution plate technique on *Trichoderma* selective medium (TSM) (25) and purified by single spore culture. The isolates were identified on the basis of their morphological characteristics (26). The purified and identified cultures of *T. harzianum* were maintained on Potato Dextrose Agar (PDA) medium and stored at 4ºC for further use. Soilborne fungal plant pathogens including *F. graminearum* (*Fusarium* head blight of wheat), *S. sclerotiorum* (*Sclerotinia* stem rot of canola), *M. phaseolina* (charcoal rot of melon) and *R. solani* AG4 (melon damping-off) were received from the Culture Collection of the Tarbiat Modares University.

3.2. T. harzianum dose Assessmentand Mutants Isolation

Spore suspension (10^7 mL^{-1}) of *T. harzianum* 65 (Th65) isolate (WT) was spread on Water-Agar (WA) plates then irradiated with gammacell (Co- 60, activity 2500 Curry, rate dose of 0.23 $Gy.second^{-1}$ by doses of 0, 50, 150, 200, 250, 300, 350, 400 and 450 Gy at Nuclear Science and Technology Research Institute of Iran and incubated at 25ºC for 7 days. The irradiated spores of each dose were transformed to PDA plates by a needle. After 24 h, percentage of germinated spores was recorded (24).

3.3. Antagonistic Activity Assay Against 4 Soilborne Phytopathogens

Mutants (24) selected and Th65 (WT) were evaluated *in vitro* against four soil borne phytopathogens into dual culture described by Dennis and Webster (27). Culture filtrates (extracellular extract or non-volatile compounds) were tested according to Dennis and Webster (28). The mutants and Th65 (WT) were inoculated in conical flasks (250 mL) containing 100 mL potato dextrose broth. Inoculated flasks were incubated at 23±1ºC at 70 rpm for 12 days. The culture was filtered through Micropore filter (0.22 µm, Syringe®) and culture filtrate was added to PDA at 42ºC to obtain a final concentration of 10% (v/v). The medium was poured into the 9 cm plates with 15 $mL.plate^{-1}$. The medium was inoculated with 7 mm discs of above mentioned pathogens and control plates with no inoculation. The plates were sealed with Parafilm tape and incubated at 27±1ºC, except for *S. sclerotiorum* that incubated at 23ºC for three days (Figure 1). Pathogens' GI% were calculated as GI%=(dc-dt)/dc×100, where dc is colony diameter of pathogen in control, and dt is colony diameter of pathogen in treatment. The experiments were conducted in completely randomized design with three replicates and analyzed by SAS (version 9.1). The means were compared with Duncan's Multiple Range Test ($P < 0.05$).

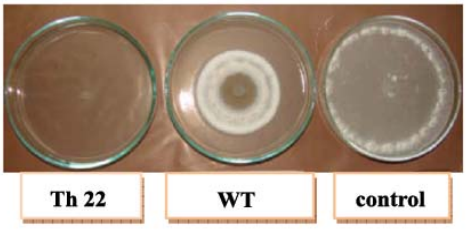

Figure 1. The effect of culture filtrate of Th22 mutant inoculated with *S. sclerotiorum* in comparition with culture filtrate of Th65 (WT) and control (without culture filtrate) after 7 days incubation at 23±1°C

Table 1. The RAPD and rep-PCR primers used

Primers	Sequence
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-14	TCTGTGCTGG
OPA-18	AGGTGACCGT
OPA-16	AGCCAGCGAA
rep 1R-I	IIIICGICGICATCIGGC-
rep 2-I	IIICGNCGNCATCNGGC
Eric 1R	ATGTAAGCTCCTGGGGATTCAC
Eric2	AAGTAAGTGACTGGGGTGAGCG
Box A1R	CTACGGCAAGGCGACGCTGACG

3.4. Biomass Production and DNA Extraction

From edges of a 3-day old fungal culture, 3-4 pieces were transferred to flat bottles with 50 mL of Potato Dextrose Broth (PDB) and placed at 25ºC for 48 h at 125 rpm. Mycelia were harvested on filter paper (Whatman No.1) by vacuum pump and stored at -70ºC for further use. DNA extraction was performed as previously described by Safaie *et al.* (29). Quality and quantity of extracted DNA were assessed by electrophoresis on 0.8% agarose gel and biophotometer (Eppendorf, Germany), respectively.

3.5. Randomly Amplified Polymorphic DNA (RAPD) Analysis

The polymerase chain reactions (PCRs) were carried out in 20 µL of master mix containing 20 ng of genomic DNA template and 10 pmols of random primer (Table 1) (Sinaclon, Iran). All PCR reactions were started at 92ºC for 3 min and 30 cycles of 92ºC:1 min; 33ºC: (except for 35ºC for OPA09) 1 min; and 72ºC: 2 min. The cycles were followed by single cycle of 5 min at 72ºC in an epgradient thermocycler (Eppendorf, Germany). Each reaction of RAPD-PCR repeated twice.

3.6. Repetitive Sequence-based PCR (rep-PCR) Analysis

PCR reaction was carried out in 20 µL and in an epgradient thermocycler (Eppendorf, Germany). The master mix included: 2 µL of buffer (10× PCR buffer) 0.5 µL *Taq* Polymerase (5 unit.µL^{-1}), 0.8 µL (0.5 mM) $MgCl_2$, 0.4 µL mixture of nucleotides (10 mM), 1 µL (10 pM) of each primer (Table 1) and 1 µL (20 ng) template DNA. In negative control 1 µL of sterile deionized water was added instead of DNA. Thermal cycles were set up with some modifications as described by McDonald *et al*. (22). For rep-PCR initial at the PCR was initiated at 94ºC for 7 min, followed by 35 (for rep 1R-I) and 40 (for rep 2I) cycles of 92ºC: 1 min; 43ºC (for rep 1R-I) and 40 (for rep 2I):1 min; 72ºC: 2 min. Final extension was 10 min at 72ºC. For BOX-PCR initial denaturation was 94ºC for 3 min that followed by 30 cycles of 94ºC: 1 min; 53ºC: 1 min; 65ºC: 2 min. These cycles were followed by single cycle at 65ºC for 8 min. For ERIC-PCR initial denaturation was at 95ºC for 2 min and 35 cycles of [94ºC:1 min; 51ºC: 1 min; 72ºC: 2 min]. These cycles were followed by single cycle at 72ºC for 7 min. The PCR products and 1 kb ladder were separated on 1.4% (w/v) agarose gel. The bands were visualized by staining with ethidium bromide (1 mg.mL^{-1}) on UV transluminator and data were analyzed using the MVSP software (with Jaccard coefficient).

4. Results

4.1. Mutagenesis and Isolation of T. harzianum 65 Mutants

Dose of 450 *Gy* completely (100%) inhibited spore germination. At 250 *Gy*, 40-50% (43.4%) of spores germinated and therefore was selected as the optimum dose for irradiation (data obtained for the other doses not shown). Mutants (115) were obtained from WT (Th65) and were all tested for growth inhibition against *R. solani* (data not shown). Accordingly, 24 mutants were selected. The gamma radiation caused differences in morphological properties of *T. harzianum* such as color, colony appearance, sporulation and growth rate of mycelia at different irradiation. *Th1*, *Th5*, *Th6* and *Th8* mutants had more sporulation in comparison to WT and *Th17* showed less after five days of incubation (data not shown).

4.2. In vitro Assays

The GI% of *S. sclerotiorum*, *F. graminearum*, *M. phaseolina* and *R. solani* AG4 after 3 days of incubation with culture filtrate in dual culture (Tables 2-5) revealed significant differences among mutants and WT ($p < 0.05$). In between, *Th15* showed maximum GI%. Other superior mutants were *Th11*, *Th1* and *Th22*. Assay of antagonistic activity against *F. graminearum* revealed that *Th11*, *Th22*, *Th2*, *Th15* and *Th17* caused more GI% than WT (Table 2). Antagonistic assay against *R. solani* revealed that *Th17*, *Th9*, *Th11*, *Th1* and *Th21* resulted in more GI% than WT (Table 3). *In vitro* assay against *S. sclerotiorum* revealed that *Th5*, *Th4*, *Th11* and *Th22* had more GI% than WT (Table 4). *In vitro* assay against *M. phaseolina* revealed that *Th1*, *Th11*, *Th15* and *Th18* had maximum GI% (Table 5). The maximum GI% by mutant culture filtrates were

Table 2. The means of GI% of *F. graminearum* exposed to mutants of *T. harzianum* (Th1- Th24) and WT (Th65) after 3 days incubation in dual culture (A) and culture filtrate (B) using Duncan's Test ($P < 0.05$). Means followed by the same letters indicate no significant difference. Variance analysis of dual culture test and culture filtrate test are shown below respectively

Mutants	Means (A)	Duncan Grouping	Mutants	Means (B)	Duncan Grouping
Th21	60.70	A	Th11	50.00	A
Th17	60.20	A	Th18	47.50	AB
Th5	60.20	A	Th1	47.50	AB
Th9	59.20	A	Th3	44.16	ABC
Th4	58.70	A	Th9	40.00	ABC
Th65 (WT)	57.20	AB	Th24	36.66	BCD
Th23	56.20	ABC	Th19	33.33	CDE
Th7	53.70	BCD	Th16	32.50	CDEF
Th16	52.20	DC	Th12	26.66	DEFG
Th12	52.20	DC	Th13	22.50	EFGH
Th20	52.20	DC	Th10	21.66	EFGHI
Th14	51.73	DC	Th21	20.00	FGHI
Th15	51.73	DC	Th65(WT)	18.33	GHIJ
Th8	51.70	DC	Th22	17.50	GHIJ
Th22	51.23	DC	Th7	17.50	GHIJ
Th19	51.23	DC	Th15	15.83	GHIJK
Th18	50.73	D	Th4	12.50	HIJKL
Th2	50.23	D	Th2	10.83	HIJKL
Th11	50.23	D	Th6	10.00	HIJKL
Th24	49.73	D	Th23	9.16	HIJKL
Th13	49.23	D	Th8	8.33	IJKL
Th10	49.23	D	Th17	8.33	IJKL
Th3	48.76	D	Th14	5.00	JKL
Th1	48.76	D	Th20	3.33	KL
Th6	42.80	E	Th5	0.00	L

Table 3. The means of GI% of *R. solani* exposed to mutants of *T. harzianum* (Th1-Th24) and WT (Th65) after 3 days incubation in dual culture (A) and culture filtrate (B) using Duncan's Test ($P < 0.05$). Means followed by the same letters indicate no significant difference. Variance analysis of dual culture test and culture filtrate test are shown below respectively

Mutants	Means (A)	Duncan Grouping	Mutants	Means (B)	Duncan Grouping
Th11	48.73	A	Th22	44.43	A
Th17	48.73	A	Th2	43.06	A
Th4	48.70	A	Th15	43.03	A
Th12	48.70	A	Th7	41.70	A
Th9	47.86	AB	Th13	40.26	A
Th7	47.86	AB	Th8	33.33	AB
Th2	47.00	AB	Th3	26.36	BC
Th22	47.00	AB	Th11	20.86	BCD
Th19	47.00	AB	Th21	16.70	CDE
Th20	47.00	AB	Th19	16.70	CDE
Th10	46.16	AB	Th4	16.66	CDE
Th21	45.33	AB	Th10	16.66	CDE
Th18	45.33	AB	Th12	15.26	CDEF
Th13	44.46	AB	Th14	15.26	CDEF
Th15	44.46	AB	Th9	12.20	CDEFG
Th6	44.46	AB	Th1	11.50	DEFG
Th14	44.43	AB	Th18	11.40	DEFG
Th23	43.63	AB	Th20	10.88	DEFG
Th3	43.60	AB	Th16	9.20	DEFG
Th24	43.60	AB	Th65(WT)	5.20	EFG
Th16	43.60	AB	Th23	3.90	EFG
Th1	42.76	ABC	Th6	2.60	FG
Th8	42.73	ABC	Th24	2.00	FG
Th5	41.86	BC	Th5	1.70	FG
Th65(WT)	36.76	C	Th17	0.20	G

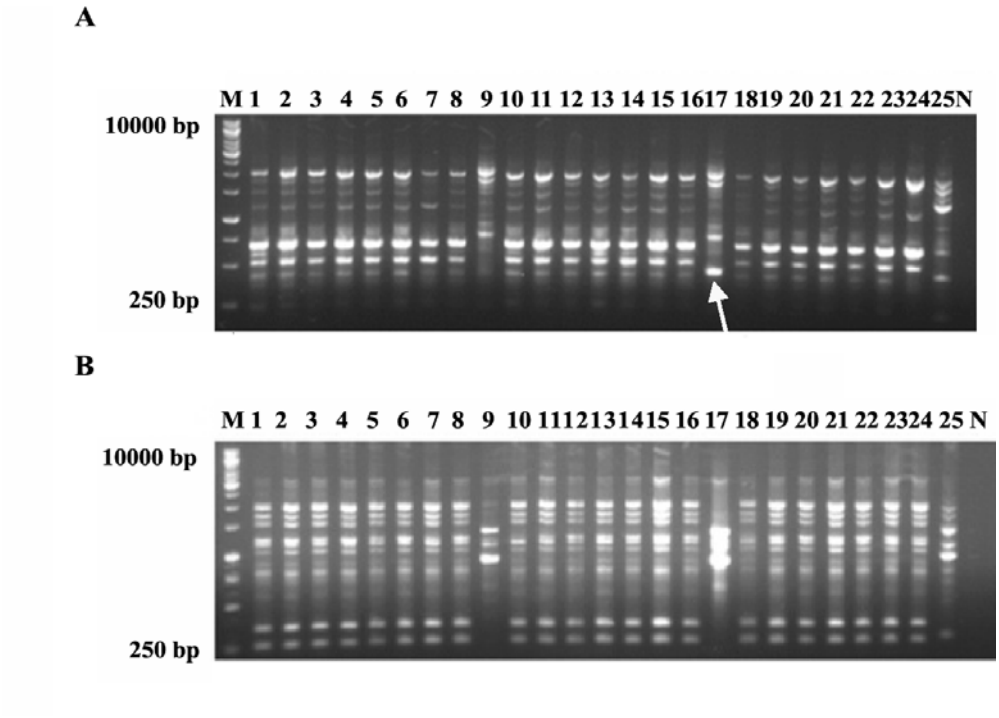

Figure 2. DNA fingerprinting of *Trichoderma harzianum* by A: OPA010 and B: OPA016; wells 1 to 24 are mutants (*Th1-Th24*) and 25 is WT. OPA010 amplified a 500 bp band, which separated *Th17* from *Th9*, other mutants and WT, N: negative control and M: 1 kbp DNA ladder

recorded in *S. sclerotiorum* (Table 4). In dual culture test, *Th4*, *Th5*, *Th9*, *Th11*, *Th15* and *Th17* demonstrated maximum GI% against studied pathogens. *Th1*, *Th4*, *Th9*, *Th11*, *Th15* and *Th18* showed more colonization rate than WT after 3 days of incubation with mentioned phytopathogens (data not shown). Culture filtrate tests of *Th1, Th2*, *Th11*, *Th15* and *Th22* showed maximum GI% against studied pathogens (Figures 2 to 5).

4.3. RAPD Analysis

Cluster analysis of RAPD amplicons using 5 random primers revealed a significant genetic diversity between the mutants and WT. OPA010, OPA011, OPA016, OPA09 and OPA014 were managed to amplify 19, 22, 22, 21 and 21 loci, respectively. The percentage of polymorphic bands detected with these primers, were 57.8, 50, 50, 61.9 and 47.6%, respec-

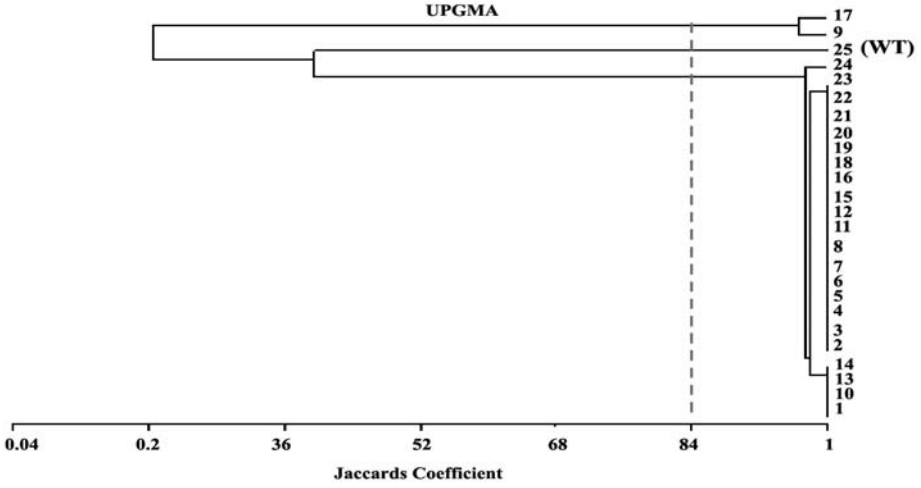

Figure 3. Dendrogram constructed with UPGMA clustering method using the MVSP software for the mutants and wild type of *T. harzianum* derived from pooled RAPD data

Table 4. The means of GI% of *S. sclerotiorum* exposed to mutants of *T. harzianum* (Th1- Th24) and WT (Th65) after 3 days incubation in dual culture (A) and culture filtrate (B) using Duncan's Test ($P < 0.05$). Means followed by the same letters indicate no significant difference. Variance analysis of dual culture test and culture filtrate test are shown below respectively

Mutants	Means (A)	Duncan Grouping	Mutants	Means (B)	Duncan Grouping
Th5	88.40	A	Th4	100.00	A
Th11	88.40	A	Th22	99.26	A
Th16	84.50	AB	Th23	98.53	A
Th24	76.70	ABC	Th2	98.53	A
Th15	76.70	ABC	Th12	98.53	A
Th17	75.26	ABC	Th8	97.80	A
Th6	72.83	ABCD	Th10	96.86	A
Th10	69.76	ABCDE	Th7	94.10	AB
Th9	68.96	ABCDE	Th6	94.10	AB
Th12	68.96	ABCDE	Th14	93.30	AB
Th19	68.96	ABCDE	Th11	91.83	ABC
Th22	67.43	ABCDE	Th5	91.10	ABC
Th21	67.43	ABCDE	Th20	91.10	ABC
Th8	65.10	BCDEF	Th3	88.13	ABCD
Th18	65.10	BCDEF	Th9	86.66	ABCD
Th7	63.56	BCDEF	Th21	86.66	ABCD
Th3	57.36	CDEF	Th15	85.40	ABCD
Th20	55.83	CDEF	Th19	82.96	ABCD
Th13	53.46	DEF	Th16	77.76	BCDE
Th2	49.63	EF	Th65(WT)	74.83	CED
Th4	49.63	EF	Th1	73.33	DE
Th23	45.76	F	Th24	73.30	DE
Th65(WT)	44.96	F	Th18	65.90	EF
Th1	14.73	G	Th13	55.53	F
Th14	9.33	G	Th17	23.00	G

Table 5. The means of GI% of *M. phaseolina* exposed to mutants of *T. harzianum* (Th1- Th24) and WT (Th65) after 3 days incubation in dual culture (A) and culture filtrate (B) using Duncan's Test ($P < 0.05$). Means followed by the same letters indicate no significant difference. Variance analysis of dual culture test and culture filtrate test are shown below respectively

Mutants	Means (A)	Duncan Grouping	Mutants	Means (B)	Duncan Grouping
Th15	61.43	A	Th1	69.03	A
Th12	59.96	AB	Th11	61.90	AB
Th1	59.50	ABC	Th15	59.93	AB
Th18	59.50	ABC	Th21	58.70	ABC
Th14	59.50	ABC	Th24	56.36	ABC
Th10	59.50	ABC	Th18	55.56	ABC
Th11	57.60	ABCD	Th3	52.36	ABC
Th17	55.93	BCDE	Th16	49.23	BCD
Th7	55.23	CDEF	Th22	49.20	BCD
Th13	54.76	DEFG	Th2	47.63	BCDE
Th20	54.30	DEFGH	Th17	45.23	BCDEF
Th24	53.83	DEFGHI	Th9	45.23	BCDEF
Th23	53.80	DEFGHI	Th19	40.46	CDEFG
Th19	53.33	DEFGHI	Th10	32.56	DEFG
Th5	52.86	EFGHI	Th65(WT)	32.53	DEFG
Th3	51.90	EFGHI	Th13	30.96	DEFGH
Th16	50.96	FHGI	Th12	29.4	EFGH
Th65(WT)	50.46	HGI	Th23	28.6	GHF
Th9	50.00	HI	Th4	25.4	GHI
Th8	49.53	I	Th8	24.6	GHI
Th6	40.50	J	Th7	23.00	GHI
Th2	40.50	J	Th14	13.50	HI
Th21	40.50	J	Th5	12.70	HI
Th4	38.10	KJ	Th20	10.33	I
Th22	35.70	K	Th6	8.70	I

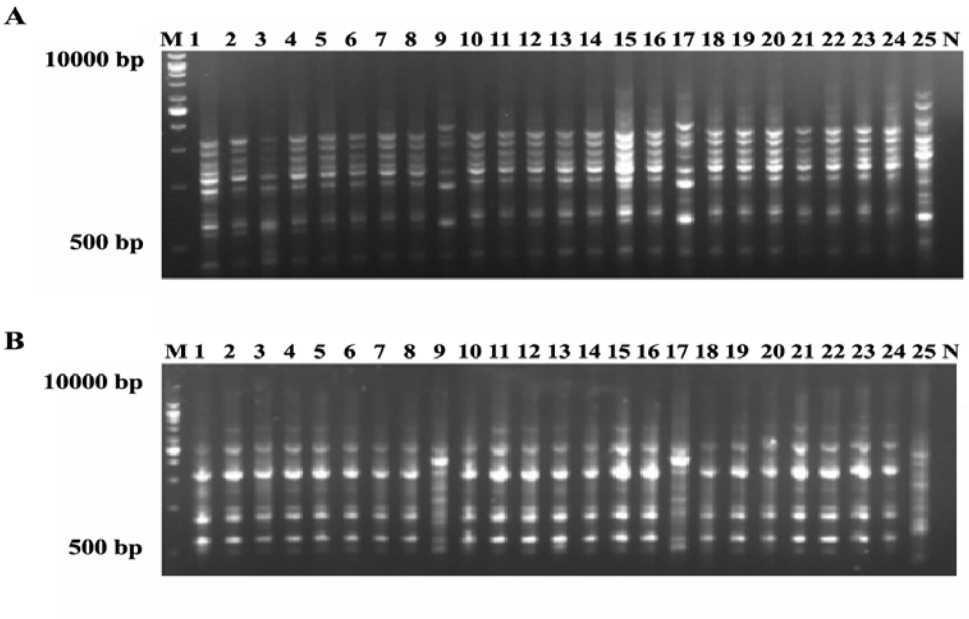

Figure 4. DNA fingerprinting of *Trichoderma harzianum* by A: rep1R-I and B: ERIC1; wells 1 to 24 are mutants (*Th1-Th24*) and 25 is WT, N: negative control and M: 1 kbp DNA ladder

tively. The most amplified loci belonged to OPA011 and OPA016 (Figure 2). DNA fingerprinting using RAPD-PCR revealed that gamma radiation induced genetic changes (Figure 2). The result obtained for combination of five primers of RAPD at similarity level of 84% divided the mutants and WT into 3 groups where the wild type and the *Th9* and *Th17* mutants were grouped in two separate clades and the rest of mutants in a group in close distance to mutants (Figure 3).

4.4. Rep-PCR Analysis

rep-PCR primers (5) with a similarity of 84% divided the mutants and WT into 3 groups: the group of WT, *Th9* and *Th17* in a group and the other mutants in other group. The mutants with improved antagonistic activity, *Th9* and *Th17*, separated from WT, in a distinct group (Figure 5). The results obtained for cluster analysis of 5 primers of rep-PCR and DNA fingerprinting revealed that gamma mutation induced genetic changes (Figure 4). Number of identified loci included

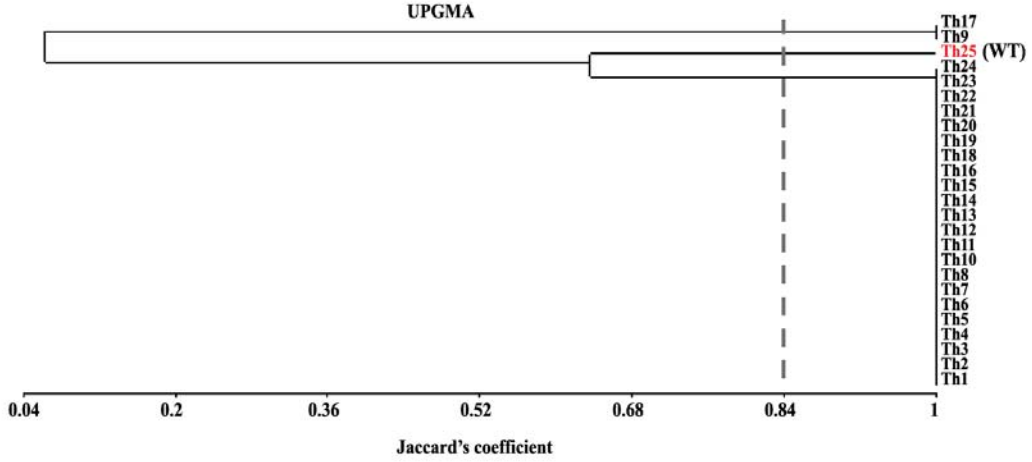

Figure 5. Dendrogram constructed with UPGMA clustering method using the MVSP software for the mutants and wild type of *T. harzianum* derived from pooled REP data

for REP1R-I, REP2I, Box, ERIC2I and ERIC1 were 26, 23, 15, 21 and 21, respectively and the percentage of polymorphic bands detected in these primers were 73.70, 56.5, 53.3, 61.9 and 66.6%. rep1R-I produced the highest number of loci (Figure 4A). Rep-PCR revealed more polymorphic bands than RAPD-PCR.

5. Discussion

Gamma rays have very high energy, causing gene mutations by replacement of nucleotides (by oxidative deamination) or chromosome breakage. Here, random mutation by gamma irradiation with optimal dose of 250 *Gy* on *T. harzianum* caused changes in the genome and induced some mutants with more growth and sporulation rates than WT, with improved antagonistic activity for some such as *Th1* mutant. *Th15*, *Th11* and *Th1* exhibited greater GI% and colonization rate on *F. graminearum*, *S. sclerotiorum*, *M. phaseolina* and *R. solani* AG4 compared to WT. The obtained data on dual culture tests of isolates like that of culture filtrate assays indicated gamma radiation significantly affected production of extracellular compounds and enzymes involved in mycoparasitism. These secreted molecules led to high antagonistic activities against pathogens in some mutants; some of which showed good performance in dual culture test. However, some others such as *Th5* demonstrated weak secretion of enzymes in culture filtrate test. Phenotypically, some other mutants demonstrated improved extracellular secretion, while their colonizations in dual culture were slow.

Induction of mutation is a genetic manipulation method to improve efficacy of biocontrol agents against soil borne plant pathogens (30). Szekeres *et al*. (31) reported that *T. harzianum* treated with UV overproduced protease, leading to much biocontrol activity against soil borne fungal phytopathogens. Similarly, Vaidya *et al*. (32) UV radiated *Alcaligenes xylosoxydans* produced more chitinase with enhanced biocontrol activity against soil borne fungal pathogens.

The DNA fingerprinting managed to distinguish between mutants and WT with well-coherency with their antagonistic capabilities similar to earlier works (33, 34, 35 and 36).

Among the RAPD primers used, OPA010 amplified a reproducible 500 bp band that separated *Th17* from *Th9* and other mutants and WT (Figure 2A). According to *in vitro* assays (Tables 2-5) GIs% of *Th9* and *Th17* in dual culture tests were similar, but *Th9* showed more colonization and growth rate than *Th17*. Furthermore, in culture filtrate assays *Th9* showed higher GI% than *Th17*, probably this band is related to gene(s) that affects expression of special mycoparasitic enzymes that needs further analysis. Dendrogram of cluster analysis of RAPD (OPA010) separated *Th9* from *Th17* with 96% coefficient (Figure 3). Zymand *et al*. (37) used RAPD and identified T-39 of *T. harzianum*. Dodd *et al.* (38) used SCAR to recognize GV4 from other *T. virens* isolates.

Rep-PCR fingerprinting was reproducible and easy to assay in *T. harzianum*. The rep1R-I produced more polymorphic bands and detected more loci than other primers in rep-PCR. Markers that were used here mangaed to differentiate WT and mutants and efficiently established these differences with the phenotype of antagonism against fungal phytopathogens. Trombert *et al*. (39) used rep-PCR to distinguish UV mutation in *Escherichia coli* and observed that it was a suitable marker to show the effects of UV mutation in microorganisms. Afsharmanesh *et al*. (40) assessed the random mutagenesis by gamma radiation on *Bacillus subtilis* UTB1 in biocontrolling *Aspergillus flavus*. Eight mutants out of the 45 were selected based on different polymorphicpatterns, obtained by rep-PCR (ERIC and BOX). Of which, six mutants showed enhanced production of biosurfactants and produced more robust biofilm than the wild type UTB1. Several studies have shown that gamma ray radiation could change the enzyme activity and other antifungal metabolites (41 , 42). Ahari *et al*. (23) applied gamma irradiation 150 $Gy.second^{-1}$ (by 0.38 rate dose) on *F. solani* f.sp. *phaseoli* and were able to induce production of non-pathogenic mutants as biocontrol agents against pathogenic isolates (*F. solani* f.sp. *phaseoli*). Here, three mutants were introduced that cauld possibly be used as effective biocontrol agents against the mentioned soil borne fungal pathogens. Further studies on enzyme assays of superior mutants, sequencing of their differential bands obtained in other molecular marker analyses are needed to provide the mechanism involved in antagonistic activity of the mutants and finally to test the biocontrol abilities of mutants in nature.

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