J. Crop Prot. 2014, 3 (4):509-521

Research Article

Evaluation of gamma-induced mutants of *Trichoderma harzianum* for biological control of charcoal rot of melon (*Macrophomina phaseolina*) in laboratory and greenhouse conditions

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Abstract: *Macrophomina phaseolina* is one of the major yield limiting factors of melons in tropical and subtropical regions. For eco-friendly and effective management of the disease, 24 gamma induced mutants from *Trichoderma harzianum* were evaluated against three isolates of the pathogen representing three geographically different regions viz. Khorasan (isolate 1), Garmsar (isolate 2) and Khuzestan (isolate 3). The isolates of *Trichoderma* (mutants and wild type) were evaluated against the pathogen in dual culture and through production of volatile and non-volatile inhibitors. Maximum growth inhibition was observed in Th1, Th4, Th15, Th9 and Th22 mutants after three days. In greenhouse evaluation against *M. phaseolina* (isolate 1) among the inoculated treatments minimum plant infection was observed in Th9 treatment (28% disease reduction) as compared to infected control and among the uninoculated treatments Th1and Th9 mutants resulted in maximum growth of roots and shoots of melon plants as compared to uninfected control. These mutants are introduced as potential candidates against *M. Phaseolina*. The results proved that gamma-mutagenesis by enhancing the antagonistic properties of *T. harzianum* 65 can be useful for the biocontrol of soil borne plant pathogens such as *Macrophomina phaseolina*.

Keywords: Charcoal rot of melon, Improvement biocontrol ability, Gamma-mutagenesis, *Trichoderma harzianum*

Introduction

Charcoal rot of melon (*Macrophomina phaseolina* (Tassi) Goid, a seed- and soil borne fungus with a wide distribution and a wide host range (Dhingra and Sinclair, 1978) is common in tropical and subtropical regions. Symptoms are typical of the vine declines in that the leaves begin yellowing and collapsing in the crown prior to harvest and the decline radiates outward. Fruit become small sized and low in sugars, crown lesions, typical of many of the vine declines, are observed infrequently. Most plants exhibit no crown lesions, small black microsclerotia (and sometime pycnidia) form within the lesion giving a dusty, charcoal appearance (Bruton and Miller, 1997). There are a few effective measures for controlling the disease including maintaining optimal soil moisture to avoid plant stress, rotation of cucurbits with a small grain crop (Etebarian, 2006), development of resistant cultivars (Abd-Elsalam, K. 2010) and biological control by some bacteria including *Pseudomonas*

Handling Editor: Dr. Vahe Minassian

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Received: 10 June 2013, Accepted: 22 February 2014
Published online: 22 April 2014

[DOR: 20.1001.1.22519041.2014.3.4.7.8]

aeruginosa, P. spp. (Etebarian, 2006; Singh *et al*., 2010), *Bradyrhizobium* spp*., Rhizobium meliloti* (Arora *et al*., 2001), *Bacillus* spp. (Valiente *et al*., 2008), *Pantoea agglomerans* (Vasebi *et al*., 2013) and *Trichoderma* spp. (Elad *et al*., 1986; Adekunle *et al*., 2001; Dobey *et al*., 2005; Vasebi *et al*., 2013). A biological control agent colonizes the rhizosphere, the site requiring protection and leaves no toxic residues as opposed to chemicals. The first requirement for biological control is the identification and development of highly effective isolates There are a number of potential biocontrol agents within the genus *Trichoderma*, including *T. harzianum* (Papavizas, 1985; Hermosa *et al*., 2000). These fungi have attracted attention because of their multiple actions against various soil borne plant pathogens (Harman *et al*., 2004). Proposed mechanisms for biocontrol are: stimulation of the defensive mechanisms of the plants (Benitez *et al*., 1998), competition for the substrate (Naar and Kecskes, 1998) as well as antibiosis by the production of antifungal metabolites and mycoparasitism by the action of cell-wall degrading enzymes (Benitez *et al*., 1998; Yedidia *et al*., 1999). Chitinolytic and glucanolytic enzyme systems involved in the mycoparasitism of *Trichoderma* isolates have been investigated in detail and are well characterized (Benitez *et al*., 1998).

Isolates with improved production of extracellular enzymes after a shorter induction period could be more effective biocontrol agents. One of the potential tools for improvement of isolates is mutagenesis. In the case of the genus Trichoderma, earlier investigations report about the improvement of antibiotic production by UV mutagenesis (Graeme-Cook and Faull., 1991) and about obtaining fungicide resistant mutants with the potential to be used in integrated pest management (Papavizas *et al*., 1982;), however, the number of studies on the improvement of the extracellular cell wall-degrading enzyme secretion abilities of mycoparasitic *Trichoderma* isolates by mutagenesis is restricted (Melo *et al*., 1997; Rey *et al*., 2001; Szekeres *et al*., 2004; Kovacs *et al*., 2008; Li *et al*., 2010; Jiang *et al*., 2011).

In the present study, we applied Gamma-mutagenesis to improve the antagonistic properties of *T. harzianum* 65. The resulting mutants were screened for the superior ones against *M. phaseolina* isolates *in vitro* and for biocontrol of charcoal rot disease of melon (*M. phaseolina*) in greenhouse experiments.

Materials and Methods

Microorganisms

Trichoderma spp. from rhizosphere of healthy plants adjacent to wilted plants (Khuzestan province, Iran) were isolated using dilution plate techniques on *Trichoderma* selective medium (TSM) (Elad and Chet, 1983) and purified by single spore method. They were identified on the basis of their morphological characteristics (Rifai, 1969). The purified and identified cultures of *Trichoderma harzianum* were maintained on Potato Dextrose Agar (PDA) medium and stored at 4 °C for further use. *M. phaseolina* isolates (Khorasan (isolate 1), Garmsar (isolate 2), Khuzestan (isolate 3) used in these experiments were received from the Culture Collection of Tarbiat Modares University.

Mutagenesis of *T. harzianum*

Spore Suspension (10^7 spore/ml) of "*T. harzianum* 65" (WT) was spread on Water Agar (WA) medium and irradiated with gamma cell (Co- 60, activity 2500 Cury, rate dose 0.23 Gray.second^{-1}, Atomic Energy organization of Iran) with 0-50, 150-200, 250-300, 350-400 and 450 Gray of doses and incubated at 25 °C for 7 days. Three irradiated spores of each dose level, after 24 hours, were transferred to the PDA using a needle. Percentages of germinated spores recorded and optimal dose of irradiation was determined based on 50% germination of irradiated spores (Moradi *et al*., 2012).

In vitro antagonistic assays

Twenty four mutants and the wild type were evaluated against three isolates of *M.*

[DOR: 20.1001.1.22519041.2014.3.4.7.8]

phaseolina from different geographical origin by dual culture technique as described by Dennis and Webster (1971c). Petri dishes (9cm) containing PDA were inoculated with three day old mycelial plugs (7mm in diameter) of from *M. phaseolina* and *Trichoderma* isolates. The plugs were placed at equal distance from the periphery of plates. Inoculated plates were incubated at 27 (± 1) °C and the radial growth of *M. phaseolina* was measured 1, 2, and 3 days after inoculation. Plates without *Trichoderma* were maintained as controls. Each treatment contained three replicates. Percent of Growth Inhibition (GI%) of *M. phaseolina* was calculated as:

$$GI\% = [(dc-dt)/dc] \times 100$$

where GI percent growth inhibition, dc colony diameter of pathogen in control, and dt colony diameter of pathogen in treatment.

Effect of volatile inhibitors

The mutants and wild type were examined in laboratory for volatile production following the technique described by Dennis and Webster (1971a). The *Trichoderma* isolates were centrally inoculated by placing 7mm plugs taken from three day old culture on the PDA plates and incubated at 27 (± 1) °C for three days. The top of each Petri dish was replaced with bottom of the PDA plate inoculated centrally with the pathogen. PDA plates without *Trichoderma* isolates inoculated by *M. phaseolina* were maintained as controls. Three replications were used per treatment. Each pairs of Petri dishes were sealed together with Parafilm tape and incubated at 27 (± 1) °C. Colony diameter of the pathogen was measured at 1, 2 and 3 days after incubation and GI% of *M. phaseolina* was calculated using above mentioned formula.

Effect of non-volatile inhibitors

The effect of non-volatile substances produced by the Trichoderma mutants was determined following method of Dennis and Webster (1971b). The isolates of *Trichoderma* were inoculated in 100 ml sterile potato dextrose broth in 250ml conical flasks. Inoculated flasks were incubated at 23 (± 1) °C for 12 days. The culture was filtered through Millipore filter (0.22 µm, Syringe®) and culture filtrate was added to molten PDA medium (at 42 °C) to obtain a final concentration of 10% (v/v). The medium was poured into the plates at 15ml/plate in three replications and inoculated after solidification with 7mm discs of pathogen isolates. Control plates were maintained without amending with culture filtrate. Petri plates were sealed with Parafilm tape and incubated at 27 (± 1) °C for 3 days. Radial growths of *M. phaseolina* isolates were recorded at 1, 2 and 3 days after incubation. GI% of *M. phaseolina* was calculated using above mentioned formula. *In vitro* experiments were conducted in completely randomized design with three replications. SAS (version 9.1) ANOVA and Duncan's Test (P-value < 0.05) were performed to analyze data of *in vitro* experiments.

Biocontrol of charcoal rot of melon in greenhouse

Pot experiment was conducted in randomized complete block design with four replications to evaluate the performance of the most efficient mutants of *T. harzianum* 65. The inoculum of *M. phaseolina* was multiplied on rice (cv. Tarom) grains. The grains were moistened (1g rice seeds: 1 ml water) in tap water for 12 h, and filled into 250 ml conical flasks (50 g /flask). The flasks containing grains were autoclaved for two subsequent days at 50 pound for 30 min and inoculated with a block (2 × 2 cm^2) of five-day-old culture of *M. phaseolina* (isolated from Khorasan) and incubated for 15 days at 27 (± 1) °C. Selected *Trichoderma* isolates were grown on PDA for 5 days and two blocks (2 × 2 cm^2) of them were added to an Erlenmeyer flask containing (100 g /flask) wheat grain (were moistened in tap water for 12 h and were autoclaved for two subsequent days and incubated at 25 (± 1) ^oC for 15 days. Surface sterile plastic pots (20 cm in diameter) were filled with sterilized soil, Perlite and peat moss

[DOR: 20.1001.1.22519041.2014.3.4.7.8]

(1: 1: 1, 2 kg/ pot) and inoculated with the inoculum of *M. phaseolina* at 7.5 g. kg^{-1} of soil five days before sowing. Ten seeds of melon (cv. Shadegan) were sown in each pot and the antagonist was added on the day of sowing the seeds. Before sowing, seeds were surface disinfested by soaking in 70% ethanol for two minutes and then rinsed three times in sterile distilled water and after 12 hours soaking were dried. Pots were irrigated at two or three day's intervals.

Data analysis

Shoot and root fresh and dry weights of the plants were recorded 40 days after inoculation and percentage of infected plants out of the total germinated seeds for each pot (recorded 15 days after sowing) were calculated. Percentages of infected plants were calculated by the following formula:

[(Number of healthy plants in healthy control– Number of healthy plants in treatment)/Number of healthy plants in healthy control] × 100

The experiments were repeated two times during 2012 from May to September. Treatments comprised: chemical control with Carboxin thiram (250 ml/100Kg seed); uninoculated control; *M. phaseolina* alone; Th65 (WT) alone, Th65 (WT) plus *M. phaseolina*; Th1 alone, Th1 plus *M. phaseolina*; Th9 alone, Th9 plus *M. phaseolina*; Th11 alone, Th11 plus *M. phaseolina* and Th4 alone, Th4 plus *M. phaseolina*. ANOVA and Duncan's Test ($P < 0.05$) were performed to analyze data of *in vivo* experiments using MSTAT-C. The percent variables were normalized using $((x/100) + 0.5)^{1/2}$

Results

Mutagenesis of *T. harzianum*

While irradiation with 450 Gray completely prevented spore germination, irradiation with 250 Gray resulted in 50% spores germination, and it was selected as optimum dose. Applying this optimum dose, 24 mutants were selected from wild type isolate (Th65) based on inhibition zone against the soil borne plant pathogen, *Rhizoctonia solani*. Th1, Th5, Th6 and Th8 mutants showed more sporulation than the wild type five days after incubation.

***In vitro* antagonistic assays**

Twenty five isolates of *T. harzianum* 65 (24 mutants and wild type) were tested for *in vitro* evaluation against *M. phaseolina* isolates.

Dual culture

The GI% of *M. phaseolina* isolate (isolate1) three days after incubation (Fig. 1a) revealed significant differences among mutants and wild type ($p < 0.05$) meanwhile mutants Th15, Th1 and Th12 showed maximum growth inhibition. Th7 and Th3 mutants were the next ones in row according to their GI%. The comparison of GI% of *M. phaseolina* isolate (isolate2) three days after incubation (Fig. 1b) revealed that mutants of Th23, Th12 and Th19 imposed maximum growth inhibition. Comparison of GI% of *M. phaseolina* isolate (isolate3) three days after incubation (Fig. 1c) indicated that maximum growth inhibition were imposed byTh22 and Th4 as well as Th1, Th9, Th13, Th15 and Th21. Growth inhibition recorded in all the *M. phaseolina* isolates significantly differed. Garmsar isolate (isolate2) was highly antagonized by *T. harzianum* (WT and mutants) isolate, while Khuzestan and Khorasan isolates were placed next to Garmsar isolate. All isolates of *M. phaseolina* differed significantly in mycelial growth inhibition caused by *T. harzianum* (WT and mutants) isolate. Maximum inhibition was observed in interaction between Garmsar isolate and Th15 (74%) and Th4 (70%) mutants after three days of incubation in dual culture. In this experiment, Th4, Th7, Th15 and Th18 mutants indicated quicker colonization than wild type three days after incubation.

Effect of volatile inhibitors

There was no significant difference ($p > 0.01$) in growth inhibition of *T. harzianum* (WT and mutants) three days after incubation (Fig. 2)

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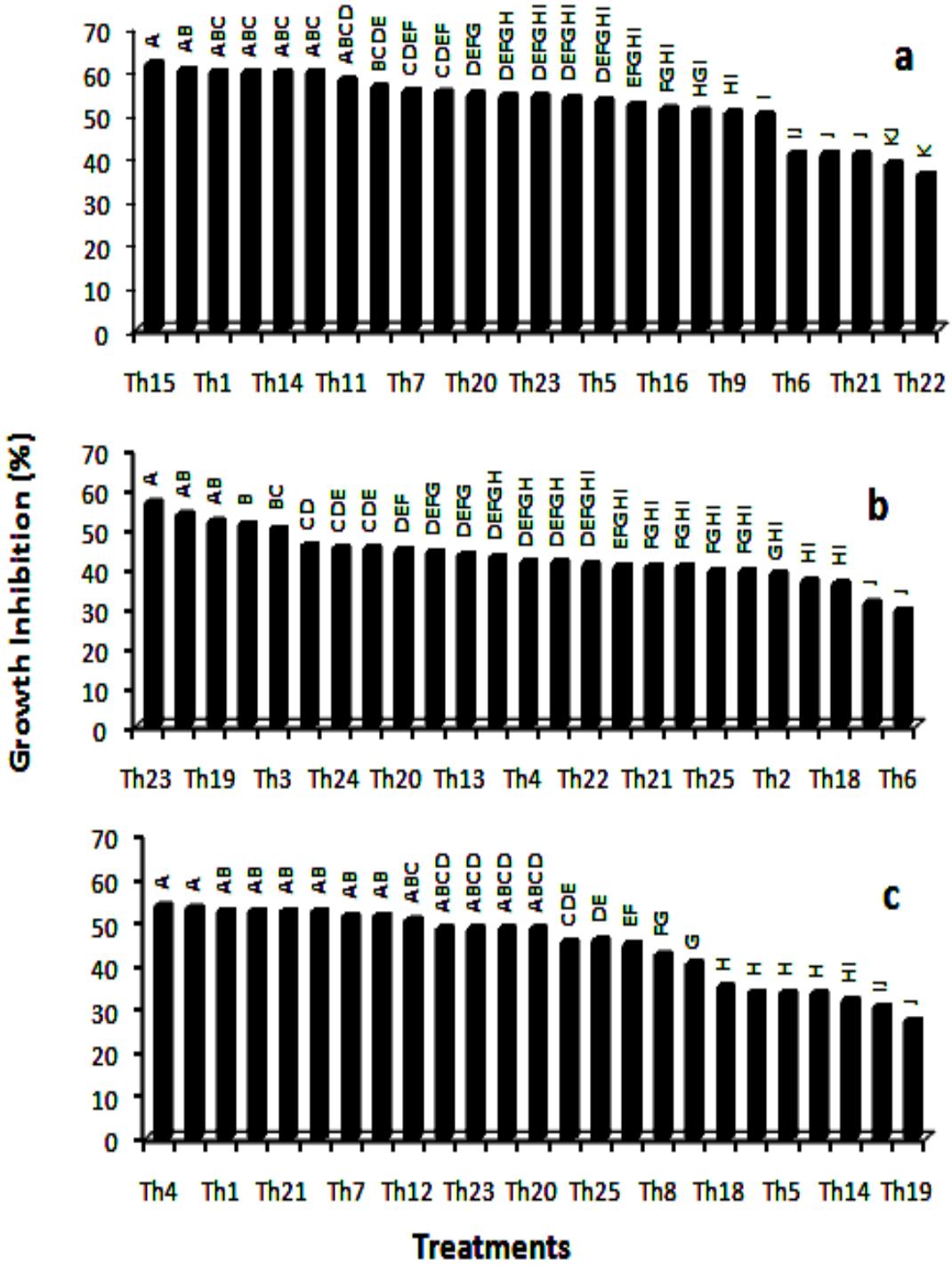

Figure 1 Radial growth inhibition of *Macrophomina phaseolina* isolate 1 (a), isolate 2 (b) and isolate 3 (c) imposed by *Trichoderma harzianum* mutants (Th1-Th24) and wild type (Th65) in dual culture test. Means followed by the same letters indicate no significant difference (Duncan's Test, $P < 0.05$).

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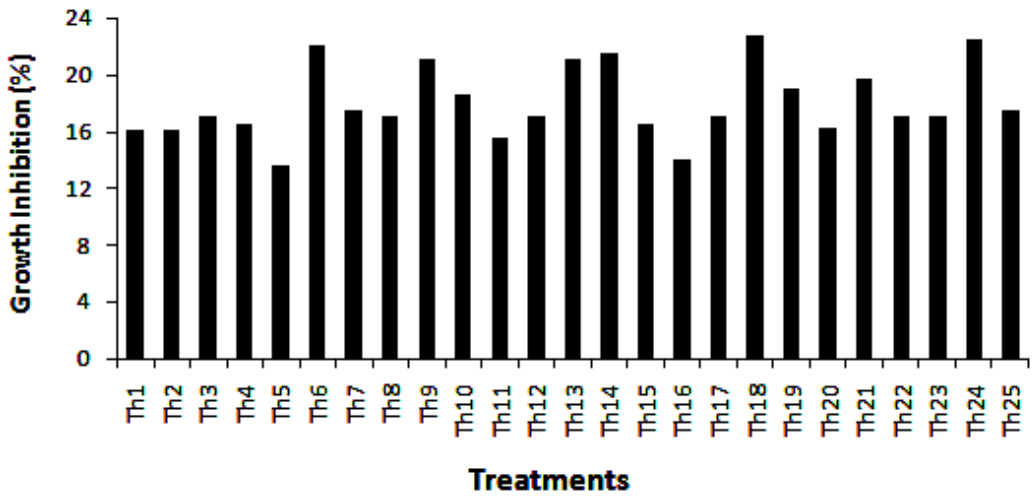

Figure 2 Average radial growth inhibition of *Macrophomina phaseolina* isolate 1, isolate 2 and isolate 3 imposed by volatile compounds of *Trichoderma harzianum* mutants (Th1-Th24) and wild type (Th65). Means did not significantly different.

Effect of non-volatile inhibitors

The GI% of *M. phaseolina* isolate (isolate 1) three days after incubation (Fig. 3a) revealed that there were significant difference in mutants and wild type ($p < 0.05$) and thatTh1 mutant showed maximum growth inhibition. Th11 and Th15 mutants were the next ones in the row with respect to GI%. The GI% of *M. phaseolina* isolate (isolate2) three days after incubation (Fig. 3b) revealed that Th15 mutant resulted in maximum growth inhibition. Th4 and Th10 were the next ones with respect to GI%. The GI% of *M. phaseolina* isolate (isolate3) three days after incubation (Fig. 3c) revealed that Th15 and Th22 mutants imposed maximum growth inhibition. Th4, Th8, Th10 and Th13 were the next ones with respect to GI%. Growth inhibition recorded in all of the *M. phaseolina* isolates significantly differed i.e. Garmsar (isolate2) and Khuzestan (isolate3) isolates proved to be highly susceptible to *Trichoderma* isolates, and Khorasan isolate showed the least growth inhibition. All the isolates of *M. phaseolina* were significantly different according to mycelial growth inhibition caused by *T. harzianum* (WT and mutants). Maximum inhibition was observed in interaction between culture filtrate of Khuzestan isolate and Th22 (88%) and Th15 (87%) mutants after three days of incubation.

Biocontrol of charcoal rot of melon in greenhouse

Four superior mutants and wild type of *T. harzianum* were examined for their biocontrol ability against charcoal rot of melon in greenhouse. The results revealed that the effect of antagonists were significant ($p < 0.05$) as compared with control (infected plant without antagonist) based on shoot and root fresh and dry weights. Among the evaluated treatments against *M. phaseolina* (isolate1), Th9 by 28% disease reduction compared to infected control supported highest plant stand and minimum infected plants were observed in Th65 (WT) plus *M. phaseolina* and fungicide plus *M. phaseolina* (Fig. 4). The percentages of infected plants in other treatments with *M. phaseolina* were not statistically different.

Fresh shoot weight was highest in Th65 (WT), among the evaluated treatments against *M. phaseolina* (isolate1), while Th9 (plus *M. phaseolina*) showed maximum dry shoot weight (Fig. 5). The results (Fig. 6a) revealed that highest root weight was observed in the soil treated with Th1 (infested and uninfested soil) and there was no significant difference between Th1 plus *M. phaseolina* and fungicide plus *M. phaseolina* (chemical

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control).The highest dry root weight was in Th65 (WT) (infested and uninfested soil) and among the evaluated treatments against *M. phaseolina* (isolate 1), Th1 and Th9 (plus *M. phaseolina*) showed maximum dry root weight and there was no significant difference between Th1 plus *M. phaseolina* and fungicide plus *M. phaseolina* (chemical control) (Fig. 6 b).

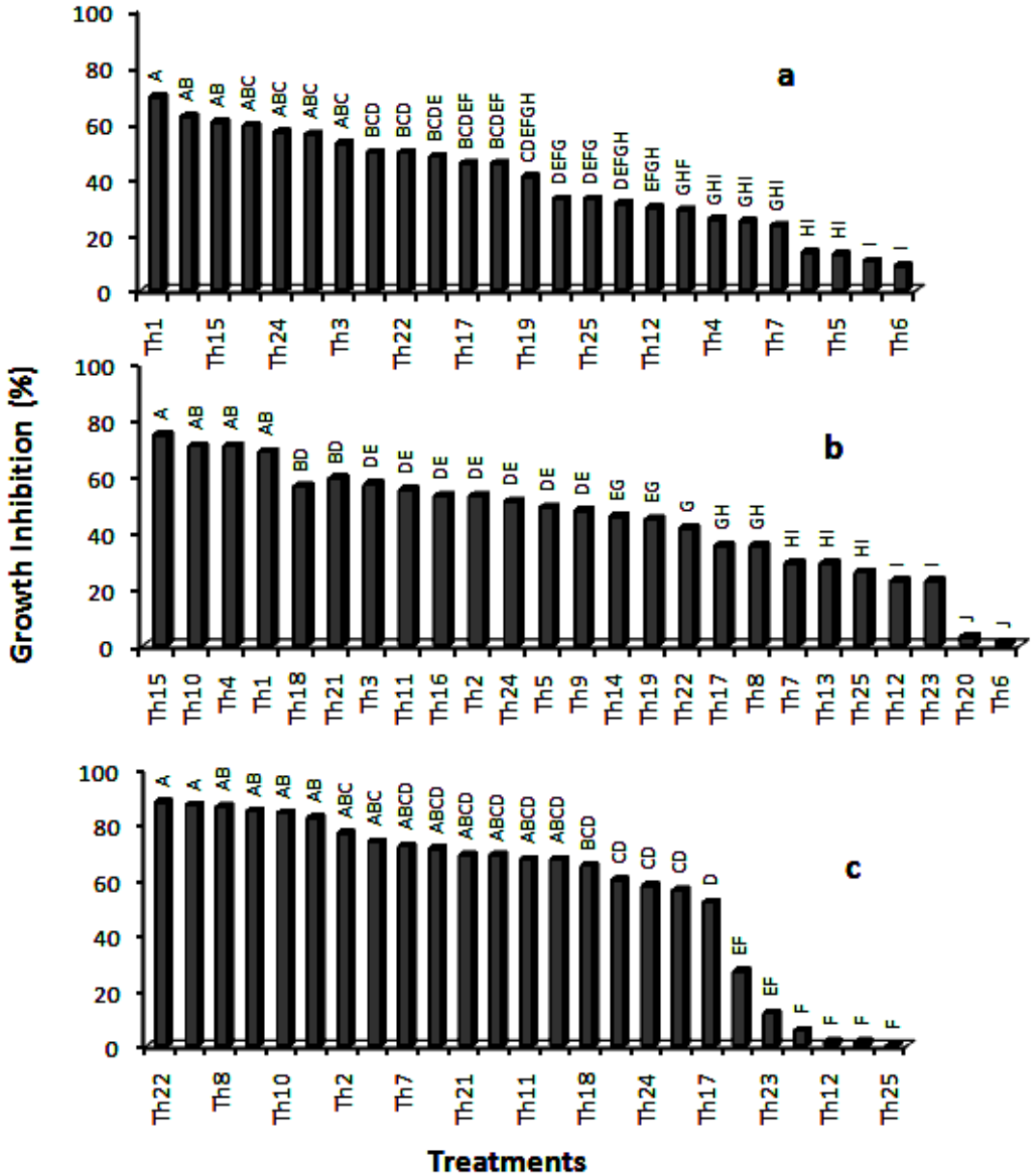

Figure 3 Radial growth inhibition of *Macrophomina phaseolina* isolate 1 (a), isolate 2 (b) and isolate 3 (c) imposed by non-volatile compounds of *Trichoderma harzianum* mutants (Th1-Th24) and wild type (Th65). Means followed by the same letters indicate no significant difference (Duncan's Test, $P < 0.05$).

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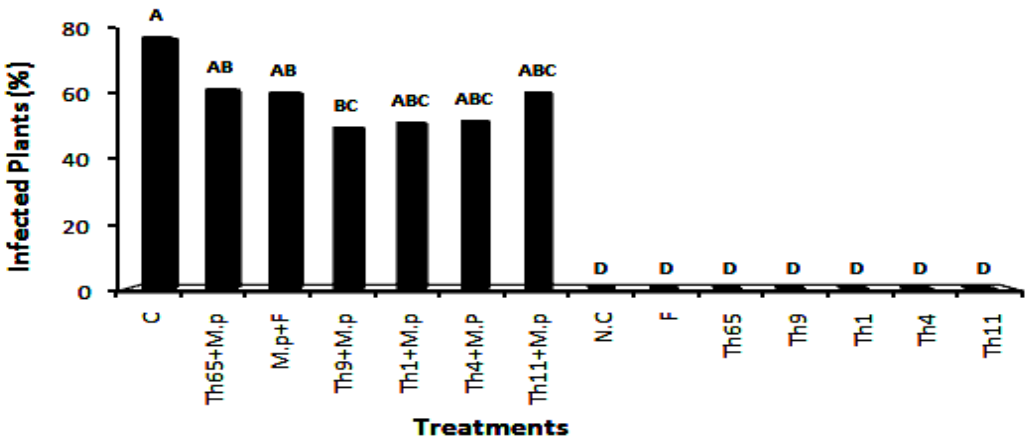

Figure 4 Effect of soil treatments with wild type and mutants of *Trichoderma harzianum* on percent infected plant of melons in pot soil inoculated with *M. phaseolina* (isolate 1) 40 days after planting. C:inoculated control, N. C: non inoculated control (healthy plant), Th65: WT and F: fungicide(Carboxin thiram). Means followed by the same letters indicate no significant difference (Duncan's Test, $P < 0.05$).

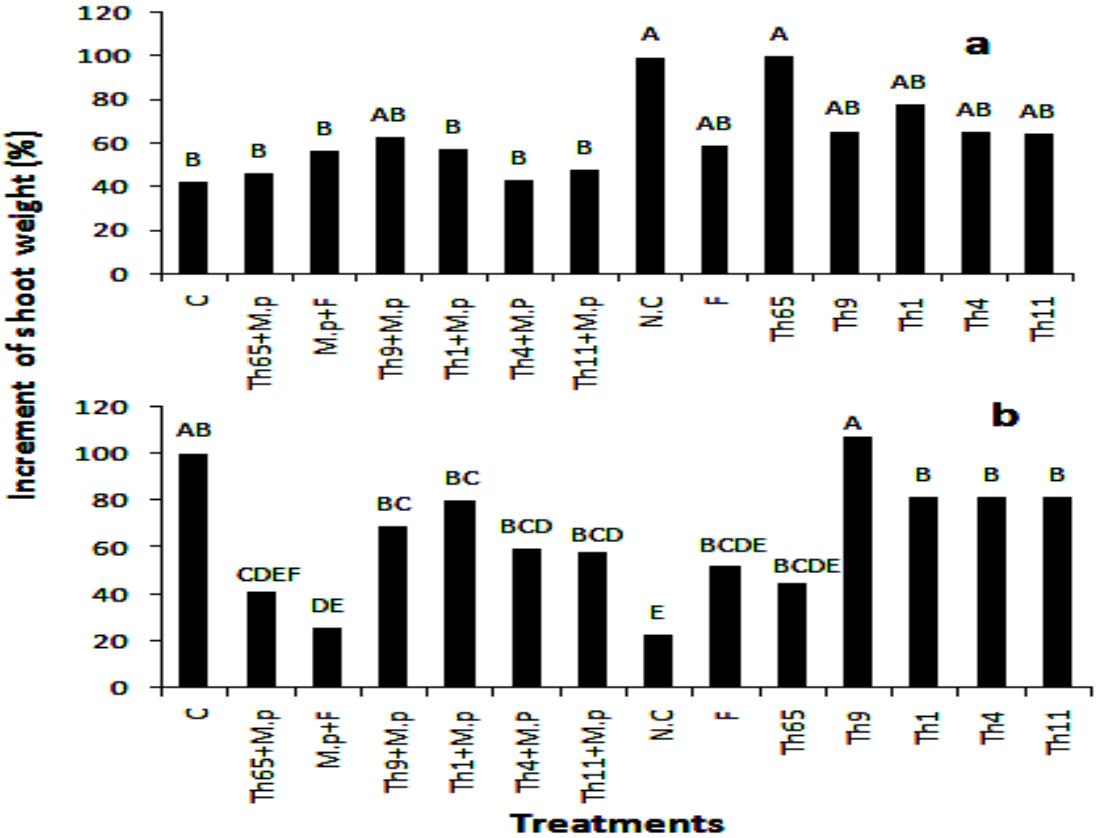

Figure 5 Effect of soil treatments with wild type and mutants of *Trichoderma harzianum* on fresh (a) and dry (b) shoot weights of melons in pot soil inoculated with *Macrophomina phaseolina* (isolate 1) 40 days after planting. C: inoculated control, N. C: non-inoculated control (healthy plant), Th65: WT and F: fungicide (Carboxin thiram). Means followed by the same letters indicate no significant difference (Duncan's Test, $P < 0.05$).

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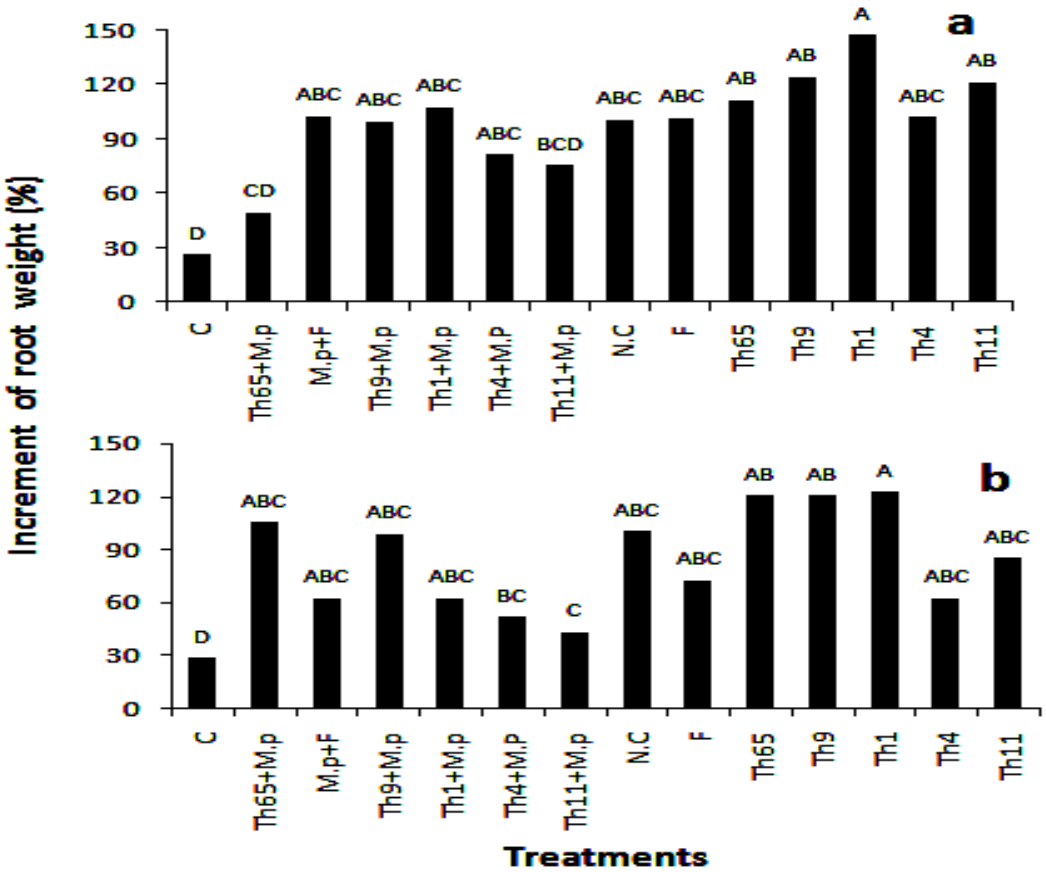

Figure 6 Effect of soil treatments with wild type and mutants of *Trichoderma harzianum* on fresh (a) and dry (b) dry root weights of melons in pot soil inoculated with *Macrophomina phaseolina* (isolate 1) 40 days after planting. C: inoculated control, N. C: non-inoculated control (healthy plant), Th65: WT and F: fungicide (Carboxin thiram). Means followed by the same letters indicate no significant difference (Duncan's Test, $P < 0.05$).

Discussion

M. phaseolina is one of the major yield limiting factors for melon cultivation in tropical and subtropical regions. Due to the soil borne nature of the disease, use of chemicals in controlling the melon charcoal rot is not recommended. Hence, the economical and feasible approach would be either to search for resistant sources or resort to biological control (Dobey *et al*., 2005). The biological control is the best alternative method especially against soil borne pathogens such as *M. phaseolina*. The present results indicate that all mutants of *T. harzianum* 65, significantly inhibit mycelial growth of the pathogen isolates. Maximum GI% was achieved by Th4, Th15, Th22 and Th23 mutants three days of incubation. Th1, Th9 and Th10 ranked as the second best antagonists next to Th15 and Th4 three days after incubation. Th1 showed more sporulation in comparison to above mentioned mutants. Th4 and Th15 showed higher colonization rate than the wild type - three days after incubation. Among the *M. phaseolina* isolates, maximum GI% was imposed on Khorasan (isolate1), and Khuzestan (isolate3) isolates. The antagonists inhibited the growth of pathogen significantly by the production of non-volatile antibiotic substances. Maximum growth inhibition of

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the pathogen was imposed by Th1 mutant. Th11 ranked as the second best antagonist after Th1. Th1 and Th9 induced maximum growth of roots and shoots in melon plants. The least infected plants were observed in chemical controls and Th65 (WT) with pathogen treatments.

T. harzianum earlier has proved as a potential bioagent of charcoal rot disease (Elad *et al*., 1986; Karthikeyan *et al*., 2006). Also, Arora *et al*. (1992) reported that root colonization by *Trichoderma* isolates frequently enhances root growth and development. The *T. harzianum* wild type and mutants increased root development in maize and several other crop plants both under greenhouse and field conditions (Harman, 2000). Mutation induction is a genetic tool to improve efficacy of biocontrol agents against soil borne plant pathogens (Spadaro and Lodovica, 2005). The present study revealed that gamma mutation by optimal dose 250 Gray causes changes in genome and induces mutants with enhanced antagonistic activity compared with wild type against *M. phasolina in vitro* and *in vivo* experiments. Mutation induction by gamma irradiation has been shown to increase; capability of *Trichoderma* species to produce enzymes like chitinase and antibiotics;colonization of tomato roots than wild type and to be a superior biocontrol agent asainst Fusarium wilt of tomato (Mohamed *et al*., 2006). other researchers have proved that mutation induces enhancement of biocontrol ability on soli borne diseases (Zekeres *et al*., 2004;Vaidya *et al*., 2003; Haggag and Mohamed, 2002; Haggag, 2008). Also, Ahari *et al*. (2010) by gamma irradiation with optimal dose 150Gy.second^{-1}(with rate dose 0.38) on *F. solani f. sp. phaseoli* induced non-pathogenic mutants to act as biocontrol agents against pathogenic *F. solani f. sp. phaseoli*. Present findings are in agreement with the above mentioned results and a superior biocontrol candidate is introduced against charcoal rot disease of melon. Gamma-mutagenesis by improvement of antagonistic properties of biocontrol agents can be used as a strategy to combat against soil borne plant pathogens such as the agent of charcoal rot disease of melon.

Acknowledgements

The authors are sincerely thankful to Dr. Shahbazi from Nuclear Science and Technology Research Institute and Dr. Eslahi from Agriculture and Natural Resources Research Center of Khuzestan for their helpful assistance.

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[DOR: 20.1001.1.22519041.2014.3.4.7.8]

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[DOR: 20.1001.1.22519041.2014.3.4.7.8]

ارزیابی جهش یافتگان *Trichoderma harzianum* القا شده توسط اشعه گاما در بیوکنترل بیماری پوسیدگی ذغالی خربزه (*Macrophomina phaseolina*) در شرایط آزمایشگاه و گلخانه

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دریافت: ۲۰ خرداد ۱۳۹۲؛ پذیرش: ۳ اسفند ۱۳۹۲

چکیده: قارچ *Macrophomina phaseolina* یکی از عوامل محدودکننده اصلی کشت خربزه در نواحی گرمسیری و نیمه گرمسیری می‌باشد. برای مدیریت مؤثر و سازگار با محیطزیست ناشی از این بیمارگر، ۲۴ جدایه جهش یافته توسط اشعه گاما از *Trichoderma harzianum* علیه سه جدایه از *M. phaseolina* مورد ارزیابی قرار گرفت. آزمون‌های کشت متقابل، متابولیت‌های فرار و عصاره‌های خارج سلولی جدایه مادری و جهش یافتگان تریکودرما علیه بیمارگر مورد مطالعه قرار گرفت. سه روز پس از کشت، جهش یافتگان Th1, Th4, Th15, Th9 وTh22 بیشترین میزان بازداری از رشد علیه بیمارگر را از خود نشان دادند. در بررسی‌های گلخانه‌ای، علیه بیماری پوسیدگی ذغالی خربزه در بین تیمارهای آلوده به ماکروفومینا تیمار Th 9 کمترین درصد گیاهان آلوده (۲۸ درصد کاهش مرگ‌ومیر در مقایسه با شاهد آلوده) و در بین تیمارهای بدون ماکروفومینا، جهش یافته‌های Th1 و Th9 بیشترین میزان رشد ریشه و قسمت‌های هوایی را در مقایسه با شاهد سالم نشان دادند. این جهش یافته‌ها به‌عنوان نماینده جهت بیوکنترل بیمارگر گیاهی مربوطه معرفی می‌شوند و پتانسیل تجاری شدن دارند. نتایج این پژوهش نشان داد که القای جهش توسط اشعه گاما با بهبود خصوصیات آنتاگونیستی *T. harzianum* 65 می‌تواند در بیوکنترل بیمارگرهای گیاهی خاک برد نظیر *M. phaseolina* مفید واقع شود.

واژگان کلیدی: پوسیدگی ذغالی خربزه، بهبود توانایی آنتاگونیستی، جهش‌زایی گاما و *Trichoderma harzianum*

[DOR: 20.1001.1.22519041.2014.3.4.7.8]