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Volatiles and functional peptides compositions of Trichoderma variants induced by a new strategy of irradiation

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ABSTRACT

Induction of mutation using gamma radiation improves the efficiency of Trichoderma against tomato fungal pathogens (*Alternaria solani, Fusarium oxysporium*, and *Rhizoctonia solani*). Results of the present study reveal the antagonistic activity of selected 2nd generation variants is higher than their 1st generation variants and the activity of latter variants is higher than their parent strains. The metabolites derived from irradiated variants are identified to understand their role in antibiosis interactions. GC/MS profile of volatiles emitted from *T. viride* Td_1 and as its variants shows they are different in composition. The present study clears the potency of volatiles emitted from 2nd generation variant; *T. viride* Td_1/2–3 to control tomato fungal pathogens using two strategies; applying the volatile compounds to tomato seeds or to soil infested with fungal pathogens. Results also showed the superiority of functional peptides originates from an irradiated variant; *T. koningii* Tk_1/3-3 to control tomato fungal pathogens especially peptide fraction; F_4 that characterized using LC/MS to fifteen sub-fractions having 12 amino acid residues or less; one of them is identified as peptabiotic.

1. Introduction

Microorganisms used as antagonists are rarely a wild-type; this is because; they are often genetically modified to maximize their antagonistic activity (Junaid et al., 2013). Induction of random mutations by ionizing and non-ionizing radiation or chemical mutagens such as acridine orange and ethyl methane sulfonate has been used as a useful tool (Griffiths et al., 2000). Gamma rays are the most energetic form of electromagnetic radiation; having energy 10 million eV or more; that cause gene mutation by replacement of nucleotides, oxidative deamination or chromosome breakage (Chakarov et al., 2014).

Trichoderma spp shares almost 70% of fungal biological control agents' (BCAs) market (Chandra and Singh, 2016). Improving their efficiency has been achieved using different strategies including; increasing salt stress (Mohamed and Haggag, 2006), pesticide resistance (Hatvani et al., 2006) and chemical mutation (Shafique et al., 2010). Gamma irradiation has been also used to create numerous variants belonging to genus Trichoderma that are effective against soil-borne plant pathogens owing to increasing their extracellular cell wall-degrading enzymes content. Mohamed et al. (2010) proved the capability of Trichoderma UV-induced variants (TvM1-UV1, TvM9-UV1, TvM1-UV2, and TvM9-UV2) to produce higher chitinase yields thus reducing the colonization of *Sclerotia rolfesii* and *Sclerotinia sclerotiorum* in bean rhizosphere compared with their parental strain and being superior biocontrol agents against rot-root and white-rot diseases, respectively.

Alternatively, many species of Trichoderma have been shown to produce a wide heterogeneous range of bioactive metabolites that play a dramatic role in the antibiosis process rather than lytic enzymes. These metabolites can be grouped into two main types: low molecular mass; volatile metabolites and high molecular mass metabolites such as bioactive peptides. In the present study, four strains of *Trichoderma*; *Trichoderma harzianum* Th_1, *T. koningii* Tk_1, *T. longibrachiatum* Tl_1 and *T. viride* Td_1; selected from a previous study (Awad et al., 2017); were irradiated by a new procedure based on successive exposing to high doses of gamma rays to select variants with a higher antagonistic activity against three fungal pathogens (*Alternaria solani, Fusarium oxysporium* and *Rhizoctonia solani*). In addition, the volatiles and functional peptides of selected variants were quantified, characterized and evaluated in applications either in *Vitro* or under greenhouse conditions.

2. Materials and methods

2.1. Microorganisms

Three strains out of 259 fungal pathogens were selected from a

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preliminary study according to their virulence and pathogenicity against healthy tomato root cultivar (GF12). They are *Alternaria solani, Fusarium oxysporium*, and*Rhizoctonia solani*. In addition, four biological control agents belong to genus Trichoderma; *Trichoderma harzianum* Th_1, *T. koningii* Tk_1, *T. longibrachiatum* Tl_1, and *T. viride* Td that showed the highest antagonism activity in a previous study against tomato fungal pathogens (Awad et al., 2017) were also selected as resources for gamma irradiated variants.

2.2. Irradiation studies

The spore suspension of selected *Trichoderma* spp were aseptically collected and irradiated using Cobalt-60 model Russian gamma cell (Issledovate) located at National center for Radiation Research and Technology (NCRRT) with increasing doses (0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 4.5 and 5.0 kGy) to determine the D_{10} value and the lethal dose for each antagonist (Atique et al., 2013). The dose rate at the time of experiment was 1.5 kGy/h.

2.3. Antagonistic studies under laboratory and greenhouse conditions

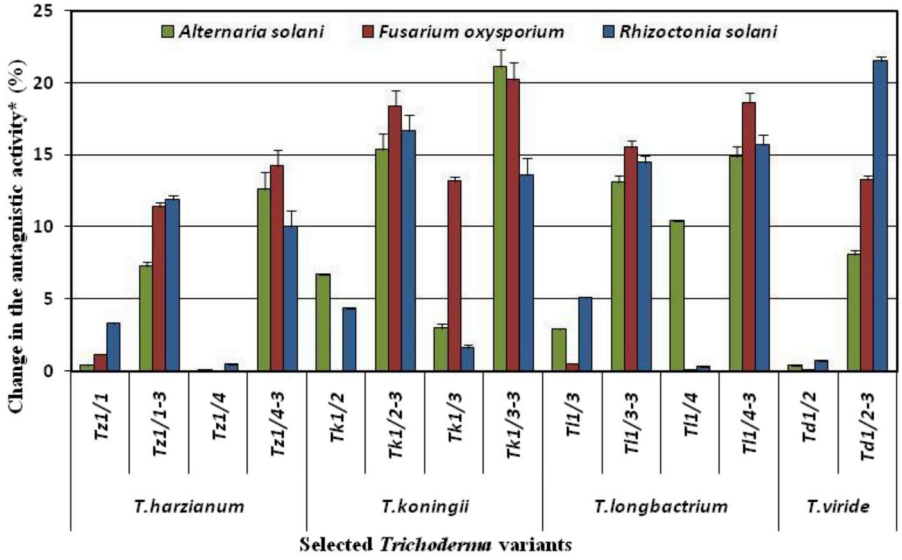
Antagonistic studies under laboratory conditions were studied using a dual culture technique and the percentage of growth reduction was determined according to a previous formula (Siameto et al., 2010).

Tomato seeds; *Lycopersicon esculentum* var. GF 12 were purchased from Maka Seeds Company. Spore suspensions of antagonists (10^6conidia ml^{-1}) were prepared using carboxy methylcellulose (0.1%) whereas fungal pathogens were grown on wheat grains. Greenhouse studies were designed using plastic pots (15 cm, diameter) containing approximately 1.25 kg of sterilized irradiated loamy clay soil. Ten tomato seeds were sown in each pot; three replicate pots were specified for each treatment in completely randomized experimental design. Each experiment included three treatments: 1) non infested soil samples (control), 2) infested soil samples with fungal pathogens at the rate of 5 g/kg soil and 3) infested soil samples with fungal pathogens were treated with 2 ml antagonist or partially purified metabolites. Pre- and Post-emergence damping off percentages as well as the percentages of survival plants and disease index were determined after 18 day of planting (Morsy et al., 2009).

2.4. Estimation the metabolites of Trichoderma spp

2.4.1. Volatile compounds

The antagonistic activity of volatile compounds emitted from *Trichoderma* spp either parent strains or their variants was studied against selected fungal pathogens according to procedure recommended by Nagamani et al. (2017). Volatiles evolved from T. viride Td_1 or its irradiated variants were collected using a modified trap setup based on activated charcoal (Cale et al., 2016). Emitted volatiles were then eluted using 0.5 ml methylene chloride and analyzed by GC (HP6890) equipped with TR-5MS 5% phenyl polysilphenylenesiloxane column (30 m × 0.25 mm × 0.25 µm) and interfaced with MS



-Bars represent standard error of data

Fig. 1. Evaluation the antagonistic activity of gamma irradiated *Trichoderma* variants against selected tomato fungal pathogens under laboratory conditions.

(HP5973) using the Wiley 275L database (Wiley-VCH, Weinheim, Germany). The carrier gas was helium at a flow rate of 1 ml min^{-1}. The oven temperature was programmed from 50 to 180 °C at a constant increase rate (5 °C min^{-1}), with a final isothermal period (10 min). Ten grams of tomato seeds (GF12) or hundred grams of sterilized soil infested with selected fungal pathogens were aseptically pursed in four layers of cheesecloth and hung out in 1 L screw-capped bottles containing 250 ml of Sabouraud's agar media previously inoculated with *T. viride* Td_1 or its variants (1st generation variant $Td_1/2$ or 2nd generation variant $Td_1/2$–1). Cultures were incubated at 25 °C for 7 days before using treated tomato seeds or infested soil samples to evaluate the efficiency of volatile compounds for controlling tomato fungal pathogens in individual experiments.

2.4.2. Functional peptides

Functional peptides produced by *Trichoderma* spp either parent strains or irradiated variants were individually quantified using the standard curve of polymyxin B (Ivashkiv, 1975). The fungus achieved the highest yield of functional peptides; *T. koningii* Tk_1 and its 1st generation variant $Tk_1/3$ that previously irradiated with 4.5 kGy or its 2nd generation variant that successively irradiated by 4.5 and 6.0 kGy; were cultivated into malt broth medium and incubated at 26 °C for 20 days. Then, growing mycelia were harvested by filtration and both of the culture filtrates and mycelia were extracted twice with ethyl acetate. The functional peptides were partially purified using column chromatography (Maddau et al., 2009).

The reduction of the radial growth of the selected fungal pathogens at different concentrations of partially purified peptides (25, 50 and 100 mg/ml) was monitored using an agar diffusion technique (Balouiri et al., 2016). Peptide fraction (F_4) that achieved the most antagonistic activity was further identified by LC/MS (UPLC) located at Center of Applied Research and Advanced Studies, Faculty of Pharmacy, Cairo University using the following conditions; detector: triple quad 6420, pump: quat. Pump 1290 infinity sampler, autosampler: 1290 infinity sampler and software: mass hunter. Chromatographic data of polypeptides were analyzed by a protein database designed by the Swiss Institute of Bioinformatics (http://web.expasy.org/protogram).

2.4.3. Statistical analysis

All data were statistically analyzed using SAS software package version 9.1.

3. Results

3.1. Irradiation studies

Exposing selected *Trichoderma* spp to increasing doses of gamma irradiation showed their resistance was increased in the following pattern; *T. viride* Td_1 > *T. koningii* Tk_1 > *T. harzianum* Tz_1 and *T. longibrachiatum* Tl_1. Consequently, their D_{10} values were calculated from the regression linear equation as 0.752, 0.739, 0.663 and 0.563 kGy in the same order. Colonies of *Trichoderma* spp which had survived to sub-lethal doses of gamma irradiation were purified and maintained as 1st generation variants. These variants were further exposed to irradiation doses higher than sub-lethal doses. Irradiated variants that couldn't survive to higher doses are neglected as unsuccessful variants whereas those tolerated to higher irradiation doses were selected as 2nd generation irradiated variants.

3.2. Evaluation the antagonistic activity of irradiated Trichoderma variants under laboratory conditions

Fig. (1) illustrates the antagonistic activity of the most active 2nd generation variants against selected tomato fungal pathogens; they are two variants of each *Trichoderma harzianum* (Tz_1/1–3, Tz_1/4–3), *T. koningii* (Tk_1/2–3, Tk_1/3-3), *T. longibrachiatum* (Tl_1/3-3, Tl_1/4–3) and one variant of *T. viride* (Td_1/2–3); as well as their 1st generation variants (Exposed once to gamma rays) and their parent strains (unirradiated *Trichoderma* spp) for comparison aim. Results revealed the antagonistic activity of the 2nd generation variants of *Trichoderma* spp was higher than their parent strains as well as their 1st generation variants.

3.3. Evaluation the antagonistic activity of irradiated Trichoderma variants under greenhouse conditions

Under greenhouse conditions, data revealed in Table 1 showed the antagonistic activity of 2nd generation variants is higher than 1st generation variants and the activity of 1st generation variants is higher than their parent strain. The most potent 2nd generation variant; *T. viride* Td_1/2-3 decreases the damping-off either pre or post-emergence caused by *A. solani* to zero and 3.3%; respectively as well as the disease index to 33% compared to 36.6, 40.0 and 95% recorded by control treatment in the same order. Regarding tomato fungal pathogen; *F. oxysporum*, Pre- and post-emergence damping-off were inhibited completely after treatment of tomato cultivar with 2nd generation irradiated variants of *T. harzianum* Tz_1/4–3 and *T. koningii* Tk_1/3-3 variants; respectively. In addition; irradiated variants *T. harzianum* Tz_1/1–3 and *T. konngii* Tk_1/2–3 increased the number of survival tomato plants infected with *R. solani* to 93.3%.

Table 1
Evaluation the antagonistic activity of the gamma irradiated variants against selected tomato fungal pathogens under greenhouse conditions.

Selected Trichoderma spp and their irradiated variants		*Alternaria solani*				*Fusarium oxysporium*				*Rhizoctonia solani*			
		Pre-emergence (%)	Post-emergence (%)	Survival plants (%)	Disease index (%)	Pre-emergence (%)	Post-emergence (%)	Survival plants (%)	Disease index (%)	Pre-emergence (%)	Post-emergence (%)	Survival plants (%)	Disease index (%)
T. harzianum	Tz_1	16.6 ± 1.8^{CD}	30.0 ± 3.8^{B}	53.3 ± 0.7^{I}	66.0 ± 4.1^{B}	20.0 ± 1.9^{BC}	20.0 ± 2.2^{BC}	60.0 ± 1.2^{I}	60.0 ± 2.6^{B}	20.0 ± 1.3^{D}	26.6 ± 3.6^{BC}	53.3 ± 3.0^{G}	62.0 ± 1.3^{B}
	$Tz_1/1$	13.3 ± 1.9^{DE}	16.6 ± 1.4^{CD}	70.0 ± 1.7^{G}	60.0 ± 5.0^{BC}	13.3 ± 0.4^{DE}	16.6 ± 1.2^{CD}	70.0 ± 1.4^{H}	56.0 ± 2.4^{BC}	10.0 ± 0.5^{FG}	16.6 ± 1.4^{D}	73.3 ± 1.6^{ED}	52.0 ± 1.5^{BC}
	$Tz_1/1$-3	10.0 ± 2.0^{EF}	10.0 ± 1.0^{EF}	80.0 ± 1.4^{EF}	40.0 ± 6.4^{DE}	10.0 ± 1.4^{EF}	6.6 ± 0.5^{F}	83.3 ± 0.7^{DE}	40.0 ± 4.0^{D}	0.0 ± 0.0^{H}	6.6 ± 0.6^{FG}	93.3 ± 2.1^{A}	42.0 ± 1.0^{DE}
	$Tz_1/4$	10.0 ± 1.7^{EF}	20.0 ± 2.0^{C}	70.0 ± 1.0^{G}	61.0 ± 6.7^{BC}	13.3 ± 0.3^{DE}	16.6 ± 1.3^{CD}	70.0 ± 2.4^{H}	57.0 ± 1.4^{BC}	16.6 ± 1.0^{E}	10.0 ± 0.6^{EF}	73.3 ± 1.7^{ED}	55.0 ± 3.9^{BC}
	$Tz_1/4$-3	10.0 ± 1.0^{EF}	6.6 ± 0.5^{FG}	83.3 ± 1.3^{DE}	39.0 ± 3.9^{DE}	0.0 ± 0.0^{H}	10.0 ± 1.2^{EF}	90.0 ± 1.0^{BC}	39.0 ± 3.0^{D}	13.3 ± 1.1^{EF}	3.3 ± 0.3^{G}	83.3 ± 3.3^{C}	34.0 ± 1.7^{E}
T. koningii	Tk_1	20.0 ± 2.4^{C}	16.6 ± 0.8^{CD}	63.3 ± 1.2^{H}	61.0 ± 4.1^{BC}	23.3 ± 0.5^{B}	16.6 ± 1.7^{CD}	60.0 ± 0.6^{I}	64.0 ± 4.2^{B}	13.3 ± 1.0^{EF}	30.0 ± 3.4^{B}	56.6 ± 2.5^{G}	57.0 ± 3.2^{BC}
	$Tk_1/2$	13.3 ± 1.8^{DE}	10.0 ± 1.4^{EF}	76.6 ± 2.2^{F}	57.0 ± 4.3^{BC}	10.0 ± 1.3^{EF}	16.6 ± 0.4^{CD}	73.3 ± 2.3^{GH}	61.0 ± 6.5^{B}	10.0 ± 0.8^{FG}	13.3 ± 1.5^{DE}	76.6 ± 0.7^{D}	57.5 ± 1.5^{BC}
	$Tk_1/2$-3	6.6 ± 1.0^{FG}	3.3 ± 0.3^{G}	90.0 ± 1.0^{BC}	39.0 ± 3.2^{DE}	6.6 ± 0.9^{FG}	6.6 ± 1.2^{F}	86.6 ± 0.9^{CD}	45.0 ± 1.7^{CD}	0.0 ± 0.0^{H}	6.6 ± 1.2^{FG}	93.3 ± 1.9^{A}	36.0 ± 4.4^{E}
	$Tk_1/3$	10.0 ± 1.6^{EF}	13.3 ± 0.5^{DE}	76.6 ± 0.9^{F}	55.0 ± 4.0^{BC}	13.3 ± 1.4^{DE}	10.0 ± 1.1^{EF}	76.6 ± 0.8^{FG}	59.0 ± 5.3^{B}	13.3 ± 1.3^{EF}	16.6 ± 2.5^{D}	63.3 ± 3.2^{F}	50.5 ± 0.8^{CD}
	$Tk_1/3$-3	3.3 ± 0.2^{GH}	3.3 ± 0.3^{G}	93.3 ± 0.8^{AB}	38.0 ± 4.0^{DE}	3.3 ± 0.2^{GH}	0.0 ± 0^{G}	96.6 ± 3.0^{A}	44.0 ± 2.4^{D}	6.6 ± 0.2^{G}	3.3 ± 0.3^{G}	90.0 ± 1.1^{AB}	40.0 ± 2.8^{E}
T. longbactrium	Tl_1	26.6 ± 3.1^{B}	16.6 ± 0.9^{CD}	56.6 ± 1.1^{I}	58.0 ± 4.4^{BC}	13.3 ± 1.0^{DE}	23.3 ± 2.1^{B}	63.3 ± 2.7^{I}	61.0 ± 3.2^{B}	23.3 ± 0.5^{C}	36.6 ± 3.0^{A}	40.0 ± 2.0^{H}	58.0 ± 4.9^{BC}
	$Tl_1/3$	20.0 ± 1.0^{C}	16.6 ± 0.7^{CD}	63.3 ± 3.4^{H}	50.0 ± 4.0^{CD}	10.0 ± 1.5^{EF}	13.3 ± 1.3^{DE}	76.6 ± 0.7^{FG}	59.0 ± 4.1^{B}	13.3 ± 0.8^{EF}	16.6 ± 2.1^{D}	70.0 ± 1.4^{E}	50.0 ± 0.1^{CD}
	$Tl_1/3$-3	13.3 ± 0.3^{DE}	10.0 ± 1.4^{EF}	70.0 ± 1.5^{G}	34.0 ± 4.5^{CD}	6.6 ± 1.1^{FG}	6.6 ± 0.6^{F}	86.6 ± 0.6^{CD}	38.0 ± 3.0^{D}	10.0 ± 1.5^{FG}	6.6 ± 1.0^{FG}	83.3 ± 1.2^{C}	35.0 ± 3.4^{E}
	$Tl_1/4$	13.3 ± 1.2^{DE}	20.0 ± 1.8^{C}	66.6 ± 1.6^{GH}	55.0 ± 6.2^{E}	10.0 ± 1.4^{EF}	20.0 ± 1.9^{BC}	70.0 ± 3.9^{H}	57.0 ± 1.8^{BC}	13.3 ± 1.0^{EF}	13.3 ± 1.2^{DE}	72.8 ± 1.5^{DE}	55.0 ± 4.3^{BC}
	$Tl_1/4$-3	6.6 ± 0.6^{FG}	13.3 ± 0.8^{DE}	80.0 ± 1.5^{EF}	37.0 ± 4.0^{DE}	0.0 ± 0.0^{H}	6.6 ± 0.2^{F}	93.3 ± 1.9^{AB}	38.0 ± 4.3^{D}	10.0 ± 1.3^{FG}	3.3 ± 0.2^{G}	86.6 ± 0.6^{BC}	37.0 ± 2.7^{E}
T. viride	Td_1	13.3 ± 1.4^{DE}	10.0 ± 1.5^{EF}	77.1 ± 1.8^{F}	59.0 ± 5.5^{BC}	16.6 ± 1.1^{CD}	13.3 ± 0.6^{DE}	70.0 ± 3.6^{GH}	62.0 ± 3.5^{B}	33.3 ± 1.4^{B}	23.3 ± 2.9^{C}	43.3 ± 1.8^{H}	60.0 ± 2.6^{BC}
	$Td_1/2$	10.0 ± 1.5^{EF}	10.0 ± 1.5^{EF}	86.0 ± 1.4^{CD}	55.0 ± 3.0^{BC}	10.0 ± 1.6^{EF}	10.0 ± 1.5^{EF}	80.0 ± 1.9^{EF}	60.0 ± 7.0^{B}	16.6 ± 1.1^{E}	10.0 ± 1.2^{EF}	73.3 ± 1.4^{DE}	55.0 ± 2.0^{BC}
	$Td_1/2$-3	0.0 ± 0.0^{H}	3.3 ± 1.8^{G}	96.6 ± 2.4^{A}	33.0 ± 3.2^{E}	3.3 ± 0.2^{GH}	6.6 ± 1.2^{F}	90.0 ± 0.3^{BC}	$38..0 \pm 2.3^{D}$	10.0 ± 1.7^{FG}	13.3 ± 1.1^{DE}	77.1 ± 1.3^{D}	36.0 ± 3.2^{E}
Control*		36.6 ± 3.3^{A}	40 ± 1.5^{A}	23.3 ± 1.0^{J}	95.0 ± 3.0^{A}	33.3 ± 2.1^{A}	36.6 ± 3.3^{A}	30.0 ± 1.6^{J}	92.0 ± 4.6^{A}	36.6 ± 1.4^{A}	36.3 ± 2.5^{A}	26.6 ± 1.7^{I}	88.0 ± 4.6^{A}
R-square at Pr < 0.0001		0.9576	0.9751	0.9906	0.9114	0.9791	0.9666	0.9834	0.9196	0.9864	0.9666	0.9887	0.9495

*Control: Tomato seeds planted in soil infested with selected fungal pathogens and untreated with antagonists.
- Values are shown as mean ± standard error. For each column, different letters represent Duncan grouping and indicate a significant difference.

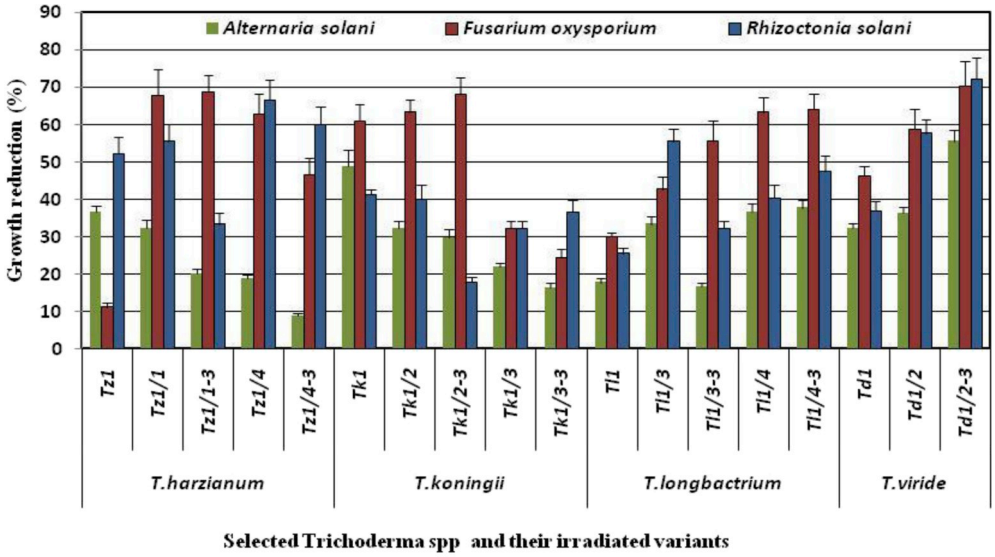


Fig. 2. Antagonistic activity of volatiles compounds emitted from irradiated *Trichoderma* variants as well as their parent strains against selected tomato fungal pathogens under laboratory conditions.

3.4. Volatile compounds

3.4.1. Evaluation the antagonistic activity of volatile compounds under laboratory conditions

The potency of volatile compounds evolved from irradiated variants of *T. longibrachiatum* and *T. viride* against *A. solani* was higher than the potency of volatiles evolved from parent strains under laboratory conditions (Fig. 2). The effectiveness of volatile compounds evolved from selected *Trichoderma* spp and their irradiated variants against *F. oxysporum* was higher than those recorded against *A. solani.* Irradiated

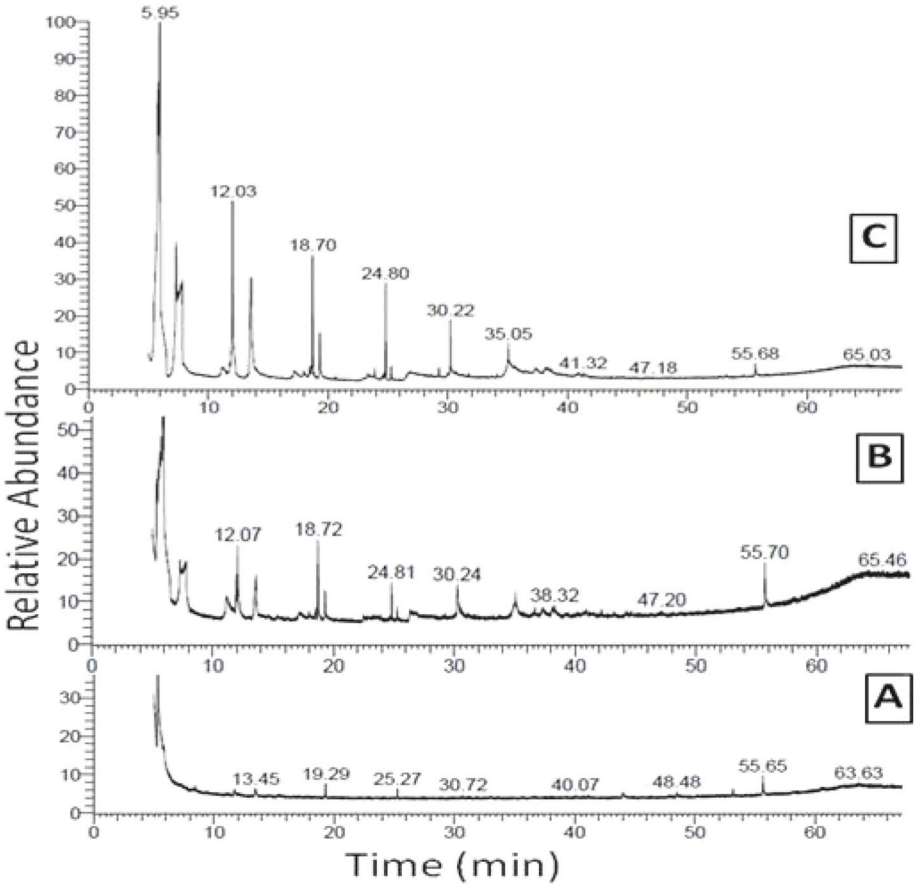


Fig. 3. Profile of volatile compounds emitted by *Trichoderma viride*, trapped in activated charcoal and identified in GC/MS. A- Parent strain of *T. viride* Td_1. B- First generation variant of *T. viride* $Td_1/2$. C- Second generation variant of *T. viride* $Td_1/2$-3.

Table 2
Application of volatile compounds emitted from the 2nd generation variant *Trichoderma viride* Td_1/2-3 on tomato seeds (GF 12) or loamy soil to control tomato fungal pathogens under greenhouse conditions.

Selected tomato fungal pathogens	Tomato seeds treated with VOCs				Soil treated with VOCs				Control*			
	Pre-emergence (%)	Post-emergence (%)	Survival plants (%)	Disease index (%)	Pre-emergence (%)	Post- emergence (%)	Survival plants (%)	Disease index (%)	Pre-emergence (%)	Post-emergence (%)	Survival plants (%)	Disease index (%)
Alternaria solani	0.0 ± 0.0^{D}	5.0 ± 0.8^{BC}	95.0 ± 1.5^{A}	41.0 ± 1.5^{D}	2.5 ± 0.3^{D}	0.0 ± 0.0^{C}	97.5 ± 1.3^{A}	46.0 ± 2.4^{BC}	7.5 ± 0.3^{D}	17.5 ± 0.2^{BC}	75.0 ± 3.2^{A}	88.0 ± 1.5^{B}
Fusarium oxysporium	5.0 ± 0.7^{B}	0.0 ± 0.0^{D}	95.0 ± 0.8^{A}	45.0 ± 3.7^{CD}	7.5 ± 0.3^{B}	2.5 ± 0.4^{C}	90.0 ± 2.6^{BC}	42.0 ± 2.0^{BC}	15.0 ± 1.2^{C}	17.5 ± 0.4^{BC}	67.5 ± 3.4^{A}	85.0 ± 1.9^{BC}
Rhizoctonia solani	2.5 ± 0.1^{C}	7.5 ± 0.9^{AB}	90.0 ± 1.8^{AB}	58.0 ± 3.3^{AB}	0.0 ± 0.0^{E}	10.0 ± 1.3^{AB}	90.0 ± 1.4^{BC}	35.0 ± 2.4^{C}	12.5 ± 2.5^{C}	20.0 ± 1.0^{B}	67.5 ± 1.7^{A}	81.0 ± 2.3^{C}
A. solani & F. oxysporium	5.0 ± 0.5^{B}	2.5 ± 0.2^{CD}	92.5 ± 1.3^{AB}	52.0 ± 2.9^{BC}	5.0 ± 0.9^{C}	7.5 ± 0.3^{B}	87.5 ± 0.8^{CD}	50.0 ± 2.8^{B}	37.5 ± 1.6^{A}	12.5 ± 1.0^{C}	50.0 ± 3.0^{BC}	90.0 ± 1.3^{A}
A. solani & R.solani	2.5 ± 0.2^{C}	10.0 ± 1.2^{A}	87.5 ± 2.2^{CD}	60.0 ± 2.0^{AB}	0.0 ± 0.0^{E}	7.5 ± 0.4^{B}	92.5 ± 1.5^{AB}	61.0 ± 6.5^{A}	15.0 ± 0.8^{C}	27.5 ± 0.7^{A}	57.5 ± 1.8^{B}	91.0 ± 1.3^{AB}
F. oxysporium & R.solani	0.0 ± 0.0^{D}	10.0 ± 1.0^{A}	90.0 ± 1.6^{BC}	64.0 ± 1.3^{A}	7.5 ± 0.4^{B}	10.0 ± 0.9^{AB}	82.5 ± 1.0^{DE}	62.0 ± 1.3^{A}	22.5 ± 0.8^{B}	32.5 ± 2.1^{A}	45.5 ± 2.1^{C}	94.0 ± 1.3^{A}
A. solani & F. Oxysporium & R. solani	7.5 ± 0.4^{A}	10.0 ± 1.6^{A}	82.5 ± 1.5^{C}	65.0 ± 1.5^{A}	10.0 ± 1.2^{A}	12.5 ± 2.3^{A}	77.5 ± 1.5^{E}	63.0 ± 2.7^{A}	25.0 ± 1.2^{B}	30.0 ± 3.2^{A}	45.0 ± 2.3^{C}	96.0 ± 1.2^{A}
Probability <	0.0001	0.0007	0.0074	0.0014	0.0001	0.0008	0.0007	0.0026	0.0001	0.0003	0.0004	0.0024
R-square	0.9799	0.9380	0.8726	0.9233	0.9731	0.9350	0.9378	0.9070	0.9786	0.9513	0.9485	0.9093

*Control: Tomato seeds planted in soil infested with selected fungal pathogens and untreated with volatile compounds.
- Values are shown as mean ± standard error. For each column, different letters represent Duncan grouping and indicate a significant difference.

Table 3
Evaluationthe antagonistic activity of different concentrations of partially purified functional peptides fractions produced by *Trichoderma koningii* Tk_1 and its irradiated variants against selected tomato fungal pathogens under laboratory conditions.

	Partially purified Functional peptides fractions	Growth reduction percentage of selected fungal pathogens (%)* when exposed to different concentrations of functional peptides fractions (ppm)								
		Alternaria solani			*Fusarium oxysporium*			*Rhizoctonia solani*		
		25	50	100	25	50	100	25	50	100
***T. koningii* Tk_1**	**Fraction 3**	7.8 ± 0.1^{F}	11.1 ± 0.5^{CD}	18.9 ± 0.4^{BC}	6.7 ± 0.8^{FG}	7.8 ± 1.1^{FG}	89 ± 1.2^{A}	5.6 ± 1.2^{FG}	11.1 ± 0.3^{BCD}	16.7 ± 0.8^{BCD}
	Fraction 8	6.7 ± 0.2^{G}	12.2 ± 1.0^{CD}	14.4 ± 0.2^{DEF}	8.9 ± 0.9^{DEF}	11.1 ± 0.8^{DEF}	14.4 ± 1.2^{EF}	6.7 ± 0.9^{EFG}	8.9 ± 1.2^{CD}	12.2 ± 1.1^{CDE}
	Fraction 10	5.6 ± 0.1^{H}	8.9 ± 0.2^{D}	13.3 ± 0.3^{EFG}	7.8 ± 1.2^{EFG}	14.4 ± 0.6^{CD}	16.7 ± 0.2^{DE}	7.8 ± 1.2^{DEF}	15.6 ± 1.2^{B}	20.0 ± 2.3^{BC}
	Fraction 11	11.1 ± 0.1^{C}	14.4 ± 0.4^{BC}	21.1 ± 0.3^{B}	12.2 ± 1.1^{BC}	21.1 ± 1.4^{B}	22.2 ± 0.3^{C}	13.3 ± 2.2^{B}	14.4 ± 0.8^{BC}	23.3 ± 2.6^{B}
***T. koningii* Tk_1/3 variant (First generation)**	**Fraction 3**	5.6 ± 0.2^{H}	8.9 ± 0.2^{D}	11.1 ± 0.4^{GH}	3.3 ± 0.9^{H}	5.6 ± 1.1^{G}	6.7 ± 0.3^{H}	11.1 ± 0.2^{CD}	12.2 ± 0.8^{BC}	13.3 ± 1.2^{CDE}
	Fraction 4	8.9 ± 0.2^{E}	14.4 ± 0.3^{BC}	21.1 ± 1.1^{B}	11.1 ± 0.7^{CD}	13.3 ± 0.7^{CDE}	13.3 ± 1.2^{EFG}	10.0 ± 0.2^{CDE}	16.7 ± 1.1^{B}	20.0 ± 2.0^{BC}
	Fraction 7	13.3 ± 0.2^{B}	16.7 ± 0.5^{B}	18.9 ± 0.4^{BC}	8.9 ± 0.7^{DEF}	10.0 ± 0.4^{EF}	11.1 ± 0.3^{FG}	3.3 ± 0.8^{GH}	5.6 ± 1.1^{D}	8.9 ± 1.7^{DE}
	Fraction 8	5.6 ± 0.1^{H}	10.0 ± 0.3^{D}	12.2 ± 0.8^{FG}	10.0 ± 0.5^{CDE}	14.4 ± 0.7^{CD}	18.9 ± 0.3^{D}	4.4 ± 0.8^{FG}	6.7 ± 1.8^{D}	10.0 ± 1.5^{DE}
	Fraction 9	4.4 ± 0.1^{I}	11.1 ± 0.3^{CD}	15.6 ± 1.3^{DE}	5.6 ± 1.2^{GH}	12.2 ± 0.4^{DE}	13.3 ± 1.1^{EFG}	11.1 ± 0.7^{CD}	13.3 ± 1.2^{BC}	14.4 ± 1.5^{CDE}
***T. koningii* Tk_1/3–3 variant (Second generation)**	**Fraction 1**	0.0 ± 0.0^{J}	0.0 ± 0.0^{E}	8.9 ± 0.9^{H}	0.0 ± 0.0^{I}	0.0 ± 0.0^{H}	10.0 ± 0.2^{G}	0.0 ± 0.0^{H}	5.6 ± 1.2^{D}	7.8 ± 1.5^{E}
	Fraction 3	10.0 ± 0.2^{D}	12.2 ± 0.3^{CD}	16.7 ± 1.2^{CD}	14.4 ± 0.8^{B}	16.7 ± 1.4^{C}	16.7 ± 1.2^{ED}	13.3 ± 1.3^{BC}	16.7 ± 1.8^{B}	18.9 ± 2.3^{BC}
	Fraction 4	22.2 ± 0.1^{A}	42.2 ± 3.3^{A}	50.0 ± 1.9^{A}	18.9 ± 1.4^{A}	41.1 ± 2.5^{A}	47.8 ± 2.3^{B}	20.0 ± 2.2^{A}	46.7 ± 4.3^{A}	54.4 ± 6.1^{A}
R-square at Pr-0.0001		0.9992	0.9881	0.9919	0.9650	0.9671	0.9979	0.9536	0.9755	0.9591

- Values are shown as mean ± standard error. For each column, different letters represent Duncan grouping and indicate a significant difference.
*Compared to control (selected tomato fungal pathogens were grown for 7 days to reach the margin of plates).-Bars represent standard error of data.

variant; *T. viride* Td_1/2–3 succeeded to decrease the radial growth of *A. solani*, *F. oxysporum* and *R. solani* by 55.5, 70.4 and 72.2%; respectively. It is important to mention that volatile compounds not only reduce the growth of selected tomato fungal pathogens completely or partially but also negatively impact hyphal pigmentation.

3.4.2. GC/MS analysis of emitted volatile compounds

As expected, volatiles emitted from the parent strain of *T. viridi* Td_1 are evolved as a mixture of organic compounds. Fig. 3-A clears the GC/MS analysis of volatiles evolved from *T. viridi* Td_1 (parent strain) displayed eight major volatile compounds and eleven minor compounds (< 1%). Bis(2-ethylhexyl) phthalate is the most abundant volatiles based on the intensity of the GC/MS analysis; 29.58%. Five volatiles were emitted from the parent strain of *T. viridi* Td_1 and are absent from the volatiles' profile of irradiated variants; they are 3-nitropropanoic acid, cathine, 1-cyclohexyl-4,4diethoxybutanone, ethyl-14-methyl hexadecanoate, and octadecanoic acid, ethylester. The volatiles profile of the 1st generation irradiated variant of *T. viride* Td_1/2 showed fifteen volatile compounds (Fig. 3-B). However, 3-Phenyl-2,3-dihydro-1-Hisoindol-1-one (25.62%), 2-methoxy-1-benzothieno-2,3-quinolin-6(5H) one (19.62%), and bis(2-ethylhexyl) phthalate (7.79%) are the most common volatiles. The profile of volatiles emitted from the 2nd generation irradiated variant *T. viridi* Td_1/2–3 is similar in chemical composition to those emitted from the 1st generation variant but their quantities are different (Fig. 3-C).

3.4.3. Evaluation the antagonistic activity of volatile compounds under greenhouse conditions

Table (2) showed the success of tomato seeds after have been treated with volatile compounds emitted from *T. viride* Td_1/2–3 variant to grow and overcome tomato diseases caused by each of *A. solani, F. oxysporum* and *R. solani* individually or combined together as a single inoculum. These volatiles completely inhibited the pre-emergence damping-off caused by *A. solani* individually or combined with *R. solani* and the post-emergence damping-off caused by *F. oxysporum*. In addition, they succeeded to decrease the disease index of tomato plants infected with *A. solani* and *F. oxysporum* to nearly half. On the same trend, volatile compounds enhanced the growth characters of tomato plants GF12 including shoot length, root length, shoot fresh weight and root fresh weight as compared to control (Data not shown).

Regarding the soil samples had treated by volatiles emitted from *T. viride* Td_1/2–3 variant before planted with tomato seeds, the incidence of the pre-emergence damping-off disease was decreased to the minimum value (0%) when soil infected with *R. solani* in individual form or combined with *A. solani* (Table 2). In addition, the disease index decreased up to 60%. The amazing result is the success of this strategy in combined fungal pathogens (As a model for soil community fungal pathogens) as well individual fungal pathogens. Consequently, the root and shoot fresh weights were increased by four folds compared to control after treating the soil with *A. solani*& *R. solani* and *A. solani*& *F. oxysporum* with volatiles (Data not shown).

3.5. Functional peptides

3.5.1. Functional peptides quantification of irradiated trichoderma variants as well as their parent strains

Exposing selected *Trichoderma* spp to gamma radiation for two successive times induces 2nd generation variants with a positive impact on their peptides yield. The highest peptides yield is achieved by the 2nd generation variant *T. koningii* Tk_1/3-3; 563 mg/ml whereas the titer of its parent strain and the 1st generation variant are 119 and 247 mg/ml; respectively.

3.5.2. Partially-purification of functional peptides

Crude functional peptides were purified by column chromatography using silica gel as a stationary phase. With increasing the polarity of the solvent system (petroleum ether and ethyl acetate), the functional peptides of *koningii* Tk_1 and its 1st generation variant; *T. koningii* Tk_1/3 were fractionated into eleven and nine fractions; respectively whereas their analog of the 2nd generation variant Tk_1/3-3 was partially purified into four fractions with a clear difference in color; light yellow (fraction 1), orange (fraction 2), light brown (fraction 3) and deep yellow (fraction 4).

Evaluation the antagonistic activity of partially purified functional peptides fractions against selected fungal pathogens under laboratory conditions.

Seven fractions of peptides partially purified from *T. koningii* Tk_1 have no impact on the growth of the selected fungal pathogens whereas four fractions showed a growth reduction in a range between 5.6% and 23.3% compared to control especially fraction 11 (Table 3). Five peptide fractions (3, 4, 7, 8, 9) produced by 1st generation irradiated variant; *T. koningii* Tk_1/3 succeeded to reduce the growth of tomato fungal pathogens at 25 ppm or higher concentrations. The most active fraction; F_4 (produced by the 2nd generation variant Tk_1/3-3) decreases the radial growth of three selected pathogens nearly by 40% and 50% at 50 and 100 ppm; respectively. This fraction was selected and applied in the greenhouse studies.

3.5.3. Evaluation antagonistic activity of functional peptides under greenhouse conditions

In a greenhouse experiment, the selected peptide fraction (F_4) succeeded to control damping-off caused by selected fungal pathogens thus the percentage of surviving plants increased up to 100% (Fig. 4). In addition, the disease index of root rot disease after treatment was decreased by nearly half or more.

Characterization of partially purified functional peptides fraction (F_4) produced by second generation variant *Trichoderma koningii* Tk_1/3-3 by LC/MS.

The selected peptide fraction was further purified using LC/MS to characterize its chemical composition. Its purification afforded fifteen sub-fractions at retention times (RT) ranging from 0.750 to 11.397 min with a single and double charged sodium adducts (Fig. 5). The b_1 fragment ion was found in three sub-fractions eluted at 9.432, 10.43 and 11.397 min. Most sub-fractions represent compounds belonging to a class of functional peptides having 12 amino acid residues or less. Three b_{13} fragments were detected at different m/z values; 1149.7, 1163.7 and 1177.7, while the fragments y_7 was observed at 774.5 and 782.6 m/z. Sub-fraction eluted at 9.432 min has a molecular weight 1606 Da with amino acids residue sequence as follows; Ser-Gln-Asp-Arg-Arg-Asp-Aib-Gln-Gln-Ala-His-Aib. Thus, it is considered a novel peptabiotic; rich in α-aminoisobutyric acid (Aib) and characterized by dimers of arginine and glutamine (uncommon character of peptabiotics).

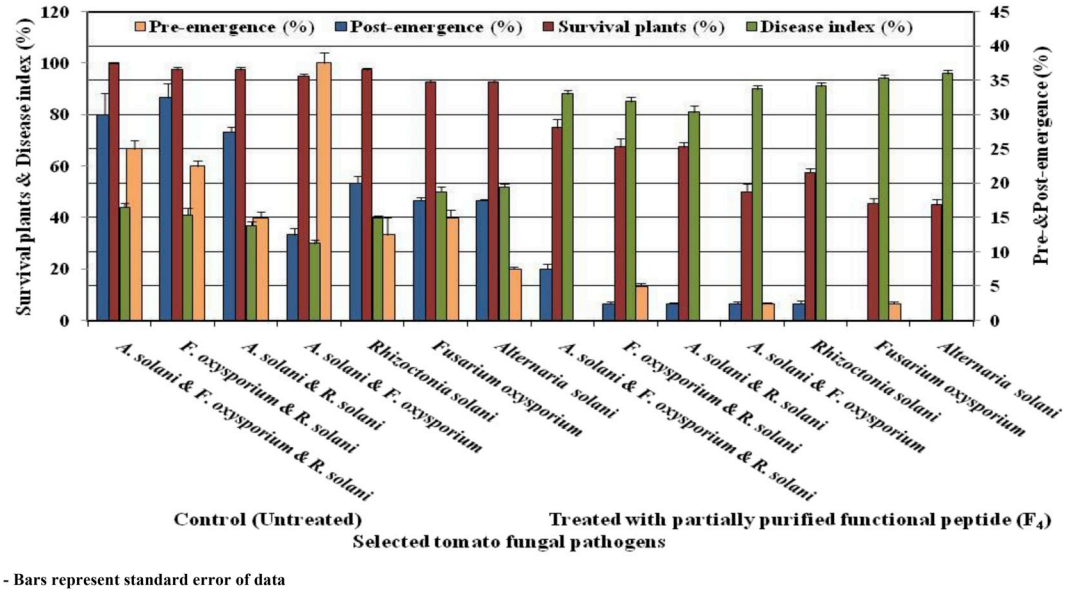


Fig. 4. Application of partially purified peptide fraction (F_4) produced by second generation variant *Trichoderma koningii* Tk_1/3-3 to control tomato fungal pathogens under greenhouse conditions.

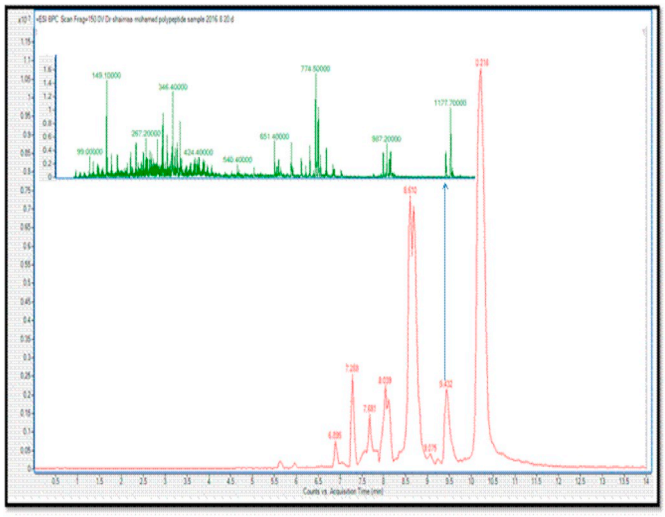


Fig. 5. LC/MS chromatogram of purified polypeptide fraction (F_4) isolated from second generation variant *Trichoderma koningii* Tk_1/3-3 showing a novel peptabiotic with molecular weight 1606 Da.

4. Discussion

Trichoderma spp are considered the best-known biocontrol agents used for almost all genera of phytopathogenic fungi including; Armillaria, Botrytis, Colletotrichum, Dematophora, Endothia, Fulvia, Fusarium, Fusicladium, Monilia, Nectria, Phytophthora, Plasmopara, Pythium, Rhizoctonia, Rhizopus, Sclerotium and Verticillium (Raut et al., 2014). *Rhizoctonia solani* is a typical fungal pathogen that causing root rots disease whereas *Fusarium oxysporium* causes wilting as well as root rots (Hassanet al. 2014). *Alternaria solani* is responsible for leaf spot disease by invading the vascular system mainly and causing tracheomycosis and subsequently root rots (Minerdi and Moretti, 2010). Therefore, the present study selects the disease index of root rots as an indication for controlling efficiency of *Trichoderma* variants.

The penetrating efficiency of highly energetic gamma irradiation is enough to ionize molecules or atoms and break different chemical bonds. Therefore, results showed a decrease in fungal survival after irradiation treatment. The lethal effect of ionizing radiation on microbial cells is due to direct and indirect effects. DNA fragmentation and corruption of the osmotic balance of microbial cell membranes are main direct effects whereas indirect effects as a result of the radiolysis of cellular water and formation of reactive oxygen species and free radicals causing single and double strand DNA breakages as well as AT-CG transversion (Jeonget al. 2016). Survival of microbial cells after irradiation is owing to suitable repair systems including scavenging enzymes; peroxidase, catalase, and superoxide dismutase that scavenge toxic free radicals (Ighodaro and Akinloye, 2017). Alternatively, a mutation that is induced as the response to irradiation is a great source for altering microbial metabolism; the present study succeeds to create new variants of Trichoderma with higher antagonism efficiency using gamma rays (Fig. 2 & Table 1).

Fungal volatile compounds (VOCs) originate from primary and secondary metabolic pathways can permeate air-filled pores of soils and travel long distances without any direct contact between the antagonist and fungal pathogens (Morath et al., 2012). They are involved in biological control and communication between antagonists and their living environment (Bitaset al. 2013). They encompassed hydrocarbons, aromatic compounds, heterocyclic compounds, and various terpenes with different molecular weights (Lee et al., 2016).

Volatiles express the individuality of species in chemical terms and their profile change according to growth stage and environmental conditions (Bailly and Weisskopf, 2012). For our best knowledge, this is the first report that monitors the change takes place for VOCs after stressing fungal producers by gamma irradiation. The present study identified Bis(2-ethylhexyl) phthalate as a effective volatile for controlling tomato fungal disease. This volatile compound showed a broad spectrum antibacterial activity against both Gram-positive (*Staphylococcus aureus* and *Sarcina lutea*) and Gram-negative (*Escherichiacoli* and *Shigella* spp) bacteria (Habib and Karim, 2009). The primary interaction of volatiles with fungal pathogens takes place at the level of the cytoplasmic membrane; as changes in membrane potential and membrane permeability due to the existence of specific receptors on the plasma membrane (Nemčovič et al., 2008). Afterward, the signal from the plasma membrane is transduced to the nucleus with the involvement of mitogen-activated protein kinase and/or G-protein signal transduction (Strehmel et al., 2017). Volatile compounds evolved from *Bacillus subtilis* induced vacuolization, granulation or morphological abnormalities such as wall thickness, conidial-shape deformation and hyphal lysis in *A. alternata*, *F. oxysporum* and *Pythium afertile* (Chaurasia et al., 2005).

T. koningii Tk_1/3-3 and *T. viride* Td_1/2–3 are considered 2nd generation variants with higher antagonistic activity than their parent strains. They are exposed twice to high irradiation doses; 4.5&6 and 5& 6.5 in the same order. In general, low doses of gamma radiation stimulated carbohydrates and inhibited protein production, whereas high doses of gamma radiation inhibited carbohydrates and stimulated protein production (Abomohra et al., 2016). Gamma irradiation also causes changes in the secondary structure of proteins, resulting in variation in physicochemical properties of proteins (Moussa &Rizk 2003). Functional peptides comprise non-volatiles compounds that synergistically play an important role in mycoparasitism of *Trichoderma* spp. Peptaibiotics as a class of functional peptides shows a high content of α-aminoisobutyric acid content and their synthesis is a mixture of isoforms ranging from 7 to 20 amino acids in length. It exhibits a wide spectrum of biological activities including antibacterial, antifungal and antiviral effects as well as inducing the systemic plant-defense responses (Contreras-Cornejo et al., 2016). Long-chain peptaibols are known to form voltage-gated or non-gated ion channels in bilayer lipid membranes, thus inhibiting the mitochondrial ATPase and uncoupling of oxidative phosphorylation (Degenkolb et al., 2006). Results also show the dimerization takes place between two monomers of arginine and glutamine in a newly identified and characterized peptabiotic. Dimerization leads to a diverse spectrum of antimicrobial activity than monomers owing to the formation of pores or channels in the lipid membranes (Lee et al., 2008).

5. Conclusion

In conclusion, the present study suggests a new procedure for selecting microbial mutants or variants based on high doses of gamma radiation rather than low doses that are intensively used before. Different *Trichoderma* spp were exposed to increasing doses of gamma radiation and the 1st generation variants were chosen according to the sub-lethal dose of their parents. Afterward, 1st generation variants were exposed to higher irradiation doses and the 2nd generation variants that tolerate the highest irradiation dose were further selected. The irradiated variants showed a higher antagonistic activity against tomato fungal pathogens; *Alternaria solani, Fusarium oxysporium* and *Rhizoctonia solani* than their parent strains either in *vitro* or under greenhouse conditions. The present study revealed the great variation in a metabolic pool of irradiated variants in comparison with their parents' special volatiles and functional peptides content. A follow-upstudy will be focused on the genetic variations of irradiated variants associated with increasing antagonistic activity of their secondary metabolites.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101261.

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