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Enhancement of antifungal metabolites production from gamma-ray induced mutants of some Trichoderma species for control onion white rot disease

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ABSTRACT

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The influence of gamma-irradiation on improving production of anti-fungal metabolites and biological proficiency of the biocontrol agent *Trichoderma* species against *Sclerotium cepivorum*, the causal organism of onion white rot disease, was studied. Mutants of *Trichoderma harzianum*, *T. viride* and *T. koningii* were generated by exposing their sporulated cultures to 20 and 50 kilo-rad doses of gamma irradiation. The isolated mutants displayed variation in growth and antagonistic activity. Two mutants with better growth rate, sporulation and antagonistic effect against the pathogen were selected from each species. Antifungal metabolites were extracted from the culture filtrates of the *Trichoderma* isolates, fractionated by PAGE or HPLC and assayed. Results showed that all selected mutant isolates produced highly active exo-enzymes compared to the wild type. Mutants derived from *T. harzianum* and *T. koningii* had the highest isozyme band number and quantity of chitinase and β-1,3 glucanase. Mutants of *T. viride* also showed higher levels of cellulase and chitinase isozymes. Fractionation of the culture filtrates via HPLC revealed that production of the metabolites included phenolic compounds and antibiotics, including gliotoxin, trichodermine and viridin, were greatly increased over the wild-type in all selected mutants. In soil naturally infested with *S. cepivorum*, *Trichoderma* mutants significantly reduced the onion white rot disease incidence and improved the plant yield. *Trichoderma* mutants were effective in reducing the pathogen growth in rhizosphere soil compared to the wild type strains. At the same time, population counts of mutants were dramatically increased in rhizosphere soil for 18 weeks compared to 7 weeks in wild types.

Key words: Antibiotics, antifungal metabolites, exoenzymes, gamma radiation, mutation, onion white rot, *Sclerotium cepivorum*, *Trichoderma species*.

INTRODUCTION

Antagonistic *Trichoderma* species are considered promising biological control agents against numerous phytopathogenic fungi [10,18]. *Trichoderma* species are known as producer of many antifungal metabolites including enzymes, antibiotics and others [19,22]. Chitinase, β-1,3-glucanase and cellulase are important in the hyper-parasitic mechanism [1,12,13,15,24,27,31]. Antibiotics such as trichodermine, gliotoxin and virindin excreted by *Trichoderma* were proven to have antifungal activity [22,28]. Phenolic compounds produced by *Gliocladium virens* were reported to be in association with gliotoxin activity. They may be degradation products diluting the biological activity of gliotoxin [17].

Mutation has been used to improve the antifungal metabolites production and antagonistic potential of biocontrol agents to control a broad spectrum of phytopathogens [25]. Several successful endeavors had been made to increase the biocontrol potential of *Trichoderma* species by exposing the spores to chemical or physical mutagens, such as gamma ray [3,6,9,14,18,23,33].

White rot caused by *Sclerotium cepivorum* was considered one of the most serious diseases of onion and garlic. The disease greatly decreased the yield [7,30].

In the present work, mutagenesis of three *Trichoderma* species including *T. harzianum*, *T. viride* and *T. Koningii* were obtained by γ-ray irradiation to enhance their biocontrol abilities against *S. cepivorum* and their production of

antifungal metabolites. The selected mutants were assessed of their antagonistic activity in comparison with that of the wild isolates *in vitro* and *in vivo*. Hydrolytic enzymes, i.e., chitinase, β-1,3-glucanase and cellulase as well as some antibiotics and total phenolic compounds were also assayed.

MATERIALS AND METHODS

Fungal isolates and growth media

Strains of *T. harzianum* Rifai, *T. viride* Pers and *T. koningii* Oudem were isolated from rhizosphere soil of onion plants at El-Kalubia Governorate (Egypt) and identified in the Plant Pathology Department, National Research Center, Dokki, Cairo, Egypt according to the method of Taylor [29]. *S. cepivorum* Berk ,the causal organism of onion white rot was isolated from diseased plants grown in Giza and El- Kalubia Governorates (Egypt). All isolates were maintained on potato dextrose agars (PDA) at 27±1℃. Czapek Dox salt solution (CDS) was used to obtain the *Trichoderma* culture filtrates. Minimal agar medium (MM) (0.6% NaCl, 0.1% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, 0.5% glucose and 2% agar in distilled water) was used for testing the auxotrophic characters. Desoxycholate agar medium (DAM) (1% peptone, 1% lactose, 0.5% NaCl, 0.1% sodium desoxycholate, 0.1% ferric citrate, 0.1% sodium citrate, 0.2% K_2HPO_4, 0.003% neutral red and 2% agar) was used for restricting the growth of fungal colonies to less than 2 mm in diameter even after long incubation to induce sporulation. The test organisms resume normal growth when transferred to PDA.

Mutants induction and isolation

Mutants were induced and isolated according to the reported methods [8,20,25] with some modification. The parent cultures were grown on PDA slants for 14 d at 30℃ to induce sporulation. The sporulated cultures were exposed to gamma-irradiation at 20 k-rad for 75 min or 50 k-rad for 180 min. A gamma cell ^{60}Co, installed at National Center for Radiation Research and Technology, Cairo, Egypt was used for irradiation. Both irradiated and non-irradiated (control) conidia were harvested from the slants after adding 10 ml sterilized physiological saline (0.85% NaCl) to each slant, and spores were separated with a sterile needle. The slant tubes were mixed by Vortex for 30 sec, and the suspension was aseptically filtered through four layers of cheesecloth to remove mycelium. Filtrate was centrifuged at 1,340 g for 10 min to harvest spore. Pellets of the prepared spores were aseptically re-suspended in 5 ml sterilized physiological saline (0.85% NaCl) containing 0.1% Tween-80 to disperse spore clumps, and mixed by Vortex for 30 sec. Hemocytometer was used for spore counting, while plate count was used for testing the viability. Approximately one-tenth ml of a suitable dilution of the conidial suspension was plated onto DAM. The surviving colonies were tested on MM to eliminate auxotrophic colonies. The colonies appeared on MM were selected and examined for some characters including sporulation and their antagonistic effects against *S. cepivorium*. The isolates, which displayed better characters than its wild type isolates, were isolated and sub-cultured 7 times on PDA to test their stability. Those isolates which retained the altered characteristics were identified as mutants. Radial growth and spores counts of the mutants and the parental isolates were tested. Sporulation was determined in liquid potato dextrose incubated with shaking at 27±1℃. One milliliter sample of the cultures were aseptically removed and spores numbers determined on solid PDA plates. Biochemical and molecular analysis of the antifungal metabolites in fungal filtrates as well as biocontrol activities against onion white rot disease were examined.

Inhibition activity

Trichoderma isolates and mutants were tested for their antagonistic effect against *S. cepivorum* by inoculating the fungal discs (5 mm in diam.) on PDA plates near the periphery opposite to each other. Both growth inhibition and covering were measured. *Trichoderma* isolates were grown on CDS for 15 days at 27±1℃ to obtain the fungal culture filtrates. Each fungal culture filtrates was added to PDA to give final concentrations of 10 and 50%, and then inoculated with a disc of *S. cepivorum*. Colonies diameters were determined after five days of growth.

Metabolite preparation

A mycelial agar disc from five days old of the wild type or mutant of *Trichoderma* isolates was transferred to 25 ml of CDS in a 100-ml Erlenmeyer flask. After incubation for 10 days at 28℃ with rotary shaking at 200 rpm, the culture filtrates were removed and stored at -20℃ until use.

Detection of enzymes on polyacrylamid gel-electrophoresis

Hydrolytic enzyme activity and isozyme patterns were detected by staining in sodium dodecyl sulfate polyacrylamid electrophoresis (SDA-PAGE) with 4% acrylamid in the stacking gel and 12% acrylamid in separating gel.

Chitinase

Chitinase activity was detected with SDA-PAGE according to the method of Kang *et al.* [11]. The gel was stained with 30 mM tris HCl buffer, pH 7.5, 32 mM N-Acetylglucosaimine, 4 mM magnesium acetate, 0.18 mg/ml phosphate serine and 1 mM UDP-N-Acetyl-D-glucosaimine. The gel in a staining solution was incubated at 30℃ for 12 hr. Zones of chitinase activity appeared as opaque bands visible in day light on clean PAG background.

β-1,3-glucanase

β-1,3-glucanase activity was analyzed on SDA-PAGE according to the method of Bertheou *et al.* [2]. The gel was stained by mixing 0.1 M with citrate buffer, pH 5.0, 1% polygaclacturoic acid and 3% agrose solution (60℃). The gel in a staining solution was incubated at 37℃ for 1 to 4 h. The gel was placed with the substrate in agrose plate containing 1% water solution of cetyltrimelyle ammonium bromide. Translucence band of β-1,3-glucanase appeared on the opaque background of the gel.

Cellulase

Cellulase activity was observed on gel according to the method described by Chernoglazov, *et al.* [4]. The gel was stained by mixing with 5 mg of bromoindoxyl-β-D-cellobioside, 20 mg nitro blue monotetra zolium (NBMZ), dimethyl formanide and 20 ml of 0.1 M sodium acetate buffer, pH 5.0. The gel in a staining solution was incubated at 40℃ in the dark until dark blue bands appeared. The time of development is 2 to 15 h depending on the concentration of the active enzyme on the gel.

Antibiotics and total phenolic compounds

Antibiotics and total phenolic compounds were extracted with chloroform and identified using high performance liquid chromatography (HPLC). Portions 20 μl of the partially purified concentrates were injected into a Backman Ultraspere ODS 5 μm column (4.6 x 250 mm) that was mounted in a Beckman HPLC system comprising a 421A controller, a 427 integrator, and a 165 variable wavelength detector set at 254 nm. The eluant was a mixture of double distilled water (65%), HPLC-grade acetonitrile (20%), and HPLC-grade methanol (15%), adjusted to pH 4.0 with acetic acid. Retention times of the major compounds had been determined and compared with standard (Sigma, Chemicals). Antibiotics and total phenolic compounds were determined as μg /mg dry weight (D.W) of mycelium in the collected samples.

Field experiments

Trichoderma wild-type isolates and their selected mutants were applied as seeds treatments to protect onion plants against the infection by *S. cepivorum*. Experiments were carried out in a sandy loam soil in Giza Govarnorate during 1998/1999 and 1999/2000 seasons. A row and column design of treatments in 6 replicates was arranged. Each plot was saturated with the *Trichoderma* suspension at a rate of 10^5 spores ml^{-1} in a buffer containing 20 mM glucose and 20 mM potassium phosphate. Daily irrigation was provided. The percentage of onion with white rot disease was calculated during the different growth periods. Yield of onion bulbs was determined at the final stage of growth. The survival of *S. cepivorum* in rhizosphere soil was assessed after 3,7,10,15 and 18 week of planting. Colonization of soil rhizosphere with the tested *Trichoderma* isolates were also assessed and colony forming units (cfu) was recorded by soil dilution plating methods.

Statistical analysis

The collected data were statistically computed using the software SPSS for Windows (release 7.5.1, Dec. 20, 1996, SPSS Inc.). Results from the experiments were expressed with the slandered error of the treatment means for 95% confidence limits.

RRESULTS

Isolation of mutants

Results showed a negative correlation between the gamma-irradiation dosage and survival of irradiated conidia on MM (Table, 1). At 20 and 50 k-rad dosages, *T. viride* showed the best survival (14.86% and 3.71%, respectively) compared to either *T. harzianum* (11.97% and 1.13% respectively) or *T. koningii* (3.46% and 1.15%, respectively).

Description of the mutants

Sporulation and the antagonistic effect of mutants against *S. cepivorum* were examined (Table 2). *T. harzianum* and *T. koningii* irradiated with 50 k-rad dosage resulted in the highest percentage of isolates with best sporulation characters including color and profuse as well as pathogen growth reduction. However, for isolates of *T. viride*, those showing sporulation and antagonistic activity much better than their parents were selected and considered as mutants. Table [3] showed the characteristics of some γ-ray inducing colonies of the three *Trichoderma* species on PDA. Colonies of eight out of 137 isolates of *T. harzianum* were presumed to be mutants and designated as TH2013, TH2039, TH2046, TH2050, TH502, TH508, TH5012, TH504 and TH5053. Among 70 colonies of *T. viride* isolated, 11 were presumed to be mutants and symbolized as; HV202, HV203, HV208, HV2010, HV2018, HV2020, HV2030, HV2039, HV505, HV5012 and HV5020. For *T. koningii*, 7 of 35 colonies were presumed as mutants and symbolized as; TK203, TK206, TK2012, TK2015, TK504, TK508 and TK509. Gamma ray irradiation altered the colony morphology and increased the antagonistic effect against *S. cepivorum* by increasing pathogen growth reduction. Mutant isolates of *T. harizanum* increased the pathogen growth reduction ranging from 84% (TH5012) to 100% (TH508) compared to 53% of (WT) isolates. *T. viride* mutants increased pathogen growth reduction ranging from 55% (TV505) to 100% (TV208) compared to 45% in WT. Also, *T. koningii* brought about pathogen growth reduction ranging from 63% (TK203) to 100% (TK508 and TK509) compared to 57% of (WT). These isolates, which maintained the original variation and showed best effects, were selected and identified as stable mutants.

Table 1. Effect of γ- irradiation on survival of three *Trichoderma* species

Trichoderma species	γ-irradiation dose (k-rad)	Number of tested conidia [1] (x 10^8)	Number of [1] survival conidia (x 10^4)	Conidia survival (%)
T. harzianum	Un-irradiate (control)	1 ±0.06	142 ±1.16	1.420
	20k-rad	10 ±0.58	17 ±2.31	0.017
	50k-rad	1.6 ±0.23	1.6 ±0.35	0.010
T. viride	Un-irradiate (control)	1.8 ±0.46	35 ±2.89	0.194
	20k-rad	1.3 ±0.17	5.2 ±1.15	0.040
	50k-rad	1.2 ±0.12	1.3 ±0.17	0.011
T. koningii	Un-irradiate (control)	0.7 ±0.20	26 ±3.46	0.371
	20k-rad	0.8 ±0.12	0.9 ±0.29	0.011
	50k-rad	0.4 ±0.17	0.3 ±0.43	0.008

[1.] Number of conidia/ml harvested from spore suspension.

Table 2. Sporulation characteristics of gamma induced mutants of three Trichoderma spp.and their antagonistic effect against Sclerotium cepivorum

		Sporulation characteristics [1]						
		Color						
Trichoderma species	γ- irradiation dosage (k-rad)	Green dark	Green	Greenish yellow	Greenish to white	Profuse sporulation	Colony diameter (mm)<0.2 [2]	*S. cepivorum* growth reduction (%)
T. harizanum	0	0.0 [3]	4.0	96.0	0.0	100.0	0.0	
	20	22.0	16.0	57.0	5.0	11	42.3	59.0
	50	46.0	36.0	16.0	2.0	19.4	66.0	76.6
T. viride	0	0.0	98.5	2.0	0.0	100.0	0.0	
	20	4.0	88.0	8.0	0.0	64.0	54.0	55.5
	50	0.0	94.0	0.0	6.0	92.0	20.0	41.0
T. koningii	0	0.0	100	0.0	0.0	100.0	0.0	
	20	28.0	62.0	6.0	4.0	72.5	62.0	61.0
	50	58.0	42.0	0.0	0.0	58.3	70.0	80.3

[1.] Data represent the percent of new biotype of *Trichoderma* spp. affecting the sporulation. Assays were carried out on PDA after 4 days incubation at 27+1℃.
[2.] Diameter of spores colony<0.2, but greater than controls.
[3.] Values represent the mean of ten replicates.

Growth rate of *Trichoderma* wild-type and their selected mutants

Periodical recordings of the radial growth of wild type and mutants on PDA indicated that there were differences in the growth rate among these isolates up to 6 days of incubation (Fig. 1). All mutant isolates were fast growing. Isolates TH508, TK509, TK508 and TV208 covered the whole plates in 4 days compared to 6 days by the wild isolates. The same trend was verified for sporulation in liquid medium up to 21 days (Fig. 2). Wild type isolates barely increased the spore count up to 15 days and then declined. Among the six mutant isolates, TH508, TK508 and TK509 were especially high in spore count compared to the wild type.

Inhibition activity bioassay

Data given in Table 4 show that mutant isolates caused the highest reduction rate against *S. cepivorum* growth compared to the wild-type which resulted in a minute growth reduction. TH508, TK509 and TK508 mutants showed high ability to restrict the size of *S. cepivorum* colonies (Fig. 3). At the same time, date also indicated that, the growth of *S. cepivorum* was significantly reduced on PDA supplemented with culture filtrates of TH508, TH509, TK508 and TV208 at rate of 10 and 50% concentrations. Growth of *S. cepivorum* was completely inhibited by 50% culture filtrate of TH508, TK509, TK508 and TV208 compared to wild type isolates of *T. harzianum* (58.5%), *T. viride* (48.0%) and *T. koningii* (57.5%).

Metabolites production

Enzymes production. Extracellular enzyme activities of wild-type and mutant isolates were carefully studied via SDS-PAGE electrophoresis analysis and enzyme activity staining. Different quantities of the exo-enzymes were found among these isolates. *T. harzianum* and *T. koningii* wild-types showed 6 and 3 isozyme bands of chitinase (Fig. 4),

Table 3. Different characteristics of the seventh subcultures of the wild types of three Trichoderma spp and their gamma induced mutants

Original Trichoderm spp.	Mutant isolates	γ- ray dosage (k-rad)	Sporulation characteristics: Colony diameter (cm) 1st.[1]	Colony diameter (cm) 7th[2]	Profuse[4] 1st.	Profuse[4] 7th	Color 1st.	Color 7th	Sclerotium cepivorum growth reduction (%) 1st.	Sclerotium cepivorum growth reduction (%) 7th
T. harzianum	WT[3]	Non	0.1	0.1	++	+	Greenish yellow	Greenish yellow	55	53
	TH2013	20k-rad	0.1	0.1	+++	+++	Green	Green	88.3	89.3
	TH2039	20k-rad	0.1	0.1	++	++	Greenish yellow	Greenish yellow	87.5	87.5
	TH2046	20k-rad	0.2	0.2	++	++	Greenish yellow	Greenish yellow	95.2	95.2
	TH2050	20k-rad	0.2	0.2	++	++	Greenish yellow	Greenish yellow	93.2	93.2
	TH502	50k-rad	0.1	0.1	++	++	Greenish white	Greenish white	85.4	88.4
	TH508	50k-rad	0.3	0.4	++++	++++	Dark Green	Greenish white	100	100
	TH5012	50k-rad	0.2	0.2	+++	+++	Dark Green	Dark Green	92.5	92
	TH5046	50k-rad	0.2	0.2	++	++	Greenish white	Dark Green	86.5	84.5
	TH2053	50k-rad	0.2	0.3	++++	+++	Greenish white	Greenish white	95.5	87.5
T. viride	WT	Non	0.1	0.1	++	+	Green	Green	44.2	45.2
	TV202	20k-rad	0.1	0.1	++	++	Green	Green	88.2	86.2
	TV203	20k-rad	0.3	0.3	++++	++++	Green	Green	100	100
	TV208	20k-rad	0.3	0.3	++++	++++	Green	Green	60	63
	TV2010	20k-rad	0.2	0.2	+++	++	Green	Green	61.2	61.2
	TV2018	20k-rad	0.2	0.2	+++	+++	Green	Green	59.3	59.3
	TV2020	20k-rad	0.2	0.2	+++	+++	Green	Green	58.6	57.6
	TV2030	20k-rad	0.2	0.2	+++	+++	Green	Green	93.3	95.3
	TV2039	20k-rad	0.1	0.1	+	+	Green	Green	66.6	68.6
	TV505	50k-rad	0.1	0.2	+++	+++	Green	Green	55	55
	TV5012	50k-rad	0.1	0.1	+	++	Green	Green	95.5	96.5
	TV5020	50k-rad	0.1	0.1	++	+	Green	Green	77.3	77.3
T. koningii	WT	Non	0.1	0.1	++	+	Green	Green	53	57
	TK203	20k-rad	0.2	0.1	++	++	Green	Green	63.3	63.3
	TK206	20k-rad	0.2	0.2	++	++	Green	Green	70	71
	TK2012	20k-rad	0.3	0.3	++++	+++	Dark Green	Dark Green	95.3	93.3
	TK2015	20k-rad	0.1	0.1	+	+	Dark Green	Dark Green	74.3	70.3
	TK504	50k-rad	0.3	0.3	+++	+++	Dark Green	Dark Green	74.3	90.3
	TK508	50k-rad	0.3	0.3	++++	++++	Dark Green	Dark Green	100	100
	TK509	50k-rad	0.3	0.3	++++	++++	Dark Green	Dark Green	100	100

[1.] (1 st) First subculture.
[2.] (7 th) Seventh subcultures.
[3.] (WT) wild type.
[4.] (-) disperse, (+) little, (++) moderate, (+++) good and (++++) compact or numerous.

respectively. Both species also displayed 3 isozyme bands of β-1,3- glucanase and cellulase. *T. viride* gave 3 isozyme bands of cellulase and chitinase (Fig. 4), and 2 bands of β-1,3-glucanase. The band numbers of the mutant isolates were increased. Chitinase (Fig. 4) and β-1,3-glucanase reached up to 9 and 4 bands (TH508), 8 and 3 bands (TK509) and 7 and 4 bands (TH5053 and TK508), respectively. Cellulase, β-1,3-glucanase, and chitinase isozymes assembled by (TV208) were reached up to 6, 4 and 5 band numbers, respectively. The activities of these isozymes seemed to increase in quantity even under γ-irradiation. A much higher levels of chitinase and β-1,3-glucanase in quantity were detected in TH508 and TK509 compared to the wild type. The quantities of cellulase, β-1,3-glucanase, and chitinase isozymes produced by TV208 and TV203 were much higher than those of the wild types.

Antibiotics and total phenolic compounds production. Data collected from HPLC charts representing the number and relative abundance of metabolite production by *Trichoderma* wild type and their mutants were given in Table 5. The wild types and mutants displayed variation in production of metabolites. All the stable mutants produced the high quantity of metabolites compared to the wild types. The amount of metabolites produced by TH508, TK509 and TV208 were 0.81, 0.748 and 0.56 μg/mg of D.W, respectively, compared to the wild types of 0.455, 0.472 and 0.378 μg/mg D.W, respectively. Production of antibiotics by wild types and mutants were also measured. When

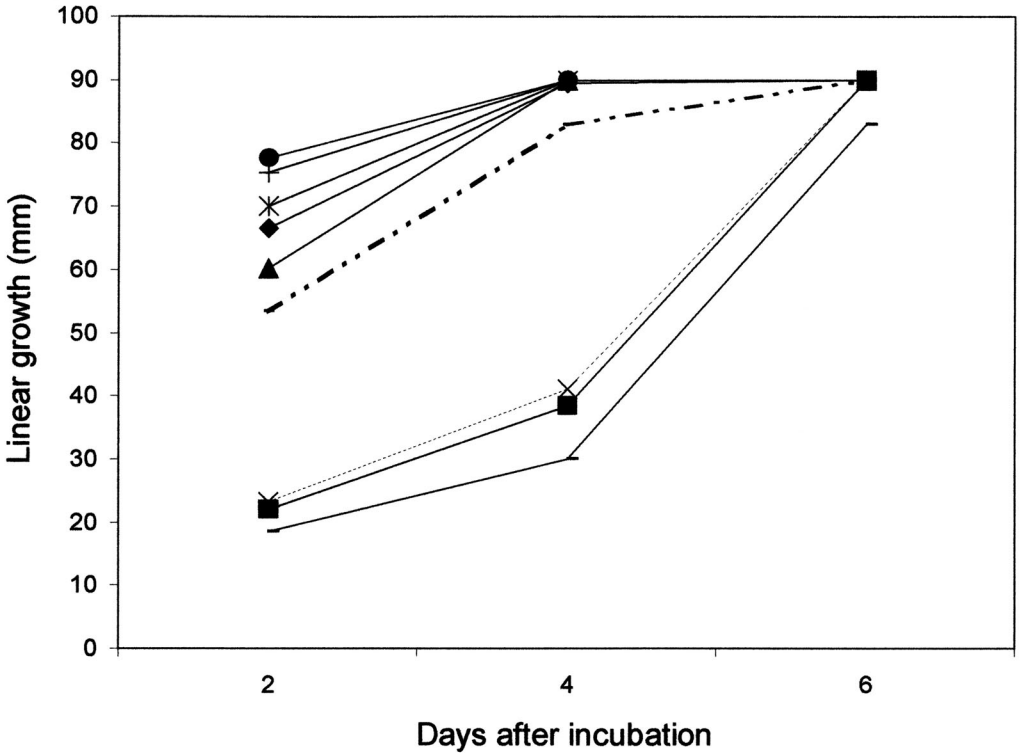

Fig. 1. Linear growth (mm) of the *Trichoderma* spp. and their mutants.
(■ : *T. harzianum* ; ◆ : GTH2035 ; ＋ : TH508 ; — : *T. viride* ; ･･ : TV203 ; ▲ : TV208 ; × : *T. koningii* ; ✳ : TK508 ; ● : TK509)

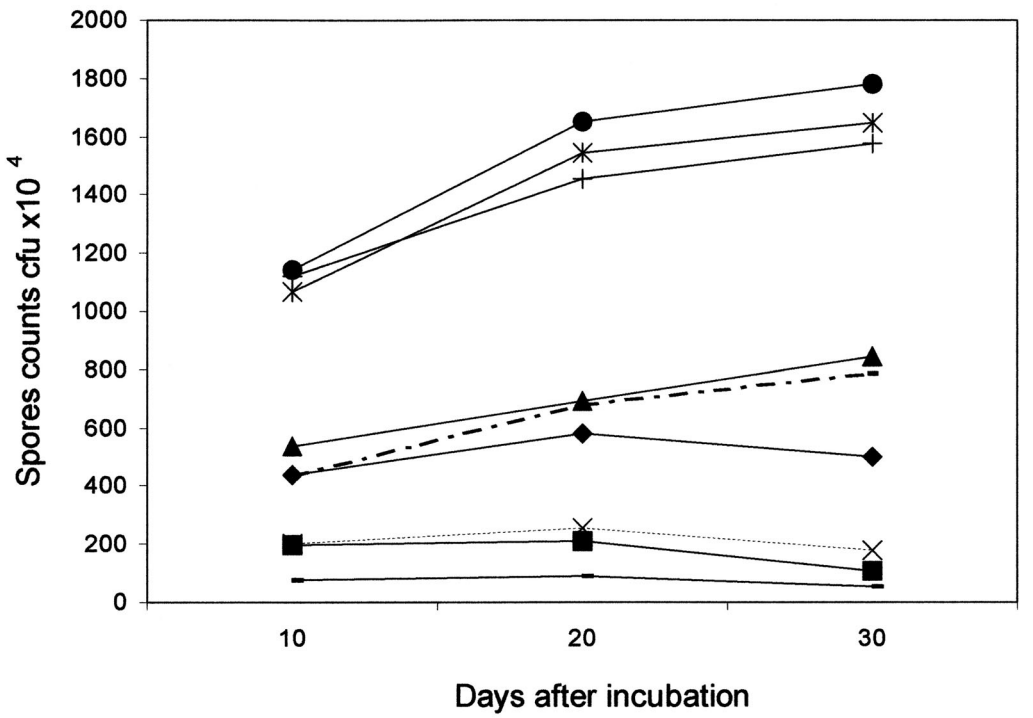

Fig. 2. Sporulation (cfu x10^4) of the *Trichoderma* spp. and their mutants.
(■ : *T. harzianum* ; ◆ : TH2035 ; ＋ : TH508 ; — : *T. viride* ; ･･ : TV203 ; ▲ : TV208 ; × : *T. koningii* ; ✳ : TK508 ; ● : TK509)

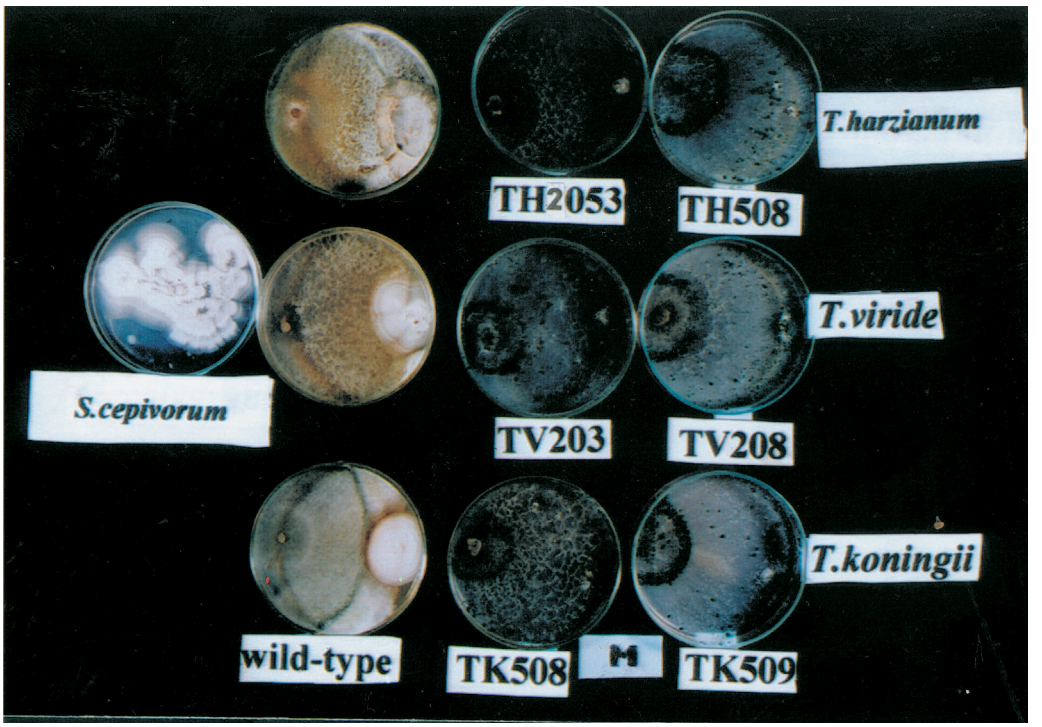

Fig. 3. Growth inhibition of *Sclerotium cepivorum* by three *Trichoderma* spp. and their mutants (Mycelial discs of *Trichoderma* spp. and *S. cepivorum* were placed at the opposite sides of PDA plates).

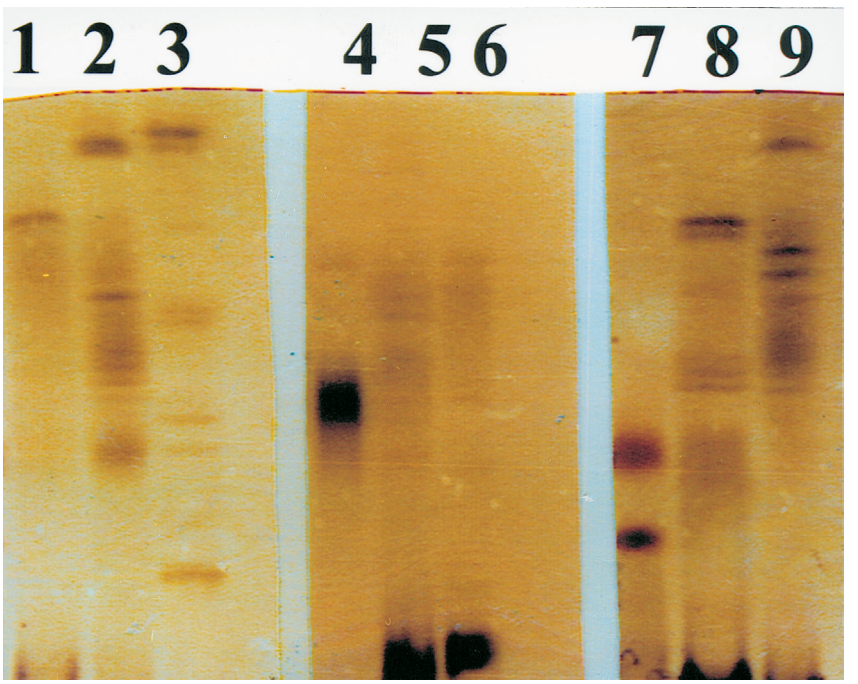

Fig. 4. Isozymes bands of chitinase in the culture filtrates of three *Trichoderma* spp. and their mutants analyzed by polyacrylamid-gel electrophoresis (SDS-PAGE).
(Lane 1: *T. harzianum*, lane 2: TH5053, lane 3 : TH508, lane 4 : *T. viride*, lane 5 : TV203, lane 6 : TV208, lane 7 : *T. koningii*, lane 8 : TK508, lane 9 : TK509.)

Table 4. Effect of wild types of *Trichoderma* spp. and their mutants on the growth of *Sclerotium cepivorum*

Trichoderma spp. (wild type/ mutant)	Antagonistic effects against *S. cepivorum*		
	Pathogen growth reduction (%)	Inhibitory effect of culture filtrate [1]	
		10%	50%
T. harizanum	38.0+0.5 bc [2]	6.7+0.1 e [3]	58.5+0.4 c [4]
TH5053	96.6+0.2 a	48.8+0.4 d	100.0a
TH508	100.0 a	64.4+0.5 b	100.0 a
T. viride	33.3+0.4 c	4.6+0.1 e	48.0+0.3 d
TV203	96.3+0.5 a	55.5+0.5 c	96.6+0.6 b
TV208	100.0 a	58.5+0.4 c	100.0
T. koningii	44.4+0.2b	5.5+0.2 e	57.5+0.4 c
TK508	100.0 a	66.6+0.5 b	100.0 a
TK509	100.0 a	57.6+0.4 a	100.0 a

1. Inhibition of *S. cepivorum* growth was determined on PDA containing 10 and 50% of *Trichoderma* culture filtrates.
2. Mean of 5 replicates.
3. Values in the same raw followed by different letters are not statistically significant different at $p<0.05$.
4. Results are given as mean + standard error.

Trichoderma isolates were irradiated with γ-irradiation, visible increase in the antibiotics induction were noticed in mutants. Some mutants of *T. harzianum* and *T. koningii* produced much more trichodermine, gliotoxin and viridin than their respective wild types (Table 5). The amount of trichodermine and viridin produced by *T. viride* were also higher than those produced by its wild type. Mutants TH508 yielded trichodermine and gliotoxin up to 0.497 and 0.355 μg/mg D.W, respectively. Whereas wild type of *T. harzianum* produced only 0.030 and 0.016 μg/mg D.W, respectively. At the same time, a new gliotoxin compound was detected in the culture filtrate of TV208. The two mutants TV208 and TK508 also yielded higher total phenolic compounds (0.047 and 0.046 μg/mg D.W, respectively) than other tested strains.

Field experiments

In a natural soil infested with *S. cepivorum*, highly significant differences in the percentage of disease incidence in both seasons (P<0.05) were achieved among untreated, and those treated with wild type and mutant isolates (Table 6). Disease incidences in soil treated with *T. harzianum* (15.4 and 17.4%), *T. koningii* (14.9 and 17.3%) or *T. viride* (26.3 and 29.8%) wild isolates were lower than that of the controls (41.3 and 45.6%) in the two seasons tested. The disease incidences diminished pronouncedly in those treated with mutants. The best treatments were those involving TH508, TK509 and TK508, which provided almost complete control in both seasons. TH5053 and TV208 were also effective in controlling disease down to 1.4 and 1.8% in two seasons, respectively. Yields of onion bulbs harvested from those treated with *Trichoderma* wild-types were higher than those from controls (Table 6). Mutant treatments were significantly better than wild type treatments as far as yields are considered. Soil treated with TH508, TK509 and TK508 gave the highest yield over the other mutants in both seasons.

In untreated plants, the population counts of *S.*

Table 5. Total metabolites, antibiotics and phenolic compounds in the culture filtrates of three *Trichoderma* spp. wild-types and their mutants (as μg/ml dry weight)

Trichoderma spp. (Wild type/mutant)	Total metabolites	Antibiotics			Total phenolic compounds
		Trichodermine	Gliotoxin	Viridin	
T.harzianum	0.46	0.03	0.016	0.015	0.010
TH5053	0.67	0.39	0.148	0.020	0.027
TH508	0.81	0.50	0.355	0.295	0.025
T.viride	0.38	0.07	ND	0.217	0.022
TV203	0.48	0.14	ND	0.232	0.026
TV208	0.56	0.18	0.067	0.395	0.047
T.koningii	0.47	0.05	0.012	0.009	0.021
TK508	0.70	0.71	0.046	0.034	0.026
TK509	0.75	0.16	0.136	0.380	0.046

1. Antibiotics and total phenolic compounds were quantified by HPLC analysis of filtrates of liquid cultures ad normalized to the dry weight of mycelium in the collected samples.
2. ND, not detected.

Table 6. Effect of application of three *Trichoderma* spp. and their mutants on disease incidence of *S. cepivorum* white rot and yield of onion bulbs

Trichoderm spp. (Wild type/mutant)	Disease incidence (%) of weeks after planting					Mean yield/plot [1] (kg)
	3	7	10	15	18	
1998-1999 season						
Control	2.4 ±0.5a [1]	13.4±1.1a	25.3 ±1.3a	35.2 ±2.3a	41.3 ±3.6a	19.2±1.32e
T. harzianum	0.8 ±0.2c	4.6±0.4c	7.5 ±0.4c	11.4 ±0.6c	15.4 ±1.5c	23.6±1.6d
TH5053	0.0d	0.0d	0.0d	0.8 ±0.2d	1.4 ±0.5d	28.4±1.7b
TH508	0.0d	0.0d	0.0d	0.0d	0.0d	31.3±1.9a
T. viride	1.8 ±0.8b	10.3±0.8b	15.6 ±1.3b	19.4 ±1.2b	26.3 ±1.6b	22.6±1.3d
TV203	0.0d	0.0d	0.8 ±0.2d	1.3 ±0.3d	2.3 ±3.8d	25.6±1.1c
TV208	0.0d	0.0d	0.4 ±0.1d	0.8 ±0.2d	1.4 ±0.3d	26.5±1.2c
T. koningii	0.9 ±0.3c	4.8 ±0.5c	7.8 ±0.7c	11.8 ±0.6c	14.9 ±1.6c	23.4±1.0d
TK508	0.0d	0.0d	0.0d	0.0d	0.0d	30.6±1.1ab
TK509	0.0d	0.0d	0.0d	0.0d	0.0d	32.3±1.0a
1999-2000 season						
Control	3.3 ±0.5a	15.6±0.9a	26.7 ±2.3a	38.4 ±3.3a	45.6 ±3.3a	17.5±1.3d
T. harzianum	0.8 ±0.2c	5.3 ±0.5c	8.0 ±4.08c	13.3 ±1.2c	17.4 ±1.0c	22.4±1.4c
TH5053	0.0c	0.0d	0.0d	0.6 ±0.2d	1.8 ±0.7d	26.5±1.6b
TH508	0.0c	0.0d	0.0d	0.0d	0.0d	30.0±2.1a
T. viride	2.6 ±0.3b	13.4±0.6b	17.2 ±0.9b	21.5 ±1.06b	29.8 ±1.2b	21.5±0.9c
TV203	0.0c	0.4±0.1d	0.4 ±0.1d	0.8 ±0.2d	2.3 ±0.3d	25.4±0.9b
TV208	0.0c	0.0d	0.0d	0.4 ±0.1d	1.8 ±0.4d	26.4±0.7b
T. koningii	0.8 ±0.2c	5.5 ±0.3c	8.3 ±0.5c	12.8 ±0.5c	17.2 ±0.8c	22.8±0.9c
TK508	0.0c	0.0d	0.0d	0.0d	0.0d	30.3±1.8a
TK509	0.0c	0.0d	0.0d	0.0d	0.0d	31.7±2.2a

[1.] Plant yield was recorded 18 weeks after planting.
[2.] Results are given as mean + standard error, and values represent the percent of six replicates.
[3.] Values in the same raw followed by different letters are not statistically significant different at $p<0.05$.

cepivorum increased during the18-week growth period from 1.8 to 8.8 propagules per gram of rhizosphere soil o (Fig. 5). All mutants clearly reduced pathogen population. TH508 and TK509 treatments led to a slight increase up to 7 weeks. Thereafter, the population decreased and down to an undetectable level at the18th week. At the same time, wild types of *Trichoderma* increased in rhizosphere soils up to 7 weeks and started a decline phase thereafter (Fig. 6). On contrast, population densities of all mutants greatly increased up to 18 weeks. TH508 (887.3 propagules/g soil) and TK509 (906.3 propagules/g soil) seemed to be more abundant in the rhizosphere soil than wild types (38.6 and 50.0 propagules/g soil, respectively).

DISCUSSION

Induced mutation is one common measures to restrain the genetic construction of microorganisms (18,25). Induced mutagenesis using γ-rays proved to be effective in enhancing antifungal metabolites production and achieving biological efficiencies in three species of *Trichoderma*, including *T. harzianum*, *T. viride* and *T. Koningii*, against *S. cepivorum*, the causal organism of onion white rot disease. Direct negative correlation between the doses of γ- irradiation and the survival of irradiated conidia on MM medium were distinct. After 7 subcultures, stable mutants with improved growth rate, sporulation and antagonistic effects were obtained. Among them, six mutants were selected due to their antagonistic activities. This is presumably a consequence of their higher constitutive of metabolites activity [4,8,18,32]. *Trichoderma* species are well known good competitors, because they produced large amounts of extracellular enzymes and antibiotics causing cell-wall degradation and growth inhibition of the pathogens [1,19,28].

Results from this study showed that γ- irradiation of *Trichoderma* spp. can alter the activities of exo-enzymes. Induced mutants not only produced more chitinase, β-1,3-glucanase and cellulase, they also produced more isozyme types than the wild types. Gamma irradiation can also increase the ability of *Trichoderma* species to produced antibiotics and phenolic compounds. TK509 and TH508 were detected to produce high levels of trichodermine, gliotoxin and viridin, while TV208 displayed the highest levels of viridin and trichodermine as well as a new gliotoxin. This may account for the ability of induced mutants to multiply in the rhizosphere and decrease the population of *S. cepivorum*

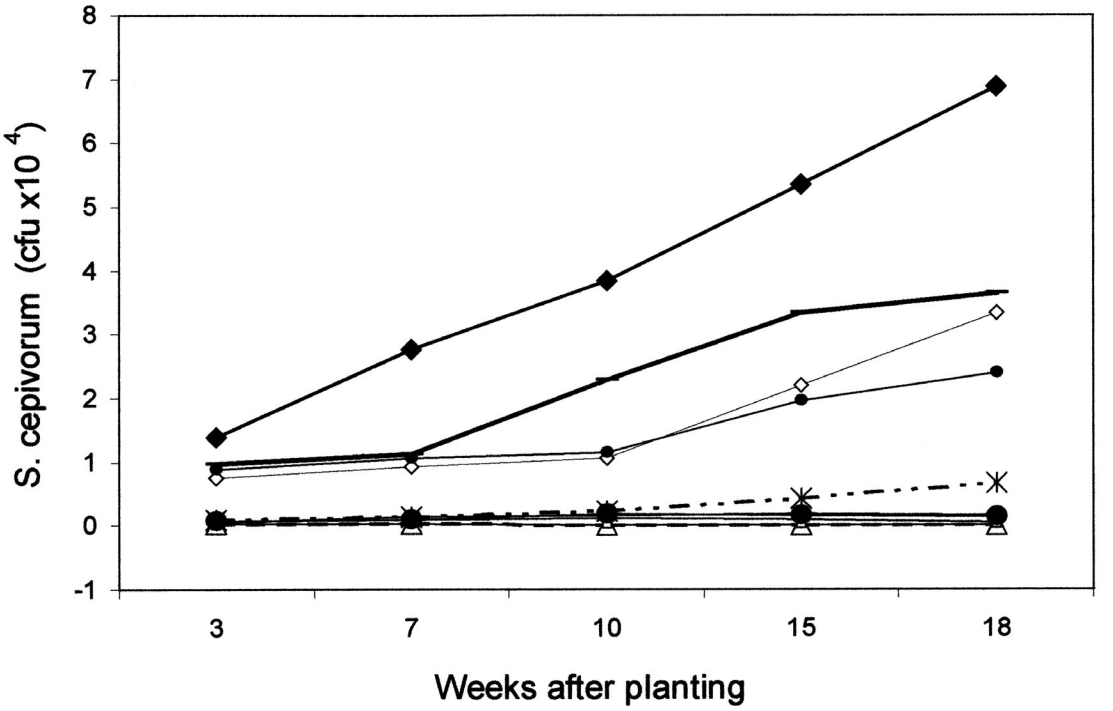

Fig. 5. Population of *Sclerotium cepivorum* (no./g soil) in the rhizosphere soils of onion plants.
(◇ : *T. harzianum* ; × : TH2035 ; △ : TH508 ; — : *T. viride* ; × : TV203 ; ● : TV208 ; ● : *T. koningii* ; ● : TK508 ; □ : TK509 ; ◆ : control)

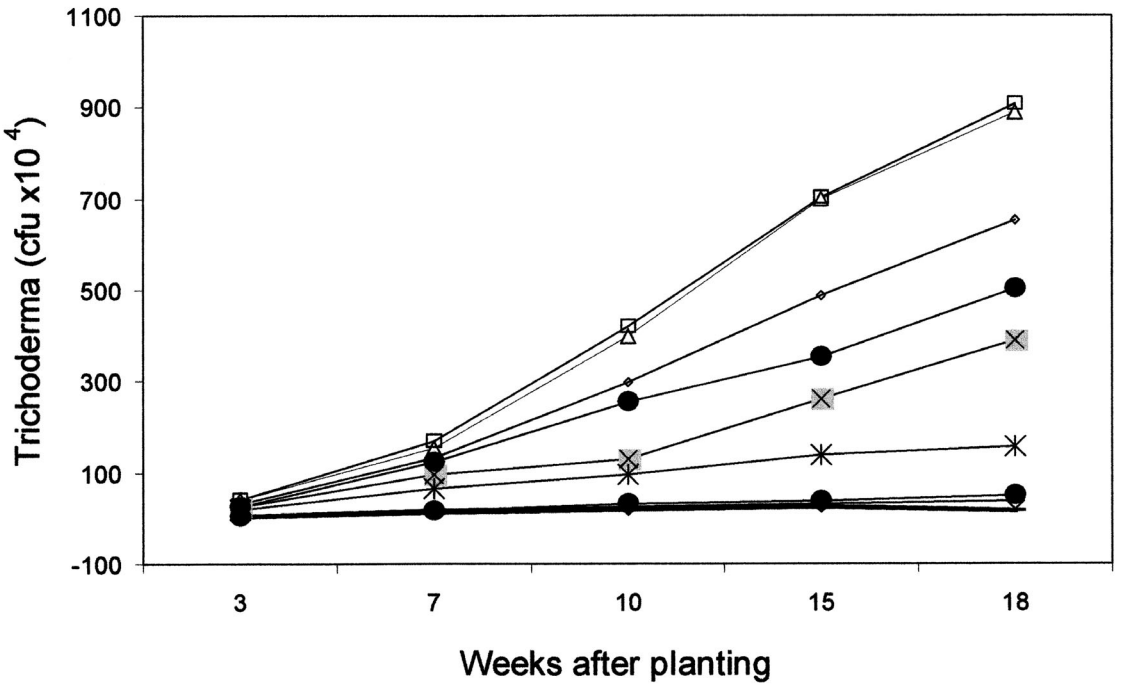

Fig. 6. Population of *Trichoderma* spp. (no./g soil) and their mutants in the rhizosphere soils of onion plants.
(◇ : *T. harzianum* ; × : TH2035 ; △ : TH508 ; — : *T. viride* ; X : TV203 ; ● : TV208 ; ● : *T. koningii* ; ● : TK508 ; □ : TK509)

in soil. All the tested mutants established a much better disease control than any of the wild types. No diseases were found in the treatments amended with the three mutants of TH508, TK509 and TV208. Population of the pathogen *S. cepivorum* was minimized in the rhizosphere soil. Meanwhile, a marked increase in plant yields was noticed in comparison with the wild isolates. These results indicated that tested mutants were more stable and effective in disease-suppress when grown in natural soil than the wild types. Results indicate that mutants, excreting high levels of enzymes and antibiotics, may display strong pathogen-suppress activities. The variation in the enzymes activities clearly reflects the levels of substrate utilization by the mutants, which in turn indicated their different mycoparasitic potential [14,18].

Mutagenic techniques for improvement of fungal biocontrol agents through regulators the antifungal metabolites production is evident. Gadgil *et al*., [8] reported that mutation may not only effect the cellulase genes directly but also affect the synthesis and secretion of the cellulase enzymes. Increases of production of chitinase [16] and cellulase [8,32] could be achieved by mutants of *Trichoderma*. Significant levels of toxic metabolites also were ascertained in *Trichoderma* mutants, which may be associated with increasing in rhizosphere competence with other micro flora and pathogen [5,20]. The study clearly shows the possibility of improving the antagonistic microorganisms for biological control of plant diseases through mutation with γ-irradiation.

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摘 要

Haggag, Wafaa M. [1,3] and Mohamed, H. A. A. [2] 2002. γ 射線誘變木黴菌增生抗菌代謝物質防治洋蔥白腐病. 植病會刊 11:45-56 ([1.] 埃及國家研究中心 (National Research Centre) 植物病理系；[2.] 埃及國家研究中心微生物遺傳系 (Dokki, Cairo)；[3.] 聯絡作者，E-mail: whaggag@link.net；傳眞：+ 002023832317)

本研究利用 γ 射線誘導木黴菌 (*Trichoderma* spp.) 產生突變株，藉以探討其對木黴菌產生抗菌代謝物質之改善情形及其防治洋蔥白腐病菌 (*Sclerotium cepivorum*) 之功效。以劑量爲 20 與 50 kilo-rad 的 γ 射線照射 *T. harzianum*, *T. viride* 及 *T.koningii* 的孢子，獲得表現不同生長速率與拮抗能力的突變株。從每一種 (species) 木黴菌，選出兩株生長較快、產孢量較多、且抗菌能力較佳者進行試驗。將木黴菌野生株 (wild type) 與突變株進行液態培養，從濾液中萃取代謝物質，並經過 SDS-PAGE 電泳分析、或 HPLC 層析及檢定。結果顯示，與野生株比較，所有供試的 γ 射線突變株均產生較高活性的胞外酵素 (exo-enzymes)。分析 β-1,3 glucanase 與幾丁質分解酵素 (chitinase) 的同功異構酵素，*T. harzianum* 與 *T. koningii* 的突變株產生的酵素量及形成的條帶 (bands) 最多。而 *T. viride* 突變株產生纖維素分解酵素 (cellulase) 與幾丁質分解酵素的活性亦較高。經由 HPLC 分析，供試木黴菌突變株產生的代謝產物 (包括酚化合物與抗生素 (gliotoxin, trichodermine 及 viridin)) 均比野生株產生者大幅度增加。在田間受白腐病菌 (*S. cepivorum*) 自然污染的洋蔥田，接種木黴菌突變株後均能顯著地降低本病的發病度，且增加洋蔥產量。試驗顯示，木黴菌突變株較野生株更能顯著抑制病原菌在根圈土壤中生長，同時，突變株在土壤中的密度可以持續上升至 18 星期，而野生株的密度在 7 星期後即開始下降。

關鍵詞：γ 射線、木黴菌、突變、抗生素、抗菌代謝物、胞外酵素、酚化合物、洋蔥白腐病、*Sclerotium cepivorum*