Biocontrol potential of salinity tolerant mutants of *Trichoderma harzianum against Fusarium oxysporum* causing tomato wilt disease

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ABSTRACT

This work aims to apply γ- irradiation in a breeding program of Trichoderma harzianum to enhance its biocontrol ability against F. oxysporum through increasing their production of antifungal metabolites (i.e., hydrolytic enzymes,antibiotics and total phenols) under salt stress conditions. Exposing a wild-type culture of the mycoparasitic fungus T. harzianum to gamma irradiation induced two stable salt-tolerant mutants (Th50M6 & Th50M11).Under saline conditions, both mutants greatly surpassed their wild type strain in growth rate, sporulation and biological proficiency against Fusarium oxysporum, the causal agent of tomato wilt disease. Tolerant T. harzianum mutants detained a capability to grow and convinced sporulation in growth media containing up to 69 mM NaCl. In comparison with their parent strain, characterization of both mutants confirmed that they have reinforced contents of proline and hydroxyproline, relatively higher sodium content compared to potassium, calcium or magnesium contents, higher level of total phenols. Data also showed that mutants produce certain active metabolites including; extracellular-enzymes of chitinases, cellulases, β-galactosidases, as well as, some antibiotics i.e., trichodermin, gliotoxin and gliovirin. Trichoderma mutants significantly reduced wilt disease incidence and improved yield and mineral contents of tomato plants under both saline and non-saline soil conditions, as well as, under infested and natural conditions. T. harzianum mutants were also more efficient in dropping the F. oxysporum growth in rhizosphere compared to the wild type strain. Population density of both mutants in rhizosphere far exceeded those of T. harzianum wild type strain.

Key words: *Antifungal metabolites, mutagenesis, γ-irradiation, salt stress.*

INTRODUCTION

Wilt disease caused by *Fusarium oxysporum* is among the most overwhelming disease in tomato (*Lycopersicon esculentum* L.) causing pensive economic expenditure (De Cal *et al*., 1997). It is a rowdy disease because of the auspicious ecosystem needed for its development (Campell and Madden, 1990). El-Abyed *et al.* (1988) and Ragazzi *et al*. (1994) reported that mycelial growth and sporulation of different *Fusarium* species including *F. oxysporum* were motivated under salt stress conditions. Gour *et al.* (1990) suggested that *Fusarium* infection might have contributed to wilting and yellowing of plants at high salinity levels.

Antagonistic *Trichoderma* species are considered as promising biological control agents against numerous phytopathogenic fungi including *F. oxysporum* (Sarhan *et al.,* 1999). These filamentous fungi are very common in nature, with high population densities in soil and plant litters (Samuels, 1996). They are saprophytic, promptly growing and easy to culture besides producing huge amounts of conidia with long lifetime.

One of the most crucial boundaries to use *Trichoderma* strains as biofungicides is their low osmotolerance level. Soil hydrological characters are considered as restraining parameters touching *Trichoderma* activities, most particularly spore germination, germ tube growth (Magan, 1988) and mycelial growth (Luard and Griffin, 1981). They also display a decisive outcome on saprophytic ability (Eastburn and Butler, 1991), on the interaction with other fungi (Jenog *et al*., 1997), as well as on enzyme production (Kredics *et al*., 2000).

Anthony (1998) noticed that the halobacteria are the only microorganisms that are tolerant to salinity at the molecular level. All other bacteria, fungi, plants and animals need to be salt tolerant for most of their macromolecules through maintaining defined and conserved conditions in the cytoplasm. Salt tolerance of a given organism depends upon the range of external salinity over which it is able to sustain these conditions in the cytoplasm.

Mutation has been manipulated to improve production of antifungal metabolites and antagonistic potential of biocontrol agents to control a broad spectrum of phytopathogens (Rey *et al.,* 2000). Several successful endeavors had been made to enhance the biocontrol potentiality of *Trichoderma* species by exposing the spores to chemical, e.g., EMS or physical mutagens, e.g., gamma ray (Youssef and Aziz, 1999). Breeding of *Trichoderma* is directed to achieve effective mycoparasitic strains for biocontrol against plant fungal pathogens under a wide range of adverse environmental conditions (Manczinger *et al.,* 2002). More attention should be paid to the mutagenic methods of breeding, because strains bred by mutagenesis can easily get registration for field use from environmental protection agencies more than strains produced by protoplast fusion, transformation or *via* gene cloning.

In the present work, mutations in *Trichoderma harzianum* were induced by γ-irradiation for the sake of enhancing its bio-control ability against *Fusarium oxysporum* and the production of antifungal metabolites under salt stress conditions. The selected salt-tolerant mutants were assessed to their antagonistic activity in comparison to their wild isolates *in vitro* as well as *in vivo*. Hydrolytic enzymes, i.e., chitinase, β-galactosidase and cellulase as well as some antibiotics and total phenols were also assayed under salt stress.

MATERIALS AND METHODS

Fungal isolates and growth conditions

A local isolate of *Trichoderma harzianum* Rifai was used throughout this study. *Fusarium oxysporum* f. sp. *Lycopersici* Schelect was isolated from diseased tomato plants. Fungal isolates were maintained on potato dextrose agar (PDA) at 27 °C ±1 °C. Potato dextrose broth (PDB) medium or czapek dox salt solution (CDS) was used to obtain the fungal culture filtrates. On desoxycholate agar medium (DAM), the growth was greatly restricted and the colonies did not exceed 2 mm in diameter.

Mutants induction and characterization

The salt-tolerant mutants were induced and isolated according to the methods of

Gadgil *et al*. (1995); Migheli *et al*. (1998) and Rey *et al*. (2000) with some modifications. PDA slants containing 14-day-old sporulated *T. harzianum* cultures were exposed to two dosages of γ-radiation (200 Gy for 75 min and 500 Gy by the dose rate 4.32 and 0.09 rad/sec, respectively) provided from ^{60}Co source, installed at National Center for Radiation Research and Technology, Cairo, Egypt. Both irradiated and non-irradiated (control) conidia were harvested, re-suspended in physiological saline solution (1.46 mM NaCl) containing 0.1 % Tween-80, to disperse spore clumps. Hemocytometer was applied for spore counting, while plate count was used for testing viability. Suitable dilution of the conidial suspension was plated onto DAM medium. The growing colonies were selected with respect to exhibiting phenotypic deviation from the parental strain and examined on PDA medium for some characters especially their antagonistic effects against *F. oxysporum* under saline stress conditions. The isolates that displayed good characters than the wild type isolate were selected and sub-cultured 7 times on PDA medium to test their stability. The tested cultures compared to the original ones and isolates, which maintained the original variation, were identified as mutants. Selected mutants were tested for their tolerance capacity to salinity.

Salt stress tests

Salt tolerance capacity of *T. harzianum* isolates was measured as percentage of reduction in linear growth that calculated by (100 x Y - X / Y), where X is the maximum radius (mm) of the isolates grown on NaCl supplemented medium and Y is the radius of the isolates grown on NaCl free medium. Fungal growth was determined as colony diameter measurement but sporulation was identified as colony forming units (cfu). Antagonistic activity was measured as zone inhibition and growth reductions through dual culture on PDA medium or filtrate inhibition, where volume of each *T. harzianum* culture filtrate was added to PDA medium to provide a final concentration of 50 %, then inoculated with equal discs of the tested pathogen. Colonies diameters were determined after four days). Oven-dried (65^{o}C, 24 hr) fungal samples were digested in mixture of sulfuric acid: perchloric acid (5:1, v/v), and analyzed for further analytical determinations. Sodium (Na) and potassium (K) concentrations were estimated by flame spectrophotometer. Magnesium (Mg) and calcium (Ca) concentrations were assayed by atomic absorption spectrophotometer. Free proline and its derivative hydroxyproline of *T. harzianum* isolates were extracted according to AAAOM (1998) and estimated by the EPPENDORF Biotronik Amino Acid analyzer LC 3000. Cultures filtrates of *T. harzianum* isolates were prepared by transferring equal discs (5-day-old) to 1-L Erlenmeyer flasks containing CDS free or amended with 69 mM NaCl. After incubation for 10 days at 28 ^{0}C on rotary shaking at 200 rpm, the cultures filtrates were removed and stored at –20 ^{0}C after extraction until used. The effect of saline medium on the activity of extracellular chitinase, cellulase and β-galactocidase were assessed *in vitro* with a microtiter plate spectrophotometer (Labsystems, Uniskan II) according to Boller *et al.* (1983), Chernolazov *et al*. (1989) and Antal *et al.* (2000), respectively. Trichodermin, gliotoxin and gliovirin antibiotics**,** as well as total phenols were determined in the filtrates according to A.O.A.C. (1975) and Roberts and Lumsden (1990) methods and identified by HPLC using a reverse phase C8 column and compared with a standard (Sigma chemicals).

Pot trials

Trichoderma wild type and the mutant isolates were used to protect tomato (var. Peto 86) against infection by *F. oxysporum* f. sp. *Lycopersici* Schelect. Their effects were determined under both control (soil collected from Gezerit El Dahal location, Giza Governorate, Egypt with EC amounting to 0.4 mhs) and saline (soil collected from Noubaria location, Behera Governorate, Egypt with EC amounting 6.4 mhs) conditions. Pots (30 cm diameter) were inoculated with *F. oxysporum* at 2 x 10^4 colony forming units (cfu) and 0.01 %Tween-80. Seeds were soaked in the *Trichoderma* suspension at a rate of 10^5 spores / ml. Pots (5 replicates) were sown with five seeds and irrigated daily with saline solution (2800 ppm), while Control treatment received only normal irrigation water. The percentage of wilt disease incidence, population counts of *Trichoderma* isolates and *F. oxysporum* in the soil rhizosphere were calculated.

Field trials

Two successive field experiments were carried out at Noubaria Region, Behera Govarnorate, Egypt during 2000 / 2001 and 2001 / 2002 seasons to evaluate the potentiality of *T. harzianum* wild-type and the selected mutant isolates in protecting tomato plants against infection with *F. oxysporum* f. sp. *lycopersici* in a sandy loam soil. Chemical constituents of the soil were as follows: Na 740 ppm, Ca 520 ppm, Mg 267 ppm, K 16 ppm, bicarbonates 13.2 ppm and EC 6.4 mmhos/ cm. A split plot design, with four replicates was used. Tomato seeds (var. Peto 86) were sown in trays containing 2% spore suspension of *Trichoderma* at a rate of 10^5 spores ml^{-1} in a buffer containing 20 mM glucose and 20 mM potassium phosphate for 45 days. Tomato seedlings were transplanted 50 cm apart in rows of 1.5 m width. Fifty seeds were used for each replicate. Disease incidences were calculated during different growth periods. Population densities of both *Fusarium* (per gram soil) and *Trichoderma* (cfu x 10^4) isolates in rhizosphere soil were scored monthly during the growth period. At harvest, yield parameters of tomato plants were gauged. Nitrogen (Jackson, 1962), phosphorus (Olsen and Sommers, 1982) and potassium (Pipper, 1950) contents in tomato shoots were determined.

Statistical analysis

The collected data were statistically computed using the software SPSS for Windows (release 7.5.1, Dec. 20, 1996, SPSS Inc.). Data were subjected to analyses of variance and treatment means were compared by an approximate Duncan's multiple-test ($P<0.05$).

RESULTS

Mutants selection and characterization

Substantial adverse consequences towards the wild type of *T. harzianum,* in terms of growth, sporulation and antagonistic activity under NaCl salt stress up to 69 mM are presented in Table (1). Percentage of reduction in the linear growth and spore production of the tested *T. harzianum* strain increased parallel to the increase in NaCl concentration. On the other hand, antagonistic activities of the tested *T. harzianum* against *F. oxysporum* were decreased with the increase in NaCl concentration. Correlations were significant at $P> 0.01$ indicating that the tested *Trichoderma* strain was sensitive to salinity.

Two dosages of γ-irradiation (200 Gy and 500 Gy) were used to mutagenize *T. harzianum* conidia. From the surviving colonies, four isolates (Th20M53, Th50M6, Th50M11 and Th50M46) maintaining the original variation and performance, were selected and identified as stable mutants.

As shown in Table (2), reduction in linear growth of the isolated mutants was not exaggerated when exposed to 0.0, 51 and 69 mM NaCl. Meanwhile, *T. harzianum* (WT) displayed a reduction in linear growth. The morphological growth of the isolated mutants and wild type was flat and cottony, respectively on natural or salt media. Furthermore, Th50M46 showed a convex type of growth at 69 mM NaCl. In natural medium, the tested isolates exhibited compact spores except for Th50M11 and Th50M46 that showed good disperse spores. In salt medium (51 mM NaCl) most of the tested isolates produced compact spores, while Th50M11 showed good disperse spores. Regarding to spore color in natural medium, the mutants Th20M53 and Th50M46 formed green spores, while Th50M6 and Th50M11 bent dark green spores, *T. harzianum* (WT), twisted yellowish green spores, and Th20M53 produced whitish green spores. In salt medium (51 mM NaCl), the isolates WT and Th50M46 formed fashioned white spores Th20M53 produced whitish green spores, Th50M6 gave yellowish green spores, and Th50M11 created green spores. In salt medium (69 mM NaCl) the tested *Trichoderma* isolates exhibited white spores, and only Th50M11 displayed green spores.

Out of the isolated mutants that retained their tolerance to salt stress even after seven subcultures, the two mutants Th50M6 and Th50M11 were selected for further investigations. The effect of saline medium on linear growth, sporulation and antagonistic potential of the tested mutants are measured and described in Table (3). Generally, the growth of the tested *Trichoderma* isolates was affected by salt stress. Nevertheless, mutant isolates were more tolerant to salt stress than their wild parents. In natural medium, while the mutant Th50M6 achieved a maximum radial growth after 4 days of incubation period, while the wild type and mutant (Th50M11) showed a delayed reduction in their linear growth reaching 57.78 and 4.44%, respectively after the same period. In saline medium, the wild type, Th50M6 and Th50M11 showed a delayed reduction in their linear growth reaching 77.78, 2.22 and 11.11%, respectively after the same period. With regard to spore formation in the wild strain expressed as cfu x 10^4, the selected mutants far exceeded their parents grown in either natural or saline medium at all incubation periods. Simultaneously, the mutant Th50M6 displayed a maximum antagonistic activity against *F. oxysporum* on either natural or saline medium compared to the wild strain that exhibited a distinct decrease in its biological control activity on saline medium than in natural one by 0.0 and 20 %, respectively. At the same time, *F. oxysporum* was completely inhibited when amended with 50 % culture filtrate of Th50M6 and Th50M11 compared with 74.5 and 12.6 % of wild type at natural and saline medium, respectively.

Table (1): Effect of salt stress on growth, sporulation and antagonistic activity of T. harzianum against F. oxysporum.

NaCl (mM)	Reduction in linear growth (%)	Spore production (cfu x 10^4)	Antagonistic activity (as zone inhibition, mm)
0.0	0.0e*	93.2a	0.3a
17	18.57d	83.3b	0.3a
34	37.14c	53.3c	0.2ab
51	55.71b	41.2d	0.0b
69	75.37a	21.2e	0.0b

*Values represent the percent of six replicates. Values in each column followed by the same letter are not significantly different ($P<0.05$).

Table (2): Characterization of some γ-ray induced mutants from T. Harzianum grown in different saline media.

Trichoderma isolates	NaCl (mM)	Reduction in linear growth (%)		Description					
				Growth		Sporulation [5]		Color [6]	
		1st	7th	1st	7th	1st	7th	1st	7th
Wild type [1]	0.0	0.0	0.0	Flat	Flat	+	+	Y g	Y g
	51	49.3	49.3	Cottony	Cottony	+	+	W	W
	69	53.1	53.1	Cottony	Cottony	+	+	W	W
Th20M53 [2]	0.0	0.0	0.0	Flat	Flat	++++	++++	W g	W g
	51	0.0	0.0	Flat	Flat	++++	++++	W g	W g
	69	0.0	0.0	Flat	Flat	++	++	W	W
Th50M6 [3]	0.0	0.0	0.0	Flat	Flat	++++	++++	D g	D g
	51	0.0	0.0	Flat	Flat	++++	++++	Y g	Y g
	69	8.89	8.89	Flat	Flat	++++	++++	W	W
Th50M11[3]	0.0	0.0	0.0	Flat	Flat	+++	+++	D g	D g
	51	0.0	0.0	Flat	Flat	+++	+++	G	G
	69	0.0	0.0	Flat	Flat	+++	+++	G	G
Th50M46 [3]	0.0	0.0	0.0	Flat	Flat	+++	+++	G	G
	51	0.0	0.0	Cottony	Cottony	-	-	W	W
	69	0.0	0.0	C. t. [4]	C. t. [4]	-	-	W	W

[1] *T. harzianum* Wild type (WT). [2 & 3] 200 and 500 Gy γ-ray induced mutants, respectively.
[4] (C. t.) Convex type. [5] (-) not disperse, (+) little disperse, (++) moderate disperse, (+++) good disperse, (++++) compact. [6] (Dg) Dark green, (G) Green, (Yg) Yellowish green, (Wg) Whitish green and (W) White. (1st) First subculture, and (7th) Seventh subcultures.

Table (3): Effect of saline medium on linear growth, sporulation and antagonistic potential of Trichoderma harzianum wild type and two of its selected mutants against F. oxysporum.

Trichoderma Isolates	Linear growth (mm)				Sporulation (cfu x 10^4)				*F. oxysporum* Growth reduction %		*F. oxysporum* Growth reduction (%) by 50% culture filtrates	
	Natural[1]		Saline[2]		Natural[1]		Saline[2]		Natural[1]	Saline[2]	Natural[1]	Saline[2]
	Days after incubation											
	2	4	2	4	10	30	10	30	6	6	6	6
T.harzianum[3]	22c*	38b	5c	20c	195c	136c	43c	57c	50b	40.4c	74.5b	12.6c
Th50M6[4]	75a	90a	73a	88a	1389a	1944a	1016a	1563a	100a	100a	100a	100a
Th50M11[4]	70b	86a	65b	80b	833b	1314b	736b	1023b	98.6a	94.3b	100a	86b

[1] PDA NaCl-free medium. [2] PDA amended with 69 mM NaCl medium. [3] Wild type. [4] γ -ray induced mutants.
*Values represent the mean percentage of six replicates. Values in each column followed by the same letter are not significantly different (P<0.05).

The tested *Trichoderma* isolates were validated by their salt tolerance capacity through evaluating some ion contents (Fig. 1), the amino acid praline (Fig. 2), under salt stresses conditions. Fig.(1) shows that magnesium (Mg), calcium (Ca) and potassium (K) contents in the mycelia of both wild fungi and its selected mutants Th50M6 and Th50M11 were reduced by salinity. On the other hand, sodium (Na) content in the mycelia showed an increase when grown under saline conditions. Generally, the tested mutants showed favor potassium (K) over sodium (Na) under saline condition than natural condition. Fig. (2) describes that the wild type, Th50M6 and Th50M11 showed increase in total proline content reached 2.0, 1.17 and 2.5 folds,

respectively when grown in the saline medium compared to natural medium.

The genetic improvement program *via* γ-irradiation made it possible to obtain the *T. harzianum* salt tolerant mutant Th50M6 that exhibited superiority in bio-controlling *F. oxysporum* compared to their wild parent under salt stress conditions.

The production of antifungal metabolites by the wild parent, Th50M6 and Th50M11 were affected negatively by salt stress (Table 4). However, the mutants showed less decrease than their wild type. In natural media, Th50M6 and Th50M11 produced the highest amounts of gliotoxin, gliovirin and trichodermin in contrast with salt conditions. Whereas, wild type showed reduction in gliotoxin, gliovirin and trichodermin production in natural media and under salt stress conditions. Furthermore, total phenols obtained from these mutants displayed increased amounts in control media and under salt stress conditions compared the with wild type. The same results hold true in extracellular hydrolysis enzymes production. Whereas, Th50M6 and Th50M11 produced more chitinase, cellulase and β-galactosidase in control media and under salt stress conditions compared.

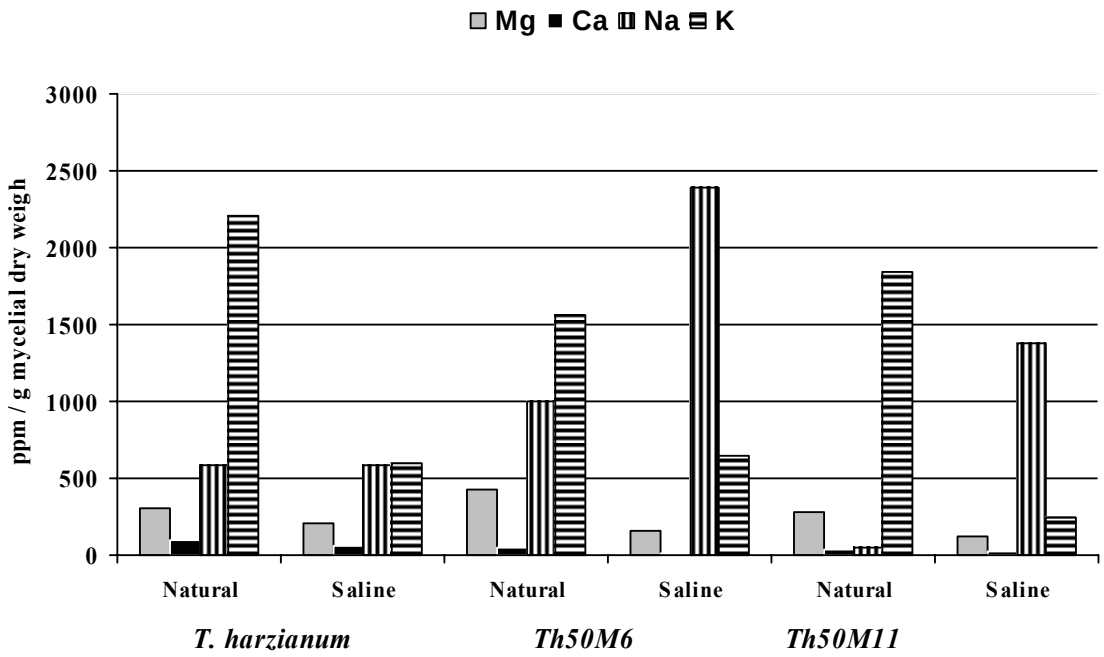

Fig. (1): Effect of saline medium on Mg, Ca, Na and K contents in the mycelium of two γ-induced mutants from T. harzianum as well as their wild type.

* Natural and saline media are referring to PDA NaCl-free medium and PDA medium amended with 69 mM NaCl, respectively.

* *T. harzianum* is wild type isolate. * Th50M6 and Th50M11 refer to γ-ray induced mutants.

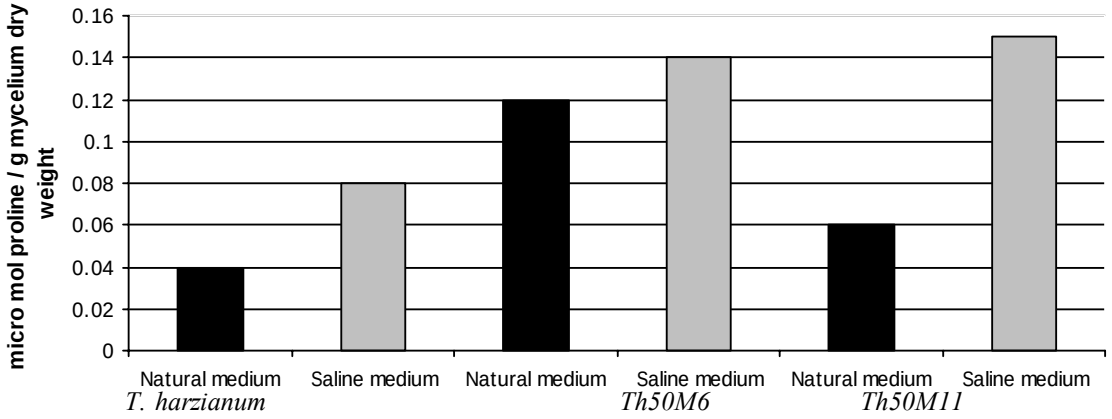

Fig. (2): Effect of saline medium on proline contents.

*Natural and saline media refer to PDA NaCl-free medium and PDA medium amended with 69 mM NaCl, respectively. **T. harzianum* is wild type isolate.* Th50M6 and Th50M11 refer to γ–ray induced mutants. **Proline content (proline and its derivative hydroxyproline) as µg / g mycelium dry weight.

Table (4): Inhibitory substances and extracellular hydrolysis enzymes produced by Trichoderma harzianum wild type and its mutants in their culture filtrates under natural and salt stress.

Trichoderma isolates	Media	Inhibitory substances				Extracellular hydrolysis enzymes		
		Gliotoxin	Gliovirin	Trichodermin	Total phenols	Chitinase	Cellulase	β -galactosidase
		µg / g mycelial dry weight						
Wild type	Natural[1]	0.015	0.012	0.03	0.009	0.213	0.143	0.170
	Saline[2]	0.005	0.004	0.01	0.002	0.080	0.010	0.020
Th50M6[3]	Natural[1]	0.148	0.216	0.49	0.025	3.980	2.140	2.870
	Saline[2]	0.133	0.189	0.43	0.019	3.750	2.120	2.541
Th50M11[3]	Natural[1]	0.135	0.187	0.40	0.021	2.540	2.300	2.560
	Saline[2]	0.112	0.163	0.34	0.018	2.330	2.150	2.424

[1] PDB NaCl-free medium; [2] PDB amended with 69 mM NaCl medium; [3] γ–ray induced mutants.

Pot trials

The competency of *T. harzianum* wild type and their selected mutants on controlling *Fusarium* wilt of tomato under non-saline and saline soils infested with *F. oxysporum* is presented in Table 5. The wilt disease incidence was higher in untreated plants either for non-saline or saline soils after 90 days of sowing. Wilt disease incidence showed highly decrease in non-saline and saline soils when the seeds dressed with the spores of wild type *T. harzianum*. But, dressing the seeds with the spores of mutants of *T. harzianum* resulted in a significantly greater decrease in the wilt disease incidence under non-saline and saline soils. Colonization of either *F. oxysporum* or *Trichoderma* isolates in tomato rhizosphere was assessed throughout 90 days growth period in control and saline soils. *F. oxysporum* propagates counts under non-saline and saline soils were non-detectable in the soils treated with the mutants Th50M6 and Th50M11 compared to soil treated with the wild type *T. harzianum* or untreated soil. At

the same time, the selected *Trichoderma* mutants Th50M6 and Th50M11 showed significantly greater increase in their population densities as compared to the wild type *T. harzianum* in non-saline and saline soils.

Field trials

The effect of *T. harzianum* wild type and its selected mutants in controlling *Fusarium* wilt in tomato under salt or natural soils infested with *F. oxysporum* was assessed (Fig. 3). Data showed that wilt disease incidence was higher in untreated plants reaching 17.5 and 19.3 % after 120 days of sowing during 2000/ 2001 and 2001 / 2002 seasons, respectively. Treated plants with the wild type of *T. harzianum* decreased the incidence of wilt disease to 12.3 and 8.1 %, respectively. The mutants Th50M6 and Th50M11 possessed considerably higher biocontrol activity. No disease incidence was monitored in tomato plants receiving Th50M6, and a minimum level of disease symptoms was distinguished in those receiving Th50M11, which reached 1.6 and 1.9% during 2000/2001 and 2001 /2002 seasons, respectively. Colonization of either *F. oxysporum* or *Trichoderma* isolates in tomato rhizosphere was assessed 120 days after sowing during 2001 / 2002 season (Fig. 4). Density of *Fusarium* was decreased with seed dressing by *T. harzianum* wild type (12.3 propagate /g soil) in comparison to untreated seeds (21.8 propagate /g soil) after 120 days. However, minimum propagates were recorded in the rhizosphere of tomato plants inoculated with Th50M6 and Th50M11 mutants reached to 1.6 and 3.6 propagate /g soil, respectively after 120 days of sowing. At the same time, density of either Th50M6 or Th50M11 mutants were highly increased through 120 days up to 542.0 and 475.3 cfu x 10^5, respectively compared to wild type (8.1 cfu x 10^5). Table (6) shows the effect of applying *T. harzianum* wild type and its selected mutants on tomato plant yield and mineral content. Generally the different growth parameters were higher in plants treated with *T. harzianum* mutants than those treated with the wild type one. The increase in each of tomato plant yield and different minerals were significantly higher when associated with the mutants isolates than with either its wild type or untreated plants.

Table (5): Application efficiency of some Trichoderma harzianum isolates (wild-type and its mutants) on tomato wilt disease incidence after sown in non-saline and saline soils infested with F. oxysporum under pot experiments.

Trichoderma treatment	Soil	Wilt disease incidence[3] %	Population count *Fusarium* propagate / g soil	Population count *Trichoderma* (cfu x 10^5)
Untreated	Non-saline [1]	24.3a[5]	19.4a[5]	--
	Saline[2]	21.6a[5]	15.2ab[5]	--
T. harzianum (WT)	Non-saline [1]	15.2b[5]	9.3b[5]	187.2d[5]
	Saline[2]	9.4c[5]	7.4b[5]	28.2e[5]
Th50M6[4]	Non-saline [1]	0.0e[5]	0.0c[5]	462.3a[5]
	Saline[2]	0.0e[5]	0.0c[5]	385.2b[5]
Th50M11[4]	Non-saline [1]	1.6e[5]	0.6c[5]	402.3b[5]
	Saline[2]	0.6e[5]	0.3c[5]	312.6c[5]

[1] Soil obtained from Gezerit El Dahab location, Giza Governorate, Egypt, EC, 0.4 mmhos/cm. [2] Soil obtained from Noubaria location, Behera Governorate, Egypt, EC 6.4 mmhos/cm. [3] Wilt disease incidence was calculated after 90 days.[4] γ–ray induced mutants.. [5] *Values represent the mean percentage of six replicates. Values in each column followed by the same letter are not significantly different (P<0.05).

Table (6): Application efficiency of Trichoderma harzianum wild type and its mutants on growth, yield and minerals contents of tomato plants growing under field conditions1 & 2.

Trichoderma isolates	2000/2001 season				2001/2002 season			
	Yield (Kg/ m^2)	Shoots mineral content (%)			Yield (Kg/ m^2)	Shoots mineral content (%)		
		N	P	K		N	P	K
Wild type	7.32c[4]	3.21b[4]	0.65b[4]	3.61b[4]	7.02b[4]	3.65b[4]	0.69b[4]	3.67b[4]
Th50M6[3]	9.04a[4]	4.11a[4]	0.92a[4]	4.03a[4]	8.75a[4]	4.42a[4]	0.98a[4]	4.21a[4]
Th50M11[3]	8.94b[4]	4.01a[4]	0.81a[4]	3.98a[4]	8.65a[4]	4.12a[4]	0.94a[4]	4.01a[4]
Untreated	6.45d[4]	2.78c[4]	0.39c[4]	3.31c[4]	6.31c[4]	2.98c[4]	0.41c[4]	3.36c[4]

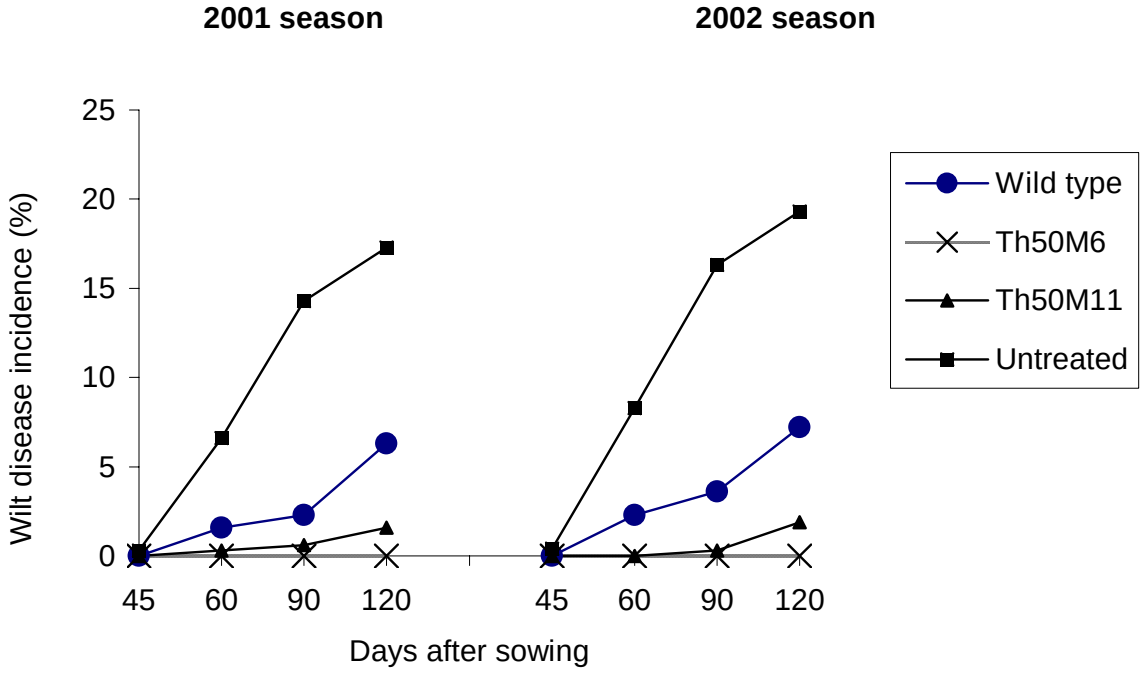

Fig. (3): Application efficiencies of Trichoderma isolates on disease incidence of Fusarium oxysporum causing tomato wilt under field conditions[1 & 2].

[1] The experiments carried out at Noubaria Region, Behera Govarnorate, Egypt, during 2000 / 2001 and 2001 / 2002 seasons. [2] (EC 6.4 mmhos/cm).,* Th50M6 and Th50M11 refer to γ–ray induced mutants.

DISCUSSION

Salinity is one of the most widespread constraints to soil fertility (Abd-Alla and Omar, 1998). Preliminary investigations of Kredics *et al.* (2000) proved that salt stress had significant detrimental effects towards growth, sporulation and antagonistic activity of the wild type isolate of *T. harzianum* against *Fusarium oxysporum*. Several successful trials had been made to increase the biocontrol potential of *Trichoderma* species under a wide range of adverse environmental conditions by exposing the spores to chemical e.g., EMS or physical mutagens, e.g., gamma rays (Kredics *et al.,* 2001). Mutation has been suggested to

improve growth characters, antifungal metabolites production and antagonistic potential of *T. harzianum* against *F. oxysporum* under saline conditions.

One of the physiological adjustments in microbial growth at reduced water activity is the accumulation of specific intercellular substances as well as changes in their biosynthetic pathways (Soliman *et al.,* 1994), which are metabolically neutral osmolytes that might also be osmoprotectent (Anthony,1998). Sodium (Na) content of the tested *Trichoderma* isolates increased at saline medium, while Ca2+, Mg2+ and K+ content decreased as previously detected by Khan *et al.* (2001). On the other hand, the wild type *T. harzianum* and its mutant isolates displayed an increase in their total proline derivatives when grown in the saline than in natural media. Peng *et al.*(1997) results suggested that free proline always accumulates under osmotic stress conditions, however, upon removal of osmotic stress, proline levels return to normal. The results reveal that the removal of free proline during the recovery from salinity or dehydration stress engages an induction of proline dehydrogenase (PDH) gene, while the activity of DELTA1-pyrroline-5-carboxylate-synthetase (P5CS) gene declines. The reciprocal regulation of P5CS and PDH genes appears to be a key mechanism in the control of proline levels during and after osmotic stress. PDH gene was also significantly induced by exogenously applied proline.

Since the production of antifungal metabolites, extra enzymes, and antibiotics appears to be responsible for the ability of *Trichoderma* strains to control the growth of pathogen –mediated wilt of tomato, it is hypothesized that superior biocontrol activity might be achieved by increasing production levels of these metabolites. In the current study, addition of NaCl to the growth medium at a rate of 69 mM NaCl increased metabolic activities of *Trichoderma* mutants including extra enzymes of chitinase, cellulose, β-galactosidase as well as the antibiotics, i.e., trichodermin, gliotoxin and gliovirin as well as total phenols compared to the wild type (Stefanova *et al*., 1999). The same results was showed by Abd-Alla and Omar (1998), who stated that enzymatic activity of ten tested moulds including *T. harzianum* was strongly decreased by the addition of NaCl to the growth medium at the rates of either 0.5 or 1%. The information about the influence of water conditions on metabolic activities of *Trichoderma* strains is essential for planning their application in biocontrol strategies. Kredics *et al*. (2000) studied the influence of water potential on linear mycelial growth, secretion and *in vitro* activities of different enzymes of *T. harzianum* strain T66. Optimal water potential values for the secretion of β-glucosidase, cellobiohydrolase, β-xylosidase, NAGase and chymotrypsin-like protease enzymes were different. Cellobiohydrolase and NAGase enzymes showed optimal secretion at the highest examined water potential, while the maximum activities of secreted β-glucosidase, β-xylosidase and chymotrypsin-like protease enzymes were transpired at lower water potential values than those optimal for growth. *In vitro* enzyme activities were exaggerated by water potential, but significant enzyme activities were measured for most of the enzymes even at –14.8 MPa, which is below the water potential, where mycelial growth ceased. These results suggest the possibility of using mutants with improved xerotolerance for biocontrol purposes in soils with lower water potential.

In biological control experiments with tomato plants infested by *F. oxysporum* under saline soil (Na 740 ppm, Ca 520 ppm and EC 6.4 mmhos/ cm), mutants established a much better disease control, plant growth, yield and mineral content than the wild type. Overall, *F.*

oxysporum population was minimized in soil rhizosphere together with a marked increase in the plant yield in comparison with the wild isolate. Such levels of plant protection were previously achieved using mutant strains through increasing the production of the extracellular antifungal metabolites (Dunne *et al.,* 2000). Overproduction of antifungal metabolite s (enzymes or antibiotics) in *Trichoderma* mutants resulted in a reduced growth rate of the pathogen *in vitro* under saline soil conditions.

In conclusion, a new approach to develop improved biocontrol strains of *Trichoderma harzianum* by increasing salinity tolerance phenotype using mutation techniques was reached. Such an approach could be useful for enhancing salt tolerant, metabolic production and biocontrol ability against *Fusarium oxysporum* and protect tomato plants under saline conditions.

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الملخص العربي

مقدرة المقاومة الإحيائية لطوافر فطر التريكودرما هارزيانم المقاومة للظروف الملحية المعاكسة ضد فطر الفيوزاريم اوكسيسبوريم المسبب لمرض الذبول في الطماطم

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يهدف هذا البحث إلي تحسين مقدرة فطر التريكودرما هارزيانم كعامل مقاومة احيائية ضد الفطر الممرض الفيوزاريم اوكسيسبوريم بزيادة إنتاجيتة من نواتج الايض الميتابوليزمية المضادة للفطريات تحت الظروف الملحية القاسية من خلال برنامج تربية وراثي بالتطفير بواسطة أشعة جاما. أدي تعريض مزرعة لفطر التريكودرما هارزيانم (الطفيل الفطري) لأشعة جاما إلى استحداث طافرين ثابتين(Th50M6 & Th50M11) مقاومين للظروف الملحية المعاكسة. تحت هذه الظروف كلا الطافرين أظهرا تفوقا عن الأب البري من حيث معدل النمو و المقدرة العالية في المقاومة الإحيائية ضد فطر الفيوزاريم اوكسيسبوريم المسبب لمرض الذبول الفيوزارمي بالطماطم. أظهر طافرا التريكودرما هارزيانم المقاومين للظروف الملحية المعاكسة مقدرة مقنعة علي النمو و التجرثم علي بيئة النمو المحتوية علي تركيز من ملح الصوديوم كلوريد يصل إلي ٦٩ مللي مول. اثبت توصيف كلا الطافرين مقارنة بالأب البري تعزيز محتوي الطافرين من الحمض الأميني البرولين واحد مشتقاته الهيدروكسي برولين مع الارتفاع النسبي لمحتواها من الصوديوم مقارنة بالبوتاسيوم و الكالسيوم أو الماغنسيوم بالإضافة لمستواها العالي من الفينولات الكلية. أظهرت النتائج أيضا أن الطوافر المقاومة للظروف الملحية المعاكسة تنتج عدد من نواتج التمثيل الايضي تشتمل علي إنزيمات الكيتينيز و السيللوليز والبيتا جلاكتوسيديز المفرزة خارج الخلية وكذلك بعض المضادات الحيوية مثل التريكوديرمين و الجيلوتوكسين و الجيلوفيرين. اختزلت طوافر التريكودرما حدوث الإصابة بمرض الذبول كما حسنت إنتاجية وكذلك المحتوي المعدني لنباتات الطماطم تحت كل من الظروف الملحية وغير الملحية للتربة وكذلك تحت كل من ظروف الإصابة الطبيعية والمستحدثة. كما كانت الطوافر أكثر فاعلية في انحدار نمو المسبب المرضي فطر الفيوزاريم اوكسيسبوريم في منطقة الريزوسفير مقارنة بالأب البري. كذلك تفوقت إلي حد ما كثافة الأفراد المستوطنة لمنطقة الريزوسفير لكلا الطافرين عن الأب البري.