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Mutagenesis and inter-specific protoplast fusion between *Trichoderma koningii* and *Trichoderma reesei* for biocontrol improvement

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ABSTRACT

The present work aimed to apply mutagenesis and inter-specific protoplast techniques of two locally isolated *Trichoderma spp.* to enhancement their biocontrol abilities against some important plant fungal pathogens which cause root-rot and damping-off diseases that attacking different crops. A combination of UV-light and nitrate treatment induced seven mutants (*T. koningii,* 3 mutants and *T. reesei*, 4 mutants). The inter-specific protoplast fusion between *T. koningii* and *T. reesei* gave fusion frequency reached 1.25% and thirteen fusants were isolated. *In vitro* bioassay of the parental *Trichoderma* spp., their selected mutants and fusants indicated the effective of both *T. koningii* and *T. reesei* in suppressive the tested fungal pathogens. On the other hand, most of the selected mutants and fusants showed superiority in their antagonistic activity against these pathogens than their parental strains. the superiority in biological control activities of the selected mutants or fusants than their parents against the tested pathogens may be due to the effect of the genetic treatments (i.e. mutagenesis an protoplasts fusion) on their genetic back ground to be varied that allow to change in genetic control of antifungal metabolites production to be more effective. The results indicated that mutation and protoplast fusion techniques are successful tools to enhance the antagonistic effects of *Trichoderma* species against several fungal plant pathogens.

Keywords: Biological control, plant pathogens, mutation, protoplast fusion.

INTRODUCTION

Several microorganisms e.g., bacteria, actinomycetes, yeasts and different genus of fungi especially *Trichoderma* spp produce many types of antifungal metabolites [1].

Biological control of several plant pathogens showed promise as an option diseases management strategy [2]. Antagonistic microorganisms represent the most diverse group of organisms on the plant. Even through the natural microflora, antagonistic fungi included *Trichoderma* and *Gliocladum* species are surmised of special share as biological control agents against numerous phytopathogenic fungi [2]. *Trichoderma* or *Gliocladum* species are known as cogent producer of many antifungal metabolites including enzymes, antibiotics and others [3 - 5].

Mutagenesis or protoplast fusion of biocontrol agents were applied to improve the antifungal production and antagonistic potential over a broad spectrum of phytopathogens, survival, longevity and activity ([6 - 13]. Protoplast fusion is a quick and easy method for combining the advantageous properties of distinct promising strains. It was successfully applied in the breeding of *T. harzianum* biocontrol strains [14,15]. Protoplasts could be produced easily from biocontrol *Trichoderma* strains and their induced fusion resulted in genetic recombinants with elevated biocontrol abilities in many instances [16].

The present work aimed to apply mutagenesis and inter-specific protoplast fusion techniques of two locally isolated *Trichoderma spp.* to enhancement their biocontrol abilities against some of important plant fungal pathogens which cause root-rot and damping-off diseases that attacking different crops growing either in open field or in greenhouses.

MATERIALS AND METHODS

Strains

Bioagents: Two locally *Trichoderma* species, *T. koningii* and *T. reesei* [17] were kindly obtained from Prof. Dr. Mohamed Fadel, Microbiological Chemistry, NRC, and Egypt.

Pathogens: Four fungal pathogens*; Fusarium oxysporum,* [18]*, Pythium ultimum* [19], *Sclerotia*

rolfsii and *Sclerotinia sclerotiorum* [13] the causal fungi of root-rot, damping-off and wilt diseases, were isolated from diseased plants, from south Giza (Gezerit El-Dahab) and El-Bohira (Noubaria districts), Egypt and identified in Plant Pathology Department, NRC, Dokki, Cairo, Egypt.

Fungi cultures were maintained on Potato Dextrose agar (PDA) medium. Minimal agar medium (MM) was used to test the auxotrophic characters, Dextrose Broth (PDB) medium or Czapek Dox Salt solution (CDS) were used to obtain the fungal culture filtrates [12].

Mutation Induction and Isolation: The mutants were obtained from *Trichoderma* spp. according to the methods of [8] with slightly modifications. The parent strains; *T. koningii* and *T. reesei* were grown on PDA slants at 30°C to induce sporulation. One week after sub-culturing conidial suspension was prepared by dislodging the conidia from the agar surface with a sterile needle to a sterile glass vial and by pouring sterilized physiological saline (0.85 % NaCl) containing 0.1 % Tween-80 to disperse spore clumps. The prepared conidial suspension was divided to two portions in two sterilized small glass vials. Conidial concentrations were adjusted to ≈ 10^6 / ml. The first glass vial was used as control. The second glass vial was treated with 500 µg/ml sodium nitrate ($NaNO_2$) and was irradiated for 80 min under ultraviolet lamp (GERMICIDAL LAMP (VL-G), UV-tube T-15C 15W 254 nm, VILBER-LOURMAT) where the distance between the agar surface and the lamp was adjusted to 30 cm. After irradiation the conidial suspensions were incubated at 30°C for 45-60 min in dark. After incubation period 0.1 ml was poured on the solidified Czapek-Dox mineral medium supplemented with 0.1% Triton X-100 to restrict the growth of the fungal colonies [8]. The plates were incubated at 30°C for 6 days until the fungal colonies were observed.

The surviving colonies were tested on MM to eliminate auxotrophic colonies. The colonies appeared on MM were selected and examined for some characters including growth rate that measured as linear growth (mm), sporulation that counted as colony forming unit (cfuX$10^{5)}$ according to [12] and their antagonistic effects against *Pythium ultimum*. The isolates, which displayed better characters than its parent isolates, were isolated and sub-cultured 7 times on PDA to test their stability. Those isolate which retained the altered characteristics were identified as mutants and designated as (Tk-UVN-No. or Tr-UVN-No.

Protoplast Fusion:

Behavior resistance of the bioagents on different antifungal agents: The parental strains; *T. koningii* and *T. reesei* were streaked on the surface of malt extract agar plates supplemented with specific antifungal agents (Benomyl, Cyclohexamide, Hygromycin, Micanazol and Nystatin) with the doses of 30, 76, 1, 60 and 100 µg / 50 ml medium, respectively were used to determine the fungal resistant behavior, and incubated for 7 days at 30°C. Then, their resistance behavior was recorded.

Protoplasting medium (PM) or (Regeneration medium): The medium of [20] was used for pre-growing fungal strains for protoplasting, contains (g L^{-1}): glucose, 80; NH_4NO_3, 2; KH_2 PO_4, 10; $MgSO_4.7H_2O$, 0.25; $FeCl_3.6H_2O$, 0.02 and $MnSO_4$, 0.014 at an initial pH of 4.5.

Protoplasting buffer (PB): The parental isolates were inoculated in PB buffer consists of phosphate buffer (0.1 M, pH 5.8 – 6) containing 0.7 M NaCl, 0.2 M $CaCl_2$ and Novozyme 234 (20 mg mL^{-1}) and incubated at 30°C with gentle shaking for up to 6 h.

Fusion buffer (FB): The buffer of FB consists of phosphate buffer (0.05 M, pH 7.5) containing 30% (w/v) polyethylene glycol (PEG) 6000, 50 mM $CaCl_2$ and 0.7 M NaCl.

Selective medium (SM): The SM medium consists of; malt extract agar containing 0.7 M NaCl and supplemented with antifungal agents (Hygromycin and Micanazol).

Protoplast Formation: Spore suspension of each parent, T. *koningii* or *T. reesei* was inoculated in 50 mL PM and incubated for 36 h at 30°C on rotary shaker (180 rpm). The mycelium formed was recovered by centrifugation (1340 *xg* for 10 min), washed twice with sterilized physiological saline (0.85 % NaCl), and re-suspended in 5 mL PB. The mixture was investigated by light microscope.

Inter-specific Protoplast Fusion: Equal volumes of crude protoplast suspension from the two parents (T. *koningii* and *T. reesei*) were mixed and centrifuged (1340 *xg* for 10 min). The protoplast pellet formed was re-suspended in 2 mL FB and incubated for 20 min at 30°C.

Fusants Isolation: PEG-treated protoplast suspension was plated onto the surface of SM supplemented with antifungal agents (Hygromycin and Micanazol) and incubated for 7 days at 30°C.

Colonies growing on the surface of the plates were considered as fusants.

Antagonistic effect of the Tested Bioagents Against the Tested Pathogens

Dual culture on PDA medium: The tested *Trichoderma* species (parental or genetically modified) were examined for their antagonistic effect against the tested pathogens by inoculating the fungal discs on 90 mm diameter PDA plates near the periphery opposite to each other. Antagonistic activity was measured as growth reductions [18]. The growth of tested mutants and fusants were compared to the growth of their wild type strains as positive control and PDA medium as negative control.

Filtrate inhibition: The tested *Trichoderma* species (parental or genetically modified) were grown on CDS medium for 10 days at 30^{0}C to obtain the fungal culture filtrates. The fungal filtrates were sterilized through a 0.45 µm sterile milipore filter. Volume of each fungal filtrate was added to (2X) PDA medium to give final concentration of 50 %, and then inoculated with equal discs (5 mm in diam) of the tested pathogen. After incubation, colonies diameters were determined and antagonistic activity was measured as growth reduction compared to their growth on PDA alone [18].

Statistical Analysis : All treatments in the previous experiments consisted of three or more replicates. The obtained data were statistically computed using the software SPSS for Windows (release 9.0.0, Dec. 18, 1998, standard version, SPSS Inc.).

RESULTS

Combined mutagenic treatments: A combination of UV-light and sodium nitrate was used to mutagenized *T. koningii* and *T. reesei* conidia where about 96.54 and 91.10%, respectively of the treated conidia had been killed (Fig. 1). Several colonies were tested after mutagenesis for their morphological or antagonisms differences. Three and four mutants were isolated from *T. koningii* and *T. reesei* strains, respectively (Table 1).

After 7 subcultures the tested isolates were fast growing. While, the selected mutants (Tk-UVN-1, Tk-UVN-2, Tk-UVN-3, Tr-UVN-1, Tr-UVN-2, Tr-UVN-3, Tr-UVN-4) covered the whole plates after incubation 4 days, compared to 6 days of the parent strains (*T. koningii* and *T. reesei*). The same trend was verified for sporulation on PDA medium up to 6 days. The parent strains barely increased the spore count up to 6 days, and then declined. Among the seven mutant isolates, Tk-UVN-1, Tk-UVN-2, Tk-UVN-3, Tr-UVN-1, Tr-UVN-2, Tr-UVN-3, Tr-UVN-4 were especially high in spore count compared to the parent strains (Table 2).

Inter-specific Protoplast Fusion: Figure (2) shows free protoplasts (B and D) after lytic digestion of mycelia (A and C) of *T. reesei* and *T. koningii*, respectively. The antifungal agents (Benomyl, Cyclohexamide, Hygromycin, Micanazol and Nystatin) with the doses of (30, 76, 1, 60 and 100 µg / 50 ml medium, respectively were used to determine the fungal resistant behavior. The results showed that *T. koningii* was sensitive to Benomyl and Hygromycin, whilst resistant to Cyclohexamide, Micanazol and Nystatin. On the other hand, *T. reesei* was sensitive to Benomyl and Micanazol whilst resistant to Cyclohexamide, Hygromycin, and Nystatin. So that Hygromycin and Micanazol were used as selective markers for the tow parental fusion.

Table (3) showed the efficiency of protoplast fusion between the two parents strains were fusion frequency reached 1.25%.

Antagonistic Activity

Antagonistic effects of selected mutants against the tested pathogens

Data given in Table (4) showed that the wild type strain, *T. koningii* caused high reduction of *Fusarium oxysporum* growth while *T. reesei* produced a minute growth reduction. The mutants caused higher reduction rate ranged between 92.6% (Tk-UVN-2) to % (TrUVN-1) against *F. oxysporum* growth except the mutants Tk-UVN-3 and Tr-UVN-3 that showed 58.2% and 56.2%, respectively.

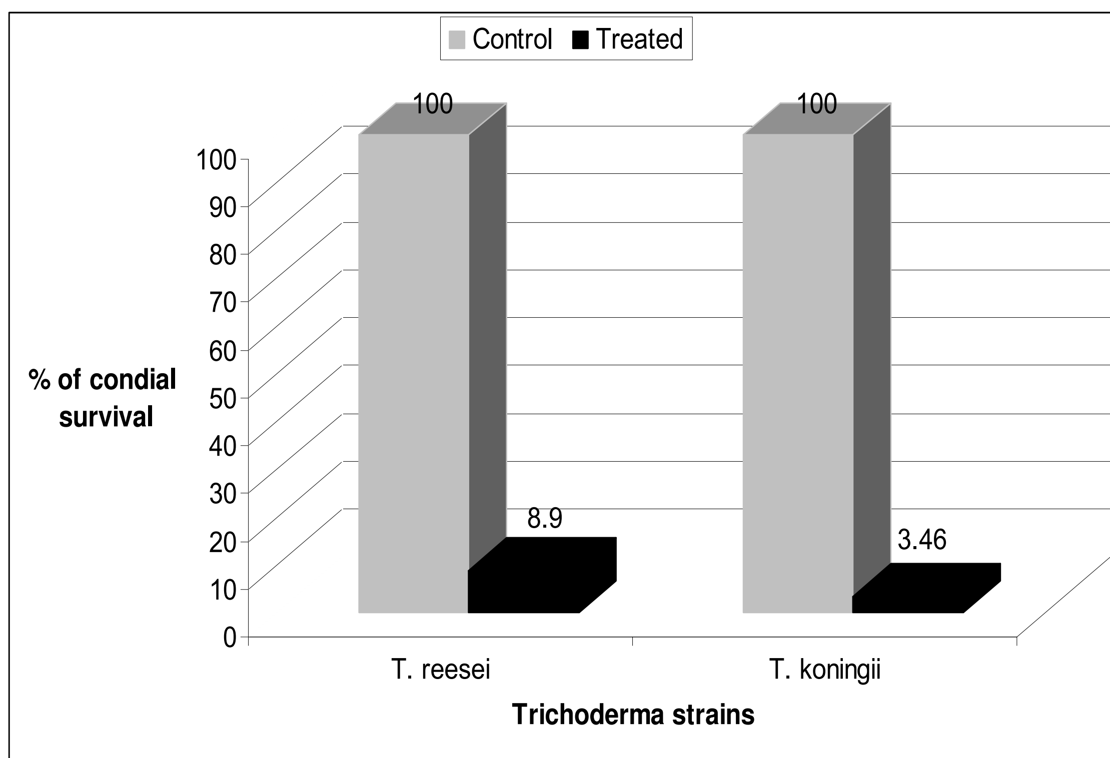

Fig 1. Effect of combination of UV-light and $NaNO_2$ treatment on survival of two *Trichoderma* species.

Table 1. Mutants obtained after treated *Trichoderma* species with a combination of UV-light and $NaNO_2$.

Trichoderma species	No of mutants	Designated as
T. koningii	3	Tk-UVN-1, Tk-UVN-2 and Tk-UVN-3
T. reesei	4	Tr-UVN-1, Tr-UVN-2, Tr-UVN-3 and Tr-UVN-4

Table 2. Growth, sporulation and antagonistic effect of the two parents (*T. koningii* and *T. reesei*) and their stable selected mutants against *Pythium ultimum* after incubation for 6 days at 30^{0}C.

Trichoderma species (Wild type / Mutant)	Growth Diameter (mm)		Sporulation (cfu)	***P. ultimum*** **Growth reduction (%)**
	After 4 days	After 6 days		
T. koningii	40b	90a	250 x 10^5	46 f
Tk-UVN-1	90a	90a	1100 x 10^5	40.6f g
Tk-UVN-2	90a	90a	1050 x 10^5	99.8a
Tk-UVN-3	90a	90a	1700 x 10^5	63.8 d
T. reesei	28c	90a	150 x 10^5	38 g
Tr-UVN-1	85a	90a	500 x 10^5	58.4 e
Tr-UVN-2	90a	90a	1010 x 10^5	60.3 de
Tr-UVN-3	90a	90a	1000 x 10^5	82.3 b
Tr-UVN-4	90a	90a	1150 x 10^5	98.2 a

[#] Means within columns followed by different letters are significantly different ($p<0.05$).

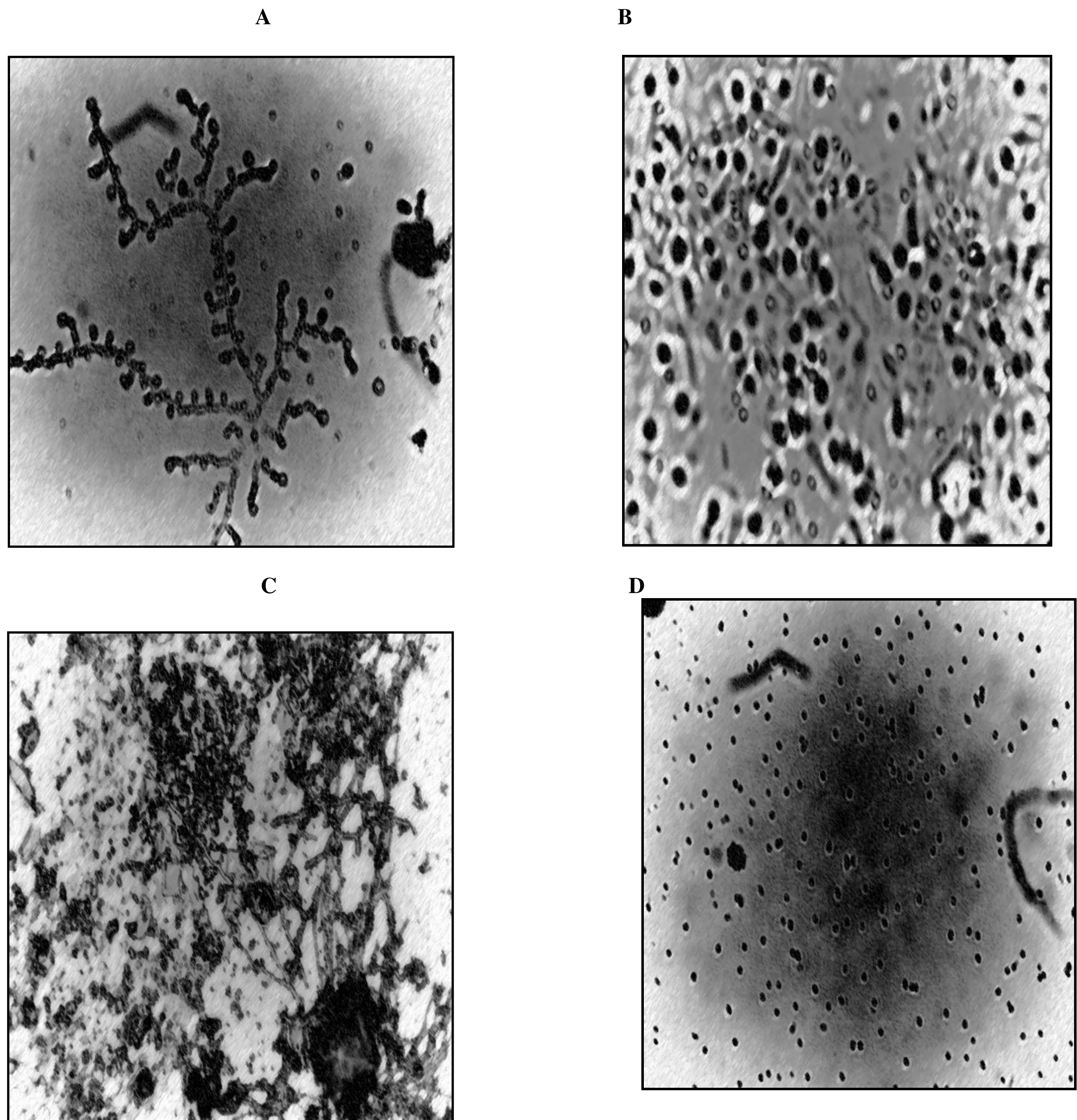

Figure 2. Protoplast formation after lytic enzyme treatment.
[A] mycelium of *T. reesei*. [B] protoplast of *T. reesei*.
[C] mycelium of *T. koningii*. [D] protoplast of *T. koningii*.

Table 3. Protoplast fusion efficiency

Parental strains	No. of starting conidia / ml	[α] No. of protoplast / ml	No. of regenerated protoplasts	[c] Regeneration frequency %	[b] No. of fusants	[d] Fusion frequency (%)
T. koningii (Tk)	10.0×10^6	12.87×10^5	5.01×10^5	0.06035×10^5	-----	-----
T. reesei (Tr)	10.0×10^6	11.27×10^5	4.39×10^5	0.06035×10^5	-----	-----
Fusants Tk x Tr	-----	$12,07 \times 10^5$	-----	-----	6.035×10^5	1.25 ± 0.85

[α] Protoplasts prepared from strains *T. koningii* and *T. reesei* were mixed in almost the same numbers and subjected to fusion treatment as described in the text. They were then placed on the regeneration medium containing Hygromycin and Micanazol as selective agents of strains *T. koningii* and *T. reesei*.
[b] The number of colonies that appeared on the regeneration medium was counted as the number of fusants.
[c] Regeneration frequency measured as no. of fusants / no. of starting conidia.
[d] Fusion frequency was calculated no. of fusants / no. of starting protoplasts.

Table 4. Reduction of the two *Trichoderma* species and their selected mutants against pathogens caused root-rot and wilt diseases.

Trichoderma species Wild / Mutant	Pathogen			
	F. oxysporum	*P. ultimum*	*S. rolfsii*	*S. sclerotiorum*
	Growth reduction (%)			
T. koningii	52.6 b	46 f	23.3 g	55.7 e
Tk-UVN-1	96.7 a	40.6f g	96.7 a	99.2 a
Tk-UVN-2	92.6 a	99.8a	98.3 a	95.4 a
Tk-UVN-3	58.2 b	63.8 d	96.7 a	80.7 bc
T. reesei	46.6 c	38 g	21.7 g	35.6 g
Tr-UVN-1	98.3 a	58.4 e	95.3 a	98 a
Tr-UVN-2	96.2 a	60.3 de	89 b	97.2 a
Tr-UVN-3	56.7 b	82.3 b	53.6 e	73.3 c
Tr-UVN-4	97.4 a	98.2 a	72.3 c	98 a

[#] the test was achieved by Dual culture technique.
[##] Means within columns followed by different letters are significantly different ($p<0.05$).

While data in Figures (3 & 4) indicated that, the growth of *F. oxysporum* significantly inhibited on PDA supplemented with 50 % culture filtrate of the mutants ranged from 88.3% in Tk-UVN-3 to 99.5% in Tr-UVN-2 compared to their wild type strains, *T. koningii* (63.4%) and *T. reesei* (58.4%).

The mutants Tk-UVN-2 and Tr-UVN-4 caused the highest reduction rate 99.8% and 98.2%, respectively against *P. ultimum* growth compared to their wild type strains, *T. koningii* (46%) and *T. reesei* (38%) which resulted in a minute growth reduction. Whilst, the other mutants caused reduction rate ranged from 40.6% (Tk-UVN-1) to 82.3% (Tr-UVN-4) Table (4). On the other hand the growth of *P. ultimum* was significantly inhibited on PDA supplemented with 50 % culture filtrate of the selected mutants that ranged from 75.3% (Tk-UVN-1) to 90.5% (Tk-UVN-2) compared to their wild type strains, *T. koningii* (56%) and *T. reesei* (44%) Figures (3 & 4).

The two parent strains *T. koningii* and *T. reesei* caused a minute growth reduction rate of *S. rolfsii* growth reached to 23.3 and 21.7%, respectively. Whilst, the mutants caused the highest reduction rate of *S. rolfsii* growth ranged between 89% (Tr-UVN-2) to 98.3% (Tk-UVN-2) Table (4). On the other hand, the growth of *S. rolfsii* significantly inhibited on PDA supplemented with 50 % culture filtrate of the selected mutants that ranged from 89.3% (Tk-UVN-1) to 100% (Tr-UVN-3) compared to their parent strains, *T. koningii* (68.6%) and *T. reesei* (48.4%) except mutant Tr-UVN-3 that showed 53.6% (Figures 3 & 4).

Also, the two parent strains *T. koningii* and *T. reesei* caused a mediate growth reduction rate of *S. sclerotiorum* reached to 55.7 and 35.6%, respectively. Whilst, the tested mutants caused the highest reduction rate ranged from 73.3% (Tr-UVN-3) to 99.2% (Tk-UVN-1) against *S. sclerotiorum* growth

compared to their parent strains Table (4). On the other hand, the growth of *S. sclerotiorum* significantly inhibited on PDA supplemented with 50 % culture filtrate of the selected mutants ranged between 84.5% (Tk-UVN-3) to 92.4% (Tr-UVN-2) compared to their parent strains *T. koningii* (67.8%) and *T. reesei* (64.5%) Figures (3 & 4).

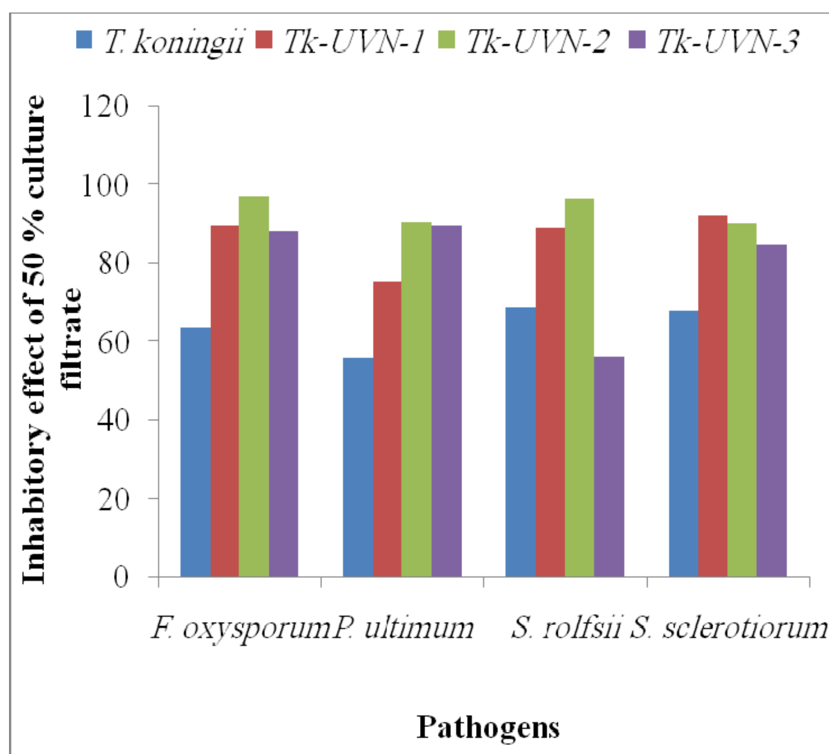

Fig 3. Inhibition of root-rot and wilt diseases pathogens by culture filtrates of *T. koningii* and their selected mutants.
[#] the test was achieved by Filtrate inhibition technique.

Antagonistic effects of selected fusants against the tested pathogens: Data given in Table (5) showed that the wild type strain *T. koningii* caused the higher reduction rate against *F. oxysporum* growth than *T. reesei* which resulted in a minute growth reduction. Whilst, the fusants; F2, F3, F5, F8, F12 and F13 caused the highest reduction rate that ranged from 96.4% to 98.6% against *F. oxysporum* growth compared to their two parents and other fusants that caused growth reduction ranged from 48.2% (F11) to 90.4% (F6).

The parent *T. koningii* caused higher reduction rate against *P. ultimum* growth than the other parent *T. reesei* which caused a minute growth reduction. Whilst, the fusants; F3, F4 and F13 caused the highest reduction rate (94.2, 90.4 and 94.6%, respectively) against *P. ultimum* growth compared to their two parents and other fusants that caused reduction ranged from 36.8% (F11) to 80.3% (F5) Table (5).

Also, the parent *T. koningii* caused higher reduction rate against *S. rolfsii* growth than the other parent *T. reesei* which resulted in a minute growth reduction. Whilst, the fusants; F2, F3, F4, F5, F8, F9, F10, F12 and F13 caused the highest reduction rate ranged between 90.2% (F13) and 98.2% (F10) against *S. rolfsii* growth compared to their two parents and other fusants that caused reduction ranged from 31.8% (F11) to 80.2% (F6) Table (5).

At the same trend, the parent *T. koningii* caused higher reduction rate against *S. sclerotiorum* growth than other parent *T. reesei* which showed reduction in a minute growth reduction. Whilst, the fusants; F3, F4, F8 and F13 caused the highest reduction rate ranged between 92.8% and 99.3% against *S. sclerotiorum* growth compared to their two parents and other fusants that showed reduction ranged from 45.4% (F11) to 87.6% (F2) Table (5).

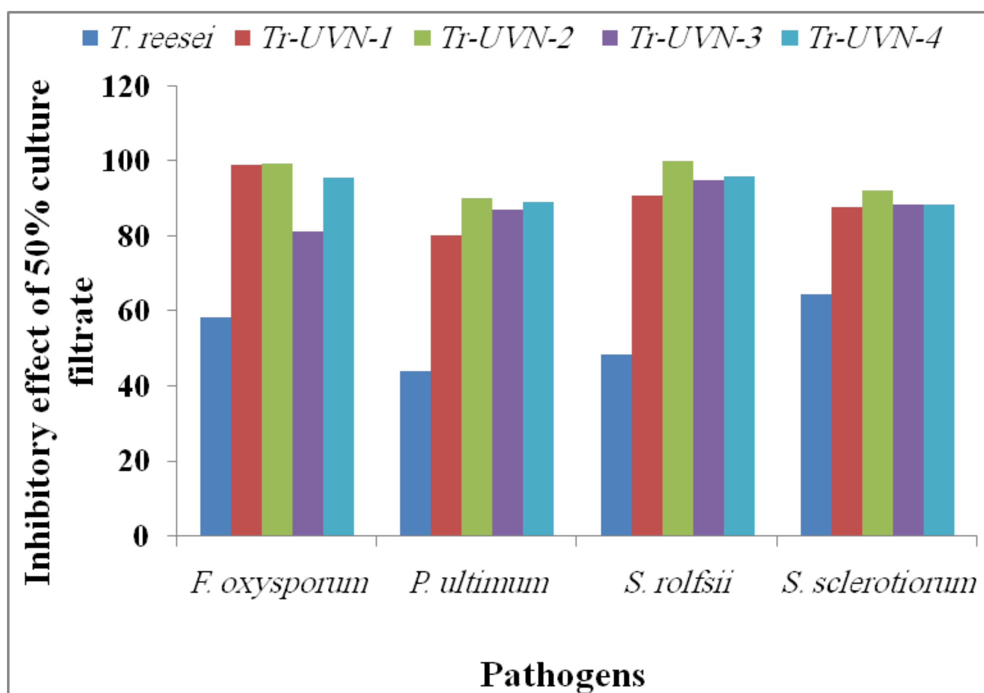

Fig 4. Inhibition root-rot and wilt diseases pathogens by culture filtrate of *T. reesei* and their selected mutants. [#] the test was achieved by Filtrate inhibition technique.

Reduction effect of the selected fusants against the tested pathogens:

Table 5. Inhibition of fusants and their parents against root-rot and wilt diseases pathogens.

Trichoderma species Wild / Fusant	**Pathogen**			
	F. oxysporum	*P. ultimum*	*S. rolfsii*	*S. sclerotiorum*
	Growth reduction (%)			
Parental strains				
T. koningii	52.6 cd	46 e	23.3 e	55.7 e
T. reesei	46.6 d	38 f	21.7 e	35.6 g
Medium	49.6 d	42 e	22.5 e	45.65 f
Fusants				
F1	48.8 d	49.4 d	71.2 c	58.6 e
F2	98.6 a	38.6 f	95.8 a	87.6 b
F3	96.4 a	94.2 a	97.6 a	99.3 a
F4	52.6 cd	90.4 a	90.8 ab	98.4 a
F5	98.5 a	80.3 b	92.7 ab	77.8 c
F6	90.4 b	82.4 b	80.2 b	78.3 c
F7	56.4 cd	50.2 d	33.2 e	65.4 d
F8	98.4 a	44.8 e	94.6 ab	97.5 a
F9	90.8 b	94.6 a	95.8 a	86.4 b
F10	60.3 c	60.4 c	98.2 a	84.7 b
F11	48.2 d	36.8 f	31.8 e	45.4 f
F12	96.4 a	60.2 c	96.4 a	86.7 b
F13	98.4 a	64.6 c	90.2 ab	92.8 a

[#] the test was achieved by Dual culture technique.
[##] Means within columns followed by different letters are significantly different ($p<0.05$). *Growth on PDA medium as negative control.

On the other hand, data in data in Table (6) indicated that, the growth of *F. oxysporum* significantly inhibited on PDA supplemented with 50 % culture filtrate of fusants; F2, F3, F5, F8, F10. F12 and F13 ranged from 90.2% (F8) to 98.4% (F13) compared to their parent strains, *T. koningii* (52.6%) and *T. reesei* (46.6%). Whilst, other fusants caused medium inhibition rate against *F. oxysporum* growth ranged from 60.2 % (F11) to 88.2% (F6), but fusant (F1) caused in a minute growth reduction than their two parents.

Also, the growth of *P. ultimum* significantly inhibited on PDA supplemented with 50 % culture filtrate of the fusants; F3, F4, F9, F10 and F13 ranged from 88.2% (F4) to 92.4% (F13) compared to their wild type strains, *T. koningii* (52.6%) and *T. reesei* (46.6%). Whilst, other fusants caused medium inhibition rate against *P. ultimum* growth ranged from 60.4 % (F8) to 78.6% (F12), but fusant (F1) caused a minute growth inhibition than its two parents and fusant F11 that caused inhibition rate ≈ the parent (Tk) Table (6).

At the same trend, the growth of *S. rolfsii* significantly inhibited on PDA supplemented with 50 % culture filtrate of fusants; F2, F3, F8, F9, F12 and F13 ranged from 90.2% (F8) to 99.8% (F13) compared to their parent strains *T. koningii* (52.6%) and *T. reesei* (46.6%). Whilst, other fusants caused a minute growth inhibition rate ranged from 38.6% (F6) to 50.2% (F11), but fusant (F7) caused medium inhibition rate than its two parents also, fusant (F5) ≈ the parent (Tk) and fusant (F10) caused inhibition rate equal the medium of the two parents Table (6).

The data was indicated that, the growth of *S. sclerotiorum* significantly inhibited on PDA supplemented with 50 % culture filtrate of the selected fusants; F3 (95.6%) and F8 (94.3%) compared to their wild type strains, *T. koningii* (52.6%) and *T. reesei* (46.6%). Whilst, other fusants caused medium inhibition rate against *S. sclerotiorum* growth ranged from 72.6% (F1) to 89.2% (F12), except fusant F6 (56.8%) caused inhibition rate ≈ the parent (Tk) and fusant F11 (67.5%) caused a minute growth inhibition than its two parents Table (6).

Table 6. Inhibition of root-rot and wilt diseases pathogens by culture filtrates of the fusants and their parents.

Trichoderma species Wild / Fusant	**Pathogen**			
	F. oxysporum	*P. ultimum*	*S. rolfsii*	*S. sclerotiorum*
	Inhibitory effect of 50 % culture filtrate			
Parental strains				
T. koningii	63.4 d	56 e	68.6 c	67.8 d
T. reesei	58.4 d	44 f	48.4 f	64.5 ef
Medium	60.9 d	50 f	58.5 e	66.2 e
Fusants				
F1	54.2 e	62.4 d	43.8 fg	72.6 d
F2	94.8 b	66.8 cd	98.6 a	88.3 b
F3	98.2 a	88.6 b	92.6 b	95.6 a
F4	76.4 c	90.8a	37.6 g	82 c
F5	96.8a	28.4 g	68.4 d	85.4 bc
F6	88.2 b	38.4 fg	38.6 g	56.8 f
F7	66.4 cd	60.4 d	72.6 c	77.8 cd
F8	90.2 b	70.4 c	90.2 b	94.3 a
F9	96.4 a	88.4 b	95.8 ab	88.5b
F10	90.4 b	90.4 a	58.4 e	74.8 d
F11	60.2 d	46.8 f	50.2 ef	67.5 e
F12	96.8 a	78.6 c	90.8 b	89.2 b
F13	98.4 a	92.4 a	99.8 a	82.6 c

[#] the test was achieved by Filtrate inhibition technique.

[##] Means within columns followed by different letters are significantly different ($p<0.05$). *Growth on PDA medium without filtrate as negative control.

DISCUSSION

The present work aimed to apply mutagenesis and protoplast fusion techniques in genetically breeding program of locally isolated *T. koningii* and *T. reesei* to enhancement their biocontrol abilities against several important plant fungal pathogens. Losses due to plant diseases may be as high as 10–20% of the total worldwide food production every year, resulting in economic losses amounting to many billions of

dollars and diminished food supplies. Whereas, chemical control involves the use of chemical pesticides to eradicate or reduce the populations of pathogens or to protect the plants from infection by pathogens. For some diseases chemical control is very effective, but it is often non-specific in its effects, killing beneficial organisms as well as pathogens, and it may have undesirable health, safety, and environmental risks [21].

So that biological control involves the use of one or more biological organisms to control the pathogens or diseases. Because biological control is more specialized and uses specific microorganisms that attack or interfere with the pathogens. The members of the genus *Trichoderma* are very promising against soil-born plant parasitic fungi. These filamentous fungi are very widespread in nature, with high population densities in soils and plant litters. They are saprophytic, quickly growing and easy to culture and they can produce large amounts of conidia with long lifetime [22].

A combination of UV-light and sodium nitrate was used to induce mutation where three and four mutants were isolated from the survivals of the mutagenized *T. koningii* and *T. reesei* strains, respectively (Table 1). After 7 subcultures the stable mutants with important morphological characters (i.e. growth, sporulation and antagonistic effect against *F. oxysporum, P. ultimum*, *S. rolfsii* and *S. sclerotiorum*) were obtained (Tables 2 & 4) and (Figures 3 & 4). This is presumably a consequence of their higher constitutive of metabolites activity. The results are harmonized with many successful attempts used the mutagenesis as tool to enhance the biocontrol efficacy of several *Trichoderma Spp.* e.g. [8, 12, 13, 18, 23, 24, 25, 26, 27].

The number of protoplast formation from the two parent strains *T. koningii* and *T. reesei* were 12.87 x 10^5 and 11.27 x 10^5, respectively. But the regeneration frequencies of the two parental strains were 5.01 x 10^5 and 11.27 x 10^5, respectively, i.e. regeneration frequency is the rate of grown colonies on CDS to rate of the protoplast formed. Hygromycin and Micanazol were used as selective markers for the tow parental fusion. The efficiency of protoplast fusion between *T. koningii* and *T. reesei* reached 1.25% (Table 3), i.e. fusion frequency that is the rate of grown colonies on SM to rate of grown colonies on CDS. The resistant behavior of the 13 selected fusants and their parental strains (*T. koningii* and *T. reesei*) was described. All the selected fusants were resistant to the selective markers (Hygromycin and Micanazol). The same trend of these results was achieved through protoplast formation isolation, fusion and regeneration of protoplasts from many *T. harzianum* species e.g. [6, 15, 28, 29, 30]. They reported that protoplast fusion have been achieved in the genus *Trichoderma,* mainly to enhance its biocontrol potential.

Subjecting the wild types of the collected bioagents *Trichoderma* spp. to *in vitro* bioassay found that, *T. koningii* and *T. reesei* were effective in suppressive the tested pathogens; *F. oxysporum*, *P. ultimum, S. rolfsii* and *S. sclerotiorum* that cause root-rot and damping-of diseases Tables (5 & 6). As noted, *Trichoderma* spp. is used for the control of plant diseases [31]. Also most of the selected mutant (Table 6 and Figures 3 & 4) or fusants (Tables 5 & 6) showed superiority in their antagonistic activity against the tested pathogens than their parental strains. In this context [12] reported that the mutagenesis of three *Trichoderma* species by exhibited high capabilities to produce efficient antibiotics, enzymes and phenols, corresponded to better onion white rot disease control in overall biocontrol ability. Also, Inter and intra hybrids of *Trichoderma spp.* by protoplast fusion were produced and evaluated of their biocontrol activity against soil-borne and foliar pathogens [9, 32, 33].

The antagonism of *Trichoderma* could be attributed to the competition for nutrients, release of toxic metabolites and extracellular lytic enzymes. Generally, *Trichoderma* species grow very faster than other fungi [34] and are reported to produce toxic substances like viridin, trichodermin, lytic enzymes, etc. [35, 36]. The secretion of extracellular enzymes such as chitinase is very well documented and its role on the biological control was established [12, 32, 37].

Conclusion: In conclusion, the superiority in biological control activities of the selected mutants or fusants than their parents (*T. koningii* and *T.* reesei) against the tested pathogens may be due to the effect of the genetic treatments (i.e. mutagenesis an protoplasts fusion) on their genetic back ground to be varied that allow to change in genetic control of antifungal metabolites production to be more effective. The results indicated that mutation and protoplast fusion techniques are successful tools to enhance the antagonistic effects of *Trichoderma* species against several fungal plant pathogens.

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