INDUCTION OF STABLE RECOMBINANTS OF *TRICHODERMA VIRENS* WITH ENHANCED BIOCONTROL CAPABILITY USING PROTOPLAST FUSION

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ABSTRACT

Two Fungicide tolerant mutants of *Trichoderma virens* (TVBen$^{R.3}$ andTVIp$^{R.7}$) were used as parental strains to improve their antagonistic activity via protoplast fusion. Protoplasts obtained from18-h-old mycelium treated by Novozyme 234 were fused by polyethylene glycol (PEG). The frequency of fusion resulting in double fungicide tolerant isolates was about 0.38 %. Analysis of fusants progeny showed that five fusants out of 21 did not exhibit any segregation but able to grow in the presence of both fungicides, indicating that these colonies are a result of a karyogamy (nuclear fusion). Two fusants resulting from a karyogamy were sable and the other three fusants were the result of temporary nuclear fusion formation that were unstable and did not loss through successive cycles of mitotic division during mycelial growth. Fusants derived from nuclear fusion were tested *in vitro* for their antagonistic activities against some plant pathogenic fungi (*Pythium altimum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Macrophomina phaseolina*). The fusants were generally equal or less active than their parents. Stability of the two double tolerant isolates (TVBen$^{R.3}$ and TVIp$^{R.7}$) was represented by their ability to maintain tolerance to fungicides after three subculturing in the absence of fungicides. However, these two isolates were superior strains with respect to antagonistic activities and biocontrol

capabilities than their parents. The present study shows that it is possible to obtain temporary nuclear fusion in which recombination events can occur. Subsequently, recombinant isolates could be obtained among segregant phenotype colonies produced from temporarily nuclear fused isolates with enhancement of biocontrol activities.

INTRODUCTION

Many *Trichoderma* species are of economic interest because of their ability to produce hydrolytic enzymes (Elad *et al.*, 1993; Schirmbock *et al.*, 1994) and antibiotic (Lin *et al.*, 1994). In fact, these fungi have been used on commercial basis as potential biocontrol fungi against different soil-borne pathogens (Papavizas 1985; Harman 1990; Anderson *et al.*, 2000). Furthermore, fungicides play a very important role in plant disease control. But chemicals, when they are applied in field, kill biological control agents, as well as disease agents, rather the pest pollution and arise of pesticide resistance biotyps. Therefore, there is now a great challenge to reduce the quantity of chemical pesticides used. Thus, biological control is one of the most attractive components of non-chemical means of plant disease control in modern agriculture. *Trichoderma* species have been identified as potential biocontrol fungi against soilborne plant pathogens (Papavizas, 1985; Harman, 1990 and Jensen and Wolffhechel 1995) and have already been used on commercial basis. However, superior strains are required for successful application. Consequently, it is useful to improve the antagonistic activities of *Trichoderma* strains for successful application in biological control.

Conventionally, improving of *Trichoderma* strains has utilized mutagenesis only (Papavizas *et al.*, 1990; Salama and Amer 1996) since crosses have not been successful and sexual stages are rare or lacking in most *Trichoderma* species (Goldman *et al.*, 1990 and Harman and Hayes 1993). Genetic recombination is a powerful means for developing strains with a broader range of antagonistic activity since the antagonistic activity of *Trichoderma* spp. is often limited to few plant pathogens (Migheli *et. al*, 1995). Protoplast fusion, provides an important opportunity for genetic recombination and manipulation of genetic materials to enhance its antagonistic activity. This approach, has been successfully used by Ogawa *et al.*, (1987) who suggested that protoplast fusion appears to be a useful

tool for combining desirable traits, suggesting the existence of a parasexual cycle and presented the possibility that diploidization occurred in *T. reesei.* Stasz *et al.*, (1988) and Peer and Chet (1990) using *T. harzianum* and Migheli *et. al.*, (1995) using *Trichoderma* spp. suggested that protoplast fusion appears to be a useful tool for combining desirable traits. Furlaneto and Pizzirani-Kleiner (1992) reported that recombinants were obtained from dikaryotic colonies suggesting the occurrence of a highly unstable diploid phase in *T. pseudokoningii.*

The present study aims to 1- prepare and fuse protoplasts of fungicide-tolerant mutants of *T. virens* in an attempt to induce heterokaryosis to isolate different recombinant fusants and obtain nuclear fusion products. 2- compare the antagonistic activity of parental, fusants and their third subculture isolates recovered from the original fusant *in vitro* in dual culture.

MATERIALS AND METHODS

Strains: *T. virens* $TVBen^{R.3}$ and $TVIp^{R.7}$ mutants were used as parental strains in fusion experiment. $TVBen^{R.3}$ mutant tolerated Benomyl up to 5 µg a.i. /ml (mycelial growth of the wild type was completely prevented at 0.5 ug a.i. / ml Benomyl). $TVIp^{R.7}$ mutant tolerated Iprodione up to 1000 µg a.i. / ml (mycelial growth of the wild type was completely prevented at 80 µg a.i./ml Iprodione). The pathogen cultures; *Pythium altimum, Rhizoctonia solani, Sclerotium rolfsii,* and *Macrophomina phaseolina,* were kindly obtained from Prof. Dr. S. Z. Khalifa, Department of Plant Pathology, Fac. Of Agric., Minufiya University.

Media: Basal medium (BM) used, was as described by Toyama *et al.*, (1984) which contained, per liter, 2.8 g $(NH_4)\ SO_4$, 600 mg urea, 4 g KH_2PO_4, 600 mg $CaCl_2.2H_2O$, 40 g glucose, 200 mg $MgSO_4$, 10 mg $FeSO_4.7H_2O$, 2.8 mg $ZnSO_4.H_2O$, 3.2 mg $MnSO_4.H2O$, 4 mg $CoCl.6H_2O$, and 2 % agar. Selective basal medium supplemented with fungicide, Benomyl and/or Iprodione and designated as BM+Beno, BM+Ip or BM+Beno+Ip was also used. Adding 100-g/l sucrose to BM as an osmotic stabilizer made protoplast regeneration minimal medium (PRMM). Protoplast regeneration selective media

were PRMM supplemented with fungicide(s), Benomyl and/or Iprodione and designated as PRMM+ Beno, PRMM+Ip or PRMM+Beno+Ip. Potato dextrose agar (PDA) and Potato dextrose broth (PDB) were employed as a growth medium. Sand-wheat bran medium was used to multiply both *F.oxysporum* f.sp. *lycopersici* and bioagent for greenhouse experiment.

Protoplast preparation: Preparation of *T. virens* protoplasts was carried out separately from $TVBen^{R3}$, $TVIp^{R7}$ and wild type according to the method of Stasz *et al.*, (1988). Young conidia were grown with shaking for 18 h in PDB containing 100 µg ampicilin + 50 µg streptomycin / ml to avoid bacterial contamination. The mycelium was harvested, washed with cold 0.7 M NaCl solution, and suspended at concentration of 100 mg wet weight / ml in lytic solution (0.7 *M* NaCl + 13.5 mg / ml of Novozyme 234), then incubated for 3 h at 30 °C with gentle agitation. Protoplasts were separated out of cell debris by filtration through four layers of cheesecloth and washed twice in 0.7 *M* NaCl solution by centrifugation at 3000 rpm for 15 min at 4 °C. Protoplasts were counted, centrifuged and resuspended in STC buffer containing 0.6 *M* sorbitol, 0.01 *M* Tris-HCl and 0.01 *M* $CaCl_2$, pH 7.5, density was adjusted up to 10^6 / ml using a heamocytometer and kept on ice until used.

Microscopic observation of nuclei: protoplasts from filamentous fungi are heterogeneous with respect to the number of nuclei they contain. The number of nuclei per *T. virens* protoplast was counted after 4,6-diamino-2-phenylindolem dihydrochloride (DAPI) staining. About 10^3 protoplasts were resuspended in 50 µl STC buffer (100µg/ml H_2O). The suspension was kept in the dark, at room temperature, for 2-3 h., then, protoplasts were observed under a fluorescent microscope at 365 nm.

Protoplast regenerability: Viable numbers of protoplasts, which were able to give rise to colonies on PRMM, were determined by preparing appropriate dilutions in STC buffer. Aliquots of 100 µl overlay PRMM + 2 % top agar were poured over a layer of PRMM or PRMM supplemented with one of the used fungicide(s). Plates were incubated for one week at 25 °C. Developing colonies were

counted. Similar dilution in distilled water provided a control to distinguish colonies produced from protoplasts and those produced from conidia and hyphal fragments (protoplast rupture upon exposure to water, whereas walled propagules do not).

Protoplast fusion: Protoplasts were fused using a procedure similar to that described by Pe'er and Chet (1990). One ml of suspension containing about 10 protoplasts in STC was prepared in 50-ml polypropylene conical tube, with about equal number of protoplast from each mutant. Then 200 µl of PEG solution (60 % w/v polyethylene glycol 4000, 10 *mM* $CaCl_2$ and 10 *mM* Tris-HCl, pH 7.5) were added in a stepwise fashion with gently rolling the tube. Then 500 µl of PEG solution were added, the mixture was gently mixed and finally 500 µl of PEG solution were gradually added to obtain 33 % final PEG concentration. After 10 min of incubation at 30°C, the mixture was diluted with 1.1 ml of STC and mixed gently, this dilution step was repeated. Finally, 2.2 ml of STC were added, protoplasts were pelleted by centrifugation at 4000 rpm at 4°C for 10 min and resuspended in 2 ml of STC. Fusion frequency was calculated by percentage of colonies appearing on PRMM + Beno + Ip in relation to mean of sum total of that appearing on PRMM + Beno or PRMM + Ip.

Fusant colony characterization: The double fungicide tolerant colonies which developed on PRMM+Beno+Ip were a consequence of either cytoplasmic fusion (heterokaryosis) of parental fused cells or their karyogamy (nuclear fusion). To distinguish between these cases, the fusant colonies were picked up and allowed to grow for sporulation on PDA supplemented with 50 µg a.i. Benomyl and 500 µg a.i. Iprodione / ml. From each fusant, about 50 single conidium colonies were isolated and analyzed for tolerance to Benomyl and/or Iprodione on PDA fungicide supplementation, since each conidium receives only a single nucleus from the conidiophore (Picataggio *et al.*, 1983).

Nuclear fusion stability: Stability of the fusants obtained by nuclear fusion was studied. The original fusant isolates were subcultured five times through their conidia on PDA medium containing both fungicides. Conidial germination percentage of five isolation

subcultures was measured on PDA medium containing Benomyl and Iprodione and compared to those of the same original fusant. Distribution of both tolerant markers was taken as indication for stability.

Antagonistic ability of fusant isolates: The antagonistic ability of both original fusant isolates and isolates recovered from their third subculture in relation to the parental strains was assessed against some plant pathogens in dual culture (Fokkema, 1973).

One week-old PDA cultures of fusant isolates, parental strains and the four pathogens were used as sources of inoculum. A disk of biocontrol fungi (4mm) diameter was placed onto 20 mm from the edge of PDA plates (9 x 20 mm). A disk of pathogen was placed 50 mm away from the biocontrol agent disk and cultures were incubated in the dark at 25 ±1 °C until growth of pathoge n completely covered the control plates. The inhibition percentage of pathogen(s) radial growth was taken as an index of antagonistic ability. Calculated by the formula [(R1 - R2) / R1]100, where R1 the maximum radius of the colony of the pathogen, R2 is the radius of that part of the pathogen colony directly opposite the colony of the biocontrol fungi (Zhou and Reeleder 1990).

RESULTS AND DISCUSSION

Protoplasts from 18-h-old mycelium of *T. virens* wild type, $TVBen^{R.3}$ and $TVlp^{R.7}$ strains were released at rate ranged from 8.2×10^5 to 1.6×10^6 protoplasts / 100 mg wet weight of mycelia using Novozyme 234. Result in Figure (1) show that the wild type and both mutant strains had similar patern of nuclei number per protoplast. About 20 % of protoplasts were anucleate. While 61, 6, 5, and 8 % had one, two, three or more than three nuclei per protoplast, respectively. Accordingly, about 76 % of the nucleated protoplasts had a single nucleus.

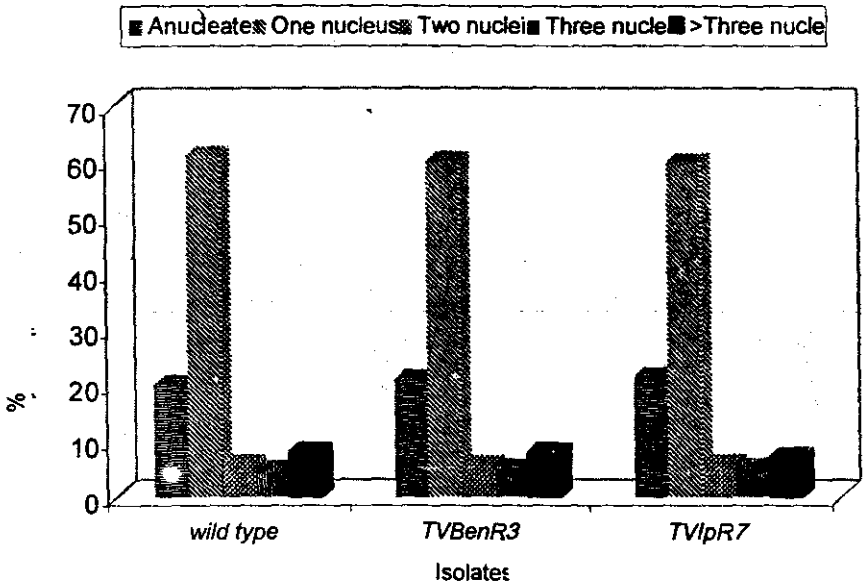

Figure 1: Percentage of nuclei number per protoplast of *Trichoderma virens* protoplasts isolated from wild type, two fungicide tolerant mutants TV.Ben$^{R.3}$ and TV.Ip$^{R.7}$.

These results are in harmony with the results of Salama (1997) using *T. lignorum* and Sal[illegible]a *et al.*, (2006) using *T. harzianum*. However, Stasz *et. al.*, (1988) reported that the protoplasts derived from 16-h-old mycelium of *T. harzianum* contained 2-12 nuclei. This disagreement could be according to differences in *Trichoderma* species.

Regeneration frequencies were determined as viable numbers of protoplasts that were able to give rise to colonies on PRMM. On fungicide(s) free PRMM, 2.0×10^4 (2 %), 1.5×10^4 (1.5 %) and 1.4×10^4 (1.4 %) colonies arose per 10^6 protoplasts from wild type and both mutants (*TVBen*$^{R.3}$ and *TV*$_{..}$$^{R.7}$), respectively (Table [illegible]). However, on PRMM containing either fungicide showed diffe[illegible] responses with mutant isolates and it was completely inhibited [illegible] wild type isolate.

Table 1: Number of regenerated colonies per 10^6 protoplasts derived from *Trichoderma virens* fungicide tolerant mutants and their wild type.

Strains-derived Protoplast	No. of regenerated colonies on PRMM supplemented with		
	None	Ben	Ipro
Wild type	2.0×10^4	0.00	0.00
TV.Ben$^{R.3}$	1.5×10^4	0.6×10^4	0.00
TV.Ip$^{R.7}$	1.4×10^4	0.00	0.9×10^4

TV.Ben$^{R.3}$, *T. virens* Benomyl tolerant mutant; TV.Ip$^{R.7}$, *T. virens* Iprodione tolerant mutant; benomyl, 3 ug a.i. (active ingredient) Benomyl/ ml; Ipro, 20 ug a.i. Iprodione / ml; PRMM, protoplast regeneration minimal medium.

Regeneration frequencies of mutants-derived protoplasts showed slight decrease compared with regeneration frequencies on fungicide(s) free PRMM (Table 1). Salama 1997 and Amer and Salama 2000 found regeneration frequencies of 1.9 and 2.0 % using *T. lignorum* and *T. hamatum*, respectively. However, it was 10 % by Stasz *et al.*, (1988) using *T. harzianum*. This difference may be due to the laboratory conditions and/or strain used.

Protoplasts of both fungicide tolerant mutants, *TVBen*$^{R.3}$ and *TVIp*$^{R.7}$ were fused and plated onto PRMM. Fusion frequencies were estimated by the percentage of number of colonies developed on PRMM + Ben + Ip over the mean number of colonies appearing on PRMM + Beno or PRMM + Ipro. In presence of both fungicides, number of colonies arose was considerably reduced by about 225-400 times compared with the presence of either fungicide (Table 2).On PRMM + Beno or PRMM + Ipro 1.14 x 10^3 or 2.26 x 10^3 colonies arose per 10^6 of fused protoplast, respectively, compared with 6.4 colonies on PRMM containing both fungicides (Table 2).

Table 2: Fusion frequency following the fusion of protoplasts from *Trichoderma virens* fungicide tolerant mutants.

Experiment	No. of colonies appearing on PRMM supplemented with				Fusion frequency (%)
	None	Ben	Ipro	Ben +Ipro	
1	5×10^3	1.1×10^3	2.5×10^3	8	0.44
2	4.1×10^3	1.0×10^3	2.2×10^3	4	0.25
3	4.4×10^3	1.3×10^3	1.9×10^3	7	0.44
4	3.8×10^3	1.1×10^3	2.4×10^3	6	0.34
5	4.0×10^3	1.2×10^3	2.3×10^3	7	0.40
Mean	5.24×10^3	1.14×10^3	2.26×10^3	6.4	0.38

Ben, 3 µg a.i. (active ingredient) Benomyl / ml; Ipro, 20 µg a.i. Iprodione / ml; PRMM, protoplast regeneration minimal medium.

Fusion frequency was about 0.38 %. Consequently, colonies appearing in presence of both fungicides are probably due to protoplast fusion or complementation between the fused parents. The complementation may be owing to either heterokaryosis or karyogamy (Pe'er and Chet 1990). Similar findings were obtained using *T. hamatum* by Amer and Salama 2000.

To distinguish between the two events, about 50 single conidium colonies produced from each of the twenty one fusant colonies that appearing in presence of both fungicides were analyzed for their tolerance to fungicides (Table 3). Five fusants out of 21 (23.8 %) did not segregate and were able to grow in the presence of either Benomyl or Iprodion ,as well as, in the presence of both fungicides, indicating that these colonies are a result of a karyogamy (nuclear fusion). Whereas, the remaining 16 fusants, (84.2 %) tended to segregate to parental types characteristic and no colonies were developed in the presence of both fungicides. Moreover, the appearance of parental types colonies in equal numbers suggests the

occurrence of cytoplasmic fusion (heterokaryosis) of parental fused cells. Similar results were obtained by Amer and Salama 2000, Peer and Chet, 1990; Salama 1997 and Stasz *et al.*, 1988, who reported that the heterokaryotic colonies appearing into two non-fusant types, in approximately equal numbers, inferring nuclei from both parental strains were present in approximately equal numbers.

Table 3: Analysis of single conidium colonies derived from *Trichoderma virens* fusant isolates to distinguish between cytoplasmic fusion (heterokaryosis) and nuclear fusion (karyogany).

Fusants	No. of tested colonies	% of colonies grown on PDA supplemented with			
		None	Ben	Ipro	Ben + Ipro
Fu-1	46	95.2	88.2	91.0	85.2
Fu-2	51	93.9	90.0	87.3	82.3
Fu-3	42	91.9	84.9	90.1	80.0
Fu-4	46	91.0	89.6	86.7	84.6
Fu-5	41	96.0	87.7	88.9	88.1
Fu-6 to Fu-21	50 to 60	~ 50.0	~ 50.0	~ 50.0	00.0

Fu, fusant produced from Benomyl tolerant mutant (TV-Ben$^{R.3}$) or Iprodione tolerant mutant (TV-Ip$^{R.7}$); Ben, 5 µg a.i. (active ingredient) Ben/ml; Ipro, 500 ug a.i. Iprodione/ml; PDA, potato dextrose agar medium.

Genetic stability of the five double tolerant (nuclear fusion) isolates was represented by their ability to maintain tolerance to fungicide after three subculturing in the absence of fungicides. Two fusants, TV-Fu.1 and TV-Fu.2, were retains the double fungicide tolerance, whereas, the remaining three fusants lost their double fungicide tolerance after the three subculturing (Table 4). These results suggested that the three fusants probably are the result of temporary nuclear fusion formation that was unstable and did not loss through successive cycles of mitotic division during mycelial growth.

Table 4: Behavior of Benomyl and Iprodionee tolerant markers in *Trichoderma virens* nuclear fusants after third successive subculturing.

Fusant	% of conidial germination on PDA supplemented with	
	None	Ben + Ipro
TV-Fu-1	93.5	92.4
Fu-1 ^{3th}	93.9	91.8
TV-Fu-2	90.4	90.0
Fu-2 ^{3th}	90.1	89.3
TV-Fu-3	91.1	91.6
Fu-3 ^{3th}	90.5	00.0
TV-Fu-4	92.1	91.2
Fu-4 ^{3th}	90.4	00.0
TV-Fu-5	95.2	92.4
Fu-5 ^{3th}	91.9	00.0

Fu, fusant produced from Benomyl tolerant mutant (TV.Ben$^{R.3}$) × Iprodione tolerant mutant (TV.Ip$^{R.7}$); Fu^{3th}, isolate produced from the third successive subculturing of original fusant; Ben, 5 µg a.i. (active ingredient) Benomyl/ml; Ipro, 500 µg a.i. Iprodione/ml; PDA, potato dextrose agar medium.

However, recombination events might have taken place during the short period of transient nuclear fusion. Subsequently, recombinant isolates could be obtained from segregant phenotype colonies produced from nuclear fusion isolates. Similar findings were observed by Amer and Salama 2000; Stasz *et al.*, (1988); Pe'er and Chet (1990) and Salama (1997). The possibility of genetic recombination that happens through a parasexual cycle and the occurrence of a highly unstable diploid phase has been discussed by Furlaneto and Pizzirani-Kleiner (1992) in *T. pseudokoningii*, Ogawa *et al.*, (1987) in *T. reesei*, and Picataggio *et al.*, (1983) in *T. reesei*.

The antagonistic activity of the parental isolates, fusant isolates and isolates recovered from the third subculture of original fusants

was assessed against soilborne pathogenic fungi in dual culture. Results in Table (5) show that the isolates gave different antagonistic activity responses against the five pathogens used. All fusants had a low significant inhibition percentage of radial growth of the pathogens in respective to parental strains, except TV-Fu.5 fusant which exhibited a significantly high inhibition percentage against *Pythium altimium* only. These results infer that the nuclear fusants may create unbalanced genotypes. On the contrary, all isolates recovered from the third subculturing significantly inhibited the growth of the four pathogens used. Salama (1997) obtained similar finding. Such results could be explained on the basis that genetic recombination occurred. Fusants produced recombinants as far as ability to inhibit radial growth of the tested pathogens are concur in spite of the fact that such subculturing resulted in instability when tested to both fungicides.

Table 5: Percent inhibition of radial growth of some plant pathogenic fungi by *Trichoderma virens* fusant and their recombinant isolates.

Parent, Fusant and recombinant isolates	Radial growth inhibition (%)			
	Pythium altimum	*Rhizoctonia solani*	*Sclerotium rolfsii*	*Macrophomina phaseolina*
TV.Ben$^{R.3}$	56.8^{g}	62.3^{f}	53.5^{f}	66.8^{f}
TV.Ip$^{R.7}$	59.3^{f}	59.0^{g}	50.9^{g}	65.3^{g}
Fu-1	52.7^{k}	54.8^{i}	49.4^{h}	58.6^{h}
Fu-2	54.6^{j}	52.1^{k}	48.7^{i}	56.8^{i}
Fu-3	56.0^{h}	50.6^{l}	47.1^{j}	55.9^{k}
Fu-4	55.4^{i}	53.2^{j}	46.8^{k}	54.1^{l}
Fu-5	60.0^{e}	57.7^{h}	45.6^{l}	56.3^{j}
Fu-1 ^{3th}	60.2^{d}	65.5^{c}	59.1^{b}	71.2^{d}
Fu-2 ^{3th}	61.3^{c}	64.1^{e}	56.8^{e}	74.2^{a}
Fu-3 ^{3th}	68.3^{a}	67.0^{b}	57.9^{c}	73.4^{b}
Fu-4 ^{3th}	61.2^{c}	69.2^{a}	57.5^{d}	69.7^{c}
Fu-5 ^{3th}	65.1^{b}	64.9^{d}	61.0^{a}	73.2^{b}

TV.Ben$^{R.3}$, *T. virens* Benomyl tolerant mutant; TV.Ip$^{R.7}$, *T. virens* Iprodione tolerant mutant; Fu, fusant produced from TV.Ben$^{R.3}$ × TV.Ip$^{R.7}$; Fu^{3th}, isolate produced from the third successive subculturing of original fusant. Means within a column followed by the same litter(s) are not significantly differ at the P = 0.05 level according to the Least Significant Difference Test (Snedecor and Cochran, 1967).

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إستحداث اتحادات وراثية ثابته و زيادة مقدرتها فى المقاومة الحيوية فى التريكودرما فيرنس باستخدام الدمج البروتوبلاستى

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أجرى هذا البحث لايجاد توليفات وراثية جديدة ثابته وذات كفاءة أفضل فى المقاومة الحيوية لفطر التريكودرما فيرنس باستخدام الدمج البروتوبلاستى. استخدمت طفرتان من الفطر الأولى؛ $TVBen^{R.3}$ المتحملة لتركيزات عالية من المضاد الفطرى بينوميل و الثانية $TVIp^{R.7}$ والمتحملة لتركيزات عالية من المضاد الفطرى إيريديون كسلالات أبوية معلمة فى تجارب الدمج الخلوى. تم عزل البروتوبلاست من خيوط ميسيليوم عمره ١٨ ساعة لكل من الطفرتين والسلالة البرية باستخدام انزيمات نوفوزيم 234 ثم تم احداث الدمج باستخدام البولى ايثيلين جليكول. وقد نتج عنه عزلات متحملة لتركيزات عاليةمن كلا المضادين الفطريين وكان ذلك بمعدل ٠,٣٨ %. وقد أظهر تحليل النسل الناتج من السلالات المدمجة خمسة عزلات من مجموع ٢١ عزلة لم يحدث بها انعزال وأمكنها النمو على فى وجود كل من المبيدين، مما يشير الى أنها ناتجة عن دمج الأنوية. بينما الـ ١٦عزلة الأخرى حدث بها انعزال الى نمط العزلات الأبوية، حيث لم تتمكن من النمو فى وجود كل من المبيدين. كما أظهرت النتائج أن عزلتان فقط من الخمس عزلات كانت ثابتة وراثيا بينما الثلاث الباقية كانت ناتجة عن دمج نووى مؤقت، حيث لم يحدث لها ثبات أثناء دورات الانقسام الميتوزى المتعاقبة أثناء نمو الميسيليوم. وتم اختبار قدرة التضاد الحيوى للعزلتين الناتجتين عن دمج نووى ضد بعض الممرضات النباتية (بيثيم التيميم و ريزوكتونيا سولانى و سكلروتيم رولفسياى و ماكروفومينا فاصولينا) وذلك تحت ظروف المعمل. وقد إظهرت النتائج أن هاتين العزلتين كانتا أعلى من الأبوين فى قدرتها على التضاد. عموما، أظهرت الدراسة أنه من الممكن الحصول على عزلات حدث لها دمج نووى مؤقت والذى خلاله يحدث اعادة توزيع وتكوين اتحادات وراثية جديدة بين التراكيب الأبوية مما يؤدى الى تحفيز أنشطة التضاد الحيوى للتريكودرما.