

Influence of Protoplast Fusion in *Trichoderma* Spp. on Controlling Some Soil Borne Diseases

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Abstract

Protoplasts from *Trichoderma harzianum* NBAIL Th 1 and *T. viride* NBAIL Tv 23 were isolated using lysing enzymes. Protoplast fusion of *T. harzianum* and *T. viride* was carried out. The fused protoplasts generated and 21 fusant isolates were used to study their antagonistic activity and RAPD-PCR characterization in comparison with their parents. Among the fusant isolates, F7 isolate produced maximum growth inhibition pathogen (one and half-fold increase as compared to the parent strains). All the tested fusants isolates exhibited increased antagonistic activity against *Fusarium oxysporum* than the parent strains except fusant F21. Specific results for fingerprinting were obtained by the seven primers of RAPD. These markers produced different fragment patterns with varied number of bands and yielded a total of 79 distinct bands. Polymorphic bands came to be 16.5% whereas monomorphic bands came to be 83.5%. Moreover, the OPO-13 primer has showed the highest polymorphism 35.3%. While, the OPA-16 primer has shown the lowest polymorphism 9.0%. The Dendrogram based on RAPD marker results grouped the two parent strains and twenty-one fusants into two different clusters with about 83% genetic similarity. The first cluster contained the two parent strains and twenty fusants, and the second cluster contained the fusant F2. Our results in this study suggested that the protoplast fusion technique is useful for developing the superior hybrid strains and enhance antagonistic activity of *Trichoderma* spp. against tested pathogenic fungi.

Keywords: Protoplast fusion; RAPD marker; Biocontrol activity; Pathogenic fungi and *Trichoderma* spp.

Introduction

Trichoderma is one of the well-known fungal used as a biocontrol agent in controlling of plant diseases caused by some fungal pathogens. *T. harzianum* and *T. viride* are filamentous soil fungi that functions as a biocontrol agent for a wide range of economically important aerial and soil borne plant pathogens [1]. The mycoparasitic activity of this organism is attributed to a combination of successful nutrient competition, the production of cell wall-degrading enzymes, and antibiosis [2,3]. Several strains of the genus *Trichoderma* are being tested as alternatives to chemical fungicides [4]. However, full-scale application of *Trichoderma* for biological control of plant pathogens has not been widespread. At a molecular genetic level, attempts to increase the biocontrol ability of *Trichoderma* have been focused on increasing chitinase or proteinase activity either by increasing the number of copies of the appropriate genes or by fusing them with strong promoters (e.g., *pcbhl::ech42*) [3-6]. These strategies have not always resulted in the expected increase in biocontrol activity. Protoplast fusion is a good tool in *Trichoderma* spp. improvement and developing hybrid strains in other filamentous fungi [3,4]. Several reports confirmed that protoplast isolation and regeneration in different fungi. Moreover, strain improvement for high yield of chitinase and cellulase by exploiting protoplast fusion system also reported [7-9]. Protoplast requires specific conditions for isolation and regeneration such as temperature, incubation time and medium, etc. [10,11]. Recent development in molecular techniques have created new possibilities for the selection and genetic improvement of life stocks [5,7,12,13]. RAPD markers are dominant, that is, it is not possible to distinguish between homozygous loci. Heterozygous RAPD loci are observed as different sized DNA segments amplified from the same locus, are detected only rarely. RAPD technique is notoriously laboratory dependent and not reproducible. Template concentration/primer mismatch may result in decreasing the amount of PCR product even total absence of PCR product, and hence the results are difficult

to interpret [12,14-16]. The present study aimed to isolation and fusion of protoplast in *Trichoderma harzianum* NBAIL Th 1 and *T. viride* NBAIL Tv 23 for enhancement their biocontrol activity against some root rot and wilt causing pathogens which cause soil borne diseases. Furthermore, fingerprinting of parent strains and their corresponding fusants using RAPD marker.

Materials and Methods

Trichoderma samples

Two parental strains (*T. harzianum* NBAIL Th 1 and *T. viride* NBAIL Tv 23) were kindly gift from IARI, New Delhi and from MTCC, Chandigarh those two strains were done and 21 fusants were selected.

Protoplast preparation and fusion

Protoplasts preparation was carried out using fungicide tolerant isolates of *Trichoderma* parental strains (*T. harzianum* NBAIL Th 1 and *T. viride* NBAIL Tv 23) according to Stasz et al. [17]. Strains of *T. harzianum* (sensitive to carbendazim and resistant to Thiophanate-methyl) and *T. viride* (sensitive to Thiophanate-methyl and resistant to carbendazim) mycelia were subjected to lytic activity with different fungal enzyme such as β -glucuronidase and driselase at different conversation for 2 hrs with intermittent agitation [18]. The enzyme

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Received August 04, 2016; **Accepted** August 18, 2016; **Published** August 22, 2016

Citation: Lakhani HN, Vakharia DN, Makhoul AH, Eissa RA, Hassan MM (2016) Influence of Protoplast Fusion in *Trichoderma* Spp. on Controlling Some Soil Borne Diseases. J Plant Pathol Microbiol 7: 370. doi: 10.4172/2157-7471.1000370

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effectively acted on mycelia mass and produced protoplasts and it released soon after the lytic activity from the mycelia structure were found to be smaller in size and later gradually enlarged to a hyaline spherical structure. Protoplasts were fused according to Hassan [5].

Antagonistic activity of *Trichoderma* strains against some pathogenic fungi

The antagonistic activity of *T. harzianum* NBAII Th 1 and *T. viride* NBAII Tv 23 against *Rhizoctonia solani*, *Sclerotium rolfsii*, *Fusarium oxysporum* and *Macrophomina phaseolina* was determined by dual culture technique according to Fahmi et al. [19].

DNA extraction

Genomic DNA from *Trichoderma* parents and fusants were isolated as described [20] using CTAB extraction buffer.

RAPD analysis

RAPD analysis was performed using seven different primers. The name of RAPD primers are as the following: OPA-16, OPA-17, OPA-18, OPB-11, OPJ-07, OPK-03, OPO-13. The RAPD amplification reactions were performed as described in [15,21].

Data analysis

RAPD profiles were screened for generating polymorphism among the *Trichoderma* parents and fusants studied. Band position for each isolate and primer combination were scored as either present (1) or absent (0) for phylogenetic analysis using NTSYS-pc (Numerical Taxonomy and Multivariate analysis) system version 2.2 by Exeter software. The dendrogram of the genetic relatedness among *Trichoderma* parents and fusants was produced by means of the unweighted pair group method with arithmetic average (UPGMA) analysis [22].

Results and Discussion

Protoplast isolation

The enzyme effectively acted on mycelia mass and produced protoplasts and it released soon after the lytic activity from the mycelia structure they were smaller in size and later gradually enlarged to a hyaline spherical structure. The enzyme reacted with the cell wall of mycelial mass became swelled up and round hyaline globules by the

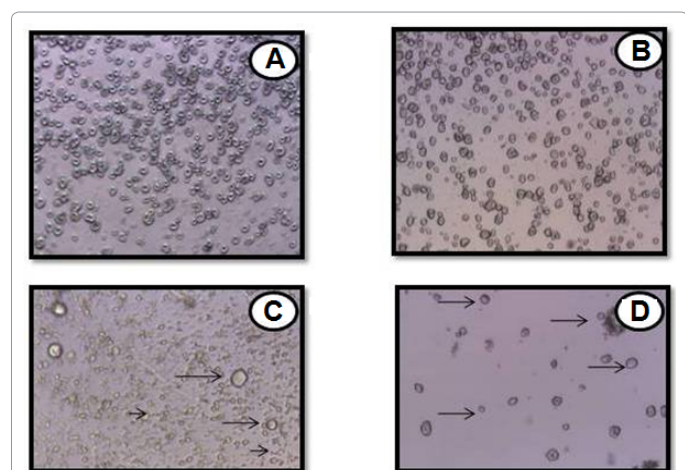


Figure 1: Protoplast isolation A) parental lysis of *T. harzianum* B) parental lysis of *T. viride* C) complete lysis *T. harzianum* D) complete lysis *T. viride*.

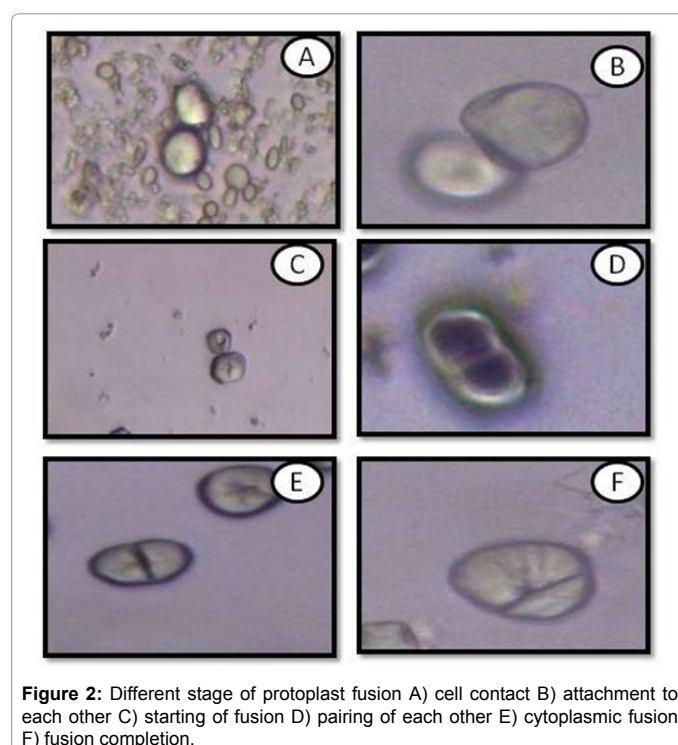


Figure 2: Different stage of protoplast fusion A) cell contact B) attachment to each other C) starting of fusion D) pairing of each other E) cytoplasmic fusion F) fusion completion.

action of these enzymes. The action of lytic enzymes on the mycelial mass depends on the used enzyme, its concentration, the incubation period and age of the mycelial mass [5,8].

The conditions for releasing the protoplasts were similar as reported earlier [5,10]. Swelling and rounding up of cell content were observed at initial stage and the lysis of mycelia started after 90 min (Figures 1A and 1B). Complete lysis of mycelia and release of protoplasts were observed after 3h (Figures 1C and 1D). The protoplasts released initially were smaller in size but later they enlarged to spherical structures.

Interestingly, the protoplasts yield was affected by many conditions as the concentrations of lysing enzymes. At low concentrations, the lysis of fungal mycelium was confined with a minimum release of protoplasts whereas at high concentrations, the mycelium lysed producing a large numbers of protoplasts. Among different concentrations of tested lysing enzymes, KCl as osmotic stabilizer was optimal for the release of protoplasts from different *Trichoderma* spp. However, in previous study highest protoplasts were obtained from *T. harzianum* using Novozym 234 at 10 mg/ml with 0.6 M KCl [18]. Other author used 10 mg/ml of Novozym 234 with 0.6 M sucrose to isolate maximum number of protoplasts from *T. harzianum* and *Trichoderma koningii* [7], and also, obtained maximum protoplasts from *Trichoderma roseum* using Novozym 234 in combination with chitinase and cellulase, each at 5 mg/ml [23].

Protoplast fusion

When adding the PEG solution to the protoplasts, they were attracted to each other and pairs of protoplasts were observed. Later, the plasma membrane at the place of contact dissolved and protoplasmic contents fused together, followed by nuclear fusion in most cases. Finally, the fused protoplasts became single, larger and round or oval shaped structures (Figure 2). Protoplasts fusion in *Trichoderma harzianum* NBAII Th 1 and *T. viride* NBAII Tv 23 has been achieved in the present study using 40% PEG. Similar concentration of PEG was reported as optimum for protoplasts fusion between different *Trichoderma* spp.,

Strains	Antagonistic averaged inhibition percentage %			
	<i>Rhizoctonia solani</i>	<i>Sclerotium rolfsii</i>	<i>Fusarium oxysporum</i>	<i>Macrophomina phaseolina</i>
<i>T. harzianum</i>	70	59	64	84
<i>T. viride</i>	69	63	65	86
Fusant 1	75	68	77	90
Fusant 2	79	76	80	89
Fusant 3	86	70	73	84
Fusant 4	86	76	87	89
Fusant 5	80	69	86	90
Fusant 6	65	69	90	93
Fusant 7	79	67	91	95
Fusant 8	58	72	83	92
Fusant 9	81	76	79	89
Fusant 10	56	78	66	86
Fusant 11	58	76	91	88
Fusant 12	80	76	87	93
Fusant 13	81	70	86	83
Fusant 14	50	56	70	91
Fusant 15	57	73	86	94
Fusant 16	79	78	76	91
Fusant 17	83	69	84	88
Fusant 18	78	66	92	84
Fusant 19	79	79	91	87
Fusant 20	80	72	80	86
Fusant 21	72	57	56	86

Table 1: Antagonistic potential of two parental strains and twenty-one of their fusants against some pathogenic fungi.

from 40% to 60% of PEG is suitable for protoplasts fusion in different fungi [23,24]. However, Pe'er and Chet [18], used 33% PEG for protoplast fusion in *T. harzianum*. Therefore, concentration of PEG is highly critical for effective fusion of protoplasts [5,24].

Antagonistic activity of fusants in dual culture technique

Antagonistic effects of all *Trichoderma* parental and fusant isolates *in vitro* were tested against *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Macrophomina phaseolina*. The antagonistic capacities of root rot and wilt causing pathogens were tested using dual culture method and the results are presented in Table 1 and Figure 3. The antagonistic activity was determined in the most fusant isolates compared to the parents. Among the fusant isolates, fusant isolate-7 produced maximum growth inhibition. It showed approximately one and half-fold increase over the parent strains. All the tested fusants exhibited increased antagonistic activity against *Fusarium oxysporum* than the parent strains except fusant-21. In all the dual culture plates, the inhibition zone appeared as a curve, with concavity oriented towards pathogens. The curvature of the inhibition area between the inoculation of *Trichoderma* fungi and the inoculation of pathogenic fungi in the same PDA plate depends on the growth rate of the inoculation. If one colony has a faster growth rate than the other, a curve in the inhibition zone will be observed easily. However, if the two inoculations have the same growth rate, a straight line would be observed when mycelia from both fungi come into contact [6,24].

Interestingly, all *Trichoderma* isolates exhibited inhibition of the mycelial growth of all pathogens. This could be due to the production of diffusible components, such as lytic enzymes or water-soluble metabolites. These components, such as chitinase, protease and glucanase, were always secreted by *Trichoderma* at low concentration [5,25]. Therefore, they can act against the pathogenic fungi before mycelial contact, thus increasing the antagonism of *Trichoderma*. From above results, it can be said that the highest growth inhibition of

the tested pathogens was detected for fusant F7 followed by fusant F6. It also can be concluded that the tested *Trichoderma* strains reduced the growth of all tested soil borne pathogens. Therefore, can be used for integrated disease management of soil borne plant pathogens. The degree of antagonism varied between and within strains of *Trichoderma* against the soil borne plant pathogens [6,19,24,26].

Molecular characterization of parents and fusants by RAPD

RAPD-PCR has been used to evaluate genetic variation and taxonomic relationships in many fungi such as *Trichoderma* [27]. This technique have been used extensively in detecting genetic variation in different microorganism species because of its simplicity [9,12,15]. Genomic diversity of parents and fusants of *Trichoderma* produced by protoplast fusion was investigated by RAPD analysis. The RAPD results are illustrated in Table 2 and Figure 4. Data showed polymorphic numbers of the genetic bands, which were the electrophoretic products of PCR for parents and fusant strains. RAPD-PCR reactions were performed among two parents (P1 was *T. harzianum* NBAIL Th 1, P2 was *T. viride* NBAIL Tv 23) and twenty one fusant isolates (F1 to F21) using seven different 10-mer primers, which were preselected for their performance with *Trichoderma* DNA. The seven RAPD primers produced different fragment patterns and numbers. The primers yielded a total of 79 distinct bands (RAPD markers), (16.5%) of which were considered as polymorphic and 83.5% of which were considered as monomorphic.

The total number of bands as shown in Table 2 varied from 17 bands with primer OPO-13 (Figure 4) to 8 bands with primer OPA-18. The total of monomorphic amplicons was 66 and the total of polymorphic amplicons was 79. It can be concluded from our study that RAPD markers are effective in detecting similarity between *Trichoderma* strains and their fusants. In fact, they provide a potential tool for studying the inter-strain genetic similarity and the establishment of genetic relationships. The RAPD-PCR results using primer (OPB-11) has showed the highest polymorphism, 12 bands in these *Trichoderma*

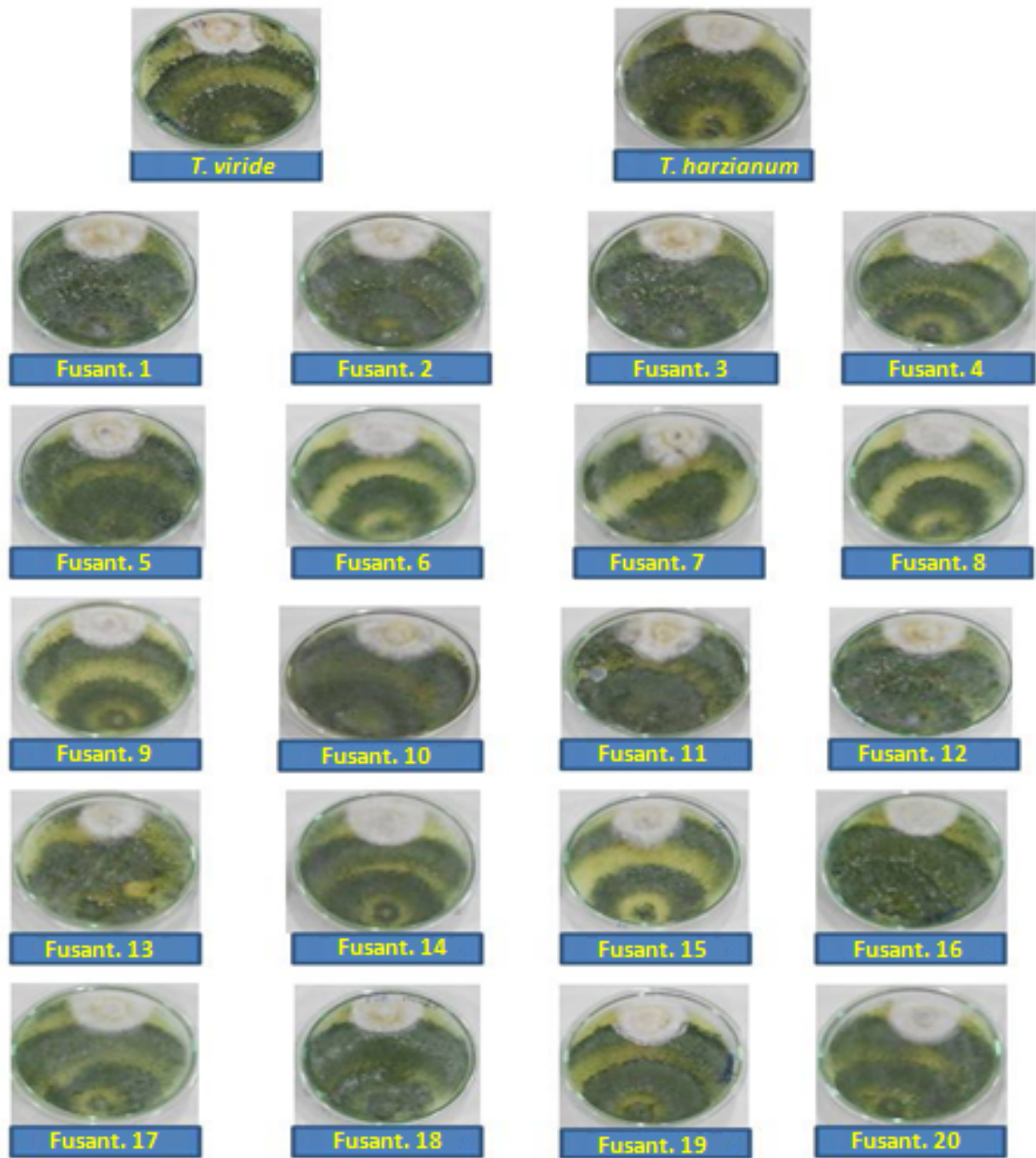


Figure 3: Antagonistic test of two parental strains (*T. harzianum* NBAlI Th 1 and *T. viride* NBAlI Tv 23) and twenty one of their fusants produced by protoplast fusion against *Fusarium oxysporum*.

Primers	Total Bands	No. of Monomorphic Bands	No. Polymorphic Bands	% Monomorphic bands	% Polymorphic bands
OPA-16	11	10	1	91.0	9.00
OPA-17	9	9	0	100	0.00
OPA-18	8	6	2	75.0	25.0
OPB-11	12	12	0	100	0.00
OPJ-07	9	7	2	77.8	22.2
OPK-03	13	11	2	84.6	15.4
OPO-13	17	11	6	64.7	35.3
Total	79	66	13	83.5	16.5

Table 2: Polymorphic bands of each RAPD primers and percentage of polymorphism in parent strains and their corresponding fusants.

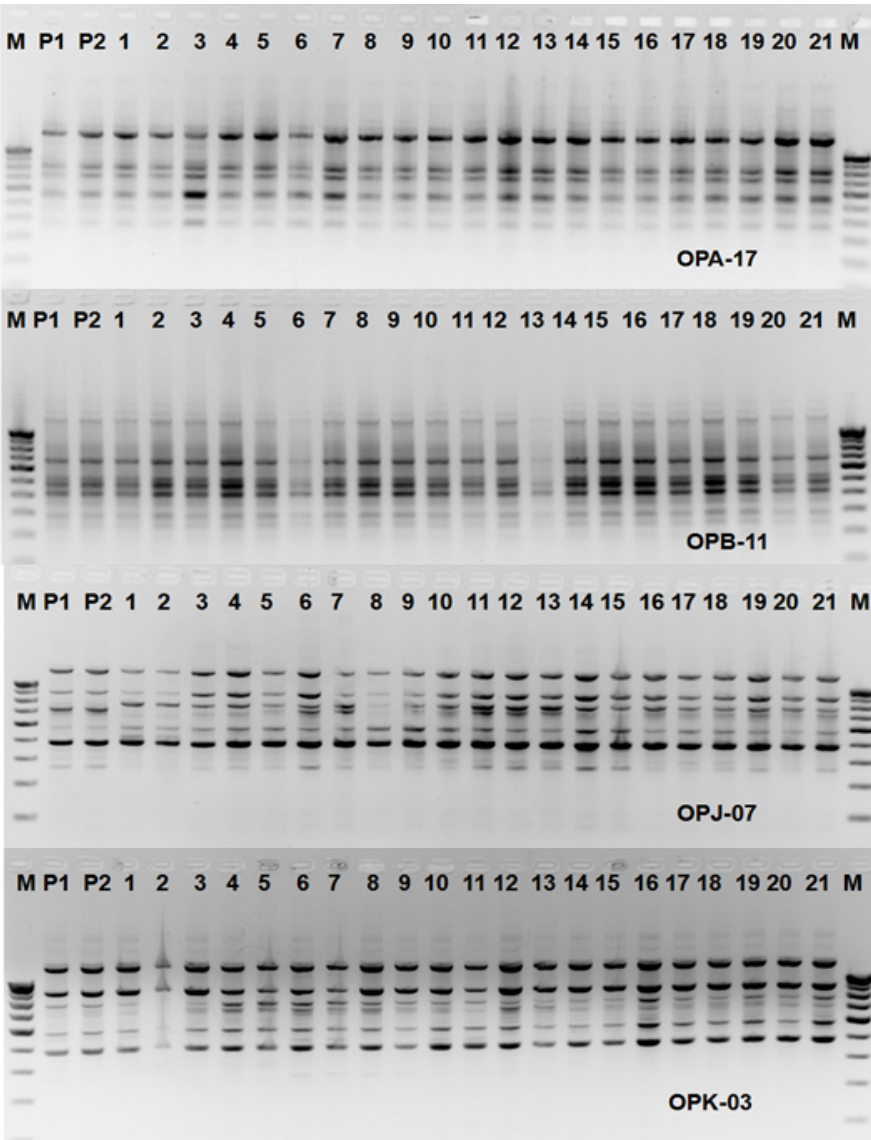


Figure 4: RAPD-PCR profile of two parental strains (*T. harzianum* NBAll Th 1 and *T. viride* NBAll Tv 23) and twenty one of their fusants produced by protoplast generated with seven RAPD primers. M: is 100 bp DNA ladder.

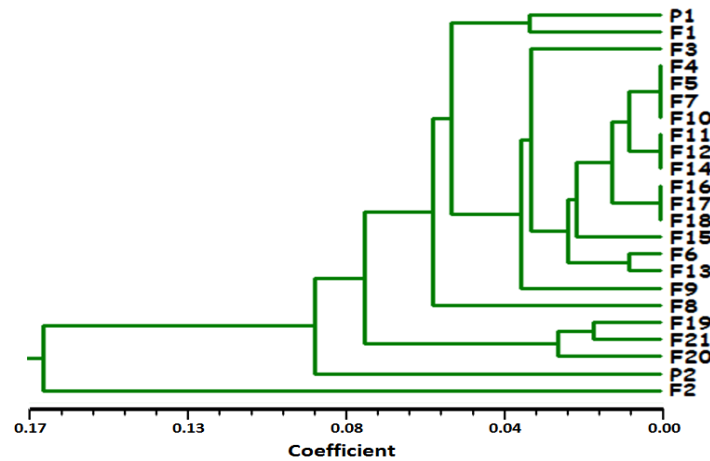


Figure 5: Dendrogram of RAPD-PCR profile of two parental strains (*T. harzianum* NBAll Th 1 and *T. viride* NBAll Tv 23) and twenty one of their fusants generated with seven RAPD primers.

strains ranged from 75 bp to 1750 bp. In case of monomorphism, two primers have showed 100 % monomorphism among two parent's strains and twenty one fusants.

Cluster analysis of *Trichoderma* parent strains and their fusants

All fragments from RAPD markers were used for molecular characterization of *Trichoderma* strains by genetic similarities and designing the phylogenetic tree for these *Trichoderma* strains. According to genetic similarity, two parent strains and twenty-one fusants were grouped into two major clusters with about 83% genetic similarity. The two parent strains and twenty fusants were grouped in the first cluster and only fusant-2 was grouped in the second cluster (Figure 5). The overall genetic distance among *Trichoderma* strains was relatively low. The smallest genetic distance (0.008) was estimated between F11 and F12, moreover F20 and F15 relatively showed the highest genetic distance, whereas the genetic distance for parent P1 and fusant F1 was marginally low. It may be suggested that the tested biological and biochemical traits caused by allelic variation of functional genes, whereas RAPD is a genome-wide fingerprinting technique and in most cases target repetitive DNA regions. Sharma et al. [28] also found no correlation between genetic variability assessed by RAPD markers and the ability of *Trichoderma* isolates to antagonize *Sclerotium rolfsii*. However, other author found a relationship between RAPD polymorphisms of *Trichoderma* strains and their antagonism against *Aspergillus niger* [29]. Moreover, the others found a high relationship between some molecular markers of *Trichoderma* strains and their antagonism against many plant pathogens as *Fusarium oxysporum*, *Pythium ultimum* and *Sclerotium rolfsii* [5,8,24,30]. Moreover, here and in a previous study, *T. harzianum* was the predominant taxon, *T. harzianum* is the most commonly reported species in the genus, occurring in diverse ecosystems and ecological niches. However, it must be borne in mind that the name "*T. harzianum*" applies to a species complex [31,32]. So we observed that most fusant strains trend to parent 1 (*T. harzianum* NBAII Th 1) characters and these haplotype fusant species may be seen to comprise a multiplicity of species when subjected to multilocus phylogenetic analysis.

Conclusion

Our results refer to combined studies, including antagonism test, protoplast fusion and molecular markers, are necessary to select indigenous *Trichoderma* strains that can be used under different environmental conditions. From the two parent strains and twenty one of their fusants produced by protoplast fusion F6 and F7 fusants showed high antagonistic activity against *Rhizoctonia solani*, *Sclerotium rolfsii*, *Fusarium oxysporum* and *Macrophomina phaseolina* comparing with parent strains.

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