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Effects of protoplast fusion on the antifungal activity of *Trichoderma* strains and their molecular characterisation

Hossein Kari Dolatabad, Mohammad Javan-Nikkah, Marzieh Safari and Thamyr Patricia Golaia

**ABSTRACT**

The main purposes of this study were to examine the effects of protoplast fusion on chitinase production, antifungal activity and major antifungal compounds in parental strains and fusants and to investigate them using the internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) method. The chitinase levels of eight fusants named Fu1-Fu8, formed by protoplast fusion between endophytic *Trichoderma harzianum* strains, were compared. Antifungal activities of the eight fusants were tested against *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Aspergillus flavus*. Pathogens were significantly controlled by the fusant Fu3. Isolation and purification of the compounds from the fusant Fu3 and parental strains were done based on the preparative thin layer chromatography method. After an antifungal activity assessment of isolated fractions, Fr5 and Fr2Fr2 fractions from fusant Fu3 and parental strains were injected into liquid chromatography-mass spectrometry (LC-MS). Harzianopyridone and chrysophanic acid compounds were identified in the Fr2Fr2 and Fr5 fractions which showed the greatest effect on *S. sclerotiorum*, *R. solani* and *A. flavus*, respectively.

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**KEYWORDS**

Biocontrol; endophytic fungi; LC-MS; preparative thin layer chromatography; protoplast fusion

**Introduction**

The pistachio (*Pistacia vera*), as a high yielding crop with a high tolerance for drought conditions and excessive salinity, plays a prominent role in farmers’ social and economic viability in the arid and semi-arid regions of Iran. Exportation of this valuable product is adversely affected...
by the toxigenic *Aspergillus flavus* which can cause aflatoxin contamination. This mycotoxin also possesses carcinogenic, mutagenic and teratogenic effects (Shenasi et al. 2002). The detrimental effects of pesticides including environmental pollution, impacts on human and animal health, and pathogenic resistance to pesticides necessitate developing suitable alternatives. With none of these dangerous effects, endophytic fungi serve as the most obvious alternative (Zucchi et al. 2008; Cardoso et al. 2010; Kusari et al. 2013).

In our previous study, we tried to isolate and identify endophytic fungi from the pistachio trees (Dolatabad et al. 2017). In brief, samples had been taken from healthy trees of Ohadi, Kale Ghochi, Akbari and Ahmad Aghaei cultivars in 13 villages of the Kerman Province in Iran. A total of 614 fungal isolates in 46 taxa were obtained and identified. Antifungal activity of these isolates against the important plant pathogenic fungi *A. flavus*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* was analyzed (Dolatabad et al. 2017). The three best strains, *Trichoderma harzianum* TH5-1-2, *T. harzianum* TH10-2-2 and *T. atroviride* TA2-2-1, were chosen for the protoplast fusion study.

*Trichoderma* spp. show biocontrol activity against different pathogenic fungi on various hosts (Papavizas 1985). The beneficial effects of *Trichoderma* include antagonistic activity using different mechanisms such as mycoparasitism, the production of enzymes that degrade the host’s cell wall, the synthesis of secondary metabolites and the increased competition for space and food with plant pathogenic fungi (Howell 2003; Harman 2006). Harman et al. (2004) reported that *Trichoderma* species are opportunistic, avirulent plant symbionts and parasitic toward other fungi.

The genus *Trichoderma* is known to produce a variety of secondary metabolites with antibiotic activity such as harzianopyridone, harzianolide, 1,8-dihydroxy-3-methyl-anthraquinone, 1-hydroxy-3-methyl-anthraquinone, t39butenolide, and t22azaphilone (Vinale et al. 2008). Harzianopyridone is a natural fungal molecule and belongs to the group of nitrogen heterocyclic compounds isolated from *T. harzianum* in 1989 (Dickinson et al. 1989). Vinale et al. (2006) reported that harzianopyridone, harzianolide and t39butenolide inhibited the growth of *Gaeumannomyces graminis* var. tritici, *Pythium ultimum* and *R. solani*. Antibacterial activity of chrysophanic acid on bacteria like *Bacillus subtilis* and *Staphylococcus aureus* (García-Sosa et al. 2006) and the antifungal activity of chrysophanic acid against plant pathogenic fungi like *Podosphaera* and *Blumeria* species has been reported (Choi et al. 2004).

Sexual states are rare in *Trichoderma* spp. (Chaverri et al. 2015) and vegetative incompatibility impedes hyphal anastomosis and
heterokaryosis in fungi (Loubradou and Turcq 2000). One way to overcome this impediment and induce parasexualism is protoplast fusion. Many researchers have demonstrated that protoplast fusion improves the parental strains and is a way to combine beneficial characteristics from different fungi (Peer and Chet 1990; Lalithakumari and Mathivanan 2003; Prabavathy et al. 2006a, 2006b; Deng et al. 2013).

In this article, we explain the effects of protoplast fusion on chitinase production, antifungal activity and major antifungal compounds in parental strains and fusants. We then investigate them using the internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) method. This is the first time we compare the effects of protoplast fusion between major antifungal compounds. The selected fusants obtained from this research will be used in future research to investigate their effects and stability against A. flavus in Pistachio orchards and other crops sensitive to R. solani and S. sclerotiorum.

**Materials and methods**

**Protoplast fusion**

Mycelium was prepared by using the Gnanam method (2013). A conidial suspension was obtained by adding 3–5 mL of sterile water to the 7-day-old culture of fungal strains. The surface was gently scratched with an inoculation loop. The resulting conidial suspension was removed with a 1-mL micropipette and transferred to a sterile 1.5-mL microtube. The conidial suspension was rinsed twice with sterile water (1.2 mL). The centrifuge was set at 500 g for 10 min at room temperature. The pellet was suspended in an appropriate volume of water to get $1 \times 10^6$ conidial density in millilitre. Subsequently, 1 mL of conidial suspension was added to the 250 mL flasks containing PDYE medium (potato: 200 g, dextrose: 20 g, yeast extract: 3 g, distilled water with pH ≈ 6.5). Flasks were then shaken for 24 h at 120 rpm at room temperature. Afterward, young mycelia (100 mg fresh weight) were harvested by passing them through three layers of sterile filter paper. They were rinsed three times with sterilised distilled water. They were rinsed three times with 5 mL of osmotic stabiliser solution (KCl 0.6 M with pH = 6.5), which was previously sterilised with an autoclave and then kept at room temperature after cooling.

Protoplast isolation was done by using the Gnanam method (2013). The wet mycelia were added to 25-mL flasks containing glucanex lytic enzyme (2, 4, 6 and 8 mg mL$^{-1}$, Sigma-Aldrich, L1412, USA) using a sterile loop. The glucanex lytic enzyme was diluted with 0.6 M of osmotic stabiliser solution (sucrose, mannitol, potassium chloride and magnesium
sulfate; pH = 5.5) and sterilised with a 0.22-micron filter (BioFil Syringe Filter, Shanghai, China). Afterward, flasks were shaken for 4 h at 120 rpm at room temperature. Every 30 min, 10 μL of samples were transferred to clean glass slides to view protoplasts under the microscope. A hemocytometer was used to count protoplasts. At this level, we noted the optimum incubation time, enzyme concentration and an osmotic stabiliser, which induced more protoplast production. Subsequently, tests were done with them. After 4 h, undigested hyphal materials were excluded by passing them through six layers of sterile filter paper. A volume of osmotic stabiliser equal to the volume of filtered liquid was added; it was then transferred to two 1.5-mL microtubes and centrifuged for 5 min at 500 g at room temperature. The upper phase was removed carefully with a micropipette and precipitated protoplasts were suspended with 1 mL of the osmotic stabiliser. The resulting protoplasts were transferred to the protoplast regeneration medium (PDA contains KCl 0.6 M) and the frequency of protoplast regeneration was calculated by using the following formula (Deng et al. 2013).

\[ P = \frac{N_c}{N_p} \times 100\% \]

\[ N_c = \text{the number of colonies in the regeneration medium; } N_p = \text{the number of inoculated protoplasts.} \]

Protoplast inactivation was done by heat and UV treatments (Zhao et al. 2008). Inactivation of T. harzianum TH5-1-2 was performed by placing it in a 60°C water bath for 2–7 min. For protoplast inactivation in T. harzianum TH10-2-2, UV radiation was produced from a 30-W bulb from 20 cm away for 120–270 s. In order to prevent reactivation, each treatment was placed in a dark area for 5 h. Inactivation was confirmed by the lack of growth in the regeneration medium (PDA, contain 0.6 M KCl) which was kept at 28°C for 3 days. While inactivated protoplasts were chosen for protoplast fusion, the inactivation rate of protoplasts was calculated using the following formula (Zhao et al. 2008):

\[ a = \frac{1 - (b - c)/(d - e)}{} \times 100\% \]

\[ a: \text{inactivation rate of protoplasts; } b: \text{the number of colonies in the regeneration medium after inactivation; } c: \text{the number of colonies in the PDA medium after inactivation; } d: \text{the number of colonies in the regeneration medium before inactivation; } e: \text{the number of colonies in the PDA medium before inactivation.} \]

Protoplast combining with polyethylene glycol 6000 (Sigma-Aldrich, USA) was carried out by using the Prabavathy et al. (2006b) method. Accordingly, polyethylene glycol was dissolved in an STC buffer (sorbitol 0.6 M, 10 μM Tris-HCl, 10 μM CaCl₂ and pH = 6.5) and then sterilised with a 0.22-micron filter (BioFil Syringe Filter, Shanghai, China). In
order to evaluate the polyethylene glycol concentration for protoplast fusion, concentrations of 30, 40, 50 and 60% were prepared. One millilitre of protoplast suspension containing equal concentrations of both strains was combined with 2 mL of polyethylene glycol solution. After 10 min, the mixture was diluted with 1 mL of STC buffer. Polyethylene glycol was rinsed with the STC buffer. The combined protoplasts were precipitated out by centrifuging 500 g for 10 min. After removing the upper phase, combined protoplasts were suspended in STC buffer. The resulting protoplasts were transferred to PDA containing 0.6 M KCl. The regeneration of fusants was examined. Protoplast fusion rate was calculated by using the following formula (Zhao et al. 2008):

\[
a = \left(\frac{(b - c)}{d}\right) \times 100\%
\]

\(a\): protoplast fusion rate; \(b\): the number of colonies produced by fusants in the regeneration medium; \(c\): the number of colonies produced by inactivated parents in the regeneration medium; \(d\): the number of colonies produced by parents in the regeneration medium.

In order to see protoplasts and nuclei, phase contrast and fluorescent microscopes were used. The Zeiss Axioplan (40X) and the Upright Microscope (100X) were used. For nuclei staining, DAPI was used (Thomas et al. 2012).

**Evaluation of fusants and parental strains activity**

The evaluation of antifungal activity was done using the dual culture method (Dennis and Webster 1971) and by mixing the ethyl acetate extract of fusants and parental strains in the PDA medium (Kumar and Kaushik 2013). Dried extracts were dissolved in methanol, mixed with 30 mL of molten PDA media, and poured into three Petri dishes. The final concentration was 250 µg mL\(^{-1}\) against *R. solani* and *S. sclerotiorum* and 500 µg mL\(^{-1}\) against *A. flavus*. In addition, for *A. flavus*, concentrations of 1000, 800, 600, 400, 200, 100, 50 and 25 µg mL\(^{-1}\) were used in the 96-well plates. One mycelial disc of each plant pathogenic fungus was placed in the centre of the Petri dishes and radial growth was measured. The control dishes contained 200 and 400 µL of methanol. The tests were performed in triplicate. Pathogenic colony diameters were measured until the control dish attained full growth. The percent growth inhibition was calculated by using the following equation (Vincent 1947):

\[
I = \frac{[(C-T)/C]}{100}
\]

In this equation, \(I\) = percent growth inhibition, \(C\) = colony growth rate in control plates, and \(T\) = colony growth rate in each treatment. The amount of chitinase activity in fusants and parental strains were also compared by enzymatic colorimetric assay according to
Rojas-Avelizapa et al. (1999). Finally, the fusant that best inhibited fungal growth was identified.

**ITS amplification and RFLP**

For DNA extraction, each fungal isolate colony was grown in 250-mL Erlenmeyer flasks containing 100-mL Potato Dextrose Broth at 25°C. After 15 days, the genomic DNA of each isolate was extracted by using the modified CTAB method (Doyle 1987).

The rDNA ITS region was amplified with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (White et al. 1990). Each reaction mixture was carried out in a volume of 25 µL, containing 50 ng of template DNA, 5 µL of 10 × PCR buffer, 3 µL of MgCl₂ (25 mM), 4 µL of dNTPs (2.5 mM), 1 µL of each primer (10 pmol), and 2.5 units of Taq DNA polymerase. PCR amplification was carried out in a Techne Genius Thermal Cycler (Canada). The amplification program was as follows: 1 cycle of 4 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C and finally 1 cycle of 7 min at 72°C. PCR products were digested by Mbo II according to the manufacturer’s instructions and fragments were electrophoresed in a 2% agarose gel.

**Thin layer chromatography (TLC)**

The best solvent system for the fractionation of the ethyl acetate extract of the most antifungal fusant and parental strains was assessed. TLC sheets (TLC plate POLYGRAM 60A polyester sheet silica 0.2-mm layer, UV254 4 cm × 8 cm) were used and ethyl acetate extracts of fusant and parental strains were loaded by capillary tubes onto the TLC sheets. Afterward, they were air dried for 5 min. The dried sheets were then placed in tanks containing different ratios of petroleum ether: ethyl acetate (4:6, 3:7, 2:8, 1:9 and 0:10). After moving the solvent and the separating the extracted components, the sheets were removed. In order to evaporate the remaining solvent, the sheets were placed out for 10 min and left under a UV lamp at 254 nm wavelength.

After identifying the best solvent system, preparative TLC was used for the isolation and purification of the components. The ethyl acetate extract was loaded linearly on TLC sheets with dimensions of 20 × 20 cm (Analtech TLC Uniplates Silica G, 20 × 20 cm). Then they were placed in a tank containing petroleum ether: ethyl acetate (4:6). The mobile phase was allowed to run along the sheet for 18 cm. The sheet was placed out to evaporate the remaining solvent and, afterward, bands were seen at 254 nm. The value of $R_f$ was calculated. The bands were scratched off of the sheets.
and dissolved in 50% ethanol. The bands were assessed for antifungal activity against A. flavus, R. solani and S. sclerotiorum using the mixed-medium method (Kumar and Kaushik 2013). Each fraction that showed antifungal activity and if the bands in that fraction were not completely separated, were loaded onto TLC sheets and placed in the tanks containing ethanol: ethyl acetate solvents (4:6) for better separation. Individual bands that were the same between parental strains and fusant with antifungal activity were injected into liquid chromatography-mass spectrometry (LC-MS) to identify the compounds.

**LC-MS**

Ten milligram of each dried sample was dissolved in 50% methanol and vortexed for 30 s. Samples were placed at room temperature for 10 min and vortexed again for 20 s. Dissolved samples were diluted with a ratio of 20:1 in 95% water and 5% acetonitrile (containing 0.1% formic acid) and vortexed for 10 s. Afterward, 15 µL of sample was injected in Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm). The samples were analyzed using LC-MS (Dionex Ultimate 3000 UHPLC + Focused) combined with the mass spectrometer (Quadrupole Orbitrap Q Exactive mass spectrometer, Thermo Scientific). Used solvents were included in the 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 0.4 mL min⁻¹. Data from LC-MS were identified with the Progenesis software (V. 4.0).

**Statistical analysis**

Statistical analysis of data was done with SAS 9.1 software while diagrams were drawn in Excel 2007. Means comparisons were performed using Duncan’s multiple test (p ≤ 0.05).

**Results**

**Protoplast fusion**

Analysis of variance for effective factors that release protoplasts showed that there were significant differences between treatments. The highest protoplast production rate was acquired with the glucanex lytic enzyme at a concentration of 6 mg mL⁻¹ with 0.6 M KCl (Table 1). The best osmotic stabiliser to produce the highest protoplast rates in both parental strains was KCl. In T. harzianum TH10-2-2, 5.9 × 10⁶ and in T. harzianum TH5-1-2, 5.54 × 10⁶ protoplasts were produced. The lowest protoplast production rate was observed when using sucrose as an osmotic stabiliser (Table 1). The optimal incubation time to release protoplasts in
both parental strains was determined to be 3 h (Table 1, Figure 1). The released protoplasts were small in size, but soon got larger and rounder.

After separation, protoplast regeneration frequencies were calculated to be 40.67% in *T. harzianum* TH10-2-2 and 47.67% in *T. harzianum* TH5-1-2. Results showed that by putting the protoplasts of *T. harzianum* TH5-1-2 in a water bath at 60°C for 5 min, the inactivation rate of protoplasts was 100%. UV radiation for 240 s (30 W, 20 cm) also caused inactivation in *T. harzianum* TH10-2-2. To increase the rate of protoplast fusion, different concentrations of polyethylene glycol (30, 40, 50 and 60%) were examined. Analysis of variance showed that polyethylene glycol concentrations had a significant effect on protoplast fusion. The mean comparison showed that the highest protoplast fusion rate was 5.7% with 40% polyethylene glycol (Figure 1).

### Evaluation of antifungal activity in fusants and parental stains through dual culture and mixed-medium methods

The eight numbers of fusants were chosen based on growth and superiority in dual culture against *R. solani*, *S. sclerotiorum* and *A. flavus*. There were significant differences between fusants and parental strains for antifungal activity against *R. solani*, *S. sclerotiorum* and *A. flavus* in both dual culture and mixed-medium methods.

Dual culture results of fusants and parental strains against *A. flavus* showed that Fu3 and Fu7 had the highest inhibition rate at 88.46%.

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**Table 1.** Comparisons between different incubation times, osmotic stabilisers (0.6 M, pH = 5.5), and concentrations of cell wall lytic enzymes (glucanex) for protoplast release in parental isolates with Duncan's multiple range test in SAS 9.1.

<table>
<thead>
<tr>
<th>Factors</th>
<th><em>Trichoderma harzianum TH5-1-2</em></th>
<th><em>Trichoderma harzianum TH10-2-2</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incubation time (h)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.64 f</td>
<td>2.33 e</td>
</tr>
<tr>
<td>1.5</td>
<td>3.3 d</td>
<td>2.99 d</td>
</tr>
<tr>
<td>2</td>
<td>3.87 c</td>
<td>3.15 cd</td>
</tr>
<tr>
<td>2.5</td>
<td>5.44 b</td>
<td>4.52 b</td>
</tr>
<tr>
<td>3</td>
<td>5.75 a</td>
<td>5.39 a</td>
</tr>
<tr>
<td>4</td>
<td>2.82 e</td>
<td>3.21 c</td>
</tr>
<tr>
<td><strong>Osmotic stabilisers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.83 d</td>
<td>3.03 c</td>
</tr>
<tr>
<td>Mannitol</td>
<td>4.33 c</td>
<td>4.61 b</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>4.91 b</td>
<td>4.78 b</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>5.9 a</td>
<td>5.54 a</td>
</tr>
<tr>
<td><strong>Glucanex (mg mL⁻¹)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.45 d</td>
<td>2.01 d</td>
</tr>
<tr>
<td>4</td>
<td>3.39 c</td>
<td>3.74 c</td>
</tr>
<tr>
<td>6</td>
<td>5.58 a</td>
<td>5.44 a</td>
</tr>
<tr>
<td>8</td>
<td>4.76 b</td>
<td>4.91 b</td>
</tr>
</tbody>
</table>

The same letter(s) in each column showed no significant differences between treatments (*p* < 0.05).
Meanwhile parental strains *T. harzianum* TH5-1-2 and *T. harzianum* TH10-2-2 showed 82.74 and 81.68% inhibition rates, respectively (Table 2). The results showed that Fu2, Fu3 and Fu4 had the highest suppression against *S. sclerotiorum* (Table 2). The dual culture results of fusants and parental strains against *R. solani* showed that parental strains had a 75–79% inhibition rate whereas Fu3 showed a suppression rate of 89.73% (Table 2).

In the mixed-medium method, 250 μg mL$^{-1}$ of ethyl acetate extract was added to the medium. The results showed that Fu3 had the highest
inhibition rates at 86.72 and 85.49% against *S. sclerotiorum* and *R. solani*, respectively (Table 2).

Mixing ethyl acetate extracts of parental strains and fusants in a medium with *A. flavus* was done in 96-well plates. Concentrations of 1000, 800, 600, 400, 200, 100, 50 and 25 μg mL⁻¹ were used in the wells. The minimum inhibitory concentration for growth in Fu2, Fu3 and Fu7 was observed at 600 μg mL⁻¹ whereas it was 800 μg mL⁻¹ in parental strains. The Fu6 and Fu8 strains had more feeble results than parents and the minimum inhibitory concentration was 1000 μg mL⁻¹.

### Evaluation of fusants and parental strains in chitinase activities

Analysis of variance showed significant differences between parental strains and fusants (Fu1-Fu8) in chitinase production. The results showed that Fu5 and Fu3 had high chitinase activities with 3.46 and 3.3 U mL⁻¹, respectively. These values were significantly higher than both *T. harzianum* TH5-1-2 (2.24) and *T. harzianum* TH10-2-2 (1.83).

There was no outcome for *T. harzianum* TH5-1-2 and *T. atroviride* TA2-2-1 fusion. Fusants of *T. harzianum* TH10-2-2 and *T. atroviride* TA2-2-1 had very little growth (data were not shown).

### ITS-RFLP

Genomic DNA of parental strains and fusants was amplified using the rDNA ITS region. The length was measured to be about 550 bp. Amplified products were subjected to restriction digestion with Mbo II. The digestion of the rDNA ITS region in *T. atroviride* TA2-2-1,
T. harzianum TH10-2-2, and fusants by Mbo II produced 1 to 4 bands (Figure 2). Two bands were seen in parental strains. The band sizes were 275 and 350 bp in the T. atroviride TA2-2-1. The band sizes were 290 and 350 bp in T. harzianum TH10-2-2. FuN1 showed three bands that were the same size as the parental strains. But FuN2 and FuN3 showed new bands at 200 bp.

The digestion of the rDNA ITS region in T. harzianum TH10-2-2, T. harzianum TH5-1-2, and fusants by Mbo II produced 1 to 3 bands. The 290 and 350 bp band sizes were seen in both parental strains and Fu3. The digested rDNA ITS region in Fu2 showed three bands with 225, 290 and 350 bp sizes (Figure 2).

**TLC**

Fusant Fu3 was chosen to separate metabolites based on antifungal activity in dual culture, mixed-medium methods and chitinase production. The best solvent system for the primary isolation of the compounds in the ethyl acetate extracts of parental strains and fusant Fu3 was determined to be a ratio of 4 to 6 petroleum ether: ethyl acetate.

After identifying the best solvent system, preparative TLC was applied to further isolate and purify the compounds. The banding patterns created in parental strain and fusant Fu3 were compared and, in total, six fractions were chosen based on similarity in banding patterns.

The $R_f$ calculations for Fr1-Fr6 were 0.083, 0.25, 0.5, 0.72, 0.88, and 0.97, respectively. The antifungal activity results showed that Fr2 and Fr5...
of parental strains and fusant Fu3 were effective against *S. sclerotiorum* (Figure 3), *R. solani* (Figure 3) and *A. flavus* (Figure 4, Table 3).

The highest percentage of growth inhibition against *R. solani* and *S. sclerotiorum* was observed in the second fraction (Fr2) of parental strains and fusant Fu3. Fu3 showed significantly higher growth inhibition than parental strains. Fu3 showed 89.84% and 87.89% growth inhibition against *R. solani* and *S. sclerotiorum*, respectively (Table 3).

The fifth fraction (Fr5) showed the highest percentage of growth inhibition against *A. flavus*. It had a growth inhibition of 88.29% in fusant Fu3, whereas in *T. harzianum TH5-1-2* and *T. harzianum TH10-2-2* were 69.83 and 72.76%, respectively (Table 3). In the 96-well plate, the minimum inhibitory concentration of *A. flavus* was 100 µg mL⁻¹ in

**Figure 3.** Antifungal activity evaluation of the Fr1-Fr6 fractions from *Trichoderma harzianum TH5-1-2, Trichoderma harzianum TH10-2-2* and fusant Fu3 against (a) *Sclerotinia sclerotiorum* and (b) *Rhizoctonia solani* using the mixed-medium method (250 µg mL⁻¹).
Meanwhile, the concentration for parental strains was 400 μg mL⁻¹ (Figure 4).

Since the second fractions (Fr2) of parental strains and fusant Fu3 were not single and showed antifungal activity, those fractions were loaded onto TLC sheets for better separation. According to the created bonding pattern, five fractions were separated and the \( R_f \) values for Fr1Fr2–Fr5Fr5 were calculated to be 0.083, 0.19, 0.25, 0.61 and 0.92, respectively.

In order to investigate the antifungal activity in the five resulting fractions, the mixed-medium method was used. The results showed that the highest percentage of growth inhibition against \( R. \ solani \) (Figure 5) and \( S. \ sclerotiorum \) (Figure 5) was observed in Fr2Fr2 of parental strains and fusant Fu3. The growth inhibition percentage of \( S. \ sclerotiorum \) by fusant Fu3. Meanwhile the concentration for parental strains was 400 μg mL⁻¹ (Figure 4).

Table 3. Comparing antifungal activity in the Fr2, Fr5, and Fr2Fr2 fractions of \textit{Trichoderma harzianum} TH5-1-2, \textit{Trichoderma harzianum} TH10-2-2, and fusant Fu3 using both the mixed-medium method with Duncan’s multiple range test in SAS 9.1.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Endophytic fungi</th>
<th>Aspergillus flavus</th>
<th>Sclerotinia sclerotiorum</th>
<th>Rhizoctonia solani</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr2</td>
<td>\textit{Trichoderma harzianum} TH5-1-2</td>
<td>52.00 b</td>
<td>62.31 c</td>
<td>74.83 b</td>
</tr>
<tr>
<td></td>
<td>\textit{Trichoderma harzianum} TH10-2-2</td>
<td>54.98 b</td>
<td>68.59 b</td>
<td>76.85 b</td>
</tr>
<tr>
<td></td>
<td>Fu3</td>
<td>79.68 a</td>
<td>87.89 a</td>
<td>89.84 a</td>
</tr>
<tr>
<td>Fr5</td>
<td>\textit{Trichoderma harzianum} TH5-1-2</td>
<td>69.83 b</td>
<td>58.64 b</td>
<td>30.26 b</td>
</tr>
<tr>
<td></td>
<td>\textit{Trichoderma harzianum} TH10-2-2</td>
<td>72.76 b</td>
<td>62.11 b</td>
<td>28.45 b</td>
</tr>
<tr>
<td></td>
<td>Fu3</td>
<td>88.29 a</td>
<td>76.57 a</td>
<td>52.33 a</td>
</tr>
<tr>
<td>Fr2Fr2</td>
<td>\textit{Trichoderma harzianum} TH5-1-2</td>
<td>53.37 b</td>
<td>90.57 b</td>
<td>82.00 b</td>
</tr>
<tr>
<td></td>
<td>\textit{Trichoderma harzianum} TH10-2-2</td>
<td>56.64 b</td>
<td>89.98 b</td>
<td>84.29 b</td>
</tr>
<tr>
<td></td>
<td>Fu3</td>
<td>81.86 a</td>
<td>94.40 a</td>
<td>88.23 a</td>
</tr>
</tbody>
</table>

The same letter(s) in each column showed no significant differences between treatments (\( p < 0.05 \)).
Fr2Fr2 of Fu3 was 94.4%. For parental strains, the growth inhibition percentage was 90.57 and 89.98%, respectively in *T. harzianum* TH5-1-2 and *T. harzianum* TH10-2-2. Furthermore, the growth inhibition percentage of *R. solani* by Fr2Fr2 in Fu3 was 88.23%, which was significantly higher than *T. harzianum* TH5-1-2 (82%) and *T. harzianum* TH10-2-2 (84.29%) (Table 3).

**LC-MS**

In order to identify compounds, Fr5 and Fr2Fr2 fractions in *T. harzianum* TH5-1-2, *T. harzianum* TH10-2-2, and fusant Fu3 were
analyzed in LC-MS. The identification of compounds was done based on the mass to charge ratio (m/z) and retention times by Progenesis V. 4.0 software. The mass spectrum images of Fr5 and Fr2Fr2 fractions of parental strains and fusant Fu3 has been shown individually (Figures 6 and 7).

In the fraction Fr5, 1,8-dihydroxy-3-methylanthraquinone or chrysophanic acid was identified with a mass to charge ratio of 299.056 m/z and a retention time of 11.34 min. The highest abundance was observed in Fu3 and the lowest abundance was observed in *T. harzianum* TH10-2-2. In the Fr2Fr2 fraction, 4-hydroxy-5,6-dimethoxy-3-[(2R, 4E)-2-methyl-4-hexenoyl]-2(1H)-pyridinone or harzianopyridone was identified with a mass-to-charge ratio of 280.119 m/z and a retention time of 9.88 min. The highest abundance was observed in Fu3 and the lowest abundance was observed in *T. harzianum* TH5-1-2.

**Figure 6.** The mass spectrum of the Fr5 fraction of *Trichoderma harzianum* TH5-1-2 (a), *Trichoderma harzianum* TH10-2-2 (b) and Fu3 fusant (c) by using liquid chromatography-mass spectrometry analysis. The production of Chrysophanic acid (arrowheads) was confirmed by identifying the presence of the pseudo-molecular ions ([M + FA-H]⁻ = 299.056 m/z⁻¹).
Discussion

Improving strains capable of bio-control through protoplast fusion could lead to the creation of superior strains. Increasing the possibility of producing recombinant is one of the benefits of protoplast fusion. We can also examine a large number of recombinants in a short span of time (Stasz et al. 1988). Protoplast fusion technology permits induction of parasexual cycles at high rates. Sundaram (1996) created isolates from two isolates of *T. harzianum* (Th-1 and Th-3). These showed similar morphological traits between Th-1 and Th-3. Protoplastic fusants have also been created from two distinct species of bio-control agents. By protoplast fusion between *T. koningii* and *T. virens*, two of the produced fusants had more bio-control potential than parents against *R. solani* in cotton (Hanson and Howell 2002). The strain T22 is one of the most successful bio-control strains against different pathogens and has different commercial formulas available on the market. This strain was produced by the protoplast integration of two strains, T95 and T12 (Stasz et al. 1988).
The highest protoplast production rate was acquired with glucanex using the concentration of 6 mg mL\(^{-1}\) with 0.6 M KCl after 3 h of incubation. With high and low enzyme concentrations, the amount of produced protoplasts decreased. In low concentrations, protoplast release happened only at the tip of the hyphae and less protoplast was produced. At the highest concentration, protoplast lysis occurred immediately after they were released. These results are in accord with Balasubramanian and Lalithakumari’s findings (2008). They reported that KCl is the best osmotic stabiliser for *T. harzianum* and *T. viride*. Prabavathy et al. (2006b) noted that the highest protoplast release rate was obtained by using 8 mg mL\(^{-1}\) of cell wall lytic enzymes and 0.6 M KCl as the osmotic stabiliser. The highest protoplast rates in *T. harzianum* and *T. viride* were obtained after 3 hours of incubation (Balasubramanian and Lalithakumari 2008). The highest protoplast fusion rate was observed with 40% polyethylene glycol. These results are consistent with the findings of Prabavathy et al. (2006b) in which 40% polyethylene glycol was used for protoplast fusion in *T. harzianum*. Mrinalini and Lalithakumari (1998) reported using similar concentrations of polyethylene glycol when creating optimal conditions for protoplasts fusion between *T. harzianum* and *T. longibrachiatum*. The most suitable concentrations of polyethylene glycol for protoplast fusion have been reported to be between 40 and 60%, although higher concentrations would cause protoplast lysis (Lalithakumari 2000; Lalithakumari and Mathivanan 2003). The results of the current experiment are in accord with Balasubramanian and Lalithakumari’s findings (2008). They noted that the optimum concentration of polyethylene glycol in protoplast fusion for *T. harzianum* and *T. viride* was 40%.

The results of this experiment in regard to increasing the biocontrol and chitinase activity of parents are consistent with other findings. Compared to parental strains, protoplast fusion between two mutants of *T. harzianum* species reduced the radial growth of *R. solani*, *Fusarium oxysporum* and *Pythium ultimum* when using the dual culture method (Fahmi et al. 2012). El-Bondkly and Talkhan’s experiment (2007) showed more antagonistic and chitinase activities in *T. harzianum* fusants against *A. niger*, *R. solani*, and *Cephalosporium acremonium* in comparison to the parental strains. The complete inhibition of mycelial growth in *C. acremonium* and *R. solani* was accomplished by four fusants. Meanwhile parental strains caused 72.2 and 75.6% growth inhibition in the mycelial growth of *C. acremonium* and *R. solani*, respectively.

The results of this experiment are consistent with the findings of Prabavathy et al. (2006b). They assayed protoplast fusion effects in
chitinase production and antagonistic activity of *T. harzianum*. They demonstrated that protoplast fusion in *T. harzianum* caused more chitinase production and antagonistic activity against *R. solani* compare to parental strains. Additionally, 100% of *R. solani* suppression was reported by five fusants. Parental strains, on the other hand, induced 67.6% suppression in *R. solani*. Balasubramanian et al. (2012) reported that *Trichoderma* HF9, a fusion between *T. harzianum* and *T. viride*, had a higher rate of chitinase activity.

Mbo II is a suitable restriction enzyme to differentiate between *T. atroviride* TA2-2-1 and *T. harzianum* TH10-2-2. Among fusants, FuN1 showed three similarly sized bands compared to the parental strains. FuN2 showed three similarly sized bands compared to the parental strains, but with an extra band. The ITS-RFLP pattern of *T. harzianum* TH5-1-2, *T. harzianum* TH10-2-2 and fusant Fu3 were the same but fusant Fu2 showed an extra band.

The Fr5 fraction, which contained chrysophanic acid compounds, showed the highest effect against *A. flavus*. In the Fr2Fr2 fraction, harzianopyridone was identified and showed the highest inhibition growth of *R. solani* and *S. sclerotiorum*. Chrysophanic acid is one of the anthraquinone compounds. Anthraquinone is a well-known compound in different species of *Trichoderma*. *T. viride* and *T. polysporum* species can produce chrysophanic acid, pachybasin, and emodin. Chrysophanic acid and pachybasin were extracted from *T. aureoviride* extract and dried mycelium (Reino et al. 2008). Chrysophanic acid has antifungal activity against *A. fumigatus*, *Candida albicans*, *Cryptococcus neoformans* and *Trichophyton mentagrophytes* (Agarwal et al. 2000). Harzianopyridone is one of the nitrogen-containing heterocyclic compounds that had been isolated from *T. harzianum* for the first time. It has antifungal activity against both *Botrytis cinerea* and *R. Solani* (Dickinson et al. 1989).

The results of this experiment are in accord with the findings of Vinale et al. (2006). They reported that chrysophanic acid and harzianopyridone were produced by the *T. harzianum* strains T22 and T39. However, 1-hydroxy-3-methyl-anthraquinone, t22azaphilone, harzianolide and t39butenolide were not found in parental strains and fusant Fu3 during our study. The harzianopyridone had the ability to control both *Gaemumannomyces graminis* var. *tritici* and *Pythium ultimum* (Vinale et al. 2006). Ahluwalia et al. (2015) reported that the T-4 and T-5 strains of *T. harzianum* were able to produce both chrysophanic acid and harzianopyridone. Although not observed in this study, these strains can produce palmitic acid, 6-pentyl-2H-pyran-2-one, 2(5 H)-furanone, stigmasterol, B-sitosterol, 1-hydroxy-3-methylanthraquinone, decanolactone, ergosterol, and 6-methyl-1,3,8-trihydroxyanthraquinone. They have
suggested that harzianopyridone as a secondary metabolite could completely control *R. solani*, *S. rolfsii*, *M. phaseolina* and *F. oxysporum*.

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**Disclosure statement**

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**References**


