Effect of Chitin Adapted and Ultra Violet Induced Mutant of *Trichoderma harzianum* Enhancing Biocontrol and Chitinase Activity

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**Abstract:** A wild strain *Trichoderma harzianum* was grown on 0.5 to 5% colloidal chitin medium for adaptation to obtain chitinase rich mutants and the same wild strain was mutatred by UV irradiation. Mutants were screened on 1% colloidal chitin medium and selected based on their ability to produce lysis zones around the colonies. Biocontrol efficacy of the mutants and wild strain were tested against phytopathogens such as *Fusarium oxysporum*, *Bipolaris oryzae*, *Rhizoctonia solani* and *Alternaria* sp. by dual culture assay on PDA medium. The overall inhibition of phytopathogens by wild, adapted and UV mutant were 69.9%, 75.5% and 82.7%, respectively. The maximum chitinase and protein content were recorded on day 6, in 1% colloidal chitin amended medium with Czapek-Dox broth pH 6.5 at 28±2 °C. Total chitinase 1.3, 1.6, specific chitinase 1.7, 2.2 and protein 1.3, 1.8 fold were increased by adapted, UV mutant respectively on the 6 days of incubation. The UV mutant strain H11 showed more extra cellular chitinase and protein production, when compared to the adapted mutant and wild strain. SDS-PAGE analysis showed, UV mutant had 20 major protein bands with up regulation was observed than adapted mutant (15 bands) and wild strains (11 bands). *T. harzianum* mutant H11 showed maximum inhibition of the above pathogens and chitinase activity with protein content confirming the superior efficacy of the mutant and it could be exploit for industrial and agricultural purposes.

**Key words:** adapted mutant, biocontrol, chitinase, *Trichoderma harzianum*, UV mutant, wild strain.

**INTRODUCTION**

*Trichoderma* is a natural biocontrol agent and it is well-known for suppressing the plant pathogens by several mechanisms, which includes production of antibiotics, competing for key nutrients, production of cell wall degrading enzymes, stimulation of plant defense mechanisms and a combination of these modes of action (Chet, 1987). Biological control is considered an important mechanism and it depends on the production of lytic enzymes including chitinases, β-glucanases and proteases (Elad et al., 1985; Lorito et al., 1994; Inbar and Chet, 1995). Correlation between the production of chitinolytic enzymes and the suppression of fungi containing chitin is the main cell wall constituents was demonstrated for many *Trichoderma* sp. (Papavizas et al., 1982; Inbar and Chet, 1995; Balasubramanian and Lalithakumari, 2008).

In the present study, we focus to enhancing chitinase production by *T. harzianum* in order to develop it as a potential biocontrol agent against certain important phytopathogens such as *R. solani* causing sheath blight in rice, *F. oxysporum* f. sp. lycopersici causing fusarial wilt of tomato, *B. oryzae* producing brown spot disease, *Alternaria* sp causing leaf spot diseases. Adapted mutant by chitin and UV mutant by UV irradiation of *T. harzianum* was obtained for overproduction of chitinase, so that it can be successfully employed as a biocontrol agent, since lytic enzymes are involved in pathogen cell wall degradation.

Microbial adaptation plays an important role in the selection of improved strains for biological processes and their novel activities based on the genetic diversity of micro organism in a better adapted environment (Oskar Zelder and Bernhard Hauer, 2000). The adapted mutant of *Penicillium chrysogenum* reported that penicillin contains increased copy number of the penicillin biosynthesis genes (Newbert et al., 1997). The successful use of microbial adaptation by continuous serial cultivation is important for industrial stains

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Jimenez-Marrero and Cameron, 1999) and improvement of industrial strains by adaptation increases product yield (Gokhale, 1992). In general, adapted mutations appear under substrate limited conditions, whereas in this study, we raised adapted mutant by increasing chitin concentration.

The wild type induced by ultraviolet (UV) irradiation mutant differed from their respective wild type strains in growth, sporulation, survival in soil and suppression of several soil borne plant pathogens (Locke et al., 1984). Moreover, mutagenesis increase enzyme production has been successful in Trichoderma (Mandels et al., 1971) and some other fungi in industrial process (Mantyla et al., 1998). Furthermore, T. harzianum mutant of Gl-2 produced more cellulase than the wild type strains (Papavizas, et al., 1990), G. virens mutants have difference in their ability to produce the antibiotic gliovirin (Howell and Stripanovic, 1983). Mutagenesis alters the production of antibiotics and mycolytic enzymes in biocontrol agent in T. viride were also confirmed (Selvakumar et al., 2000).

Chitinases are well known for their performance in several biological functions as they are required for fungal morphogenesis. Furthermore, chitinases and chitooligomers produced by enzymatic hydrolysis of chitin can also be used as chitoheaxaose and chitoheptaose for anti tumor activity in human health (Murao et al., 1992). These enzymes have been used in industries to hydrolyze chitinous material and yeast Pichia kudriavzevii for single cell protein (SCP) production (Revah et al., 1981), as well as genomic and genetic studies like chitinase gene transformation to plants (Ann and Ko, 1990).

In view of multifarious usage of chitinase, we attempt to investigate a possible role of enhancing biocontrol and chitinase activity by a chitin adapted and UV irradiated mutant. Many reports are available in mutation study in fungi however; not much work has been focused on adapted mutants by colloidal chitin. Recently, the use of enzyme based treatment technology for biocontrol activity is gaining popularity. Therefore, the present study was aimed to isolate the potential adapted and UV mutants with the objective of enhancing the biocontrol and chitinase activity.

**MATERIALS AND METHODS**

**Culture Maintenance and Inoculum Preparation:**
Lab culture collection of T. harzianum was maintained on PDA (Potato 200 g; Dextrose 20 g; Agar 20 g; distilled water 1000 ml, pH 6.5) slants. The inoculum was prepared by adding 5 ml of sterile distilled water in a 5 days old slant culture and scrapped with an inoculation needle. The suspension was transferred into 100 ml molten PDA, mixed thoroughly and poured into sterile Petri plates and incubated at room temperature (28±2°C). Mycelial discs of 9 mm were cut randomly from 5 days old cultures with sterile cork borer and used throughout the investigation.

**Adapted Mutant:**
T. harzianum wild strain mycelia disc (9 mm) was placed on 0.5 % colloidal chitin amended medium (pH 6.5) and incubated at 28±2 °C for 5 days. The 5 days old fungus was grown on a gradually increasing (0.5 to 5 %) colloidal chitin to adapt the strain to higher concentration of chitin and to obtain chitinase rich adapted mutants.

**Isolation of UV Mutants:**
Conidia from a 5 days old culture of T. harzianum were suspended at a concentration of 1×10⁶/ml in sterile distilled water and exposed to UV light (254 nm, UV Philips) at a distance of 15 cm for 10, 20 and 30 min (99 % kill). The conidia were plated directly on 1 % chitin medium (selective medium) and incubated at room temperature. Dilutions were made until single colonies were purified.

**Growth of T. Harzianum on Different Solid Media:**
The growth rate and sporulation was assessed on potato dextrose agar (PDA), chitin medium (NaNO₃ 2g, K₂HPO₄ 1 g, MgSO₄·H₂O 0.5 g, KCl 0.5 g, FeSO₄·7 H₂O 0.01 g, colloidal chitin 10 g, agar 20 g, distilled water 1000 ml, pH 6.5). Inverted mycelia disc (9 mm) was placed at the centre of the Petri plate containing media and incubated at 28 ±2°C. Colony diameter was measured at 24 h intervals.

**Dual-plate Assay for Inhibition of Pathogens:**
The wild and mutant strains of T. harzianum were evaluated for their antagonistic potential against F. oxysporum, R. solani, B. oryzae, Alternaria sp. by dual culture technique of Huang and Hoes (1976).
Preparation of Cell Free Culture Filtrates:
The 25 ml Czapek-dox amended with 1% colloidal chitin (pH 6.5) was dispensed in 100 ml Erlenmeyer flasks and sterilized for 20 min at 15 psi. Conidial suspension (1 × 10^6 conidia / ml) was served as inoculum. The fungus was grown at 28±2°C in shaken condition (100 rpm). The culture filtrate was collected by passing it through filter paper (0.22 μm, Millipore, USA). The pH of culture filtrates was monitored using an Orion pH meter (Orion Research, USA) at 28±2°C. The culture filtrate was used for enzyme and protein assay.

Chitinase Assay:
Colorimetric assay of chitinase was followed by Reissing et al., (1955). Briefly, the reaction mixture contained 1 ml of 0.1% colloidal chitin in sodium acetate buffer (0.05 M, pH 5.2) and 1 ml of culture filtrate (enzyme) incubated at 37°C for 2 h. Respective substrate and enzyme blanks were included. Heat killed enzyme (culture filtrate) was also used as control. The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetra borate (pH 9.2) to 0.5 ml of reaction mixture and boiled in a water bath for 3 min. Liberated N-acetylglucosamine was determined at 585 nm (Beckman, DU-40 Spectrophotometer, USA). Specific activity of chitinase was expressed as units of N-acetylglucosamine liberated per milligram of protein per hour.

Protein estimation:
Protein content of the culture filtrate was estimated by dye binding method of Bradford (1976), using Bovine Serum Albumin (Fraction-V, Sigma, USA) as standard. An aliquot of 1ml culture filtrate with 5 ml of dye binding reagent (G-250 Coomassie Brilliant Blue) was added and mixed well. The absorbance was read at 595 nm in a spectrophotometer (Beckman, DU-40 Spectrophotometer, USA).

Preparation of Extra Cellular Proteins and Protein Variations Analysis by SDS-PAGE:
The culture filtrate was concentrated by adding 80% ammonium sulphate and dialyzed with distilled water over night to remove the salts and other external contaminants. The dialyzed sample was lyophilized and stored at -20°C until use.

SDS-PAGE was carried out in mini slab gels (Bio-rad) according to Laemmli (1970). The lyophilized samples were dissolved with 1x sample buffer, and boiled for 3 min in boiling water bath. Each well 80 μg sample was loaded. The gels were subjected to electrophoresis at 120 V for 2 hrs. Following electrophoresis the gels were stained with Coomassie Brilliant Blue (R250) to visualize protein bands. Bio-Rad molecular weight standards marker was used (161-0374).

Statistical Analysis:
All the data were subjected to statistical scrutiny by one way ANOVA. The values P<0.05 was considered as significant and P<0.01 was considered as a highly significant.

RESULTS AND DISCUSSION

Adapted Mutant Strains:
T. harzianum wild strain was adapted to chitin from 0.5 to 5%. The experiments show that the adapted mutant strain could grow up to 5% chitin amended medium. We choose the chitin adapted mutant strain for further studies from 5% colloidal chitin amended medium.

Development of T. harzianum Mutant Strains by UV Irradiation:
The number of mutants developed varied with the time of UV exposure. The percentage of mutant survival decreased with increased UV exposure time. Twenty minute UV irradiation was optimum and more conidial survival. These mutant colonies were selected based on growth, sporulation and lysis of chitin around the colony. These mutants were designated as numbers H1 to H15, of these H11 was used for further studies (Table 1). The wild type isolates and mutant were transferred to PDA, chitin medium to allow for growth and sporulation.

Colony Morphology of Wild and Mutants Strains of T. harzianum:
The UV mutant strain of T. harzianum showed marked difference in their spore colour, morphology and pigmentation. The UV mutant showed a puffy pale green pigmentation when compared with wild strain, where as the adapted mutant showed brown colour colony and pigmentation, since it was grown in colloidal chitin medium (Fig. 1).
Growth Kinetics on Solid Medium:

The growth of wild strain of *T. harzianum* on PDA and chitin medium clearly indicated that maximum growth was observed in PDA medium only. Where as the adapted mutant maximum growth was on chitin medium followed by PDA medium. UV mutant strain showed fast growth in PDA and chitin medium when compared to adapted mutant and the wild strain (Table 2). The UV mutants revealed that it could survive in both medium.

Dual Plate Assay:

Wild and both mutants strains were evaluated against four phytopathogens. The results clearly showed that the enhanced biocontrol activity by UV mutant strains followed by adapted mutants and wild strain. It was further observed that the UV mutant strain was over grown on the phytopathogens after 4 days of incubation followed by adapted mutant (Table 3).

Table 1: Germination percentage of mutants obtained by UV irradiation at different time intervals on 1% chitin medium.

<table>
<thead>
<tr>
<th>Time of UV exposure (min)</th>
<th>No. of conidia plated</th>
<th>Mutant colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1200</td>
<td>73.7±0.21</td>
</tr>
<tr>
<td>20</td>
<td>1200</td>
<td>61.2±0.17</td>
</tr>
<tr>
<td>30</td>
<td>1200</td>
<td>35.6±0.11</td>
</tr>
</tbody>
</table>

Note: Values are mean of triplicates with ± standard error.  
P<0.05 is significant;  
P<0.01 is highly significant.

Table 2: Growth of wild and mutant strains on PDA and chitin medium.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Days</th>
<th>Radial Growth (cm)</th>
<th>UV Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild</td>
<td>Adapted mutant</td>
</tr>
<tr>
<td>PDA</td>
<td>1</td>
<td>2.1±0.23</td>
<td>1.9±0.40</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.2±0.19</td>
<td>5.2±0.73</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.1±0.38</td>
<td>7.1±1.8</td>
</tr>
<tr>
<td>Chitin medium</td>
<td>1</td>
<td>1.7±0.12</td>
<td>2.0±0.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.3±0.33</td>
<td>5.6±1.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.5±1.21</td>
<td>8.1±1.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.1±0.92</td>
<td>Over growth</td>
</tr>
</tbody>
</table>

Note: Values are mean of triplicates with ± standard error.  
P<0.05 is significant;  
P<0.01 is highly significant.

Table 3: Biocontrol assay with wild and mutants strain of *T. harzianum* against phytopathogens.

<table>
<thead>
<tr>
<th>Trichoderma Strains</th>
<th>Phyto pathogens</th>
<th>Growth of antagonist (cm)</th>
<th>Growth of pathogen (cm)</th>
<th>Pathogen inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td><em>F. oxysporum</em></td>
<td>6.7</td>
<td>2.3</td>
<td>74.4±0.18</td>
</tr>
<tr>
<td></td>
<td><em>B. oryzae</em></td>
<td>7.0</td>
<td>2.0</td>
<td>77.7±0.23</td>
</tr>
<tr>
<td></td>
<td><em>R. solani</em></td>
<td>4.3</td>
<td>4.7</td>
<td>47.7±0.17</td>
</tr>
<tr>
<td></td>
<td><em>Alternaria</em> sp</td>
<td>7.2</td>
<td>1.8</td>
<td>80.0±0.47</td>
</tr>
<tr>
<td>Adapted Mutant</td>
<td><em>F. oxysporum</em></td>
<td>7.4</td>
<td>1.6</td>
<td>82.2±0.42</td>
</tr>
<tr>
<td></td>
<td><em>B. oryzae</em></td>
<td>7.3</td>
<td>1.7</td>
<td>81.1±0.38</td>
</tr>
<tr>
<td></td>
<td><em>R. solani</em></td>
<td>4.9</td>
<td>4.1</td>
<td>54.4±0.51</td>
</tr>
<tr>
<td></td>
<td><em>Alternaria</em> sp</td>
<td>7.6</td>
<td>1.4</td>
<td>84.4±0.47</td>
</tr>
<tr>
<td>UV mutant</td>
<td><em>F. oxysporum</em></td>
<td>7.9</td>
<td>1.1</td>
<td>87.7±0.35</td>
</tr>
<tr>
<td></td>
<td><em>B. oryzae</em></td>
<td>7.8</td>
<td>1.2</td>
<td>86.6±0.32</td>
</tr>
<tr>
<td></td>
<td><em>R. solani</em></td>
<td>6.1</td>
<td>2.9</td>
<td>67.7±0.19</td>
</tr>
<tr>
<td></td>
<td><em>Alternaria</em> sp</td>
<td>8.0</td>
<td>1.0</td>
<td>88.8±0.47</td>
</tr>
</tbody>
</table>

Note: Values are mean of triplicates with ± standard error.  
P<0.05 is significant;  
P<0.01 is highly significant.

Chitinase Assay:

Chitinase activity was quantified by colorimetric method. Maximum chitinase was recorded on day 6 in 1 % colloidal chitin amended medium for wild and mutant strains. However, UV mutant and adapted mutant produced more chitinase when compared to wild strain. The chitinase activity gradually decreased after 6 days of incubation at 28±2°C (Fig. 2 and 3). Enhanced production of chitinase by both mutants could be directly related to strain improvement in *T. harzianum*.

Estimation of Protein:

The extra cellular protein was quantified using the cell free culture filtrate of three strains of *T. harzianum*. UV mutant had more protein content on day 6 compared to the adapted and wild strains. The protein activity gradually decreased after 6 days of incubation similar to enzyme activity (Fig. 4).
**Fig. 1:** Colony morphology of wild, adapted and UV mutant of *Trichoderma harzianum*

**Fig. 2:** Total chitinase activity in wild and mutant strains of *T. harzianum*

**Fig. 3:** Specific chitinase activity in wild and mutant strains of *T. harzianum*
Fig. 4: Protein content of wild and mutant strains of *T. harzianum*

**Analysis of Protein by SDS-PAGE:**

Profiles of the secreted proteins from the three selected strains, visualized on 12% SDS-PAGE, displayed notable differences further indicating the effect of the mutagen effect (Fig. 5). Protein profiles showed around 10-15 secreted proteins were clearly dominant. Of these three stains, the UV mutant H11 clearly showed additional protein band appeared than the adapted mutant and wild strain. Fifteen and twenty major protein bands with up regulation were identified in the adapted and UV mutant H11 respectively. This was conformed the over production of extra cellular protein by UV mutant followed by adapted mutant strain over the wild strain.

Fig. 5: Analysis of extra cellular protein variation by SDS-PAGE

**Discussion:**

In the present study, we observed different mycelia color and growth pattern among the mutants and wild strain. The chitin adapted mutant was brown color pigmentation, while UV mutant H11 was buffy in appearance with pale green pigmentation, when compared to wild strain. However, a high yielding mutant’s mycelium showed a violet-brown color compared to the parent strains was reported (Ullrich Keller, 1983). On the other hand, Kim *et al.*, (1983) reported *M. purpurea* ultraviolet induced mutants produced desirable products with different mutant color. The adapted and UV mutant of *T. harzianum* grown on chitin agar
medium and the mutants exhibited normal hyphae with similar radial growth pattern but UV mutant considerably narrowed hyphae than the wild strain in PDA medium. We used PDA and chitin medium to check the growth rate for mutant and wild strain, UV mutant and wild strain fast growth in PDA medium, where as adapted mutant was slow growth. However, in chitin medium both mutants were fast growth and wild strain was slow growth. The results suggested that UV mutant was well adapted to both medium.

Several colonies with different growth patterns were also observed. However, selection of colonies, based on the clearing zones on colloidal chitin agar constitutes a qualitative screening method, which allows rapid examination of a large number of colonies after mutagenesis. We suggest that this method could be employed for other fungi known for chitinase production by inducing mutants. We describe the mutants are differing from the wild type in their ability to utilize carbon sources and produce enzymes and disease suppression. Changes in the colony morphology of adapted strain reported in Mycoplasma pulmonis, Candida albicans, Thiobacillus ferroxidans (Slutsky et al., 1987; Byvbig, et al., 1989).

The UV H11 mutant and adapted mutant were showed increased biocontrol activity when compared to wild strain. The overall mycelia growth inhibition of F. oxysporum, B. oryzae, R. solani and Alterneria sp were 69.9 %, 75.5 % and 82.7 % by wild, adapted and UV mutant respectively. Graeme-cook et al., (1991) reported that high antibiotic production by two T. harzianum mutant strains, BC10 and BC63, increased inhibition of hyphal growth of R. solani and P. ultimum, while Papavizas et al., (1982) have shown UV-induced benomyl resistant mutant to suppress the saprophytic activity of R. solani more effectively than the wild strain. We observed wild strain was slow growth when compared to both mutants and their inhibitory effect also less against four phytopathogens. Furthermore, the antagonism of these two mutants with F. oxysporum, R. solani, B. oryzae and Alternaria sp. were varied and could be related with lytic enzyme production with fast growing ability. However, Lorito et al., (1993) reported chitinolytic enzymes contributing to the ability of Trichoderma sp to act as biocontrol agents.

Our results exhibited that UV mutant H11 utilized the chitin effectively and also lysis the chitin around the colonies. It is important to note that mutant H11 prominent lysis was observed followed by adapted mutant (date not shown). We optimized suitable conditions for the production of enzyme and protein was 1 % chitin at 28°C, pH 6.5 and was independent of the age of the mycelium. However, for the enzyme assay by adapted mutant we used from 5 % colloidal chitin adapted strain. Production of chitinase and protein was started to secrete after 24 h and it reached maximum on day 6 with chitin medium and gradually declined further incubation. Similar findings were reported in T. viride by Mathivanan et al., (2000) on 6 days of growth with chitin. On the other hand, Katragadda and Murugesan (1996) have reported release of chitinase on second day after incubation, while maximum production on eighth day and subsequent decline thereafter. We recorded total chitinase 1.3, 1.6 specific chitinase 1.7, 2.2 protein 1.3, 1.8 fold increased by adapted and UV mutant respectively on the six days of incubation was observed. Similar findings in mutant QM9123 produced two fold protein and cellulase on cellulose when compared to parent strain of T. viride (Mandels et al., 1971). However, in A. album mutantE3 chitinase and protein increased 26-fold (Vasseur et al., 1990), in Ophiostoma flocosum mutant MQ.5.1 was increased chitinase by six fold (Wu et al., 2006) and in T. lanuginosus UV mutagenesis showed 2.29-fold increased in phytase activity (Gulati et al., 2007) greater than their parental strain. The EMS induced mutant with an enzyme activity of 25.56 U/ml was obtained further exposure to UV radiation and yielded same mutant an activity of 34.12 U/ml (Ul-Haq et al., 2008).

SDS-polyacrylamide gel electrophoresis (PAGE) analysis showed additional protein bands appeared with up regulation in UV mutant followed by adapted mutant than their respective wild strain, which could be attributed to mutagenic effect. The increased protein was also confirmed by quantitative assay, SDS-PAGE analysis was done to compare identity among the wild and mutant strains with same amount of protein was loaded. These results showed 11, 15 and 20 major protein band in wild, adapted and UV mutant respectively, furthermore it suggested that, the number of protein bands of both the mutants were more than their counterpart of wild strain. Similarly SDS-PAGE showed additional protein band and increased extracellular enzymes by the application five rounds of mutation were reported in Ophiostoma flocosum (Wu et al., 2006). Furthermore, Graeme Cook and Faull (1991) showed the altered antibiotic production in mutants of T. harzianum with two new antifungal compounds in addition to antibiotics and additional bands in mutant strains were spotted on TLC plates, which indicate the possibility of involvement of extra antifungal compounds in mutants.

We confirmed increased biocontrol activity and chitinase and protein production by UV and adapted mutants over the wild strain. An additional protein band in UV mutant followed by adapted mutant was added weight to the probability of enhanced biocontrol activity, chitinase and protein production in the mutant strains. However, the purification of protein from both mutants is in progress to analyse variations of amino acids among the mutants. There could be no doubt using improved strains as a biocontrol agent for plant disease...
control by UV irradiated and adapted mutants. This technique can also be used in other fungi to develop potential mutants with different applications.

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REFERENCES


