

## STRAIN IMPROVEMENT OF TRICHODERMA HARZIANUM BY UV MUTAGENESIS FOR ENHANCING IT'S BIOCONTROL POTENTIAL AGAINST AFLOTOXIGENIC ASPERGILLUS SPECIES.

### ABSTRACT

Trichoderma is an opportunistic, avirulent plant symbiont. Chitin is the main structural component of fungi, enzyme responsible to metabolize the chitin and various techniques to enhance the chitinolytic enzyme production at the genetic level are receiving great attention to develop the efficient biocontrol agent. UV mutagenesis was found to be one of the novel strategy for developing Trichoderma mutants with enhanced chitinolytic activity.

Four mutant strains were obtained from the wild *T. harzianum*, after UV treatment of different time period ranging from 2min, 4min, 6min, 8 min. Chitinase overproducing mutants were selected by growing on basal chitinase detection medium, based on the diameter of purple color zone formation around the colony in a shorter time. Selected mutants were tested for in vitro inhibition of test pathogen (*A. flavus* and *A. parasiticus*) by using various plate culture techniques (dual culture, pathogen at center, inverted plate technique) for their bio-efficacy.

**KEY WORDS** *Trichoderma harzianum*, UV mutants, Biocontrol mechanisms, *Aspergillus* species, Chitinases.

### INTRODUCTION

*Trichoderma* classificationally is a genus of fungi within the division Deutromycetes, which are a heterogeneous group of fungi in which sexual stages (perfect stages) are not known or rarely found, and reproduction is limited to the production of conidia.<sup>1, 2</sup> Unfortunately, many of the microorganisms that have gained industrial importance do not have a clearly defined sexual cycle (e.g. fungi imperfecti). This has meant that the only way to change the genome with a view to enhancing productivity has been indulged in massive mutational programs, followed by screening and selection to detect the new variants that might arise.<sup>3,4,5</sup> Strain improvement usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbes to physical mutagens such as X-rays,  $\gamma$ -rays, UV-rays and chemical mutagens such as NTG, EMS, etc.<sup>6</sup>

Plant diseases are limiting factors in the production of most crops. *Aspergillus* species are among the devastating diseases causing phytopathogens in different crops causing thoughtful economic outlay. We know that. a fungicide program is required to manage fungal diseases. However, fungicide application caused many negative effects on environment. The consequence been directed primarily towards identified new control methods that could be effective, reliable and safe in the environment. Only the option is biological control and also a potent means of reducing the damage caused by plant pathogens<sup>7, 8</sup>. Biological control of several plant pathogens has shown promise as an optional disease's management strategy.<sup>9</sup>

Antagonistic microorganisms represent the most diverse group of organisms on the plant. Even through the natural microflora, antagonistic fungi included *Trichoderma* species are surmised of special shares as biological control agents against numerous phytopathogenic fungi.<sup>9</sup> *Trichoderma* species are known as a cogent producer of many antifungal metabolites, including enzymes and others<sup>10,2</sup>. Biological control provides an alternative mean over the chemical control with the advantages of greater public acceptance, reduced environmental impact, nontoxic to human being, host specific and once colonized can last for years.<sup>11</sup>

Keeping this in view, the present study was conducted for screening the biocontrol potential of *Trichoderma harzianum* and its mutants, specifically with chitinolytic enzyme overproducing capacity against aflatoxigenic *Aspergillus* species viz. *A. flavus* and *A. parasiticus*.

## MATERIALS AND METHODS

### Isolation of efficient *Trichoderma* species

*Trichoderma* species used in this study were isolated from the soil sample of area, where the disease occurrence is suppressed<sup>12</sup>. Triplicate soil sample were randomly collected and the isolation of antagonistic fungal species were performed by using serial dilution plate technique (pour plate method) on Rose Bengal Agar and Potato Dextrose Agar, emerging fungal colonies were isolated, stained with lacto-phenol cotton blue, based on the microscopic observation and colony characteristics. Colonies of *Trichoderma harzianum* were selected and transferred to new PDA plates for further experiments. Genus and species-level identification of *Trichoderma* were carried by studying the microscopic features such as conidiophores, branching, phialides shape and position, spore size and shape<sup>13</sup>(Gams and Bisset, 1998) and additional confirmation of results carried by the support of ITCC, Indian Agriculture Research Institute, New Delhi (India).

### Production and Characterization of UV Mutants

The UV mutants were obtained according to method described by Awad et al. (2005) with slight modifications.<sup>14</sup> The plates without lids containing spore suspension of wild *Trichoderma harzianum* was exposed to UV light ( $\lambda = 254$  nm). The suspension of spores prepared in normal saline (0.9%) of  $10^6$  spore/ml concentration. The sporulation was evaluated using Neubaur chamber. The 10ml of spore suspension was exposed to UV light for different time interval of 2 min, 4 min, 6 min and 8 min. The distance between spore suspension and UV source was adjusted to 15 cm.

Then 20  $\mu$ l of sample from treated spore suspension was taken from stock in 50 sterile eppendorff tubes. After that 980 $\mu$ l of TSTW (Trypton Salt Tween Wash, 0.95 g/l trypton salt broth and 3.3 $\mu$ l of tween 80) was added to it. Then these UV irradiated samples of each time the interval were spread on petriplates containing PDA (Potato Dextrose Agar). The plates were incubated at 25° C for 5 days. The slants were prepared and stored in the refrigerator for further use.

### Screening of Chitinase overproducing *Trichoderma harzianum* and its mutants

The strains of *Trichoderma* were screened for overproducing chitinolytic activity, by using the method of Kotasthane and Agrawal (2009),<sup>15</sup> which is the basis of selection, performance and in vivo bio efficacy. The wild and mutant strains of *Trichoderma harzianum* were screened for chitinase production on Chitinase Detection Medium. The final composition: [(all amounts are per liter) 4.5 g of colloidal chitin, 0.3 gm of MgSO<sub>4</sub>. 7H<sub>2</sub>O, 3.0 g of (NH<sub>4</sub>)SO<sub>4</sub>, 2.0 g KH<sub>2</sub>PO<sub>4</sub>, 1 g of citric acid monohydrate, 15 g agar, 0.15 g bromocresol purple and 200  $\mu$ l of tween-80], pH was adjusted to 4.7. After autoclaving the medium was poured into the petri plates and allow it to solidify. The fresh culture plugs of the *T. harzianum* as wild and mutants to be tested for chitinase activity was inoculated and incubated at 25° C for 3-4 days. Formation of the purple colored zone was observed and recorded. The diameter of the zone was taken as main criteria to determine the chitinase activity.

### Mycoparasitic Activity of *Trichoderma* species and its Mutants

*Trichoderma harzianum*, and its mutants were selected to assess their mycoparasitic efficiency against the selected plant pathogens i.e. *A. flavus* and *A. parasiticus*. Aflatoxigenic nature of *Aspergillus* isolates were again screened for the ability to produce aflatoxins based on the detection of UV-induced fluorescence and specifically orange to yellow pigmentation on the coconut agar medium and also on *Aspergillus* differentiation media (ADM).<sup>16</sup>

### Dual culture technique

In vitro antagonistic activity of *Trichoderma harzianum* and its mutants against *A. flavus* and *A. parasiticus* was studied in dual culture technique. This technique was developed for studying the effect of diffusible metabolite produced by the antagonist against a pathogen. From the edge of 7 days old culture 5mm diameter, mycelial disc of antagonistic fungi and the phytopathogen were inoculated on the opposite to one another and equidistance from the periphery (fig.5). The plate with pathogen disc alone was used as a control; plates were incubated at 25° C. The colony diameter of pathogen, and antagonist was measured. The radial growth of the pathogen colony was measured on 7<sup>th</sup> day and percentage inhibition was calculated by using given formulae.

$$\% \text{ inhibition} = C-T/C \times 100$$

Where, C = Radial growth in control set;

T = Radial growth in treated set.

### Pathogen at centre technique

Competitive interaction by pathogen at centre technique was developed for studying the effect of diffusible metabolite produced by the antagonist. In this technique 5mm, mycelial discs of the pathogen colony were placed aseptically in the centre of Rose Bengal Agar plate. Four 5mm disc of each antagonist were placed at the distance 35mm away from pathogen disc on sterile Rose Bengal Agar plates (fig.6). For control, each test pathogen alone without the antagonist was used. The plates were then incubated at 25° C for 7 days, and radial growth of pathogen was recorded. Percentage inhibition was calculated in relation with the growth of control<sup>17,18</sup> by using same formulae mentioned above.

### Inverted plate technique

Effect of volatile metabolites produced by *Trichoderma harzianum* and its mutants against test pathogens were evaluated by method of Dennis and Webster (1971)<sup>19</sup>. The mycelial disc (5mm) of *Trichoderma harzianum* and its mutants as well as test pathogens were centrally placed on separate Rose Bengal Agar plates and are incubated for 25°C for 4 days. After the completion of incubation period, lids of all plates were replaced with each other's bottom (sealed), so as test pathogens were directly exposed to antagonistic environment created by *Trichoderma harzianum* and its mutants. Radial growth of pathogens was recorded after 4 days of incubation and percentage inhibition was calculated in relation to control.

## RESULTS AND DISCUSSION

*Trichoderma harzianum* was isolated from rhizosphere soil collected from Amravati region. Preliminary selection of genus *Trichoderma* was carried out by using morphological characteristics such as growth of mycelia, colony color, sporulation time and the sporulation potential on different solid media (fig 1). Conidiophores and conidia morphology help for the further characterization of *Trichoderma harzianum*. *Trichoderma harzianum* is characterized by rapid growth, repetitively branched conidiophores structure and frequently bright green conidia.<sup>13</sup> The confirmations of results were also done by ITTC, IARI New Delhi.

### Development of mutants

A wild type isolated *Trichoderma harzianum* were subjected to UV mutagenic treatment. Colony morphology of wild species changing them into mutants, due to small or point mutations. Growth rate and sporulation potential of all the mutant strains was found to be higher than that of their respective wild species. All mutant isolates were found to be fast growing, covered the whole plate in 4 days compared to 6 days by the wild isolates. After four mutagenic treatment of different time interval, four mutant strains *T. harzianum* were selected and were named according to the time period *Trichoderma harzianum* (WH) as wild and mutants 2H,

4H, 6H and 8H, The varied range of phenotypic and microscopical differences was observed (fig. 2), which are assessed by their growth and sporulation potential.

In case of *T. harzianum* and its mutants, diameter of wild type was 30mm, which found to be less than mutants, with very less sporulation on 4<sup>th</sup> day. Colony was slightly submerged with watery white color mycelia, having a smooth surface. While all the mutants show the significant rate of sporulation along with growth rate ranging from 2H=33mm, 4H=39mm, 6H=29mm and 8H=37mm. 4H and 8H show the high yellow to green sporulation on Potato Dextrose agar with white–yellow color mycelia showing rough colony texture, predominantly effused with powdery granular conidia. *T. harzianum* and its mutants are shown in (fig. 3).

Development of mutants is one of the strategies in the strain improvement program of biocontrol agents. Papavizas et al. (1990) obtained UV induced mutants of *T. harzianum* tolerant of Benomyl, and they suppressed the growth of *R. solani* in soil more effectively than the wild strains. Several mutant strains of *Trichoderma* have been developed with enhanced biocontrol activity against plant pathogens.<sup>20</sup> Selvakumar et al. (2000) studies the three stable mutants of *Trichoderma* that shows different colony morphology, and antagonistic potential against *U. segetum-tritici* as compared to their parents<sup>21</sup>. In Zalidivar et al. (2001) developed a mutant strain of *Trichoderma* with enhanced production addition to biocontrol potential, the above mutant also exhibited increased fungicide tolerance than the wild parents<sup>22</sup>.

### Screening of Chitinase overproducing *Trichoderma* and its mutants

*T. harzianum* and mutant's strains were screened and selected for their chitinolytic enzyme production based upon the diameter of purple color zone surrounding the colony on chitinase detection media in a shorter time shown in (fig.4) In case of mutant 4H and 8H showed a larger purple color zones as compared to wild type and other mutants. Mutants exhibiting higher chitinase activity on chitinase detection media were selected maintained.

Purple color zone was due to bromocresol purple that was supplemented with media as a pH indicator. As the media was containing colloidal chitin, *Trichoderma* wild and mutants break down the chitin with chitinolytic enzymes, changing chitin to N-acetylglucosamine, which is basic in nature. Thus, change in the pH from acidic to basic, color of media also changes from yellow to purple color.<sup>15</sup> Chitin agar plate has been used earlier for isolating chitinolytic microorganisms by observing clear zone around the colony of microorganism.<sup>23</sup> Growth and sporulation differences were observed in case of mutants of *T. harzianum* along with higher zonation of purple color on chitinase detection media. These results indicate that the enhancement of chitinolytic activity might be due to the increase in growth rate. Formation of the purple color zone was found to be the easier alternative method to the selection of chitinolytic strains. UV induced mutation may not only affect the chitinase genes directly but also affect the synthesis and secretion of chitinase enzymes.

### Pathogen growth inhibition assay

The use of chitinolytic enzymes in the antagonistic activity of *Trichoderma* spp. against several plants pathogen is still debated<sup>24,25</sup>. It has been suggested that antagonistic *Trichoderma* strains produce chitinolytic enzymes only to colonize saprophytically hosts who have been previously killed by antibiotics (Belanger et al. 1995)<sup>24</sup>, and a correlation between in vitro chitinolytic activity of various *Trichoderma* strains and biocontrol efficacy in vivo has not yet been demonstrated. In addition, mechanisms other than parasitism have been suggested for some biocontrol strains<sup>26, 27, 28, 29</sup>. Formation of the purple color zone supports the involvement of chitinolytic enzymes in mycoparasitism and biocontrol activity.

### Dual culture technique

In vitro antagonistic property studies were carried out to evaluate the effect of wild and their UV mutants on the growth of two aflatoxigenic *Aspergillus* species by direct competitive interaction shown in (fig. 5). The antagonistic activity has often been depends

on the production of secondary metabolites<sup>30</sup>. Results for the four mutant strains with their respective wild type are presented in Table 3 and 4. UV mutants of *T. harzianum* proved to be an effective antagonist against the examined plant pathogen, showing range of percentage inhibition ranging from 56.52-71.01% against *A. flavus* and 50.00-64.00% against *A. parasiticus*. Mutant 4H and 8H show highest percentage inhibition (71.01%) against *A. flavus* (Table-3) and 2H and 4H (64.00, 60.00%) shows significant inhibition against *A. parasiticus* than wild type (52.00%) (Table-4).

### Competitive interaction by Pathogen at Centre Technique

Pathogen at centre technique shows the higher growth restriction than dual culture method (Fig. 6). Percentage inhibition of *A. flavus* growth by UV mutants of *T. harzianum* showed significant differences (2H-78.26%, 4H-81.15%, 6H- 76.81% and 8H-79.71%) when compared to the 76.81% inhibition caused by wild type (Table-3). Similar results were observed by UV mutants against *A. parasiticus*, mutants show 69.00%(2H), 76.00% (4H), 68.00%(6H), and 72.00% by 8H as compared to be wild (70.00%) (Table-4). In both the cases 4H, and 8H was found to be an efficient mutant strain as compared with wild and other mutants.

### Volatile metabolites (inverted plate technique)

The interactive study between the test pathogen along with *Trichoderma harzianum* and its mutants in inverted culture technique (volatile metabolites) showed acceptable results. In case UV mutants of *T. harzianum*, significant growth inhibition of *A. flavus* was observed (2H-76.00 %, 4H-84.00 %, 6H-70.00 % and 8H-78.00 %) when compared to the 75.00% inhibition caused by wild type(Table-3).. Similar inhibitory results were observed by UV mutants against *A. parasiticus*, mutants show 76.68% (2H), 84.00% (4H), 70.00%(6H), and 76.68% by 8H as compared to be wild (75.34%) (Table-4).. In both the cases 4H, and 8H was found to be an efficient mutant strain as compared with wild and other mutants. When we study the antagonistic activity of wild and its mutants against aflatoxigenic fungi *A. flavus* and *A. parasiticus*, *A. flavus* shows higher sensitivity towards the volatile metabolites produced by mutants of *T. harzianum*.

Doi and Mori (1994) deduced the volatile compounds of *Trichoderma* species that control the mycelium growth of different fungal pathogens on agar plates<sup>31</sup>. This finding also supports the research of Calistru et al. (1997), which revealed that volatile metabolites and extracellular metabolites produced by *Trichoderma* species can significantly suppress the growth of *A. flavus* and *F. moniliforme* rather than mycoparasitism.<sup>32</sup>

Claydon et al. (1987) reported antifungal properties of volatile compounds (alkyl pyronres) produced by *T. harzianum*<sup>33</sup>. Similarly, Rathore et al. (1992) reported volatile activity of *T. viride* against *F. solani* which vacuolated most hyphae of pathogen and that the hyphae of pathogen were comparatively thin as compared to control.<sup>34</sup> Workers like Michrina et al. (1995) and Pandey and Upadhyay (1997) have also reported the effectiveness of diffusible volatile compounds by *T. viride* and *T. harzianum* in vitro.<sup>35,36</sup>

## CONCLUSIONS

The aim of the work presented within this paper was to develop *Trichoderma harzianum* mutants that show better chitinolytic activity, which intern should possess more inhibitory activity. This opens the new possibilities for better application of chitinase. The availability of enzyme preparations with high chitinase activity could be useful not only in biological control but also in bioconversion of chitin waste materials and in production of chito -oligosaccharides for various applications. Results of this study demonstrate that stains with increased chitinase activity and biocontrol effectiveness could be easily produced by simple UV-mutagenesis.

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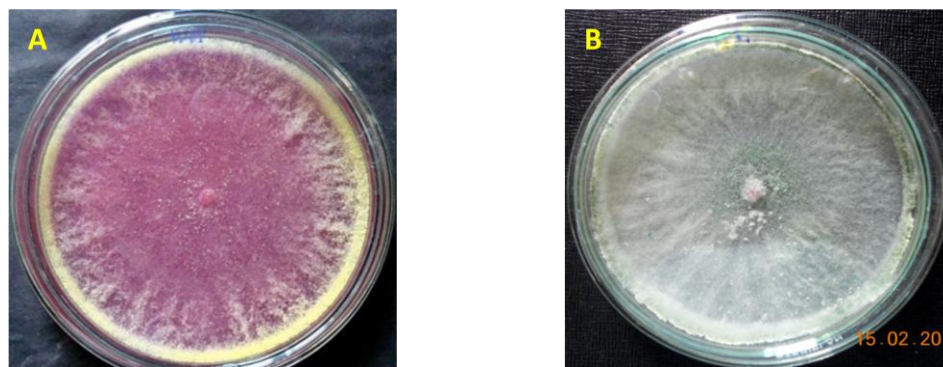
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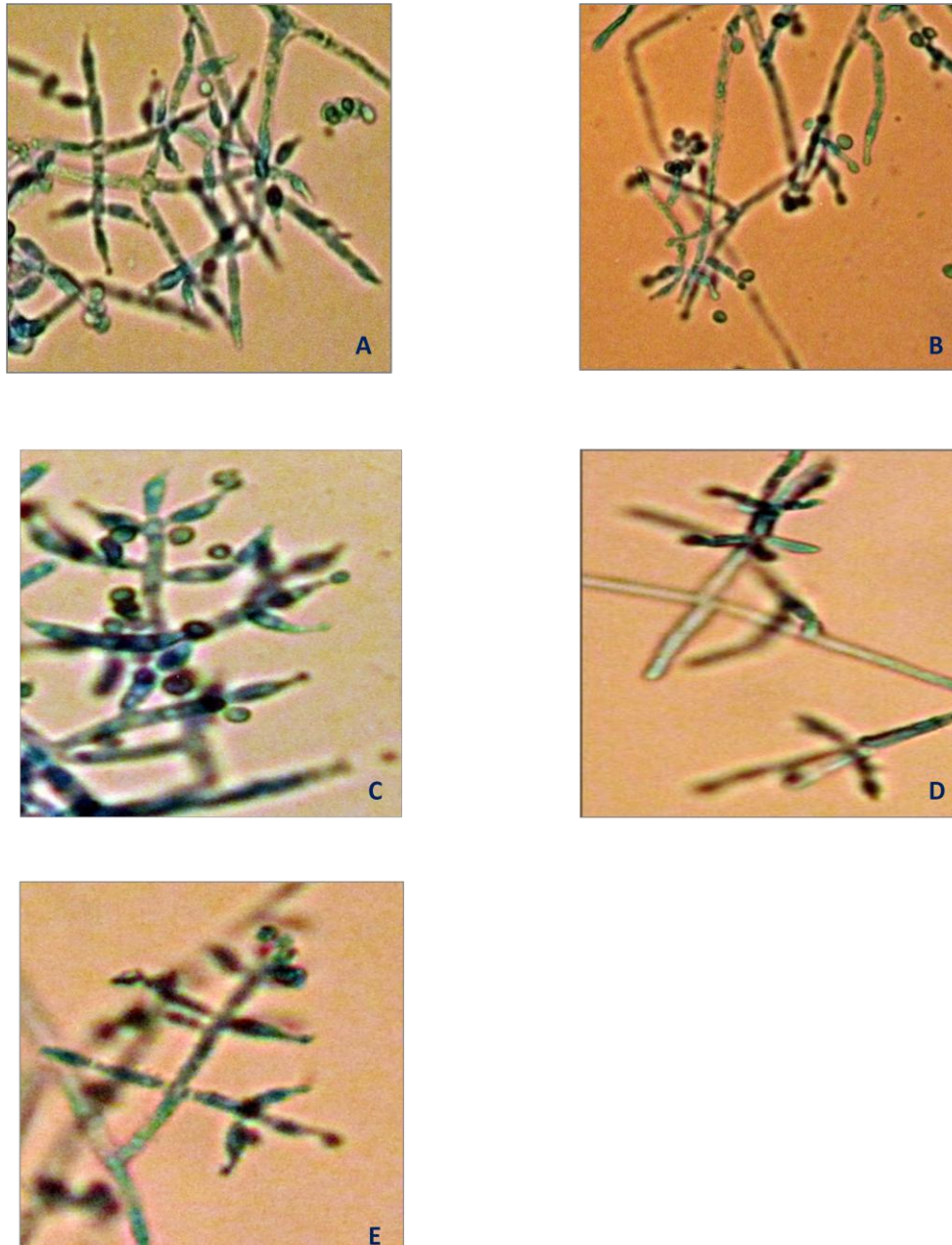
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#### Photoplates-

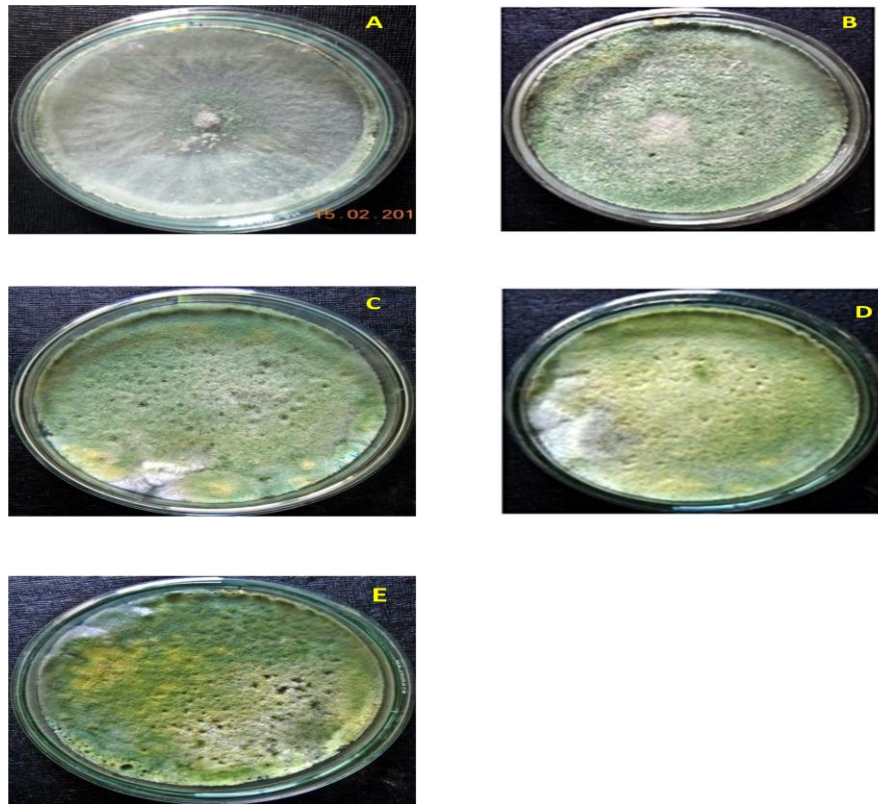


**Figure.1:** *Trichoderma harzianum* grown on Rose Bengal Agar (A) and Potato Dextrose Agar (B)

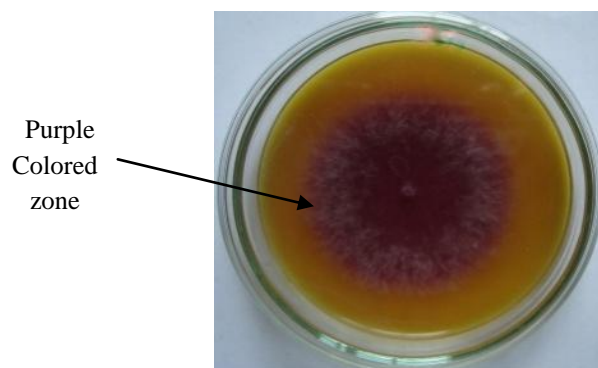




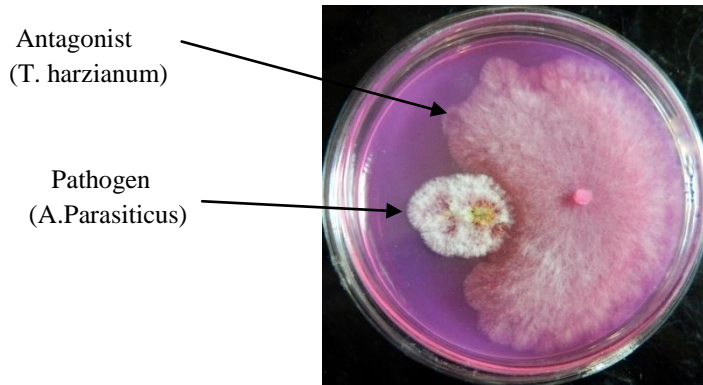
**Figure.2:** Microscopic variation in *Trichoderma harzianum* (A- WH) and its UV mutants (B- 2H, C- 4H, D -6H and E- 8H)



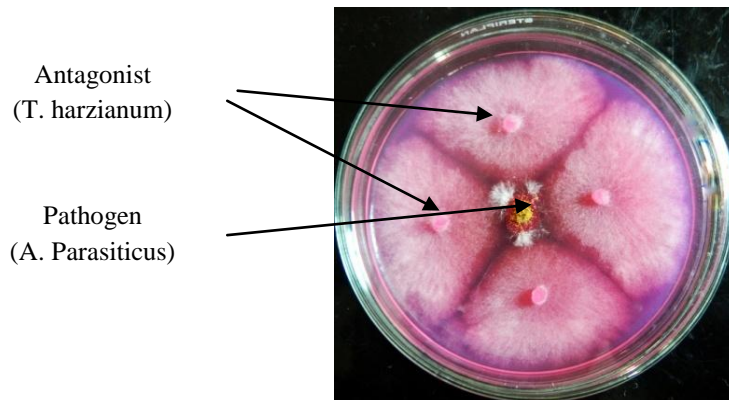
**Figure 3 - Photoplate of *T. harzianum* and its mutants A - WH, B - 2H, C- 4H, D - 6H, E- 8**



**Figure 4- Purple color zone shown by mutant of *Trichoderma harzianum* on Chitinase Detection Medium.**



**Figure 5-** Interaction of *Trichoderma harzianum* mutant and *A. parasiticus* in Dual culture technique.



**Figure 6-**Interaction of *Trichoderma harzianum* mutant and *A. parasiticus* in Pathogen at centre technique.

**Tables:-**

SN.	Characteristics	T. harzianum
1.	Growth rate (cm), colony color	9.5cm in 4days, whitish green-bright green.
2.	Colony mycelia	Floccose, compact, whitish
3.	Colony reverse	Colorless to drab color.
4.	Conidiophores branching	Much branched, form loose tufts which arise in ring like zone.
5.	Conidiation, conidial color	Moderate, compact, yellow-pale green
6.	Conidia shape	Smooth, subglobose.
7.	Exudates/Pigmentation	Colorless to pale amber, yellowish in some species.

**Table 1. Comparative accounts of macroscopic characters of Trichoderma species**

Sr. no.	Species	Spore size	Spore structure	Filament/fruiting bodies
1.	WH	2.5-2.7µm	Produced singly, accumulate at the tip of phialide, small size, not in chain, highly stained, chlamydo spores were abundant, intercalary or terminal, subglobose and pale to brownish at age.	Hyphae was septate, branched and smooth walled, conidiophores was smooth walled, straight, branched, conidiation started at the base while tip was still growing.
2.	2H	2.0-2.2µm	Pale green to brown colored spores with circular to oval shape. Sometimes appears in bunches with reduced size and numbers.	Hyphae were septate, branched. Hyphal size was found to be reduced than wild species. cell wall was smooth with conidiation started at the base of cell.
3.	4H	2.6-2.9µm	Retain original structure, in chain faint yellow to pale green, globose to subglobose, some of them was found to be double layered, number of spores increases as compared to wild species, less stain circular	Retain original structure of hyphae with septate and branched. Cell wall was slightly rough and straight. Conidiation started at the base of the cell.
4.	6 H	2.4-2.7 µm	Moderate spores small chains, circular, few oval, dark green to pale brown, rough walled, double layered. Disruption in spore structure also appears, chlamydo spores were abundant, intercalary or terminal	Retain original structure of hyphae with septate and branched. Cell wall was slightly rough and straight. Conidiation started at the base of the cell. Number of conidiogeous cell was found to be reduced
5.	8H	2.6-2.8 µm	Number of spores highly reduced, few fruiting bodies, side chain present, few were oval to circular, chlamydo spores were less in number, Produced singly, accumulate at the tip of phialide.	Septation of hypahe was disrupted, cell wall was smooth, and Number of conidiogeous cell was found to be reduced, hyphal size slightly increased.

**Table 2 : Microscopic variabilities of *Trichoderma harzianum* and its UV mutants.**

Percentage (%) of growth inhibition of *A. flavus* (pathogen) in mm (7<sup>th</sup> day)

S.N	Trichoderma	Dual culture	Pathogen at centre	Volatile metabolites
1.	WH 25	63.76% 16	76.81%25	63.76%
2.	2H 21	69.56% 15	78.26%24	62.32%
3.	4H 20	71.01% 13	81.15%19	72.46%
4.	6H 30	56.52% 16	76.81%30	56.52%
5.	8H 20	71.01% 14	79.71%22	68.11%

S.E. ±1.0954	S.E. ±1.151	S.E. ±1.678
C.D. =3.572	C.D. =3.753	C.D =5.473

**Table 3. Effect of *Trichoderma harzianum* and its UV mutants on radial growth inhibition of *A. flavus* on RBA medium in variable culture techniques. (Control AF=69mm)**

Percentage (%) of growth inhibition of *A. parasiticus* (pathogen) in mm (7<sup>th</sup> day)

SN.	Trichoderma	Dual culture	Pathogen at centre	Volatile metabolites
1	WH	24 52.00%	15	70.00% 12.33 75.34%
2	2H	18 64.00%	15.5	69.00% 11.66 76.68%
3	4H	20 60.00%	12	76.00%08 84.00%
4	6H	25 50.00%	16	68.00% 15 70.00%
5	8H	24 52.00%	14	72.00%11.66 76.68%

S.E. ±0.774	S.E. ±0.632	S.E. ±0.802
C.D. ±2.526	C.D. ±2.062	C.D ±2.617

**Table 4. Effect of *Trichoderma harzianum* and its UV mutants on radial growth inhibition of *A. parasiticus* on RBA medium in variable culture techniques (Control AP=50mm)**





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