The Bioscan 11(4): 2783-2789, 2016
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

# INDUCED MUTANTS OF NATIVE NON-PATHOGENIC *ALTERNARIA* SPP. SHOW IMPROVED BIO-CONTROL POTENTIAL AGAINST *ALTERNARIA LINI*

**ROSHAN D. KADU AND ASHWINI M. CHARPE***
Department of Plant Pathology,
Dr. Panjabrao Deshmukh Krishi Vidyapith, Akola - 444 104, Maharashtra
e-mail: ashwinicharpe@yahoo.com

**KEYWORDS**
UV-irradiation
Gamma-irradiation
Ethidium Bromide
*Alternaria lini*
Linseed
Bioefficacy

**Received on :**
31.08.2016

**Accepted on :**
29.10.2016

***Corresponding author**

**ABSTRACT**
Linseed (*Linum usitatisimum*), an important oilseed crop faces 28-60% losses due to blight disease caused by *Alternaria lini*. For the first time, native nonpathogenic fungal isolates of *Alternaria* spp. were previously identified as potential biocontrol agents against foliar pathogens *Alternaria lini* of linseed and *Alternaria helianthi* of sunflower. Currently, native isolates NPAL-5 and NPAH-2 from previous work with limited bioefficacy *i.e.* 41.92% and 43.74%, respectively, were treated with physical mutagens; Ultra Violet rays, Gamma rays, and chemical mutagen; Ethydium Bromide and tested against *Alternaria lini*. Six mutants namely NPALE3c, NPAHE3c, NPALUe, NPAHUe, NPALG2c and NPAHG3c exhibited improved bioefficacy. Among these, culture filtrate of NPALE3c supplemented as 10% has reduced radial mycelial growth of *Alternaria lini* by 63.15% and was found significantly superior over other treatments followed by NPAHE3c supplemented as 10% with 61.55% inhibition *in vitro*. Pot trial demonstrated that spraying of 10% culture filtrate of NPALE3c after 30hrs of pathogen inoculation was significantly superior showing 60.46% disease control followed by spraying of 10% NPAHE3c after 30hrs of inoculation with 53.31% disease control. Overall, Ethydium Bromide induced mutant NPALE3c exhibited maximum improvement in bioefficacy with 12.14 to 19.51% reduction in disease over native isolate NPAL-5.

## INTRODUCTION

Linseed (*Linum usitatissimum* L.) is among the oldest cultivated crops. It belongs to family Linaceae and is presumed to be originated in southwest Asia particularly India (Vavilov, 1935; Richharia, 1962). Among >200 species belonging to the Genus *Linum* (Gill, 1987) *Linum usitatissimum* L. is the only economically important specie (Kumar and Paul, 2015). Due to richness of linseed oil in alpha linolenic acid (ALA) an essential Omega-3 fatty acid and lignin oligomers accounting for 57 % of total fatty acids in its biochemical composition and commercial utility of linseed oil in paints and polymers linseed is amongst the important oilseed crops of the world (Reddy *et al.,* 2013). Linseed is grown extensively in most of the parts of India covering an area of 292.1 thousand hectares with 141.2 thousand tons production and 484 kg per hectare productivity (Anonymous, 2015) but *Alternaria lini* a facultative parasite responsible for causing blight disease (Day, 1933) affect all the aerial parts of plant and due to the necrotrophic behavior of *Alternaria* spp., it destroys chlorophyll of much larger area of leaf lamina than actual site of infection and hence drastically reduces photosynthetic area, ultimately affecting the yield greatly (Anilkumar and Hegade, 1974). Alternaria blight disease reduces the productivity of linseed by 28-60 % (Chaudhari and Shrivastava, 1975).

To manage such devastating diseases, repeated spraying of fungicides is done that not just increase the cost of production but also has adverse environmental implications (Lal *et al.*, 2015). In this view, scientific community is searching for ecofriendly approaches. Encouraging results are obtained through research work focused around use of native nonpathogenic isolates of *Fusarium* spp. against pathogenic Fusaria of soil borne nature (Couteaudier, 1992). *Alternaria* spp. is also reported to have a very high level of variability including pathogenic as well as nonpathogenic species (Ambesh *et al.*, 2014; Marak *et al.*, 2014). For the first time native nonpathogenic fungal isolates of *Alternaria* spp. were identified as potential biocontrol agents of foliar pathogens *i.e. A. lini* of linseed (Bhoye, 2009) and *A. helianthi* of sunflower (Sarnaik, 2010) in our laboratory. Most of these isolates had demonstrated limited bioefficacy. To restrict devastating pathogens mutagenesis is frequently used to widen resistant sources of host plant (Meena *et al.*, 2013). It is found that the frequency of desirable mutations depends upon the effectiveness and efficiency of mutagens used (Choudhary *et al.*, 2015 and Singh *et al.*, 2015). Not only on plants but mutagenesis has also been performed on fungi (Rajappan, 1996). There are evidences that sufficient desirable biochemical changes can be induced in fungal isolates including improvement in their bioefficacy using physical mutagens like Ultra-Violet (UV)-irradiation (Ximena and Araya, 2007; Balasubramanian *et al.*, 2010 and Patil and Kamble, 2011); Gamma (ϒ)-irradiation (Haggag, 2002; Shathele, 2009; Iftikhar *et al.,* 2010 and Mutwakil, 2011) and chemical mutagen Ethidium Bromide *i.e.* EtBr (Patrick and Catley, 1977; Chand *et al.*, 2005 and Chandra *et al.*, 2010).

Therefore, with a hypothesis that the techniques of mutagenesis using UV-irradiation, ϒ-irradiation and EtBr treatment will be useful for improving bioefficacy of native Alternaria isolates as well, the current work was conducted to improve the bioefficacy of native isolates with the objective to treat NPAL-5 and NPAH-2 isolates from previous work with UV and ϒ-irradiation and EtBr followed by *in vitro* testing of resulting mutants against *A. lini* and testing of their culture filtrates by spraying in pot trial on plants inoculated with *A. lini*.

## MATERIAL AND METHODS

### Culture collection and pathogenicity test

Pathogenic as well as nonpathogenic isolates of *Alternaria* spp. were collected from the previous work conducted in the Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra (Bhoye, 2009 and Sarnaik, 2010). In linseed, when the plants of variety 'Kiran' started producing buds, small cotton swab dipped in $10^4$ spore suspension of individual isolates were wrapped around the neck portion of the buds. Five buds were inoculated with every isolate. Buds inoculated with one isolate were covered in a single polythene bag for 30 hrs and inside them 80 to 90 per cent relative humidity (RH) was maintained by making light spray of sterilized distilled water (SDW) before sealing the bags (Bhoye, 2009 and Charpe *et al.*, 2014). In sunflower, when the plants of variety 'Morden' attained six leaf stage $10^4$ spore suspension of individual isolates were sprayed on plants. Five plants were inoculated with every isolate. Inoculated plants were individually covered with polythene bag for 30 hrs and inside them 80-90% relative humidity was maintained by making light spray of SDW before sealing the bags (Chenulu and Singh, 1964 and Sarnaik, 2010). After 30 hrs the bags were opened and plants were sprayed with SDW during morning and evening hours till the expression of symptoms both in Linseed and Sunflower. Observations were recorded on seventh day after inoculation for bud blight symptom in Linseed and leaf blight symptom in Sunflower. Alternaria isolates were categorized as nonpathogenic based on the absence of blightening and pathogenic due to appearance of corresponding blight symptoms (Bhoye, 2009; Sarnaik, 2010 and Charpe *et al.*, 2014).

### Mutant selection and stabilization

### Treatment of physical mutagens

### UV irradiation

Spore suspensions of nonpathogenic *Alternaria* spp. were irradiated with UV as per method used by Ximena and Araya (2007). For UV treatment 10 Petridishes having flat bottom were filled with 2ml spore suspension of $1x10^7$ spores/ml and placed in UV-chamber (260nm) for 5, 10, 15, 20, 25 and 30 min. Irradiated plates were kept in dark overnight to avoid photo reactivation. After dilution to 100 spores/ml irradiated spore suspensions were placed on PDA, incubated at room temperature and colonies were counted. Colonies from dose less than the lethal dose having different colony characters than original isolates were selected as first generation mutants *i.e.* M1. They were studied for stability of morphological characters till sixth generation *i.e.* M6. Colonies showing uniform characters for six generations were tested for bioefficacy (Kadu, 2013).

### Gamma irradiation

Spore suspensions of nonpathogenic *Alternaria* spp. were irradiated with Gamma at BARC, Mumbai with 100, 120 and 140 krad doses for 30, 45 and 60 min each. Rest of the procedure was same as explained for UV (Mukhopadhyay *et al*, 1992).

### Treatment of chemical mutagens

### Ethidium bromide (EtBr) treatment

Spore suspensions of nonpathogenic *Alternaria* spp. were treated with EtBr at 100, 150, 200 and 250 µl/ml concentrations for 30, 45 and 60 min each. The individual tubes were centrifuged at 5000 rpm for 10 min to remove the EtBr traces of spores with distilled water and rest of the procedure was same as explained for UV (Chand *et al.*, 2005).

### Annotation of alternaria isolates

On the basis of pathogenicity test and morphological characters, pathogenic isolates were annotated as ALP *i.e. Alternaria lini* Pathogen (ALP-1 to ALP-10); nonpathogenic isolates of linseed were annotated as NPAL *i.e.* Non Pathogenic Alternaria of *Linum usitatisimum* and nonpathogenic isolates of sunflower were annotated as NPAH *i.e.* Non Pathogenic Alternaria of *Helianthus annus*. NPAL-5 and NPAH-2 isolates previously recorded to have limited bioefficacy (Bhoye, 2009 and Sarnaik, 2010) were used for developing mutants. The six stabilized mutants finally selected for evaluation were annotated as NPALE3c, NPAHE3c, NPALUe, NPAHUe, NPALG3c and NPAHG3c, where E is EtBr treated, U is UV-irradiated, G is Gamma-irradiated, 3 is third dose, c is third exposure time and e is fifth exposure time (Table 1, 2 and 3; Kadu, 2013).

### Preparation and bioassay of culture filtrates

Culture filtrates were prepared by growing selected mutants on 150 ml potato dextrose broth (PDB) for 20 days. Broth containing mycelial mat were filtered through Whatman filter paper No.4. Filtrates were centrifuged at 5000 rpm for 10 min. Cell free supernatant was collected considering 100% concentration and was later used as 5% and 10% to test for bioefficacy *in vitro* and under pot trial. *In vitro* testing was done by poisoned food technique (Bhoye, 2009; Sarnaik, 2010; Kadu, 2013 and Charpe *et al.*, 2014). In pot trial 65 days old plants of variety 'Kiran' were used on pre flowering stage. Twenty seven treatments having 3 replications each included spraying of culture filtrates of 6 mutants, 2 native isolates and an untreated inoculated control at three time intervals. An equiproportional spore suspension ($10^4$ spores/ml) of ten *A. lini* isolates was used to inoculate plants by spraying in evening for disease development and then pots were covered with polythene bags for next 30 hrs. Culture filtrates (10%) were sprayed on plants as pre-inoculation (30hrs before inoculation) and post-inoculation (30 and 60 hrs after inoculation). Observations were recorded on $10^{th}$ day of inoculation (Bhoye, 2009; Kadu, 2013 and Charpe *et al.*, 2014) using 0-5 scale given by Mayee and Datar (1986). Percent disease intensity (PDI) was calculated by formula given by McKinney (1973).

**Experimental design and statistical analysis**

Ten *in vitro* trials were done with individual *A. lini* isolates (ALP-1 to 10) for testing bioefficacy of developed mutants and data was pooled for analysis. In pot culture one trial was done for an equi-proportional mixture of 10 *A. lini* isolates (Kadu, 2013). Data were analyzed by Factorial Completely Randomized Design (Gomez and Gomez, 1983).

## RESULTS AND DISCUSSION

Induced mutation is one of the most widely used tools to get variants to improve bioefficacy of fungi and their ability to colonize plant parts (Baker, 1991). Therefore, nonpathogenic Alternaria isolates tested by Bhoye (2009) and Sarnaik (2010) to have limited bioefficacy were mutated with EtBr, UV and ϒ-irradiations for improved bioefficacy against *A. lini* (Kadu, 2013).

When spore suspension of native isolates (wild type) were exposed to EtBr solution of 200 µg ml-1 for 60 min, 99-100% mortality and 0-1% survival of spores was recorded, as 3 log kill of fungal spores (Table1). Exposure to higher concentrations resulted in death of 100% spores. Hence, mutants were selected from treatment NPALE3c and NPAHE3c having a concentration less than the lethal dose for *in vitro* and pot trials (Chand *et al.,* 2005). Alamri *et al*. (2016) shown that EtBr solution of either 0.5 or 1.0 mg mL-1 can be used for enhancing bioefficacy of *T. harzianum* JF419706. In current study lesser dose of 200 µg ml-1 is found sufficient to enhance bioefficacy of *Alternaria* spp. as compared to its wild type.

When, isolates were treated with UV, it was found that 25 min exposure caused 97-100% death of spores of NPAL-5 isolate and 97-99% of NPAH-2 isolate, as 3 log kill (Table 2). Further exposure completely killed the spores. Among 12 treatments NPALUe gave least survival followed by NPAHUe. Hence,

**Table 1: Survival of native non-pathogenic Alternaria isolates on exposure to EtBr**

| SN | Treatments | Survival of NPAL-5 (%) | Annotation | Survival of NPAH-2 (%) | Annotation |
|---|---|---|---|---|---|
| 1 | 100 µg ml-1 for 30 min | 56-58 | NPALE1a | 62-65 | NPAHE1a |
| 2 | 100 µg ml-1 for 45 min | 51-53 | NPALE1b | 55-57 | NPAHE1b |
| 3 | 100 µg ml-1 for 60 min | 42-45 | NPALE1c | 47-49 | NPAHE1c |
| 4 | 150 µg ml-1 for 30 min | 40-42 | NPALE2a | 45-47 | NPAHE2a |
| 5 | 150 µg ml-1 for 45 min | 35-37 | NPALE2b | 38-40 | NPAHE2b |
| 6 | 150 µg ml-1 for 60 min | 20-22 | NPALE2c | 22-25 | NPAHE2c |
| 7 | 200 µg ml-1 for 30 min | 10-12 | NPALE3a | 13-15 | NPAHE3a |
| 8 | 200 µg ml-1 for 45 min | 1-4 | NPALE3b | 4-6 | NPAHE3b |
| 9 | 200 µg ml-1 for 60 min | 0-1 | NPALE3c | 0-1 | NPAHE3c |
| 10 | 250 µg ml-1 for 30 min | 0 | NPALE4a | 0 | NPAHE4a |
| 11 | 250 µg ml-1 for 45 min | 0 | NPALE4b | 0 | NPAHE4b |
| 12 | 250 µg ml-1 for 60 min | 0 | NPALE4c | 0 | NPAHE4c |
| 13 | Untreated Control | 100 | NPAL-5 | 100 | NPAH-2 |

**Table 2: Survival of native non-pathogenic Alternaria isolates on exposure to UV-rays**

| SN | Treatments (Exposure Time) | Survival of NPAL-5 (%) | Annotation | Survival of NPAH-2 (%) | Annotation |
|---|---|---|---|---|---|
| 1 | 5 min | 74-75 | NPALUa | 78-80 | NPAHUa |
| 2 | 10 min | 50-52 | NPALUb | 55-57 | NPAHUb |
| 3 | 15 min | 35-37 | NPALUc | 40-42 | NPAHUc |
| 4 | 20 min | 14-15 | NPALUd | 15-17 | NPAHUd |
| 5 | 25 min | 0-3 | NPALUe | 1-3 | NPAHUe |
| 6 | 30 min | 0 | NPALUf | 0 | NPAHUf |
| 7 | Untreated control | 100 | NPAL-5 | 100 | NPAH-2 |

**Table 3: Survival of native non-pathogenic Alternaria isolates on exposure to ϒ-rays**

| SN | Treatments | Survival of NPAL-5 (%) | Annotation | Survival of NPAH-2 (%) | Annotation |
|---|---|---|---|---|---|
| 1 | 100 krad for 30 min | 62-65 | NPALG1a | 65-70 % | NPAHG1a |
| 2 | 100 krad for 45 min | 55-58 | NPALG1b | 58-60 % | NPAHG1b |
| 3 | 100 krad for 60 min | 47-50 | NPALG1c | 50-52 | NPAHG1c |
| 4 | 120 krad for 30 min | 40-43 | NPALG2a | 44-46 | NPAHG2a |
| 5 | 120 krad for 45 min | 37-40 | NPALG2b | 40-42 | NPAHG2b |
| 6 | 120 krad for 60 min | 30-33 | NPALG2c | 33-35 | NPAHG2c |
| 7 | 140 krad for 30 min | 15-20 | NPALG3a | 20-22 | NPAHG3a |
| 8 | 140 krad for 45 min | 5-7 | NPALG3b | 7-9 | NPAHG3b |
| 9 | 140 krad for 60 min | 0-3 | NPALG3c | 0-4 | NPAHG3c |
| 10 | 160 krad for 30 min | 0 | NPALG4a | 0 | NPAHG4a |
| 11 | 160 krad for 45 min | 0 | NPALG4b | 0 | NPAHG4b |
| 12 | 160 krad for 60 min | 0 | NPALG4c | 0 | NPAHG4c |
| 13 | Untreated Control | 100 | NPAL-5 | 100 | NPAH-2 |

**Table 4: Effect of culture filtrates of mutants on growth of ten *Alternaria lini* isolates *in vitro*.**

| Treatments | Doses | | | | Mean of Factor A (Treatments) |
|---|---|---|---|---|---|
| | 5% | | 10% | | |
| | Radial Mycelial Growth (cm) | Inhibition (%) | Radial Mycelial Growth (cm) | Inhibition (%) | |
| NPALE3c | 22.8 | 59.64 | 20.83 | 63.15 | 21.82 |
| NPAHE3c | 23.89 | 57.73 | 21.72 | 61.55 | 22.8 |
| NPALUe | 27.33 | 51.62 | 25.05 | 55.68 | 26.19 |
| NPAHUe | 27.78 | 50.84 | 25.72 | 54.49 | 26.75 |
| NPALG3c | 25.05 | 55.66 | 22.88 | 59.52 | 23.97 |
| NPAHG3c | 25.66 | 54.58 | 23.7 | 58.05 | 24.68 |
| NPAL-5 | 32.3 | 42.51 | 30.65 | 45.76 | 31.47 |
| NPAH-2 | 30.13 | 46.67 | 29.29 | 48.15 | 29.71 |
| Untreated Control | 56.5 | 0 | 56.5 | 0 | |
| Mean of Factor B (doses) | 26.86 | | 24.98 | | |
| | Factor A | | Factor B | | Factor A*B |
| F test | S | | S | | S |
| SE (m) | 0.06 | | 0.03 | | 0.09 |
| CD (P=0.01) | 0.24 | | 0.11 | | 0.34 |

**Table 5: Effect of culture filtrate of mutants on Alternaria blight of linseed in pot trial**

| Treatments | Formulation | Dose | Before 30hrs of inoculation | | After 30 hrs of inoculation | | After 60 hrs of Inoculation | | Mean of PDI for Factor A |
|---|---|---|---|---|---|---|---|---|---|
| | | PDI | PDC | PDI | PDC | PDI | PDC | | |
| NPALE3c | Culture Filtrate | 10 % | 38.08 | 41.71 | 25.83 | 60.46 | 35.17 | 46.17 | 32.10 |
| NPAHE3c | Culture Filtrate | 10 % | 40.58 | 37.88 | 30.50 | 53.31 | 37.50 | 42.60 | 32.34 |
| NPALUe | Culture Filtrate | 10 % | 48.33 | 26.02 | 36.17 | 44.64 | 39.67 | 39.28 | 33.48 |
| NPAHUe | Culture Filtrate | 10 % | 49.50 | 24.23 | 37.00 | 43.36 | 40.25 | 38.39 | 32.73 |
| NPALG3c | Culture Filtrate | 10 % | 43.83 | 32.90 | 34.00 | 47.96 | 38.08 | 41.71 | 32.53 |
| NPAHG3c | Culture Filtrate | 10 % | 45.25 | 30.74 | 35.17 | 46.17 | 40.08 | 38.64 | 32.58 |
| Control | Spray Inoculation | $10^4$ spore suspension | 65.33 | | 65.33 | | 65.33 | | 65.33 |
| Mean of PDI for Factor B | | | 39.48 | | 30.34 | | 34.91 | | |
| F test | | | S | | S | | S | | |
| | | | Factor A | | Factor B | | A*B | | |
| SE (m) | | | 0.22 | | 0.15 | | 0.38 | | |
| CD (P=0.01) | | | 0.84 | | 0.55 | | 1.45 | | |

Where, Factor A = Treatments; Factor B = Time of application; PDI = Per cent Disease Intensity; PDC = Per cent Disease Control; A*B = Interaction of both the factors; S = Significant

colonies from these two treatments were picked for *in vitro* and *in vivo* studies (Ximena and Araya, 2007 and Kadu, 2013). Alamri *et al*. (2016) obtained enhanced mycoparasitic activity of *T. harzianum* JF419706 by 3 or 5 min exposure to UV. In the current work 25 min exposure was required to enhance bioefficacy of native *Alternaria* isolates. Requirement of higher exposure time as compared to *T. harzianum* is possibly due to multicellular spore type of Alternaria.

When spores were exposed to ϒ-rays at 100, 120, 140 and 160 krad for 30, 45 and 60 min each (Mukhopadhyay *et al.*, 1992) it was found that dosage of 140 krad for 60 min produced 97-100% killing of NPAL-5 and 96-100% killing of NPAH-2 spores, as 3 log kill (Table-3). Further exposure to ϒ-rays caused death of 100% spores. Hence, colonies from NPALG3c and NPAHG3c were picked for *in vitro* and *in vivo* studies. This approve the findings of Mukhopadhyay *et al* (1992) who developed 7 stable mutants of *T. virens* with improved bioefficacy over its wild type by using 125 k rad of gamma and reported that mutant 'M-7' with profuse chlamydospores but no conidia aggressively antagonized *Sclerotium rolfsii* and *Fusarium oxysporum f. sp. ciceri*. Haggag (2002) carried out mutagenesis of *T. harzianum* and *T. koningii* with 50 and 75 k rad of gamma to get 4 mutants of each species capable of producing high level of chitinase, and were stable and superior to wild type for bioefficacy against *Botrytis cinerea*. In current study high dose of gamma (140 k rad) was required. These results support Sommer (1964) who explained that multi- or bi-cellular spores are more tolerant to gamma than the unicellular spores of *T. viride*. The *Alternaria* spp. mutated here possess multicellular spores.

After preliminary screening (Data not shown) culture filtrates of 6 selected mutants were evaluated *in vitro* on ten *A. lini* isolates (ALP-1 to 10) individually. The data of their pooled response are presented in Table-4. Mutants obtained by all the three mutagenesis showed significant improvement over their wild type for biocontrol of *A. lini*. Among them NPALE3c used at 10 % dose was significantly superior over other mutants and has inhibited the pathogen *A. lini* by 63.15 % over control with an increment of 17.39 % over native isolate. It was followed by NPAHE3c that inhibited *A. lini* by 61.55 % with an increment of 13.40 % over native isolate as 10 % dose. Factorial analysis shows 10 % supplement of culture filtrates

in growth media was significantly superior to 5 % supplements in inhibiting *A. lini*.

For the first time *Alternaria* spp. is mutated for improving its bioefficacy. As there are many reports available for *Trichoderma* spp., the results obtained with Trichoderma mutants developed for improved bioefficacy are discussed with Alternaria mutants developed through current study.

Present investigations support previous reports demonstrating utility of UV to improve bioefficacy of fungi. Earlier, Papavizas *et al*. (1982) has developed UV-mutants of *T. harzianum* wild strain WT-6 that has effectively repressed the growth of *Pythium ultimum* of pea, *Rhizoctonia solani* of cotton and radish and *Sclerotium* spp. of onion. Selvakumar *et al*. (2002) and Sreeramakumar and Palakshappa (2005) has reported UV-mutants of *T.viride* with improved bioefficacy due to wide variation in β-1,3-glucanase and chitinase activity from their parent strain. Mohamed and Haggag (2010) developed seven UV-mutants of *T. koningii* and *T. reesei* against important fungal pathogens causing root rot and damping off diseases of different crops as shown by *in vitro* bioassay. Mohammed *et al*. (2010) has used UV mutagenesis for enhanced production of hydrolytic enzymes chitinase, ß-1,3-galacturonase and cellulases of *T. viride* for improved suppression of *S. rolfsii* and *Sclerotinia sclerotiorum*. Patil and Kamble (2011) obtained five *T. koningii* mutants by UV exposure of different durations and demonstrated improved antagonism by *T. koningii*-2 against charcoal rot pathogen *in vitro*. Lunge and Patil (2012) after UV treatment of 2, 4, 6 and 8 min has developed *T.harzianum* mutants with enhanced chitinolytic activity for *in vitro* inhibition of *Aspergillus flavus* and *A. parasiticus*.

Current results also confirm the utility of ϒ-irradiation for improving antagonism. These findings are in agreement with Haggag (2002) who developed eight ϒ-mutants of *T. harzianum* and *T. koningii* capable of producing high level of chitinase and enhanced bioefficacy against *Botrytis cinerea, S. rolfsii*, *R. bataticola*, *Fusarium solani* and *F. udum* as compared to its wild type. Sreeramakumar and Palakshappa (2005) have improved the bioefficacy of *T. viride* by ϒ–irradiations. Coventry *et al* (2006) has been reported that irradiation with ϒ–rays was effective to increase the biocontrol of *S. rolfsii* and *R. solani* by strains of *T. harzianum*. Mohamed and Haggag (2006) obtained two stable salt tolerant mutants having great bioefficacy against *F. oxysporum* the causal agent of tomato wilt disease. Induced mutation in *T.virens* using gamma to increase strength, competitiveness, growth and sporulation of the fungus with increased resistance to *R. solani* has been reported by Haggag and Mohamed (2007). Recently, Abbasi *et al*. (2014) compared the mutated strains of *T. harzianum* developed by gamma and their wild types against *M. phaseolina* in dual culture and detected the maximum growth inhibition in Th1, Th4, Th15, Th9 and Th22 mutants those were three days faster than the wild type. Baharvand *et al*. (2014) has developed the ā-mutants of *T. viride* with increased production of cell wall degrading enzymes, chitinases and ß-1, 3 glucanases and in turn improved bioefficacy against *M. phaseolina*. Shahbazi *et al*. (2014) has also confirmed increased bioefficacy and chitinase and protein production of *T.harzianum* and *T.viride* by ϒ-irradiation against *M. Phaseolina*.

Current study support the findings of investigators those advocate use of EtBr for improving bioefficacy of fungi. Javed *et al*. (2011) has developed improved strain TASEB-2 of a thermophilic fungi *Humicola insolens* for improved cellulase production using EtBr. Khandoker *et al*. (2013) developed EtBr-mutant of *T. viride* for enhanced cellulase production. Akbar *et al*. (2013) has enhanced pectinase production in *As. carbonarius by* multistep mutagenesis using EtBr. Alamri *et al* .(2016) has developed EtBr-mutant of *T. harzianum* JF419706 for improved bioefficacy by inducing the production of cell wall degrading enzymes.

On the basis of *in vitro* study culture filtrates of mutants were tested by spraying of 10 % dilution against Alternaria blight of linseed in pot trial (Bhoye, 2009 and Charpe *et al*., 2014) and results are presented (Table 5). It shows that all the treatments had significant reduction in disease intensity over control. NPALE3c was significantly superior over other treatments showing 60.46 % reduction in disease intensity followed by NPAHE3c that has shown 53.31 % reduction. Both these results are found when treatments were applied after 30 hrs of pathogen inoculation. The least reduction (24.23 %) in disease was observed with NPAHUe when it was applied 30 hrs before inoculation. Under pot culture condition as well, mutants have shown improvement over native isolates. NPALE3c has shown 12.77% increment and NPAHE3c has shown 10% increment of reduction in disease intensity over their native isolates NPAL-5 and NPAH-2, respectively.

In all success stories, *Trichoderma* isolates were used against a number of pathogens, but it is observed that complexity of presence of pathogens in nature reduces their effectiveness (Alamri *et al*., 2016). Therefore, to obtain an efficient biocontrol agent to suppress the disease on plant system as well, mutants of *Alternaria* spp. were tested under pot trial on linseed plants artificially inoculated with *A. lini*. In a study conducted by Haggag (2002) ϒ-mutants of *T.harzianum* and *T.koningii* protected tomato and cucumber plants from grey mould disease caused by *B. cinerea* by increased production of extracellular chitinase. Under natural infested conditions, two foliar sprays of Trichoderma mutants to tomato and cucumber plants in a plastic house at 30 days interval showed improved bioefficacy against *B. cinerea* compared to wild type. TH12, TH18 TK5 and TK15 mutants were the most effective in controlling grey mould disease in fruits and stems, reduced pathogen sporulation and improved yield. Mohamed and Haggag (2006) has demonstrated bioefficacy of two stable salt tolerant ϒ-mutants of *T.harzianum i.*e. Th50M6 and Th50M11 under saline and non-saline, infested and natural soil conditions. Both mutants greatly surpassed their wild type in growth rate, sporulation, population density in rhizosphere, suppressing pathogen growth in rhizosphere thus biological proficiency against *F. oxysporum* causing tomato wilt disease, and improved yield and mineral contents of tomato plants. Abbasi *et al*. (2014) evaluated ϒ-mutants of *T. harzianum* for biocontrol of charcoal rot of melon (*M. phaseolina*) in greenhouse conditions and mutant Th9 showed 28% disease reduction as compared to inoculated control and maximum growth of roots and shoots of melon plants as compared to uninfected control. All above references explain the enhanced bioefficacy of ā-mutants that are supported by the current

findings. During current study apart from ϒ-mutants UV and EtBr-mutants have also demonstrated their bioefficacy *in vitro* and in pot trial.

This is the first report of mutation study conducted for improving bioefficacy of *Alternaria* spp. for a foliar pathogen *A. lini*. The results indicate that EtBr treatment was the most effective followed by ϒ and then UV-irradiation both *in vitro* and in pot trial. By far UV, ϒ and EtBr mutants have not been compared in a single experiment. Although comparison of UV and EtBr mutants have been reported. Bapiraju (2004) had shown that EtBr is more effective mutagen than UV rays for enhanced biosynthesis of lipase. Javed *et al*. (2011) has compared UV and EtBr induced mutants of *H. insolens* for enhanced enzyme production. Among the mutants a UV induced strain TAS-13UV-4 NG-5 proved to be the best mutant, which produced 43.19 % CMC-ase, 60.15 % FP-ase and 59.78 % β-glucosidase. Akbar *et al*. (2013) reported better pectinase production ability (65U/ml) of EtBr-mutant 'E8' of *As. carbonarium* over UV-mutants, thus showing 1.8 fold increase over wild strain. Khandoker *et al*. (2013) reported that UV-mutant of *T. viride* produced 11.28 U/ml cellulase where as EtBr-mutant showed 14.61 U/ml cellulase activities. Alamri *et al*. (2016) developed UV and EtBr-mutants of *T. harzianum*. They reported EtBr in concentration either 0.5 or 1.0 mg mL-1 enhanced the bioefficacy of *T. harzianum* JF419706 more than UV-irradiation. In current study for the first time all the three types of mutagenesis have been compared with the same isolates of *Alternaria* spp. and EtBr induced mutants are found to have better performance over UV and Gamma induced mutants both *in vitro* and in pot culture conditions.

## REFERENCES


**Abbasi, S., Safaie, N. and Shamsbakhsh, M. 2014.** Evaluation of gamma-induced mutants of *Trichoderma harzianum* for biological control of charcoal rot of melon (*Macrophomina phaseolina*) in laboratory and greenhouse conditions. *J. Crop Protection*. **3(4):** 509-521.

**Akbar, S., Prasuna, R. G. and Khanam, R. 2013.** Multistep mutagenic strain improvement in *Aspergillus carbonarius* to enhance pectinase production potential. *International J. Applied Biology and Pharmaceutical Technology*. **4 (2)**.

**Alamri, S., Mostafa, Y. S., Hashem, M. and Alrumman, S. 2016.** Enhancing the biocontrol efficiency of *Trichoderma harzianum* JF419706 through cell wall degrading enzyme production. Interational *J. Agriculture Biology*. **18:** 765- 772

**Ambesh, B. S., Ngomle, S., Marak, T. and Das, S. 2014.** Morphological and biochemical variation of some Alternaria species infected on different floricultural plants. *The Ecoscan. Special issue*. **4:** 361-368.

**Anilkumar, T. B. and Hegade, R. K. 1974.** Alternaria leaf spot of sunflower. *Current Science*. **43(3):** 93-94.

**Anonymous. 2015.** Annual Report 2014-15, All India Coordinated Research Project on Linseed, Project Coordinating Unit (Linseed), *C. S. Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India*. p. 269.

**Baharvand, A., Shahbazi, S., Afsharmanesh, H., Ebrahimi, M. A. and Askari, H. 2014.** Investigation of gamma irradiation on morphological characteristics and antagonist potential of *Trichoderma viride* against *Macrophomina phaseolina*. *International Journal of Farming and Allied Sciences*. **3(11):** 1157-1164.

**Baker, R. 1991.** Induction of rhizosphere competence of benomyl tolerant mutants of *Trichoderma* spp. *Canadian J. Microbiology*. **34:** 694-696.

**Balasubramanian, N., ThamilPriya, V., Gomathianayagam, S., Shanmugaiah, V., Jashnie, J. and Lalithakumari, D. 2010.** Effect of chitin adapted and Ultraviolet induced mutants of *Trichoderma harzianum* enhancing biocontrol and chitinase activity. *Australian J. Basic and Applied Sciences*. **4(10):** 4701-4709.

**Bapiraju. 2004.** Mutation induced enhanced biosynthesis of lipase. *African J. Biotechnology*. **3(11):** 618-621.

**Bhoye, B. B. 2009.** Investigation into management of Alternaria blight of linseed through biological sources. *M.Sc. Thesis (Unpub.), Dr. PDKV, Akola, Maharashtra, India*. p. 94.

**Chand, P., Aruna, A., Maqsood, A. M. and Rao, L. V. 2005.** Novel mutation method for increased cellulase production. *J. Applied Microbiology*. **98(2):** 318-323.

**Chandra, A., Kalra, N. S., Sangwan, S. S., Gaurav, Darokar, M. P. and Sangwan, R. S. 2010.** Development of mutant of *Trichoderma citrinoviride* for enhanced production of cellulases. *Biosource Technology*. **100:** 1659-1662.

**Charpe, A. M., Bhoye, B. B. and Gade, R. M. 2014.** Fungistasis of Trichoderma culture filtrates against *Alternaria lini* that causes bud blight of linseed. *J. Plant Disease Sciences*. **9(1):** 86-90.

**Chaudhari, L. S. and Srivastava, K. N. 1975.** Estimation of loss of yield caused by blight diseases of linseed. *J. Karnataka Agricultural Science*. **3:** 107-109.

**Chenulu, V. V. and Singh, A. 1964.** A note on estimation of losses due to leaf blight of wheat caused by *Alternaria triticina*. *Indian Phytopathology*. **17:** 254-256.

**Choudhary, H. P., Singh, V. and Meena, A. K. 2015.** Effect of induced mutation in sheath blight disease resistance of rice. *The Ecoscan*. **9(1&2):** 369-372.

**Couteaudier, Y. 1992.** Biological control of Fusarium wilts. In: Biological control of plant diseases, E.S. Tjamos, A.C.Papavizas and R.J.Cook (Eds), *Plenum Press, New York*. pp. 99-104.

**Coventry, E., Noble, R., Mead, A., Marin, F. R., Perez, J. A. and Whips, J. M. 2006.** Allium white rot suppression with composts and *Trichoderma viride* in relation to sclerotia viability. *J. Biological Control*. **96(9):** 1009-1021.

**Dey, P. K. 1933.** An Alternaria blight of the linseed plant. *Indian J. Agricultural Science*. **3:** 881-895.

**Gill, K. S. 1987.** Linseed. *ICAR Publication, New Delhi, India*. p. 386.

**Gomez, K. A. and Gomez, A. A. 1983.** Statistical procedures for agricultural research, *IRRI, Manila, Philippines*. p. 680.

**Haggag, W. M. 2002.** Induction of hyper producing chitinase Trichoderma mutants for efficient biocontrol of *Botrytis cinerea* on tomato and cucumber plants growing in plastic houses. *Arabian J. Biotechnology*. **5(2):** 151-164.

**Haggag, W.M and Mohamed, H.A.A. 2007.** Biotechnological aspects of microorganisms used in plant biological control. *American Eurasian J. Sustainable Agriculture*. **10(1):** 7-12.

**Mohamed, H. A. A. and Haggag, W. M. 2010.** Mutagenesis and inter-specific protoplast fusion between *Trichoderma koningii* and *Trichoderma reesei* for biocontrol improvement. *American J. Scientific and Industrial Research*. **3(1):** 540-515.

**Iftikhar, T., Niaz M., Abbas, S. Q., Zia, M. A., Ashraf, I., Lee, K. J. and Ikram-Ul-Haq. 2010.** Mutation induced enhanced biosynthesis of lipases by *Rhizopus oligosporus* var. *microsporus*. *Pakistan J. Botany*. **42(2):** 1235-1249.

**Javed, M. M., Ikram-ul-haq and Irfana, M. 2011.** Multistep mutagenesis for the over-expression of cellulase in *Humicola insolens*.

*Pakistan J. Botany.* **43(1):** 669-677.

**Kadu, R. 2013.** Improving the biocontrol potential of native non-pathogenic *Alternaria* spp. through induced mutation. *M.Sc. Thesis (Unpub.), Dr. PDKV, Akola, Maharashtra, India.* p. 84.

**Khandoker, N., Mamun, A. A., Nafiz, T. N., Khan, S. N. and Hoq, M. M. 2013.** Strain improvement of *Trichoderma viride* through mutation for enhanced production of cellulase. *Bangladesh J. Microbiology.* **30(1&2):** 43-47.

**Kumar, N. and Paul, S. 2015.** Genetic analysis of yield and yield contributing traits in linseed (*Linum usitatissimum* L). *The Bioscan.* **10(4):** 1951-1955.

**Lal, K., Yadav, R. S. and Chand, S. 2015.** *In vitro* management of rice blast caused by *Pyricularia oryzae* with *Pseudomonas fluorescens* and fungicides. *The Ecoscan.* **9(3&4):** 1001-1006.

**Lunge, A. G. and Patil, A. S. 2012.** Strain improvement of *Trichoderma harzianum* by UV mutagenesis for enhancing it's biocontrol potential against aflotoxigenic aspergillus species. *The Experiment.* **4(2):** 228-242.

**Marak, T. R., Ambesh, B. S. and Das, S. 2014.** Cultural, morphological and biochemical variations of *Alternaria solani* causing diseases on solanaceous crops. *The Bioscan.* **9(3):** 1295-1300.

**Mayee, C. D. and Datar, V. V. 1986.** Phytopathometry, Technical Bulletin 1. *Marathwada Agricultural University, Parbhani, Maharashtra, India.* p. 146.

**McKinney, H. H. 1973.** A new system of grading plant diseases. *J. Agricultural Research.* **26:** 195-218.

**Meena, S. C., Singh, V., Adhipathi, P. and Chand, R. 2013.** Screening for sheath blight resistant genotypes among mutated population of rice cv. Pusa Basmati-1. *The Bioscan.* **8(3):** 919-924.

**Mohamed, H. A. and Haggag, W. H. 2006.** Biocontrol potential of salinity tolerant mutants of *Trichoderma harzianum* against *Fusarium oxysporum*. *Brazilian J. Microbiology.* **37:** 181-191.

**Mohamed, H. A., Haggag, W. H. and Attallah, A. G. 2010.** Genetic enhancement of *Trichoderma viride* to overproduce different hydrolytic enzymes and their biocontrol potentiality against root rot and white mold diseases in plants. *Agriculture and Biology J. North America.* **1(3):** 273-284.

**Mutwakil, M. H. Z. 2011.** Mutation induction in *Aspergillus terrus* using N-Methyl-N'-Nitro-N-Nitrosoguanidine (NTG) and Gamma Rays. *Australian J. Basic and Applied Sciences.* **5(12):** 496-500.

**Mukhopadhyay, A. N., Shrestha, S. M. and Mukherjee, P. K. 1992.** Biological seed treatment for control of soilborne plant pathogens. *FAO Plant Protection Bulletin.* **40:** 21-30.

**Papavizas, G. C., Lewis, J. A. and Abd-El-Moity, T. H. 1982.** Evaluation of new biotypes of *Trichoderma harzianum* for tolerance to benomyl and enhanced biocontrol capabilities. *Phytopathology.* **72:** 126-132.

**Patil, V. B. and Kamble, S. S. 2011.** The influence of Ultraviolet light on antagonistic activity of *Trichoderma koningii* against *Macrophomina phaseolina* causing charcoal rot of sweet potato. *International J. Academic Research.* **3(1):** 702-704.

**Patrick, J. K. and Catley, B. J. 1977.** The effect of ethidium bromide mutagenesis on dimorphism, extracellular metabolism and cytochrome levels in *Aureobasidium pullulans*. *J. General Microbiology.* **102:** 249-254.

**Rajappan, K. 1996.** Induction of mutants in *Trichoderma viride* by UV-irradiation. *Plant Disease Research.* **12(1):** 1-5.

**Reddy, M. P., Arsul, B. T., Shaik, N. R. and Maheshwari, J. J. 2013.** Estimation of heterosis for some traits in linseed (*Linum usitatissimum* L.). *J. Agriculture and Veterinery* Sicence. **2:** 11-17.

**Richharia, R. H. 1962.** Linseed. The Indian Central Oilseeds committee, Hyderabad, India. p. 155.

**Sarnaik, P. S. 2010.** Management of Alternaria blight of sunflower through biological sources. M.Sc. Thesis (Unpub.), *Dr. PDKV, Akola, Maharashtra, India.* p. 91.

**Selvakumar, R., Srivastava, K. D., Aggrawal, R., Singh, D. V. and Dureja, P. 2002.** Studies on development of *Trichoderma viride* mutants and their effect on *Ustilago segetum tritici*. *Indian Phytopathology.* **52(2):** 185-189.

**Shahbazi, S., Karimi, M. and Ebrahimi, M. A. 2014.** Investigation of sequence-tagged site (STS) in chitinase gene of gamma radiated *Trichoderma viride* isolates. *International J. Farming and Allied Sciences.* **3(11):** 1129-1136.

**Shathele, M. S. 2009.** Effects of Gamma irradiation on fungal growth and associated pathogens. *Research Journal of Environmental Toxicology.* **3:** 94-100.

**Singh, S., Sharma, R. K., Singh, P. and Chakravarti, S. K. 2015.** Gamma ray and EMS induced effectiveness and Efficiency of chlorophyll mutations in aromatic rice (*Oryza sativa L.*). *The Ecoscan.* **9(3&4):** 975-979.

**Sommer, N. F., Maxie, E. C. and Fortlage, R. J. 1964.** Quantitative dose response of prunes fruit decay to gamma irradiation. *Radiation Botany.* **4:** 309-316.

**Sreeramakumar, P. and Palakshappa, M. G. 2005.** Effect of Polyethylene glycol (PEG) on the biomass characteristics of *Trichoderma harzianum* and *Trichoderma viride* in liquid culture. *Indian Phytopathology.* **58(4):** 466-469.

**Vavilov, N. I. 1935.** Studies on the origin of cultivated plants. *Bulletin of Botany and Plant Breeding.* **16:** 39-145.

**Ximena, A. B. and Araya, A. 2007.** New strains obtained after UV treatment and protoplast fusion of native *Trichoderma harzianum* their biocontrol activity on *Pyrenochaeta lycopersici*. *Electronic J. Biotechnology.* **10(4):** 3347-3458.