*J. Mycopathol. Res.* **59(4)** : 425-429, 2021; (ISSN 0971-3719)


# EMS mutation in *Trichoderma harzianum* and effects of putative mutants on plant pathogens *in vitro*

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Received : 07.6.2021 Accepted : 17.09.2021 Published : 27.12.2021

One week old culture of *T. harzianum* was used to prepare conidial suspension that was treated with Ethyl Methane Sulphonate (EMS) @ 50, 100, 150, 200 and 250 µg/ml. Putative mutants obtained were studied for their morphological characters like colony diameter and sporulation. *In vitro* evaluation on radial growth (mm) and sporulation rate of wild strain and EMS mutants after three days of inoculation showed abundant sporulation as recorded by *T. harzianum*/200µg/ml EMS and*T. harzianum*/250 µg/ml EMS. Both the putative mutants and parental *T. harzianum* were tested for their efficiency against soil-borne plant pathogens *viz*., *Rhizoctonia solani*, *Fusarium oxysporum* and *Scleroum rolfsii* by dual culture technique. The putative mutants were observed to be significantly more effective against the tested plant pathogens. *T. harzianum*/250 µg/ml EMS exhibited highest inhibition of 82.96% against *R. solani* followed by *T. harzianum*/200 µg/ml with 74.81%, and 54.81% inhibition against *F. oxysporum* and *S. rolfsii* respectively.

**Key words:** Ethyl methane sulfonate, chemical mutagenesis, *Trichoderma*, mutation, *Fusarium*, *Rhizoctonia*, *Sclerotium*

## INTRODUCTION

The fungal genus *Trichoderma* was identified in 1930s. Overtime the genus *Trichoderma* has been developed as a natural bio control agent.It is well known for suppression of several plant pathogens by employing different mechanisms like competition for nutrients, synthesis of antibiotics, activation of plant defense mechanisms, production of cell wall degrading enzymes and a combination of the above.Plant diseases which are seed-borne, soil-borne, storage rots and even diseases of phyllosphere have been successfully managed by *Trichoderma*. It is a soil-borne fungus and its ability to produce extracellular enzymes determine its antagonistic potential (Thrane *et al.,* 2000).

For past few decades it has been proved to be one of the most successful fungal biocontrol agents for plant disease management (Dwivedi and Tewari, 2017).Though most natural strains of *Trichoderma* are effective and competitive, often they show low rhizosphere competency and low enzyme production ability. So, strain improvement becomes imperative in *Trichoderma*.Efficacy of Trichoderma can be improved by molecular approaches like genetic modification and recombination techniques (Raghuchander *et al*. 2011), mutation, transformation and protoplast fusion, development of consortia and better formulation. In the past, several efforts have been made to induce mutation in *Trichoderma* (Zaldivar *et al*. 2001; Kredics *et al.* 2003). Mutation which can be performed by chemicals like EMS, NTG or physical mutagens like X-rays, ?-rays, UV-rays (Parekh *et al.* 2004) is known to enhance the efficiency of some genes through duplication and deletion mechanism. Disease control ability of Trichoderma can be improved by using UV rays and chemical mutations (Bhargavi and Singara, 2009). *Trichoderma* mutants with better biocontrol efficacy against phytopathogens as compared to their wild strains have been found (Hatvani *et al.,* 2006; Walunj *et al*. 2013). Production and development of such mutants of *Trichoderma* can be a better option in plant disease management under sustainable agriculture. Therefore, in order to obtain beneficial mutants, the present study was undertaken to use one isolate of *Trichoderma*

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*harzianum* for chemical mutagenesis using EMS and testing the putative mutants against some common soil-borne pathogens compared to its wild strain in vitro.

## MATERIALS AND METHODS

### *Chemical mutagenesis*

Chemical mutagenesis was carried out according to the protocol given by Morikawa *et al*. (1985) with slight modifications. Appropriate dilutions of 50, 100, 150, 200 and 250 were prepared from stock solution of 300 µg /ml of EMS. One ml of adjusted conidial suspension ($10^5$cfu/ml) was treated with each concentration of EMS solution for 30 min at 37°C in a water bath shaker. Two hundred microliters of mutagenized sample was plated to obtain survivors. The treated plates were covered and incubated at 28±1°C.The experiments were conducted in Complete Randomised Design (CRD) and three replications were maintained for each treatment.

The putative mutants were grown in pure culture by single spore isolation. Morphological study and cultural characterisation were done by mainly observing the colony diameter, sporulation and colour of the culture. All these features were compared with wild strain to ensure the persistence of the characteristics in the mutants.

### *Testing efficacy of mutants of T. harzianum against different pathogens*

Efficacy of the putative mutants and parent isolate was tested for their antagonistic potential (mycoparasitism) by dual culture technique against plant pathogens. Activity of mutants of *T. harzianum* was evaluated against different soil-borne pathogens *viz.*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Fusarium oxysporum* under *in vitro* condition by adoption of dual culture technique (Morton and Stroube1955). Twenty millilitre sterilized PDA was aseptically poured in sterilized Petriplates and allowed to solidify. Mycelial discs (5 mm) taken from the actively growing colonies of the test pathogens (7 days old culture) and *T. harzianum* were placed simultaneously on the PDA plates opposite to each other, 1 cm apart from the periphery. Each treatment was replicated thrice. The inoculated Petriplates were incubated at 27±2°C. Observation was taken just after contact of pathogen and antagonist *T. harzianum.* Radial growth of the test pathogen was measured and per cent inhibition was calculated following the formula as mentioned below.

$$\% \, I = \frac{C - T}{C} \times 100$$

where, I= Percent inhibition of pathogens by mutants, C= Radial growth in control, T= Radial growth in the treatment (mm).

## RESULTS AND DISCUSSION

Chemical mutagenesis was carried out by preparing dilutions of 50,100,150,200 and 250 µg /ml of EMS. One millilitre of adjusted conidial suspension ($10^5$/ml) was treated with each concentration of EMS solution for 30 min at 37°C in a water bath shaker. Two hundred microliters of mutagenized sample was plated to obtain mutants.

As per the results mentioned in Table 1 and Table 2, fast growth was shown by all the putative mutants created. Growth was less in parental strain compared to EMS-induced mutants. Wild strain showed 8.87cm colony diameter after 4 DOI with poor sporulation as compared to the putative mutants which showed colony diameter of9.00 cm with good or abundant sporulation.

Similar research was conducted by Hamad *et al.* (2001), Mohsin (2006) and Shafique *et al*. (2011) where they reported that chemical treatment is more efficient in inducing high level mutations as compared to UV irradiation.Many of the successful endeavours had been made to improve the potential of *Trichoderma* species by exposing the spores to physical mutagen, UV rays and chemical mutagen EMS (Jairaj and Radhakrisnan, 2003; Li Xinghue *et al*. 2010; Shafique *et al*.2011; Anita and Ashwin, 2012).

### *Comparison of putative mutants of T. harzianum with its wild type*

The mutants were grown in pure culture by single spore isolation. Morphological study and cultural characterisation were done by mainly observing the colony diameter, sporulation and colour of the culture ( Fig.1). All these features were compared with wild strain to ensure the persistence of the characteristics in the mutants.

Data on *in vitro* evaluation on colony diameter of

**Table 1:** *In vitro* evaluation on colony diameter (cm) of parent strain and EMS putative mutants

| TREATMENTS | 24 h | 48 h | 72 h | 96 h |
|---|---|---|---|---|
| $T_0$ :Parental strain | 1.20 | 4.80 | 8.07 | 8.87 |
| $T_6$:T. harzianum /50 µg/ml EMS | 1.33 | 5.67 | 8.87 | 9.00 |
| T7:T.harzianum/100 µg/ml EMS | 1.55 | 5.73 | 8.93 | 9.00 |
| $T_8$:T.harzianum/150 µg/ml EMS | 1.53 | 6.07 | 9.00 | 9.00 |
| $T_9$:T.harzianum/200 µg/ml EMS | 1.53 | 5.87 | 8.87 | 9.00 |
| $T_{10}$:T.harzianum/250 µg/ml EMS | 1.13 | 5.13 | 7.20 | 9.00 |
| SEm± | 0.18 | 0.20 | 0.12 | 0.03 |
| CD (p=0.05) | 0.56 | 0.63 | 0.37 | 0.08 |

**Table 2**: *In vitro* evaluation on radial growth (mm) and sporulation rate of wild strain and mutants after 4 DOI

| Treatments | Radial growth(mm) on PDA plates after 4 DOI | Sporulation rate after 4 DOI |
|---|---|---|
| $T_0$ (Control) - Parental Strain of Th | 88.7 | Poor |
| $T_1$-Th/50µg/ml EMS | 90.0 | Good |
| $T_2$-Th/100µg/ml EMS | 90.0 | Good |
| $T_3$-Th/150µg/ml EMS | 90.0 | Moderate |
| $T_4$-Th/200µg/ml EMS | 90.0 | Abundant |
| $T_5$-Th/250µg/ml EMS | 90.0 | Abundant |

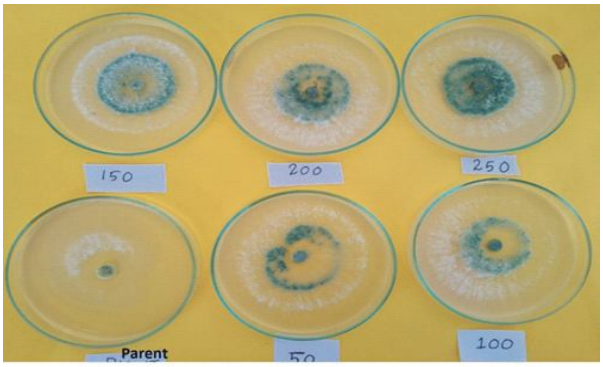


**Fig.1:** Growth differences between wild and EMS-exposed isolates of *Trichoderma harzianum*

parent strain and EMS mutants is depicted in Tables 1 and 2. EMS treatments at all concentrations under study have shown significant effect on colony diameter of *T. harzianum* recording 9.00 cm of radial growth 96 hours after inoculation compared to wild type (8.87 cm) that are statistically significant. *In vitro* evaluation on radial growth (mm) and sporulation rate of wild strain and mutants after 3 DOI revealed that *T. harzianum*/20 min., *T. harzianum*/200µg/ml EMS and*T. harzianum*/250µg/ml EMS showed abundant sporulation (Table 2) compared to poor sporulation in control. After 3 DOI, radial growth(mm) ranged between 78.60 mm in control to 90.00 mm in mutagenic treatments.

The findings of the present investigation are in agreement with Abdollah *et al*. (2014) and Sreenivasalu *et al.* (2014). Abdollah *et al*. (2014) observed that the morphological characteristics like colonies shape, color, sporulation and mycelia growth rate could be changed by UV-irradiation of different duration. Sreenivasalu *et al.*(2014) also reported that ethyl methane sulfonate mutagenesis was generated altered characteristics compared to its wild. Number of obtained mutants showed morphological changes in growth properties such as colony appearance, colony color, sporulation rate and pigmentation.

### *Efficacy of putative mutants of T. harzianum against different pathogens*

Putative mutants and the parents were tested *in vitro* against different phytopathogens mainly *Rhizoctonia solani, Fusarium oxysporum and Sclerotium rolfsii* to study their antagonistic potential.

### *Effect of antagonistic activity of wild strain and EMS mutants against Rhizoctonia solani*

Both parent and mutants showed antagonistic action against *R. solani*. *T. harzianum*/250 µg/ml EMS

**Table 3**: *In vitro* antagonism of putative *T. harzianum* mutants against plant pathogens

| Treatments | Mycelial inhibition percentage over control | | |
|---|---|---|---|
| | *R. solani* | *F. oxysporum* | *S. rolfsii* |
| $T_0$: T. harzianum parent isolate | 53.32(46.91) | 54.07(47.33) | 38.89(38.57) |
| $T_1$: T. harzianum/50 µg/ml EMS | 64.44(53.40) | 56.66(48.83) | 46.29(42.86) |
| $T_2$: T. harzianum/100 µg/ml EMS | 66.66(54.85) | 56.29(48.61) | 42.96(40.94) |
| $T_3$: T. harzianum/150 µg/ml EMS | 69.25(54.33) | 61.48(51.64) | 47.40(43.51) |
| T4: T. harzianum/200 µg/ml EMS | 72.59(58.44) | 74.81(59.91) | 54.81(47.76) |
| T5: T. harzianum/250 µg/ml EMS | 82.96(65.81) | 63.70(53.01) | 45.18(42.23) |
| SEm± | 3.94 | 2.84 | 2.20 |
| CD@5% | 11.87 | 8.57 | 6.64 |

found highly effective which exhibited 82.96% percent mycelial inhibition over control. Other EMS mutants also found effective in antagonism against *R. solani* over control and was in the range of 66.44% to 72.59 % (Table 3).

### *Effect of antagonistic activity of wild and EMS mutants against Fusarium oxysporum*

*In vitro* evaluation on effect of antagonistic activity of wild *T. harzianum* and its EMS mutants against *F. oxysporum* is depicted in the Table 3. The highest mycelial inhibition was recorded in *T. harzianum*/ 200 µg/ml EMS, 74.81% and all the treatments were statistically at par.

The present findings are in agreement with Waghmare(2019) who studied the antagonistic capability of chemical mutant *T. harzianum* against *F. oxysporum*f.sp*. dianthi*. All mutants of the *T. harzianum* shown growth inhibition against the pathogen. *Trichoderma* mutants are good natural enemies against the *F. oxysporum*f.sp.*dianthi* as compared to the wild strain. Biocontrol efficacy of mutant Trichoderma was found higher compared to wild strain against *F. oxysporum* and *R. solani* when tested *in vitro* (Balasubramanian *et al.,* 2010)

### *Effect of antagonistic activity of wild and EMS mutants against Sclerotium rolfsii*

The data pertaining to effect of antagonistic activity of wild and EMS mutants against *S. rolfsii* are presented in Table 3. The maximum mycelial inhibition was shown by $T_4$ (*T. harzianum*/200 µg/ml EMS) with 54.81 % followed by $T_3$ (*T. harzianum*/ 150 µg/ml EMS +*S. rolfsii*) with 47.40%.

The results of the present investigation are in accordance with Singh *et al*. (2016) who exposed *T. harzianum* to chemical mutagenesis in order to enhance antagonistic potential against *S. rolfsii* (collar rot of chickpea), resultantly mutant*Th-m*$_1$ and were found to be more effective than their parent strains showing 42.2 percent mycelial inhibition of the pathogen respectively. Higher capability of mutant *Trichoderma* to produce enzymes and thereby control of white rot disease of onion was reported by Mohamed and Haggag (2006).

Gaikwad *et al*. (2017) induced mutation by EMS and colchicines treatment in *Trichoderma* species isolated from soils of Maharashtra resulted in development of mutants that gave better inhibition of *S. rolfsii in vitro* as compared to wild types. Mutants of *T. virens* were significantly better than parent isolated against *S. rolfsii* and *R. solani* recording 76.6 % and 78.3% growth inhibition respectively (Alfiky, 2019).

## CONCLUSION

The putative mutants of *T. harzianum* induced by chemical EMS recorded faster mycelial growth and sporulation compared to the parent isolate. The putative mutants also showed higher antagonism against three plant pathogens tested in the study. Such enhancement in performance of the biocontrol agent *T. harzianumin vitro* offers a promise in developing more efficient strain for field application. Such benefits can be realized after thorough screening against more plant pathogens and field testing. However, the genetic changes in the bioagent due to exposure to chemical mutagens needs to be worked out fully for better understanding of biocontrol mechanism and broadening future scope as well.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge the facilities and other assistance provided by the HoD, Dept. of Plant Pathology, SASRD for carrying out this piece of research work.

## REFERENCES

Abdollah, B., Samira, S., Hamideh, A., Mohammad, A.E., Hamed, A. 2014. Investigation of gamma irradiation on morphological characteristics and antagonist potential of *Trichoderma viride* against *M. phaseolina.Inter. J. FarmingAllied Sci.* **3**:1157-1164.

Alfiky, A. 2019. Effects of ultraviolet irradiation on the i*n vitro* antagonistic potential of *Trichoderma* spp. against soil-borne fungal pathogens. *Heliyon***19**:5-7.

Anita, S. P., Ashwin, G. L. 2012. Strain improvement of *Trichoderma harzianum* by UV- mutagenesis for enhancing its biocontrol potential against aflatoxigenic *Aspergillus* species. *Res. Desk* 1: 9-25.

Balasubramanian, N., Thamil Priya, V., Gomatinayagam, S. ,Shanmugaiah, V. 2010. Effect of chitin adapted and ultraviolet induced mutant of *Trichoderma harzianum* enhancing bio control and chitinase activity. *Aust. J. Basic Appl. Sci.***4**:4701-4709.

Bhargavi, M., SingaraCharya, M.A. 2009. Strain improvement in dye decolourising mutants of *Mucor mucedo* by protoplast fusion**.** *Afr. J.Biotechnol.***8**: 6908–6912.

Dwivedi, M. and Tewari, A.K. 2017. Crop specific effect of native *Trichoderma* species. Indian Phytopathology. 70(4):457-462.

Gaikwad, M., Mane, S. S., Gawande, S.,Suryawanshi,S., Dalal, S. R. 2017. Antagonistic potential of *Trichoderma* against some soil borne soil pathogens. *Inter. J. Trop. Agric.***35**: 911-919.

Hamad, A., Haq, I., Qadeer, M.A., Javed, I. 2001. Screening of *Bacillus licheniformis* mutants for improved production of al

pha-amylase. *Pak. J. Bot.***33**: 517-525.

Hatvani, L., Manczinger, L., Kredics, L., Szekeres, A., Antal, Z., Vagvolgyi, C. 2006. Production of *Trichoderma* strains with pesticide-polyresistance by mutagenesis and protoplast fusion. *Antonie van Leeuwenhoek***89**:387-393.

Jayaraj, J., Radhakrishnan, N. V. 2003. Development of UV-induced Carbendazim-resistant mutants of *Trichoderma harzianum* for integrated control of damping-off disease of cotton caused by *Rhizoctonia solani*. *J. Plant Dis. Protect.***110** : 449–460.

Kredics, L., Manczinger, L., Antal, Z. 2003. Influence of Water Potential on growth, enzyme secretion and *in vitro* enzyme activities of *Trichoderma harzianum* at different temperatures. *Curr.Microbiol.* **40**: 310-314.

Li, X-H., Yang, H-J., Roy, B., Park, E.Y., Jiang, L-J., Wang, D., Miao, Y-G. 2010. Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. *Microbiol. Res.***16**: 190-198.

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

Mohsin, M.J. 2006. Improvement of a thermophilic fungal strain for cellulase production by chemical and UV mutagenesis, Ph. D. thesis, Dept. of Bot. GC Univ. Lahore. Ph. D

Morikawa, Y., Kawamori, M., Ado, I., Shinsha, I., Oda, F., Takasawasa, S. 1985. Improvement of cellulase production in *Trichoderma reesei. Agricult. Biologic. Chemicals.* **49**: 1869.

Morton, D. T., Stroube, N. H. 1955. Antagonistic and stimulatory effects of microorganism upon *S. rolfsii*. *Phytopathology* **45:** 419-420.

Parekh, S., Vinci, V.A., Strobel, R.J. 2004. Improvement of microbial strains and fermentation processes. *Appl. Microb.Biotechnol.***59**: 287-301.

Raguhchander, T., Saravankumar, S., Balasubramanian, P. 2011. Molecular approaches to improvement of biocontrol agents of plant diseases. *J. Biol.Contr.* **25** :71-84.

Shafique, S., Bajwa, R., Sobiya S. 2011. Strain improvement in *Trichoderma viride* through mutation for overexpression of cellulase and characterization of mutants using random amplified polymorphic DNA (RAPD) *Afr. J.Biotechnol.* **10**: 19590-19597.

Singh, R.,Maurya, S., Upadhyay, R. S. 2016.The improvement of competitive saprophytic capabilities of Trichoderma species through the use of chemical mutagens. *Braz J Microbiol* **47**:10-17.

Sreenivasalu, Y., Gopal, K., Gopi, V.,Gouri Shankar, T., Shalini Devi,S., Palanivel. 2014. Improvement of antagonism and carbendazim tolerance in native *Trichoderma* isolates through ethyl methane sulfonate (EMS) mutagenesis. *Arch. Phytopathol. Plant Protect.* **47**: 1645-1657.

Thrane, C.,Lubeck, M.,Green, H.,Degefu, Y., Allerup S.2000.Atool for monitoring *Trichoderma harzianum*;transformation with the GUS gene by protoplast technology. *Phytopathology***85**:1428-1435.

Waghmare, R.M. 2019. Antagonistic capability of UV and chemical mutant *Trichoderma harzianum* against *Fusarium oxysporum*f.sp.*dianthii* causing wilt of *Dianthuschinensis*. *Inter.J. Recent Scient. Res.* **9**: 34741-34743.

Walunj, A. A., Abhang P.B., John P. 2013.*In vitro* evaluation of mutant and wild strain of *Trichoderma harzianum* against soil borne plant pathogen. *Inter.J.Plant Protect.* **8:**108-111.

Zaldivar, M., Velasquez, J.C., Contreras, I., Perez, L. M.2001.*Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or bio control.*Electronic J.Biotechnol.* **4:**1-9.