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In this study, chemical mutagenesis of *Trichoderma asperellum* and *T. harzianum* was carried out with colchicine and the putative mutants were evaluated in terms of nuclear status, growth rate and antagonism against two soil-borne pathogens *Fusarium oxysporum* f.sp. *pisi* and *Rhizoctonia solani*. The mutation induced by 0.2% concentration of colchicine provided an observation of multinucleate conidia. The conidia produced atleast 3 nuclei in both of the *Trichoderma* spp. under study. Concentration of 0.1% colchicine developed 2-3 nuclei in the conidia of the *Trichoderma* spp. In concentration of 0.01% colchicine, development of binucleate condition in some conidia of the *Trichoderma* spp. was observed while parent strains had one nucleus in the conidia. The mutants of *T. asperellum* and *T. harzianum* obtained with 0.2% concentration of colchicine showed 9cm (against 7 cm in control) and 8.78 cm (against 4.84 cm in control) mycelial growth in 72 h respectively. *T. asperellum* mutants obtained with 0.1% concentration of colchicine showed the highest efficacy with 93.39% inhibition of *F. oxysporum* f. sp. *pisi* while *T. harzianum* mutants with 0.1% colchicine recorded the highest inhibition of 86.08% for the same pathogen compared to 46.37% and 41.77% in control respectively.The highest inhibition of *R. solani* was 87.71% with *T. asperellum* and 85.66% with *T. harzianum* both obtained with 0.2% colchicine treatment.

Keyword: Colchicine, *Fusarium*, Mutation, *Rhizoctonia, Trichoderma*

INTRODUCTION:

Trichoderma is considered as one of the most effective fungal biocontrol agents for a very long time owing to its ability to antagonize plant pathogens, promote plant growth, inducing systemic and localized resistance in plants and its ability to continue living in different environments (Dwivedi and Tewari, 2017). Biological control is an alternative method of plant disease management that can be used in agriculture under the scenario of constant changes and circumstances in climate and food security (Manczinger *et al*., 2002).

Trichoderma has been identified as a biocontrol agent for plant diseases since the 1930's . For the management of various fungal diseases of crops, *Trichoderma* spp. are widely used as they have the capability of mycoparasitism against a wide range of plant pathogens. Their utility against soil-borne diseases has been very much successful (Kumar *et al*. 2014). Soil pathogens like *Rhizoctonia* and *Fusarium* are well known plant pathogenic fungi that cause economically important diseases to many horticultural and agricultural crops worldwide (Benavides *et al*. 2021). On varying hosts symptoms produced by *Rhizoctonia* include stem canker, black scurf, pre- and post-emergence damping off, seedling/leaf blight, stem rot, pod rot etc.*Fusarium oxysporum* is the most economically important member of *Fusarium* with a world-wide distribution commonly found in diverse scale of soils, horticultural and food crops (Rahman *et al.* 2021).

Due to the disease prevalence caused by the soil borne pathogens *viz., Rhizoctonia* and *Fusarium*, recorded yield losses may be as high as 50% in critically permeated areas (Estevez *et al*. 2001). The potentiality of *Trichoderma* sp. in restricting disease of crop plants caused by *Fusarium* spp. and *Rhizoctonia solani* has been demonstrated by

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many researchers (Dubey *et al*. 2007; Rojo *et al*. 2007; Elshahawy *et al*. 2019). Treating the seeds or soil with *Trichoderma* has shown great potential in managing several foliar diseases as well as inducing resistance of the disease in number of crops (Siemering *et al*. 2016).

For the improvement of biocontrol activities against plant pathogens, genetic manipulation of biocontrol agents extends a potential. Mutation induced by physical or chemical mutagens can also be an effective strategy to generate more efficient biocontrol strains (Mirmajlessi *et al*. 2018). The chemical mutagen colchicine was discovered in 1930. It is extracted from the seeds and bulbs of the autumn Crocus which possesses a poisonous alkaloid character. It is a recognized transcendent chemical agent that has a profound mutagenic attribute. In animals, plants, and microbes colchicine has been used to induce polyploidy. It coheres to tubulin resulting in arrested mitosis, preventing the usual allocation of nuclear chromosomes resulting in polyploidy. Considering the potential of colchicine as mutagen and the prospect of developing superior biocontrol strain of *Trichoderma* for more effective plant disease management the present work was undertaken.

MATERIALS AND METHODS

Chemical mutagenesis with colchicine

The wild strains of *Trichoderma harzianum* and *T. asperellum* collected from Department of of Plant Pathology, Nagaland University were treated with colchicine at three different concentrations *viz*., 0.01%, 0.1% and 0.2% using Mandel's medium (Mandels and Sternberg, 1976). The method given by El-Bondkly*et al*. (2010) as described here was used for colchicine treatment on the *Trichoderma* spp.

Green matured conidia of the parent strain *Trichoderma harzianum* and *Trichoderma asperellum* (10-days-old culture) were added to conical flasks, each containing 50ml of Mandel's medium, 1.0% glucose and 0.5% peptone. Flasks were incubated at 28ÚC in shaking incubator at 120rpm for 18h. Different concentrations of colchicine (0, 0.01%, 0.1% and 0.2% w/v) were prepared and the conidia were treated and incubated at 28° C in the rotary shaker (200 rpm) for 10 days. For each treatment five replications were maintained. The conidia incubated in the rotary shaker were harvested with the help of centrifugation at 1000 x g for 10 min. They were spread on agar plates and incubated in the B.O.D adjusted to 26^0C for two weeks to allow maturation and conidial growth (Toyama and Toyama, 1995a and 1995b). Selected number of colonies each generated from the conidia were chosen and examined. The matured conidia of these colonies were treated with 5N, 3N and 1N HCl at 60^0C in a water bath before nuclear staining (Toyama and Toyama, 1995a and 1995b). For observation, slides were prepared. The treated conidia were stained with Giemsa solution to stain the nuclei of the conidia. The number of nuclei in conidia was observed through light microscope and photomicrographs were taken after nuclear staining with Giemsa solution.

The condition of the nuclei, culture characteristics, antagonism were observed, evaluated and recorded. The nuclei were observed at (10, 40×) magnification under brightfield light microscope (Debro microscope DX- 600) and the nuclei were photomicrographed using the software piximetre 5.2.

In vitro assay on the antagonistic ability of the putative mutant

The mutants obtained from colchicine treatment at various concentrations and the parental strains of *Trichoderma* spp. were used for antagonism against the plant pathogens *F. oxysporum*f. sp. *pisi* and *R. solani* using the dual culture technique. *In vitro* activity of antagonism was tested by measuring radius of the pathogens at 24 hours interval. Using the standard derivation given by Vincent (1947), the percent inhibition was calculated:

$$\text{Percent inhibition (I \%)} = \frac{C-T}{C} \times 100$$

Where,

I= Percent inhibition of pathogens by mutants,

C= Radial growth in control (cm)

T= Radial growth in Treatment (cm)

Nuclear condition and colony diameter of the biological control agent as influenced by colchicine treatments were observed and data were recorded. Antagonistic effects of *T. asperellum*, *T. harzianum* and its colchi mutants against *F. oxysporum* f. sp. *pisi* and *R. solani* were observed and recorded.

RESULTS AND DISCUSSION

The two parental strains of bioagents namely *T. asperellum* and *T. harzianum* were taken to induce mutation and to observe the variation in the characteristics of the parental strains and the putative mutants. These two isolates were treated with three concentrations of colchicine *i.e.* 0.01%, 0.1% and 0.2% (w/v). The colchi mutants were cultured in PDA medium and the nuclei were stained by working Giemsa solution and the nuclear condition were observed and photomicrographed. The result mentioned in Table1 (Figs. 1 and 2) indicates that the colchicine treatment caused mutation resulting in increase in the nucleus number within the conidia of the *Trichoderma* spp. under study. It was observed that treatments with colchicine at 0.01% (w/v) resulted in binucleate conidia in both the *Trichoderma* spp. while that in parent isolate uninucleate conidia was observed. Further increase in colchicine concentration to 0.1% (w/v) resulted in two to three numbers of nuclei in the conidia and colchicine at 0.2% concentration produced three nuclei in the conidia of the *Trichoderma* spp.

Table 1: Effect of colchicine treatment on nuclear condition in conidia of *T. asperellum* and *T. harzianum in vitro*.

Treatment	Nuclear number in conidia
T_1 *T.asperellum* + 0.01% concentration colchicine	2
T_2 *T.asperellum* + 0.1% concentration colchicine	2-3
T_3 *T.asperellum* + 0.2% concentration colchicine	3
T_4 *T.harzianum* + 0.01% concentration colchicine	2
T_5 *T.harzianum* + 0.1% concentration colchicine	2-3
T_6 *T. harzianum* + 0.2% concentration colchicine	3
T_{0a} *T.asperellum* parent	1
T_{0b} *T. harzianum* parent	1

Note: No of conidia observed, n=20

Similar research carried out earlier also supported the fact that the reaction of colchicine is significantly determined by its concentration and duration resulting in micro nucleation or multinucleate cells which is caused due to reconstitution of the nucleus in every chromosome or several chromosomes. Improved development of binucleate as well as smaller number of multinucleate conidia depended on higher concentration and increase in duration of colchicine treatment.

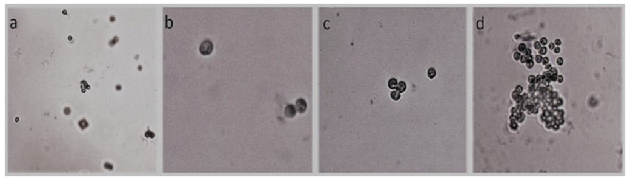

Fig. 1: Nuclear changes in *T. asperellum*after colchicine treatment. a. The original nucleus of *T. asperellum* after nuclear staining. b. Bi-nucleate conidia obtained from 0.01% concentration colchicine mutants after nuclear staining. c. Multinucleate conidia obtained from 0.1% concentration colchicine mutant after nuclear staining. d. Multinucleate conidia obtained from 0.2% concentration colchicine mutants after nuclear staining.

Evaluation on mycelial growth

The mycelial growth of the parent strain as well as the mutants was measured at 24, 48 and 72 hours after inoculation. Faster growth of the mutants was observed which resulted in faster coverage of the Petri plates compared to the parental strain (Table 2). *T. asperellum* mutants treated with 0.2% concentration of colchicine provided the best outcome with mycelial growth of 9cm, followed by 0.1% concentration of colchicine with 8.88cm and 0.01% (8.2cm) compared to the parent strain which had mycelial growth of 7cm in 72h (Figs. 3 and 4). The growth rate of *T. asperellum*, treated with 0.1% and 0.2% colchicine were statistically at par.

T. harzianum on the other hand, attained mycelial growth of 8.78cm when treated with 0.2% concentration of colchicine followed by 0.1% (7.84cm) and 0.01% (6.82cm) mycelial growth compared to the parent strain with 4.84cm mycelial growth in 72h (Table 2).

Increase in the growth rate was obtained due to increase in DNA content, chromosome number and the nuclei number induced by the polyploidy inducer colchicine (Sutthisa and Sanaomuang, 2017). Adaniya and Shirai (2001) induced mutation in ginger which resulted in significant mechanisms like very high pollen fertility and germination.

Antagonism of T. asperellum and T. harzianum parent strain and its colchi mutants against F. oxysporum f. sp. pisi

T. asperellum treated with different concentrations of colchicine *viz.*, 0.01% w/v, 0.1% w/v and 0.2% w/v were used for comparison with the parent strain.

Table2: Mycelial growth rate (cm) of *T. asperellum* and *T. harzianum* parental strain and their colchicine mutants *in vitro*.

Treatment (% colchicine)	Mycelial growth rate (cm)					
	24 hrs		48 hrs		72 hrs	
	T. asperellum	*T. harzianum*	*T. asperellum*	*T. harzianum*	*T. asperellum*	*T. harzianum*
Control	2.74	1.32	4.78	2.84	7.00	4.84
0.01%	3.92	2.40	6.48	4.74	8.20	6.82
0.1%	4.84	3.82	7.86	6.24	8.88	7.84
0.2%	5.20	4.78	8.66	7.52	9.00	8.78
SEm±	0.084	0.085	0.109	0.106	0.091	0.129
CD (p=0.05)	0.25	0.25	0.32	0.31	0.27	0.38

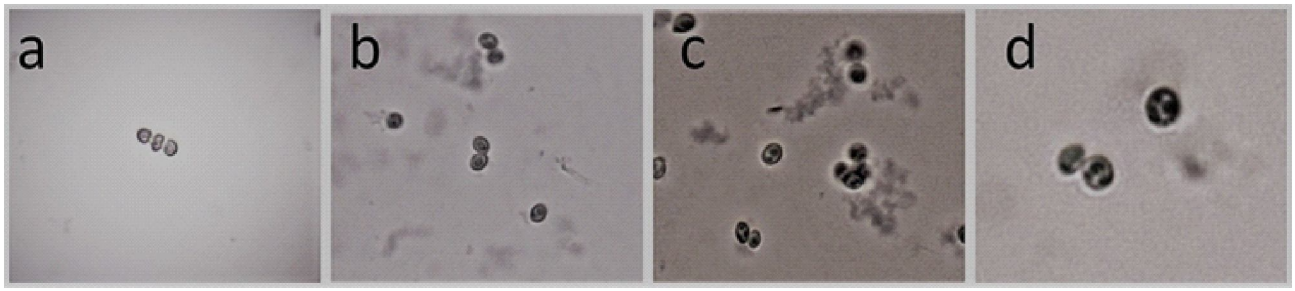

Fig. 2: Nuclear changes in *T. harzianum* after colchicine treatment a. The original nucleus of *T.harzianum* after nuclear staining. b. Bi-nucleate conidia obtained from 0.01% concentration colchicine mutants after nuclear staining. c. Multinucleate conidia obtained from 0.1% concentration colchicine mutant after nuclear staining. d. Multinucleate conidia obtained from 0.2% concentration colchicine mutants after nuclear staining.

Table 3: Evaluation of antagonistic effect of colchicine mutants of *T. asperellum* and *T. harzianum*on plant pathogens *F. oxysporum*f. sp. *pisi* and *R. solani*

Treatment	Percent inhibition of mycelial growth (%) of plant pathogens in vitro			
	By *T. asperellum*		By *T. harzianum*	
	F. oxysporum f. sp. *pisi*	*Rhizoctonia solani*	*F. oxysporum* f.sp. *pisi*	*Rhizoctonia solani*
T_1 (parent strain)	46.37	42.68	41.77	44.42
T_2 (0.01% conc. Colchicine mutant)	63.25	62.56	61.98	60.77
T_3 (0.1% conc. Colchicine mutant)	93.39	77.13	76.11	73.74
T_4 (0.2% conc. Colchicine mutant)	80.74	87.71	86.08	85.66
SEm±	2.03	1.88	1.75	2.36
CD (p=0.05)	6.10	5.66	5.27	7.09

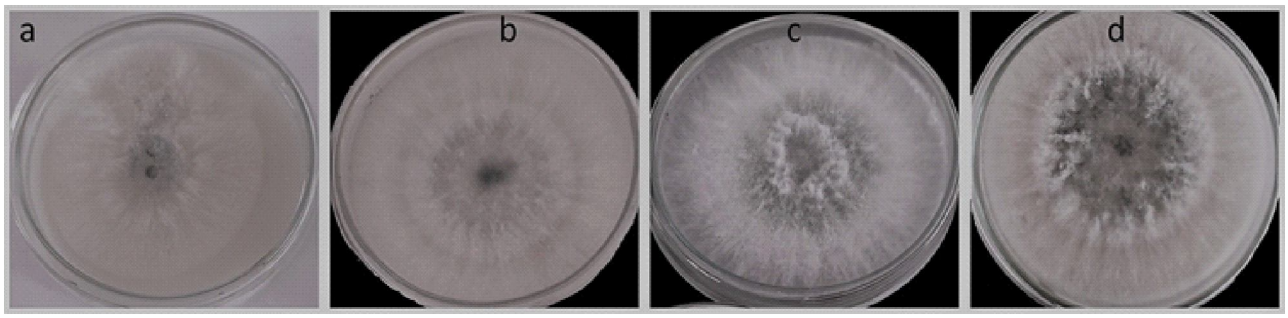

Fig. 3: Difference in mycelial growth (3 DAI) of *T. asperellum*a. Parent strain b. 0.01% concentration colchicine mutant c. 0.1% concentration colchicine mutant d. 0.2% concentration colchicine mutant.

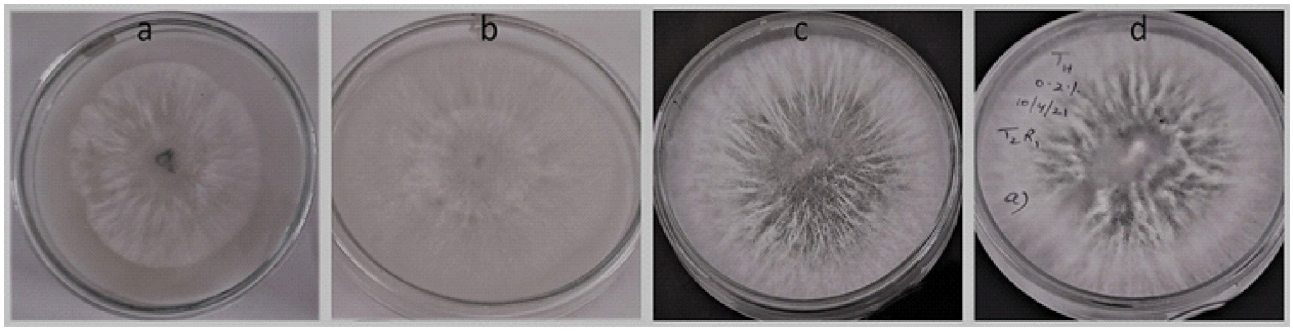

Fig. 4: Difference in mycelial growth (3 DAI) of *T. harzianum* a. Parent strain, b. 0.01% concentration colchicine mutant c. 0.1% concentration colchicine mutant d. 0.2% concentration colchicine mutant.

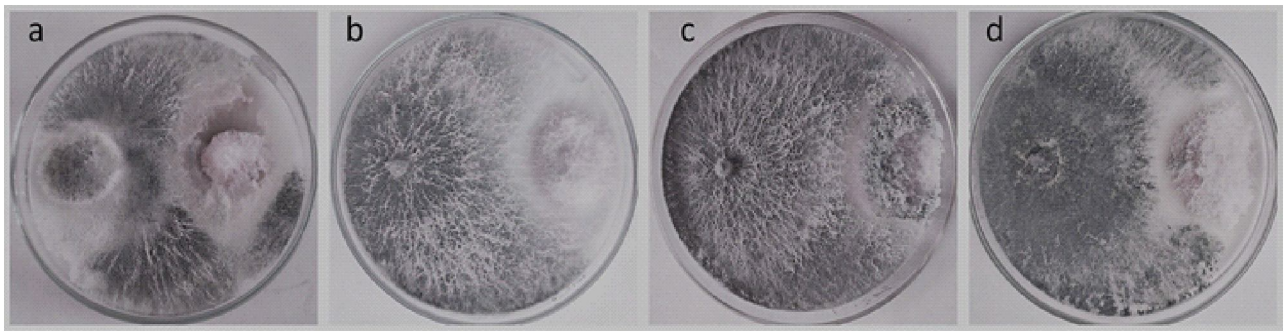

Fig. 5: Antagonism of *T. asperellum* and its mutants against *F. oxysporum*f. sp. *pisi*(4 DAI) a. Parent b. 0.01% c. 0.1% d. 0.2%

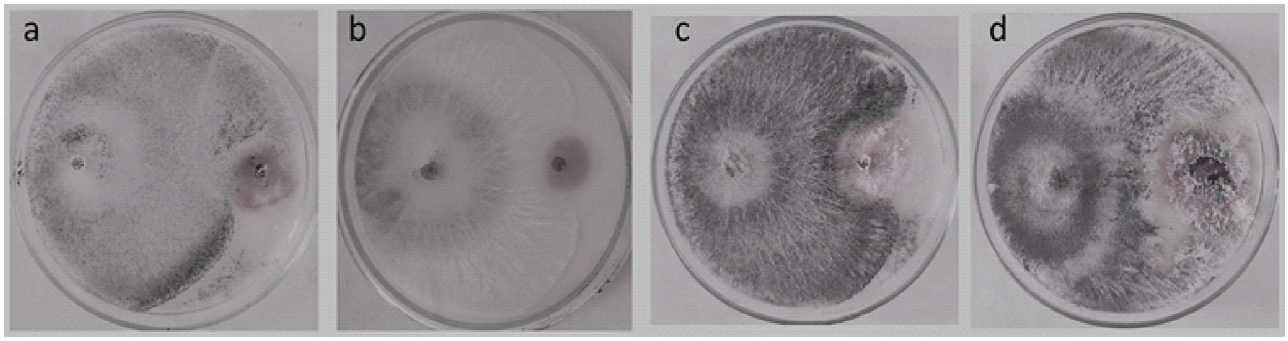

Fig. 6: Antagonism of *T. harzianum* and its mutants against *F. oxysporum* f. sp. *pisi* (4 DAI) a. Parent b. 0.01% c. 0.1% d. 0.2%

In the dual culture assay, the antagonism of the mutants surpassed the parent strain. The data on antagonistic activity (Table 3) in reference with the pictures (Fig.5) was recorded 96 h after inoculation. The highest antagonistic effect of *T. asperellum* was recorded in colchicine mutants which were obtained from 0.1% colchicine concentration (93.39% inhibition) followed by colchicine mutants with 0.2% concentration of colchicine (80.74% inhibition) and then colchicine mutants with 0.01%

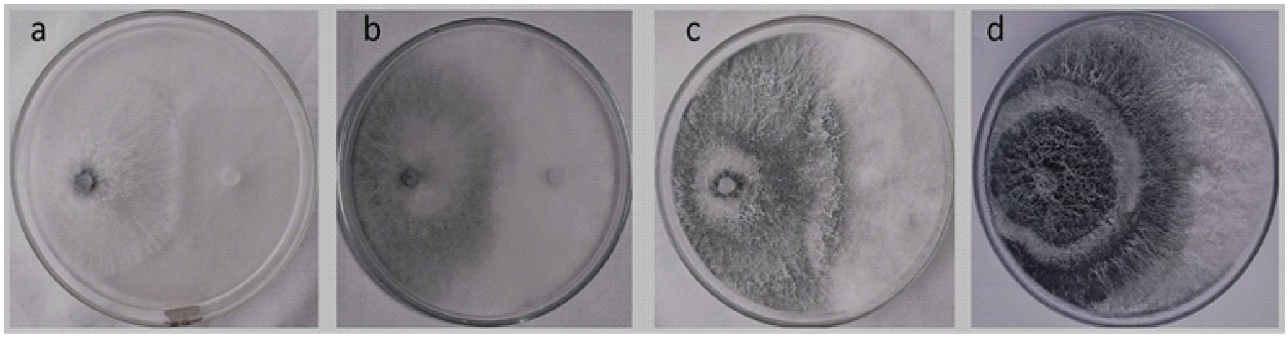

Fig. 7: Antagonism of *T. asperellum* and its mutants against *R. solani* (5 DAI) a. Parent b. 0.01% c. 0.1% d. 0.2%

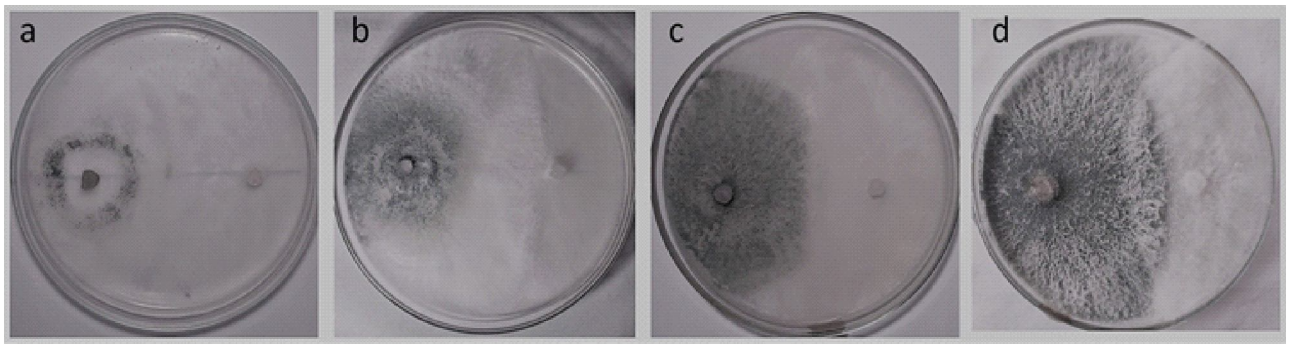

Fig. 8: Antagonism of *T.harzianum* and its mutants against *R. solani* (5 DAI) a. Parent b. 0.01% c. 0.1% d. 0.2%

concentration of colchicine (63.25% inhibition). All the mutants of *T. asperellum* showed significantly higher inhibition of the pathogen *F. oxysporum*f. sp. *pisi*than the parent strain (46.37% inhibition).

Likewise, antagonism by *T. harzianum* colchicine mutants (Table 3) obtained from 0.2% concentration colchicine treatment was the most significant which recorded 86.08% inhibition of test pathogen followed by 76.11% inhibition recorded in colchicine mutants of 0.1% concentration and 61.98% inhibition in 0.01% concentration of colchicine which also showed significantly higher antagonism compared to the parent strain (41.77% inhibition) (Fig.6). The data were recorded at 96 h after inoculation in dual culture test.

A similar study was also conducted by Gaikwad *et al.* (2017). They used the superior colchicine-mutated *Trichoderma* and the parent strain against soil borne phytopathogens of *Arachis hypogaea* and *Vigna radiata* and found that mutated *Trichoderma* gave better inhibition *in vitro* as compared to parent types. It showed that the mutants obtained efficiently and with diversity inhibited the phytopathogens tested.

In order to control the disease severity as well as incidence, it is essential for the microorganisms whether pathogenic or non-pathogenic to compete for nutrients. Infecting through mycelial contact, are more susceptible to competition for soil borne pathogens such as species of *Pythium*, *Fusarium* and plant associated microbes in soil (Khokhar *et al.* 2012).

Shete (2019) also reported similar finding about the antagonistic ability of *Trichoderma asperellum* by using gamma rays and chemical mutagenesis against *Sclerotium rolfsii, Rhizoctonia bataticola* and *Fusarium oxysporum* using dual culture technique. The resultant *Trichoderma asperellum* mutants obtained were found effective against predominant soil-borne pathogens.

Waghmare (2019) investigated the use of chemical and physical mutation in *Trichoderma harzianum* and tested its antagonism with *F. oxysporum* f. sp. *dianthi* which caused wilt of *Dianthus chinensis* L. and concluded that all variants of *Trichoderma harzianum* showed good antagonistic potential.

Antagonism of *T. asperellum* and *T. harzianum* parent strain and its colchi mutants against *R. solani*

In the test parent strains and the colchicine mutated *T. asperellum* against *R. solani* (Fig.7) were used.

The results obtained after 120 hours of inoculation depicted in Table 3 showed that 0.2% concentration colchicine mutants of *T. asperellum* produced the best result (87.71% inhibition), while 0.1% and 0.01% concentration colchicine mutants resulted in 77.13% and 62.56% inhibition respectively compared to the parent strain that caused 42.68% inhibition of *R. solani.*

Similarly, from the data recorded for *T. harzianum* parent strain and its colchicine mutants against *R. solani*(Fig.8 8) after 120 h of inoculation revealed that, the highest inhibition of 85.66% was recorded by colchicine mutants of 0.2% concentration, followed by colchicine mutants of 0.1% concentration with 73.74% inhibition and colchicine mutants of 0.01% concentration with 60.77% inhibition. The parent strain inhibited only 44.42%. *Trichoderma* mutants obtained showed better inhibition against the pathogens due to the increase in the growth rate leading to faster colonization. Several authors such as El-Bondkly*et al*. (2010) and Patil *et al*. (2016) have observed that the antagonistic capability of *Trichoderma* isolates has strong selectivity towards a specific pathogen. Coyote *et al.* (2021) determined the antagonistic potential of *T. harzianum* mutants against phytopathogenic fungi *viz., Fusarium oxysporum*, *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Sclerotium rolfsii* and they showed greater antagonistic ability against the three pathogens in Jalapeno pepper plants.

Colchicine increases the chromosome number, DNA content, nuclei number and enzyme production (Khan *et al*. 2015; Aboshosha *et al*., 2009; Toyama and Toyama, 2001).It can also be speculated that improved mechanisms may have led to increased production of antimicrobial enzymes, antibiotics, etc. which may be the reason for the mutants to show improved efficacy against the pathogens than the parent strains. They secrete several metabolites which are essential in suppressing growth of plant pathogens as well as stimulating the plant growth (Zin and Badaluddin, 2020; Contreras-Cornejo *et al*. 2015). In the fungus *Trichoderma,* its abilityas well as altered conditions obtained by chemical mutagens to antagonize phytopathogens has been evaluated and studied by many researchers.

CONCLUSIONS

From the current study carried out it can be concluded that the chemical mutagen colchicine successfully induced mutation in *Trichoderma* spp. as it increased the nucleus number within the conidia. Growth rate as well as the antagonistic potential of putative mutants was significantly better than the parent strains. The potential of the mutagen colchicine seems to be broad which might have impacted in various mechanisms, morphological and genetic characteristics that needs to be further apprehended and studied. Lethal dose count of the mutagen can be tested and studied. The need for field testing of the mutants with the phytopathogens used in this study as well with various other plant pathogens is deemed necessary. A molecular study to assess the enhanced level of DNA content in the nuclei of the conidia due to colchicine treatment can be undertaken with the aim to determine the multiplicity in the number of genes required for biocontrol mechanisms of the *Trichoderma* spp. Furthermore, the mutants can be evaluated for their enzyme production, antibiotic production, hyperparasitism, plant growth promotion activities and other useful parameters for utilization in better plant health management.

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