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Mutagenesis of *Trichoderma asperellum* for Fungicide Tolerance

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A B S T R A C T

Mutagenesis of *Trichoderma asperellum* through chemical and physical means and tolerance of *T. asperellum* mutants to commonly used fungicides was carried out at Department of Plant Pathology, Dr. PDKV, Akola. The treatments with chlorothalonil 50 per cent WP @ 0.25 per cent, carbendazim 50 per cent WP @ 0.1 per cent, propiconazole 25 per cent EC @ 0.1 per cent, thiophanate methyl 70 per cent WP @ 0.25 per cent and carboxin 37.5 per cent + thiram 37.5 per cent @ 0.25 per cent, recorded complete growth inhibition of mutants and mother culture of *T. asperellum.* Mycelial growth of mutants and mother culture in the range of 20 to 85 mm was recorded in azoxystrobin 23 per cent SC @ 0.1 per cent. It proved that fungicide azoxystrobin 23 per cent SC @ 0.1 per cent concentration was more compatible with *T. asperellum* mutants and mother culture. Among the mutants, $TaU_4(T_{12})$ was recorded 84.4 mm mycelial growth with azoxystrobin 23 per cent SC @ 0.1 per cent at 7 DAI, where as $TaU_1(T_{11})$**,** $TaG_2(T_{14})$, $TaU_2(T_{10})$ and $TaG_1(T_{13})$ recorded 80.5, 79.9, 75.8 and 75.00 mm growth respectively. The mother culture TaMc ($_{T17}$) recorded 29.1 mm growth.

Introduction

Trichoderma, is a filamentous fungus which have attracted the attention because of their multi prong action against various plant pathogens (Harmam *et al.,* 2004). Several modes of action have been proposed to explain the bio-control of plant pathogens by *Trichoderma,* these include production of antibiotic and cell wall degrading enzymes, competition for key nutrients, parasitism, stimulation of plant defense mechanisms and combination of those possibilities (Harman, 1999). However, in order to continue management of new strains/races of soil borne fungal pathogens being evolved in the nature, there is necessity to evolve new effective biocontrol agents. Amongst *Trichoderma* spp., *Trichoderma asperellum* is one of the

effective biocontrol species but sexual reproduction is absent in it and reproduction is limited to production of conidia. Fungicides are used frequently to manage plant diseases effectively, but human health and environmental concerns often put pressure on growers to reduce the number of applications. One approach is to integrate biological control agents with fungicide use. In order to be effective, it is important that the BCA is compatible with the desired fungicide. Therefore, mutagenesis in *T. asperellum* by using chemical and physical means is the best way to create efficient strains with enhanced tolerence potential against soil fungicides. In integrated plant protection, which is based on the combined application of physical, chemical and biological means of control. Compatibility of these *T. asperellum* mutants with fungicides will make them highly suitable for exploitation in future within framework of integrated disease management program. Therefore it is important to study about the effects of fungicides on the biocontrol agent. The combined use of biocontrol agents and chemical fungicides has attracted much attention as a way to obtain synergistic or additive effects in the control of soil borne pathogens (Locke *et al.,* 1985). Considering this possibilities, present study was conducted.

Materials and Methods

Chemical mutagenesis

Induction of chemical mutagenesis was carried out according to procedure of Durand *et al.,* (1988) and Chandra *et al.,* (2010)with slight modifications. Conidial suspension of the seven days old sporulated *Trichoderma asperellum* mother culture was prepared in 10ml sterile distilled water. Centrifuged twice at 10000 rpm for 5 minutes and pallets of the suspension subsequently washed in 0.067M phosphate buffer (pH-7.0) to separate mycelium. After second washing, the conidia were resuspended in 10ml of phosphate buffer and conidial concentration was adjusted with sterile water to 10^5 /ml with haemocytometer. The 1ml conidial suspension ($1x10^5$ conidia per ml) was transferred into a sterile ependrop tubes of size 2 ml. Stirred the ependrop tubes on magnetic stirrer for 5 minutes to break the conidial chains. Conidial suspension of *T. asperellum* treated with hydroxyl amine (HA) and ethyl methyl sulphonate (EMS) @ 200 µl/ml for 30,45,60 and 75 minutes seperately in orbital shaker. An untreated control tube was also treated with sterile distilled water. The treated and untreated conidia were washed three times in centrifuge machine at 5000 rpm speed for 10 minutes with sterile distilled water to remove the traces of chemicals. 1ml treated and untreated conidial suspensions were spread separately on the surface of PDA medium in petri plates under aseptic condition and incubated at 28^Oc for 72 h. After incubation, colony developed from single conidium was transferred on fresh PDA medium. Growth of *Trichoderma asperellum* mother culture and mutants was checked up to seventh generation to check its stability.

Physical mutagenesis

Mutation induced by gamma radiation

Induction of mutation by gamma radiation was carried out according to the procedure of Haggag et.al (2002). Irradiation facility available at Department of Chemistry, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur was utilized for experiment.

Mother culture of *Trichoderma asperellum* grown on PDA petri plate for seven days to induce sporulation. The sporulated culture of *Trichoderma asperellum* was irradiated with Cobalt – 60 @250gry for 30, 45, 60 and 75 minutes respectively. Both irradiated and non

irradiated (control) conidia were harvested from the petri plates after adding 10ml sterilized physiological saline (0.85 per cent NaCl) to each plate and conidia were separated with a sterile needle and inoculated on fresh PDA medium under aseptic condition. After 72 h colony developed from single conidium was transferred on fresh PDA medium. Growth of *Trichoderma asperellum* mother culture and mutant was checked upto sixth generation to check it's stability.

Induction of mutation by UV radiation

Conidial suspension of the seven days old sporulated *Trichoderma asperellum* mother culture was prepared in 10ml sterile distilled water. Centrifused twice at 10000 rpm for 5 minutes and pallets of the suspension subsequently washed in 0.067M phosphate buffer (pH-7.0) to separate mycellium after second washing, the conidia were resuspended in 10ml of phosphate buffer and conidial concentration was adjusted with sterile water to 10^5 /ml with haemocytometer.

Then 1 ml conidial suspension ($1x10^5$ conidia per ml) was poured on the solidified PDA medium. The plates without lid were treated for 30, 45, 60 and 75 minutes separately under the ultraviolet light (15W 254 nm). Distance between the agar surface and tube was adjusted to 30 cm under aseptic condition in laminar air flow. An untreated plate containing conidia was maintained as a control.

After irradiation, the treated plates were covered and incubated at 28°C for 72 h. After incubation, colony developed from single conidium was transferred on fresh PDA medium. Growth of *Trichoderma asperellum* mother culture and mutants was checked up to sixth generation to check its stability (Mech *et al.,* 2006).

Tolerance with fungicide

The fungicide were evaluated for their compatibility with carbendazim 50 per cent WP0.1 per cent, chlorothalonil 75 per cent WP 0.25 per cent, propiconazole 25 per cent EC0.1 per cent, thiophanate methyl 70 per cent WP 0.25 per cent, azoxystrobin 23 per cent SC 0.1 per cent and vitavax 0.25 per cent concentration respectively in PDA medium by employing poison food technique. Potato Dextrose Agar (PDA) medium was prepared, equally distributed 100 ml in 250 ml conical flask and sterilized in autoclave. Requisite quantity of each of the fungicides (as per concentration) was added in sterilized melted (45^0C) PDA separately so as to obtain desired concentration. Flask containing poisoned medium was shaken well to have even and uniform distribution of fungicides. About 20 ml of melted poisoned PDA was poured in each sterilized Petri plate and allow to solidify. These Petri plates were inoculated by *Trichoderma asperellum* mother culture and mutants separately. Five mm disc of one week old *Trichoderma asperellum* culture was cut with sterilized cork borer, lifted and transferred aseptically in the centre of Petri plate containing the medium poisoned with test fungicide. The control plates were kept the culture disc grown in same condition on PDA without fungicides. Inoculated plates were incubated at room temperature (25 ± 2^0C) for a period of seven days. Colony diameter was recorded in mm and per cent mycelial growth inhibition was calculated as per Vincent's formula based on the average colony diameter.

Results and Discussion

An experiment was conducted on compatibility of *T. asperellum* mutants and mother culture with fungicides by adopting poison food technique. The observations were recorded at 7 DAI, the data showed that all fungicides were found to arrest radial mycellial growth of test culture except

azoxystrobin 23 per cent SC @ 0.1 per cent concentration. The treatments with chlorothalonil 50 per cent WP @ 0.25 per cent, carbendazim 50 per cent WP @ 0.1 per cent, propiconazole 25 per cent EC @ 0.1 per cent, thiophanate methyl 70 per cent WP @ 0.25 per cent and carboxin 37.5 per cent + thiram 37.5 per cent @ 0.25 per cent, recorded complete growth inhibition of mutants and mother culture of *T. asperellum.* Mycelial growth of mutants and mother culture in the range of 20 to 85 mm was recorded in azoxystrobin 23 per cent SC @ 0.1 per cent. It proved that fungicide azoxystrobin 23 per cent SC @ 0.1 per cent concentration was more compatible with *T.asperellum* mutants and mother culture. Among the mutants, $TaU_4(T_{12})$ was recorded 84.4 mm mycelia growth with azoxystrobin 23 per cent SC @ 0.1 per cent at 7 DAI, where as $TaU_1(T_{11})$**,** $TaG_2(T_{14})$, $TaU_2(T_{10})$ and $TaG_1(T_{13})$ recorded 80.5, 79.9, 75.8 and 75.00 mm growth respectively. The mother culture $TaMc(_{T17})$ recorded 29.1 mm growth.

Madhusudhan *et al.,* (2010) evaluated six fungicides, carbendazim (50 per cent WP), propiconazole (25 per cent EC), tridemorph (BO per cent EC), chlorothalonil (75 per cent WP) and hexaconazole (5 per cent EC) for their compatibility with *T. viride*. Among them chlorothalonil (75 per cent WP) was found little inhibition at 40 ppm. Other fungicides were found complete inhibition of *Trichoderma viride* even at very lower concentration.

Similar observation was also reported by Bagwan (2010), Sharma *et al.,* (1999) and Bindu *et al.,* (2011). Nongmaithem (2015) also reported *T. viride* was compatible with mancozeb and tebuconazole while systemic fungicides like carbendazim, hexaconazole, carbendazim and propiconazole were found incompatible. Wafa *et al.,* (2016) reported compatibility between strains of *Trichoderma harzianum* (Tcomp, TH1 and TH3) and *T. viride* (TV1) which produced conidia in the presence of low concentrations of chlorothalonil and in the recommended dose of fenhexamid, showing compatibility respectively ranging from 50.5 to 89.7 per cent and 51.5 to 97.1 per cent corroborates the results of present studies. Yuan *et al.,* (2007) reported that the strain of *Trichoderma* T-21 has shown pyrimethanil resistance and the mycellial growth and the conidial production of the strain were good. Caron *et al.,* (2016) have established the compatibility chart of *Trichoderma* MAUL-20 with the most commonly used pesticides in greenhouses. They showed that among the active ingredients the fenhexamid is compatible with *Trichoderma* MAUL-20. Bagwan (2010) showed that the thiram (0.2%) was compatible with *Trichoderma harzianum* and *T. viride*. However, strains of *Trichoderma* spp. tested could not grow in the presence of chlorothalonil.

The results are similar with the work of Mclean *et al.,* (2001), which showed by the test of spore germination *in vitro* that *T. harzianum* is very sensitive to chlorothalonil fungicide. Poddar *et al.,* (2004) found that effective management of chickpea disease might be done through combined use of fungicides and bio-control agents. In the experiments, *Trichoderma harzianum* and carbendazim were successfully used against wilt disease. Similar types of results were also observed by Sallam *et al.,* (2008) corroborates the results of present studies. Timothy (2019) reported 12 different *T. asperellum* isolates along with one *T. koningiopsis* isolate was also studied for their response to soil fungicides. In a poison plate assay, there was a significant interaction between the isolate and fungicide at the recommended rate. No fungicides completely inhibited growth, but some significantly suppressed it (Table 1–3).

Table.1

Trichoderma asperellum mutants	Mode of Mutagenesis
TaH_1	Treated with hydroxyl amine (HA)@ 200 µl/ml for 30 minutes.
TaH_2	Treated with hydroxyl amine (HA) @ 200 µl/ml for 45 minutes.
TaH_3	Treated with hydroxyl amine (HA) @ 200 µl/ml for 60 minutes.
TaH_4	Treated with hydroxyl amine (HA) @ 200 µl/ml for 75 minutes.
TaE_1	Treated with ethyl methyl sulphonate (EMS) @ 200 µl/ml for 30 minutes.
TaE_2	Treated with ethyl methyl sulphonate (EMS) @ 200 µl/ml for 45 minutes.
TaE_3	Treated with ethyl methyl sulphonate (EMS) @ 200 µl/ml for 60 minutes.
TaE_4	Treated with ethyl methyl sulphonate (EMS) @ 200 µl/ml for 75 minutes.
TaU_1	Treated with ultraviolet light (15W 254 nm) for 30 minutes .
TaU_2	Treated with ultraviolet light (15W 254 nm) for 45 minutes .
TaU_3	Treated with ultraviolet light (15W 254 nm) for 60 minutes .
TaU_4	Treated with ultraviolet light (15W 254 nm) for 75 minutes .
TaG_1	Treated with Cobalt – 60 @ 250gry for 30 minutes.
TaG_2	Treated with Cobalt – 60 @ 250gry for 45 minutes.
TaG_3	Treated with Cobalt – 60 @ 250gry for 60 minutes.
TaG_4	Treated with Cobalt – 60 @ 250gry for 75 minutes.
TaMc	*Trichoderma asperellum* mother culture.

Table.2

Sr. no.	Fungicides	Trade name	Company	Concentration (%)
1	Carbendazim 50% WP	Starbenz	Swal co-operation Ltd.	0.1
2	Chlorothalonil 75 % WP	Kavach	Syngenta	0.25
3	Propiconazole 25% EC	Tilt	Syngenta Pvt. Ltd	0.1
4	Thiophanate Methyl 70% WP	Roko	Nippon soda Co. Ltd.	0.25
5	Azoxystrobin 23% SC	Amistar	Syngenta	0.1
6	Carboxin 37.5% + Thiram 37.5%	Vitavax power	Dhanuka Agritech Ltd.	0.25

Table.3 Tolerence of *Trichoderma asperellum* mutants and mother culture with commonly used fungicides

Treatments	*T.asperellum* mutants	Carbendazim 50% WP @0.1%	PGI	Chlorothalonil (75 WP) @0.25%	PGI	Propiconazole 25% EC @0.1%	PGI	Azoxystrobin 23% SC@0.1%	PGI	Thiophanate Methyl 70% WP @0.25%	PGI	Carboxin 37.5% + thiram 37.5% @0.25%	PGI	Control	PGI
		Mean radial growth.		Mean radial growth.		Mean radial growth.		Mean radial growth.		Mean radial growth.		Mean radial growth.		Mean radial growth.	
T1	TaH_1	00.00	100	00.00	100	00.00	100	21.1	**76.56**	00.00	100	00.00	100	90.00	00.00
T2	TaH_2	00.00	100	00.00	100	00.00	100	35.2	**60.93**	00.00	100	00.00	100	90.00	00.00
T3	TaH_3	00.00	100	00.00	100	00.00	100	39.8	**55.81**	00.00	100	00.00	100	90.00	00.00
T4	TaH_4	00.00	100	00.00	100	00.00	100	60.2	**33.07**	00.00	100	00.00	100	90.00	00.00
T5	TaE_1	00.00	100	00.00	100	00.00	100	21.2	**76.44**	00.00	100	00.00	100	90.00	00.00
T6	TaE_2	00.00	100	00.00	100	00.00	100	36.8	**59.07**	00.00	100	00.00	100	90.00	00.00
T7	TaE_3	00.00	100	00.00	100	00.00	100	30.4	**66.26**	00.00	100	00.00	100	90.00	00.00
T8	TaE_4	00.00	100	00.00	100	00.00	100	30.3	**66.30**	00.00	100	00.00	100	90.00	00.00
T9	TaU_1	00.00	100	00.00	100	00.00	100	59.3	**34.07**	00.00	100	00.00	100	90.00	00.00
T10	TaU_2	00.00	100	00.00	100	00.00	100	75.8	**15.81**	00.00	100	00.00	100	90.00	00.00
T11	TaU_3	00.00	100	00.00	100	00.00	100	80.5	**10.56**	00.00	100	00.00	100	90.00	00.00
T12	TaU_4	00.00	100	00.00	100	00.00	100	84.4	**6.19**	00.00	100	00.00	100	90.00	00.00
T13	TaG_1	00.00	100	00.00	100	00.00	100	75.0	**16.67**	00.00	100	00.00	100	90.00	00.00
T14	TaG_2	00.00	100	00.00	100	00.00	100	79.9	**11.22**	00.00	100	00.00	100	90.00	00.00
T15	TaG_3	00.00	100	00.00	100	00.00	100	74.8	**16.85**	00.00	100	00.00	100	90.00	00.00
T16	TaG_4	00.00	100	00.00	100	00.00	100	69.5	**22.78**	00.00	100	00.00	100	90.00	00.00
T17	TaMc	00.00	100	00.00	100	00.00	100	29.1	**67.63**	00.00	100	00.00	100	90.00	00.00
		SE(m)±						**0.75**							
		CD (P=0.01)						**2.89**							

PGI –Per cent growth inhibition.

Plate.1

Plate 1. Pure culture of *Trichoderma asperellum* (mother culture)

Plate.2

Plate 2 (a).Chemical mutagenesis of *Trichoderma asperellum* with hydroxyl amine (HA)

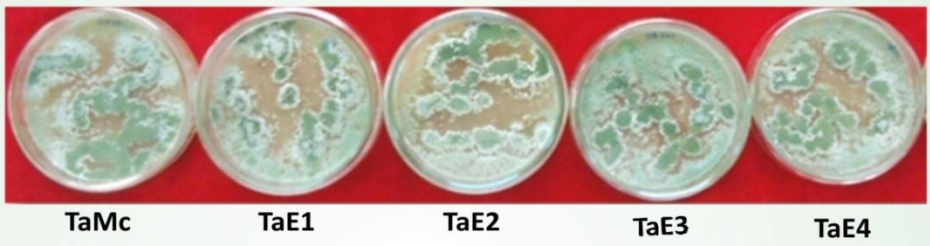

Plate 2(b).Chemical mutagenesis of *Trichoderma asperellum* with ethyl methyl sulphonate (EMS)

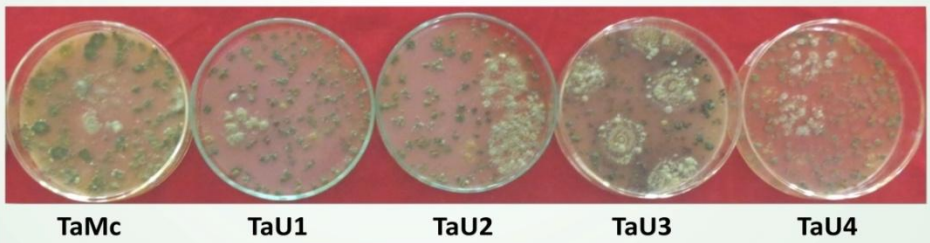

Plate 2(c). Physical mutagenesis of *Trichoderma asperellum* with U.V. radiation

Plate 2(d). Physical mutagenesis of *Trichoderma asperellum* with gamma radiation

Plate.3

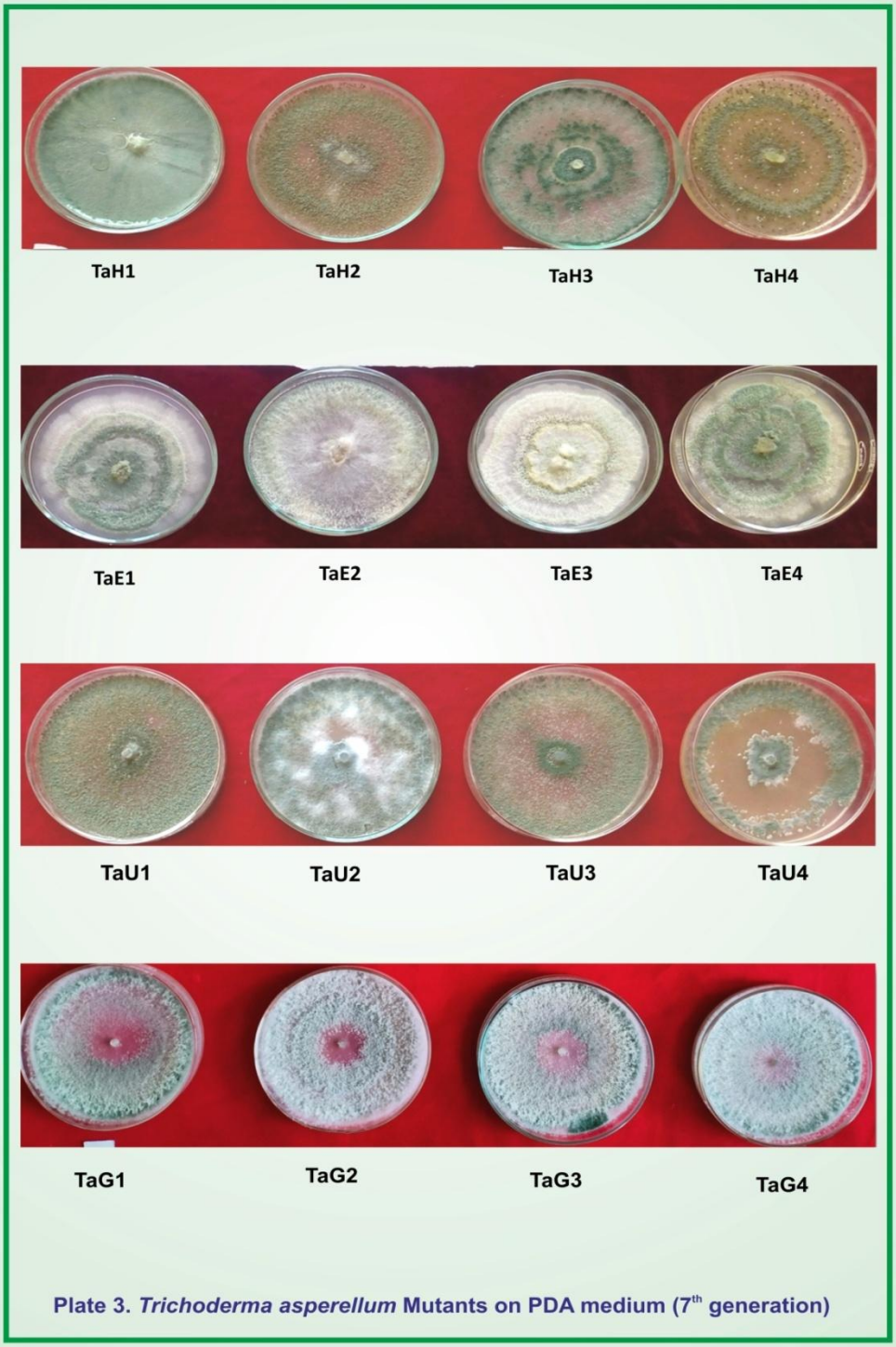

Plate 3. *Trichoderma asperellum* Mutants on PDA medium (7th generation)

Plate.4

Plate 8. Compatibility of *Trichoderma asperellum* mutants with fungicide Azosystrobin @ 0.1%

Plate.5

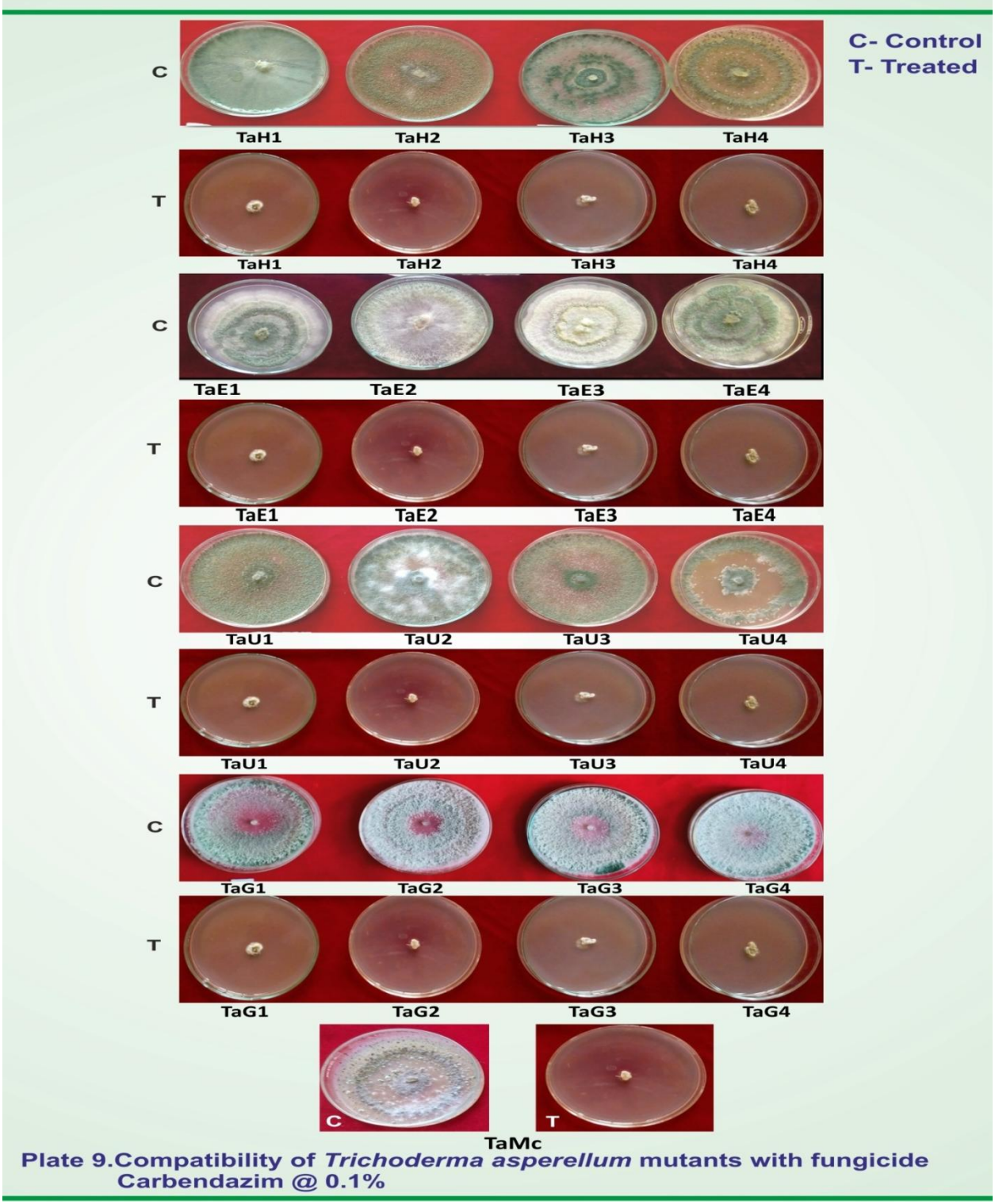

Plate 9.Compatibility of *Trichoderma asperellum* mutants with fungicide Carbendazim @ 0.1%

Plate.6

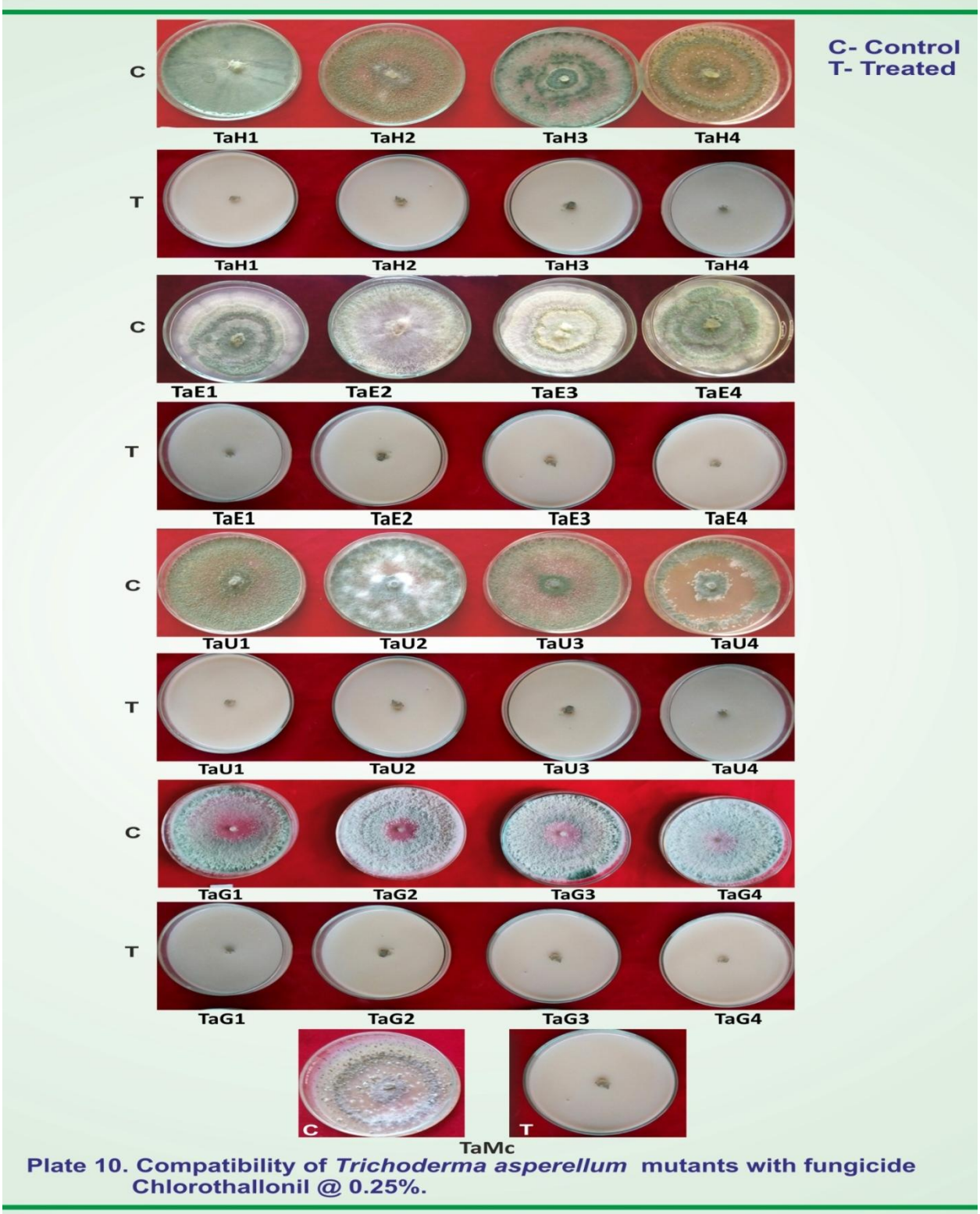

Plate 10. Compatibility of *Trichoderma asperellum* mutants with fungicide Chlorothallonil @ 0.25%.

Plate.7

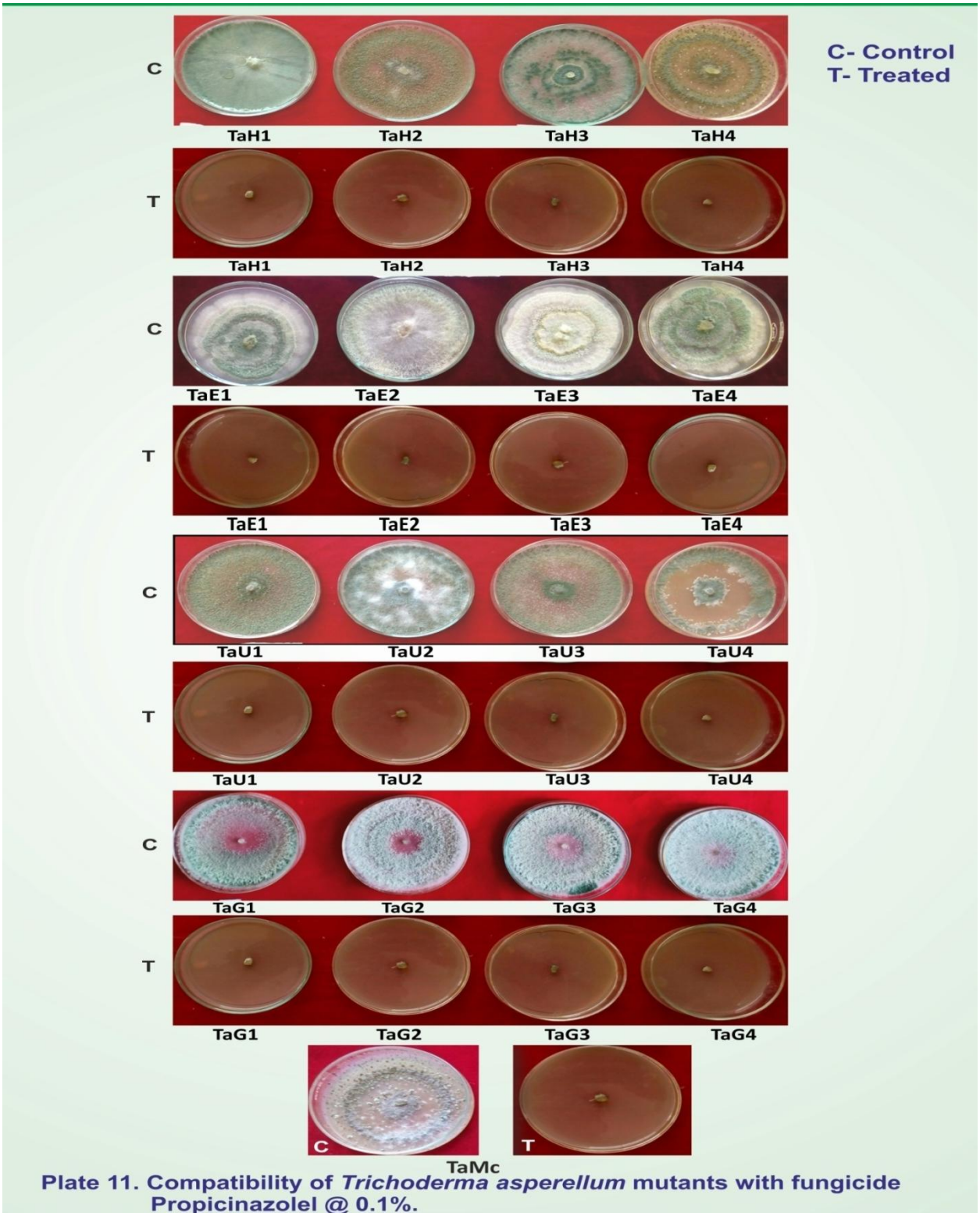

Plate 11. Compatibility of *Trichoderma asperellum* mutants with fungicide Propicinazolel @ 0.1%.

Plate.8

Plate 12. Compatibility of *Trichoderma asperellum* mutants with fungicide Thiophanate methyl@ 0.25%

Plate.9

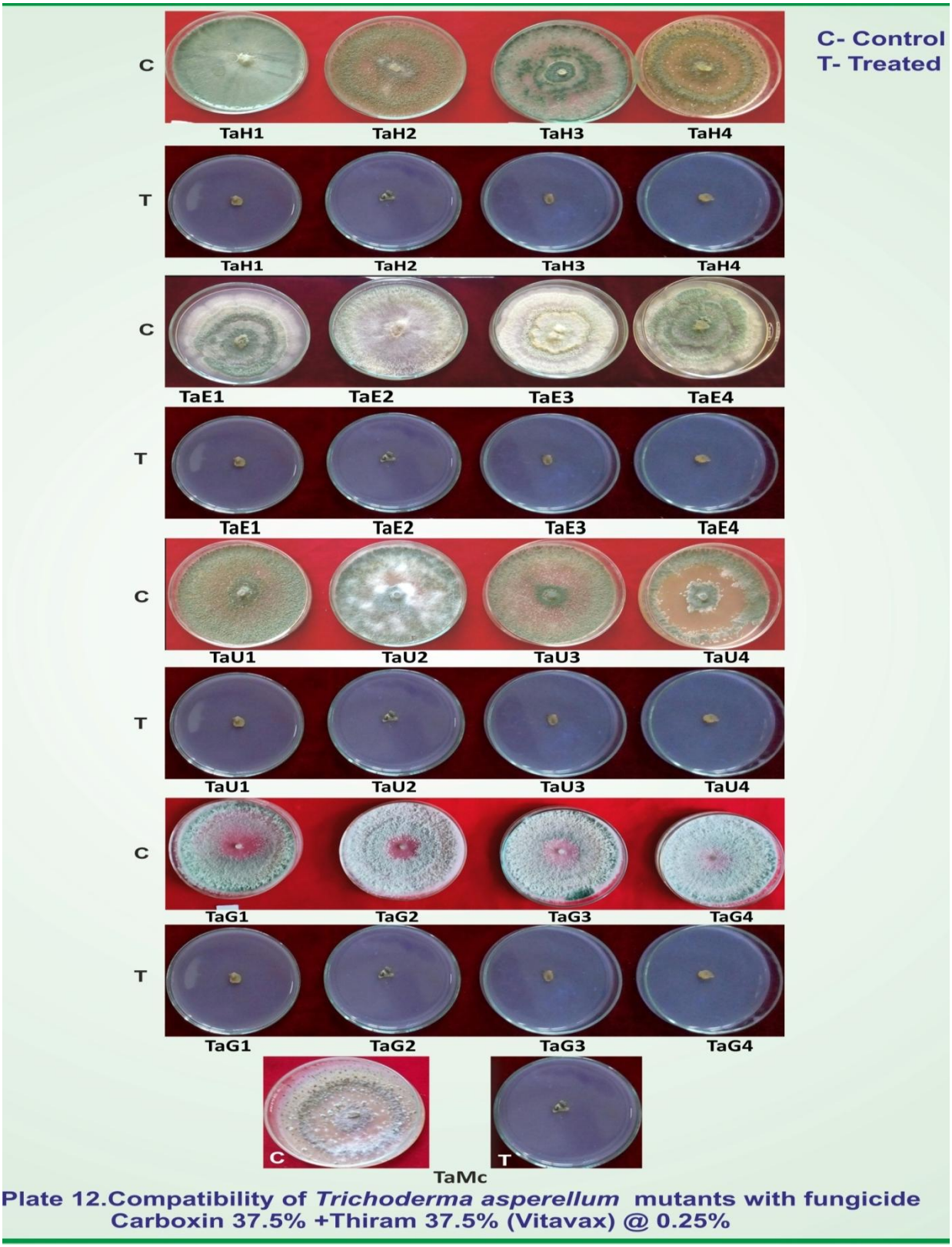

Plate 12.Compatibility of *Trichoderma asperellum* mutants with fungicide Carboxin 37.5% +Thiram 37.5% (Vitavax) @ 0.25%

Desai *et al.,* (2002) also reported that mancozeb at 500 ppm recorded a lower inhibition of hyphae (5.70 per cent) and sporulation (11.02 per cent) of *Trichoderma harzianum.* Ramarethinam *et al.,* (2001) reported that the fungicides like carbendazim (50 per cent WP), hexaconazole (5 per cent EC) completely inhibited the growth of *Trichoderma viride* centration *in vitro*. Similar observations were made by Bheemaraya et.al (2012) tested five fungicides at 0.1 and 0.2 per cent concentration and found that metalaxyl + mancozeb and mancozeb were compatible with growth of *Trichoderma* spp. while carbendazim, captan and propiconazole completely inhibited radial mycelial growth hence, were not compatible with *Trichoderma* spp. The results are also in agreement with the works of Mukhopadyay *et al.,* (1986) Sharma and Mishra (1995); Agarwal and Tripathi (1999), who also found good growth of *Trichoderma* isolates at low and medium concentrations of various fungicides.

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