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Development of Tolerant Strains of *Trichoderma harzianum* to Reduce the Use of Synthetic Pesticides in Agriculture Practices

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Abstract
Biocontrol agents for the control of plant pathogenic microorganisms, especially fungi, are widely used to reduce the health hazards of pesticides. These microbial antagonists not only control the growth of pathogens but also promote the growth of plants. The development of pesticide-tolerant strains of biocontrol agents, with reduced doses of pesticides, enables them to control more effectively than alone. The current study provides the development of tolerance in *Trichoderma harzianum* with fungicides, *viz.* Topsin-M and Carbendazim reduce chemical use in the field and provide an eco-friendly environment. Initially, Topsin-M and Carbendazim inhibit the growth of *T. harzianum* even @ 0.1 and 0.01 ppm, respectively, but after a gradual increase in the concentration of fungicides with repeated sub-culturing, tolerance was developed in *T. harzianum,* and it is able to grow on media amended with Topsin-M and Carbendazim even @ 10,000 ppm. Inhibition of pathogens more strongly with dual action, *i.e.,* reducing the dose of pesticides with tolerant strains of *T. harzianum*.

Keywords: tolerance, fungicides, growth, biocontrol agents

Highlights
- Fungal biocontrol agents, which stop the growth of pathogenic fungi, are used to enhance the biocontrol activity and synergistic effects with chemicals to inhibit the growth of pathogens.
- This biocontrol agent, i.e., *T. harzianum*, is used to create tolerance against fungicides Topsin-M and Carbendazim.

1. Introduction
Macrophomina phaseolina, a soil-borne pathogen, poses a significant threat to economically vital crops due to its destructive root-infecting nature (Baker & Cooke 1974). It was also reported for the first time to cause charcoal rot in adzuki beans (*Vigna angularis*) (Sun et al., 2016). The use of fungicides for the control of soil-borne diseases is costly and also produces environmental and health hazards to users, and adversely affects the beneficial microorganisms in the soil (Dłużniewska, 2003). *Trichoderma harzianum* is one of the known biocontrol agents used against several plant pathogens. *Trichoderma* strains with practical antagonistic abilities are potential candidates for the biological control of plant diseases (Papavizas, 1985; Manczinger, 1999). *T. asperellum* is also reported to be resistant to root rot diseases caused by *Pythium myriotylum* and reduces 60% of the growth of the pathogen (Mbarga et al., 2012). Biocontrol agents can tolerate agrochemicals successfully and also provide benefits to plants against diseases (Sun et al., 2019). Species of *Trichoderma* reduce the severity of plant diseases. *Trichoderma* strains can interact directly with roots, increasing plant growth potential, resistance to disease, and tolerance to abiotic stresses. (Hermosa, et al., 2012). The endophytic *Trichoderma* strains provide less harm to the microbiome in soil. (Kovacs, et al., 2021). *T. longibrachiatum* HL167 showed maximum salt tolerance effect and highest antifungal activity against *F. oxysporum* (Liu, et al., 2023). As a substitute for chemical pesticides, *Trichoderma* fungi can also be used as an effective biocontrol agent against stem rot (*S. rolfsii*) in groundnut (Hirpara et al., 2017)

The present research work describes the development of pesticide-tolerant strains of *Trichoderma harzianum*, which will be more helpful in controlling plant pathogens with reduced doses of fungicides.

2. Materials and Methods
Tolerance to fungicides, i.e., Topsin-M and Carbendazim in *Trichoderma harzianum* was developed using the food poison method (Grover & Moore, 1962; Mondal et al., 1995). Isolates of *T. harzianum* were exposed to gradually increasing concentrations of the fungicides in the medium to develop tolerant strains. The initial concentration of Topsin-M and Carbendazim used for *T. harzianum* was 0.01 ppm. PSA (potato sucrose agar) without fungicide serves as a control. A 5.00 mm inoculum disc of *T. harzianum* was cut from the margin of an actively growing colony and placed in the center of each of the 3 replicate Petri plates. Petri plates were incubated at 28±2 ºC. Radial growth of the biocontrol agent was observed daily. Mycelium from the colony of *T. harzianum* growing at 0.01 ppm concentration was transferred to a medium containing Topsin-M @ 0.1 ppm repeatedly until good growth of *T. harzianum* was observed. The process was repeated by gradually increasing the concentration of Topsin-M in the medium to 1, 10, 100, 1000, and 10,000 ppm.

Trichoderma harzianum showed more sensitivity to fungicides; each time, a lower concentration of the fungicide was also used while transferring the mycelium to the next higher concentration. The transfer was repeated until a good growth of *T. harzianum* was observed at a given concentration.

3. Results and Discussion

3.1. Development of Tolerance in *Trichoderma harzianum* with Topsin-M

The growth of *T. harzianum* on potato sucrose agar containing Topsin-M at 0.01 ppm was not significantly different from the control, with complete plate coverage observed after 96 hours. At 0.1 ppm, growth was slower, while at 1 ppm, it was significantly reduced compared to the 0.1 ppm treatment. However, when *T. harzianum* was repeatedly sub-cultured from 1 ppm to both 0.1 ppm and 1 ppm treatments, its growth increased significantly. A similar trend was observed in sequential transfers from 1 ppm to 0.1, 1, and 10 ppm; from 10 ppm to 1, 10, and 100 ppm; and from 100 ppm to 10, 100, and 1000 ppm. No growth was observed when the fungus was transferred from 1000 ppm to 10,000 ppm. However, sub-culturing from 100 ppm to 1000 ppm and subsequently from 1000 ppm to 10,000 ppm led to significant increases in *T. harzianum* growth (Table 1).

Table 1. Development of tolerance in *Trichoderma harzianum* against Topsin-M

Concentrations (ppm)		Different intervals of Hours for proliferation (hrs)						No. of times to transfer the growth to next concentration.
		24	48	72	96	120	144	
0	Mycelium proliferation in mm	1.32	3.41	7.02	9	9	9	(1*)
0.01		0.68	1.24.	3.29	6.71	9	9	
0.1		0	0.82	1.12	3.46	5.28	7.16	
1		0	0.6	0.72	0.98	1.06	1.32	
0		1.41	3.29	6.71	8.86	9	9	(1*)
0.01		0.69	1.39	4.26	7.32	9	9	
0.1		0.55	0.92	1.12	3.9	6.12	8.19	
1		0	0.62	0.9	1.06	2.59	3.79	
0		1.41	3.29	6.71	8.86	9	9	(2*)
0.01		0.91	1.62	5.03	8.16	9	9	
0.1		0.6	1.16	2.16	4.01	6.52	8.82	
1		0.52	0.79	1.26	3.52	4.99	6.36	
0	Mycelium proliferation in mm	1.69	3.16	7.19	9	9	9	(2*)
0.1		0.98	1.99	5.52	8.26	9	9	
1		0.55	0.9	1.99	3.62	5.71	7.41	
10		0	0	0.55	0.67	0.9	1	
0		1.38	4.41	6.92	8.69	9	9	(2*)
0.1		1	3.14	5.71	8.3	9	9	
1		0.58	1.2	2.26	4.59	6.17	8.52	
10		0	0.59	0.9	1.16	2.56	3.2	
0		1.46	5.29	8.18	9	9	9	(2*)
0.1		1.2	3.42	6.01	8.38	9	9	
1		0.81	1.42	3.21	5.16	6.8	8.71	
10		0.56	0.95	1.52	2.81	4.16	6.02	
0	Mycelium proliferation in mm	1.62	4.8	6.59	9	9	9	(3*)
1		1.29	3.4	5.28	7.16	9	9	
10		0.59	1.02	1.55	3	4.29	6.5	
100		0	0	0.55	0.69	0.82	1	
0		1.58	5.2	7.41	9	9	9	(1*)
1		1.29	3.5	5.59	7.82	9	9	
10		0.68	1.4	3.28	5.82	7	9	
100		0	0.59	0.99	1.42	3.2	4.91	
0		1.69	5.02	8.49	9	9	9	(1*)
1		1.4	3.92	6.01	8.2	9	9	
10		0.72	1.56	3.5	6	8.29	9	
100		0.56	1.06	2.82	5.19	7	9	
0	Mycelium proliferation in mm	1.71	5.21	7.8	9	9	9	(3*)
10		0.8	1.72	3.82	6.52	8.5	9	
100		0.6	1.16	3.19	5.5	7.2	9	
1000		0.5	0.9	1.02	1.16	2.29	3	

0		1.68	4.92	7.91	9	9	9	
10		0.88	1.9	4.01	7.12	9	9	(3*)
100		0.66	1.28	3.29	5.82	7.59	9	
1000		0.55	1	2.52	2.61	3.8	5.11	
0		1.6	4.56	8.2	9	9	9	
10		0.88	1.9	4.01	7.12	9	9	(3*)
100		0.7	1.91	3.83	5.9	8.2	9	
1000		0.59	1.52	2.26	4.16	6.29	8.8	
0		1.82	4.26	7.14	9	9	9	
100		0.99	2.18	4.26	6.71	9	9	(4*)
1000		0.6	1.79	2.9	4.82	7.16	9	
10000		0	0	0	0	0	0	
0	Mycelium proliferation in mm	1.62	4.52	8.19	9	9	9	
100		1.02	2.39	4.5	7.1	9	9	(3*)
1000		0.6	1.8	3.19	4.87	7.42	9	
10000		0	0.61	0.82	0.98	1.2	1.7	
0		1.69	4.79	8.2	9	9	9	
100		1.22	2.57	4.82	7.5	9	9	(3*)
1000		0.78	1.89	3.27	5.12	7.71	9	
10000		0.58	0.89	1.28	2.56	3.49	5.21	

* = mycelium transferred to next conc.
The number indicates the number of sub-culturing after which this growth was achieved.

3.2.1. Development of Tolerance in *Trichoderma harzianum* with Carbendazim

The growth of *T. harzianum* on PSA containing Carbendazim @ 0.01 and 0.1 ppm was significantly less than that in control. Growth was started after 48 hours at both concentrations. The growth of *T. harzianum* significantly increased when it was repeatedly transferred from 0.1 ppm to 0.01 and 0.1 ppm treatments. A similar trend of *T. harzianum* growth was observed when it was moved from 0.1 ppm to 1 and 10 ppm, from 1 ppm to 10, 100, and from 10 ppm to 100 and 1000 ppm treatments. Since inferior growth of *T. harzianum* was observed on medium containing Carbendazim @ 1000 ppm even after repeated culturing, the mycelium growing on 1000ppm plates was transferred to PSA containing Carbendazim @ 100, 200, 300, 400, 500, 1000 ppm. Very little growth was observed on media containing Carbendazim @ 200 ppm or more. However, repeated sub-culturing resulted in a gradual increase in growth at higher concentrations. Then, the mycelium from 1000 ppm was transferred to medium with Carbendazim @ 500, 1000, 2000, 3000, 4000, and 5000 ppm. In another experiment, mycelium from 5000ppm treatment was transferred to medium with 500, 1000, 3000, 5000, 7000, and 9000 ppm and then from 9000ppm to medium with 100, 1000, 3000, 5000, 7000, 9000, and 10,000 ppm. It was observed that after these experiments, *T. harzianum* developed tolerance to Carbendazim @ 10,000 ppm and the growth decreased thereafter with an increase in concentration (Table 2).

Table 2. Development of tolerance in *Trichoderma harzianum* against Carbendazim.

Concentrations (ppm)		Different intervals of Hours for proliferation (hrs)						No. of times to transfer the growth to next concentration.
		24	48	72	96	120	144	
0		1.28	4.26	7.16	9	9	9	
0.01		0	0	0.55	0.62	0.65	0.79	(2*)
0.1		0	0	0.5	0.59	0.61	0.65	
0	Mycelium	1.3	4.62	6.81	9	9	9	
0.01	proliferation	0	0.62	0.81	1.56	2.21	3.19	(2*)
0.1	in mm	0	0	0.59	0.62	0.69	0.75	
0		1.42	5.26	8.19	9	9	9	
0.01		0.69	0.97	1.61	3.542	4.99	6.21	(2*)
0.1		0	0.61	0.82	0.99	1.2	1.92	
0		1.38	5.16	7.57	9	9	9	
0.1		0.72	0.98	1.82	4.01	5.26	7.19	(2*)
1	Mycelium	0	0	0.69	0.78	0.92	1.2	
0	proliferation	1.46	4.21	8.19	9	9	9	
0.1	in mm	0.8	1.91	3.56	5.16	7	8.5	(1*)
1		0	0	0.7	0.92	1.02	1.52	

0		1.46	5.26	7.91	9	9	9	
0.1		0.82	2.19	3.86	5.82	7.71	9	(1*)
1		0.62	0.91	1.06	1.82	2.69	3.25	
0		1.6	5.12	8.19	9	9	9	
1		0	0	0.7	0.92	1.02	1.52	(3*)
10		0	0	0	0.61	0.72	0.88	
0	Mycelium	1.42	4.69	8.16	9	9	9	
1	proliferation	0.71	0.98	1.26	2.91	3.62	5.16	(3*)
10	in mm	0	0.59	0.62	0.86	1.2	2.56	
0		1.42	4.69	8.16	9	9	9	
1		0.81	1.21	2.16	3.59	5.12	8.01	(2*)
10		0	0.66	0.79	0.97	1.42	2.86	
0		1.51	4.56	8.19	9	9	9	
10		0	0.81	1.02	2.21	4.41	7.29	(4*)
100		0	0	0.6	0.81	0.99	1.42	
0	Mycelium	1.6	4.19	8.2	9	9	9	
10	proliferation	0.86	0.99	1.21	3.26	4.91	7.72	(4*)
100	in mm	0	0.59	0.86	1.02	2.19	3.51	
0		1.49	4.26	7.92	9	9	9	
10		0.9	1.06	1.67	4.26	5.91	8.26	(2*)
100		0	0.6	0.91	1.06	2.69	4.19	
0		1.56	5.16	8.19	9	9	9	
100		0.97	1.91	2.17	5.28	7.46	8.86	
200		0	0.67	0.82	0.98	1.25	1.46	
300		0	0.59	0.66	0.79	0.96	1.02	(5*)
400		0	0	0.56	0.72	0.86	0.95	
500	Mycelium	0	0	0	0.68	0.8	0.9	
1000	proliferation	0	0	0	0.66	0.75	0.8	
0	in mm	1.68	5.59	8.26	9	9	9	
100		1.06	2.18	3.69	5.82	8.19	9	
200		0.81	1.86	2.81	3.86	5.28	7.86	
300		0.69	1.18	1.82	2.19	4.17	6.18	(5*)
400		0.58	0.85	1.08	1.86	3.59	5.86	
500		0	0.75	0.95	1.23	2.16	4.56	
1000		0	0.69	0.82	0.97	1.25	1.38	
0		1.52	5.38	8.16	9	9	9	
500		0.86	1.2	1.89	2.5	3.06	5.46	
1000	Mycelium	0	0.76	0.96	1.26	1.96	2.57	
2000	proliferation	0	0.7	0.88	1.02	1.48	2	(3*)
3000	in mm	0	0.66	0.82	0.98	1.18	1.59	
4000		0	0.6	0.79	0.85	1.06	1.14	
5000		0	0.55	0.65	0.78	0.98	1.1	
0		1.46	5.28	7.49	9	9	9	
500		0.92	1.28	1.86	3.48	5.46	8.19	
1000	Mycelium	0.75	1.02	1.34	2.15	2.91	3.62	
2000	proliferation	0.69	0.95	1.12	1.86	2.46	3.25	(5*)
3000	in mm	0.58	0.9	1.12	1.86	2.46	3.25	
4000		0	0.73	0.89	1.18	1.79	2.5	
5000		0	0.69	0.82	1.06	1.52	2.18	
0		1.46	5.28	7.49	9	9	9	
500		0.92	1.28	1.86	3.48	5.46	3.62	
1000	Mycelium	0.75	1.02	1.34	2.15	2.91	3.62	
2000	proliferation	0.69	0.95	1.12	1.86	2.46	3.25	(5*)
3000	in mm	0.58	0.9	1.08	1.42	1.99	3.01	
4000		0	0.73	0.89	1.18	1.79	2.5	
5000		0	0.69	0.82	1.06	1.52	2.18	

0		1.56	4.86	7.82	9	9	9	
500		0.95	1.46	2.08	3.89	5.89	8.86	
1000	Mycelium	0.86	1.18	1.82	2.48	3.16	4.19	
3000	proliferation	0.68	1.01	1.67	2.18	3.06	3.86	(4*)
5000	in mm	0.6	0.82	1.08	1.8	2.42	3.19	
7000		0.55	0.76	0.98	1.17	1.99	2.56	
9000		0	0.68	0.82	1.06	1.76	2.19	
0		1.68	5.19	8.26	9	9	9	
1000		0.98	1.42	2.16	3.59	6.28	9	
3000	Mycelium	0.82	1.19	1.97	3	5.56	7.16	
5000	proliferation	0.8	1.06	1.5	2.59	4.26	6.11	(4*)
7000	in mm	0.69	0.96	1.4	2.16	3.2	5	
9000		0.59	0.86	1.19	1.82	2.56	4.36	
10000		0	0	0.92	1.06	2.19	3	

* = mycelium transferred to next conc.
The number indicates the number of sub-culturing after which this growth was achieved.

Several *Trichoderma* species exhibit the potential to control root-infecting plant pathogens, including *Macrophomina phaseolina* (Ramazan et al., 2014; Gajera et al., 2012), *Fusarium* spp. (Ramazan et al., 2014), and *Rhizoctonia solani* (Ramazan et al., 2014; Asad et al., 2014). Experimental studies have shown that *Trichoderma* spp. reduce infections when applied alongside *Toxocara canis* (a roundworm) eggs (Filho et al., 2017).

Researchers have successfully induced tolerance in biocontrol agents through UV irradiation of conidial suspensions (Papavizas et al., 1982), while others achieved similar results by gradually increasing fungicide concentrations (Abd-El Moity et al., 1982; Benyaqoub et al., 1995). *Trichoderma* species are among the most widely used biocontrol agents (Elad & Kapat, 1999; Harman, 2000; Spiegel & Chet, 1998; Sun & Liu, 2006). Mutant strains of *T. harzianum* and *T. atroviride* resistant to carbendazim and tebuconazole have also been developed using UV exposure (Hatvani et al., 2006).

A similar approach was employed in this study. Carbendazim inhibited all tested isolates at experimental concentrations, leading to complete growth suppression (Linta et al., 2024). Initially, *T. harzianum* exhibited limited growth on media supplemented with Topsin-M and carbendazim. However, gradual increases in fungicide concentrations induced tolerance in *T. harzianum*. Observations revealed that tolerance emerged after 34 consecutive exposures to Topsin-M at 10,000 ppm and after 57 exposures to carbendazim.

These findings validate earlier studies, demonstrating that enhanced carbendazim tolerance in mutant strains did not compromise the mycoparasitic or plant growth-promoting activities of *Trichoderma* (Ramangouda et al., 2023). The mutants retained the beneficial traits of their wild-type counterparts.

Conclusion

Based on observation, it was concluded that the tolerant strains of *T. harzianum* could potentially be applied in conjunction with reduced doses of pesticides, such as Topsin-M and carbendazim, to manage plant pathogens like *Macrophomina phaseolina,* and the tolerant strains of *T. harzianum* are able to control plant pathogens, especially *M. phaseolina*. These strains reduce the use of chemicals in the field or crops and prevent hazardous effects on the environment

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Conflict of Interest

It is declared that there is no conflict of interest among Authors

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