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RESEARCH ARTICLE

In vitro evaluation of (*Trichoderma harzianum*) for virulence efficacy on (*Ganoderma lucidum)*

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Abstract

Antimycotic activity of *Trichoderma harzianum* to inhibit the growth of *Ganoderma lucidum* was evaluated under laboratory conditions. In the dual cultures, 20.18% growth inhibition was recorded on the fifth day of incubation. The volatile compounds released by *T. harzianum* have supressed the growth of *G. lucidum* in the range of 21.79%- 56.15% *T. harzianum* released non-volatile metabolites into the liquid media, which inhibited the growth of *G. lucidum* upto 34.13±0.05 percent. The rate of growth inhibition increased with the increase in the age of antagonist and concentration of culture filtrate in media. *Trichoderma harzianum* contained phytochemicals such as alkaloids, phenolics, tannins, flavonoids, carbohydrates, reducing sugar and proteins, but terpenoids and amino acids were not detected.

Keywords: *Ganoderma lucidum, Trichoderma harzianum,* Biocontrol agent, Phytochemicals

Introduction

Ganoderma is a type of polypore macrofungus that grows on decaying logs or tree stumps (Kirk et al., 2011). The majority of *Ganoderma* species are pathogenic, causing root and stem rot in a wide range of monocots, dicots, and gymnosperms, resulting in tree mortality (Tattar, 1989). *G. lucidum* infects the root of the *Dalbergia sissoo* trees through damaged surfaces, causing bark degradation, and causes white fibrous rot in the stems. This fungus spreads from tree to tree through the root contact, with symptoms appearing as early as the third year of plantation (Bakshi et al., 1972). *G. lucidum* attacks both natural and planted sissoo forests, extending more rapidly on light-textured soil than on heavy soil; as a result, trees are killed instantaneously in such areas (Bakshi, 1974). When this fungus infects a tree, it damages the stems and affects the water and nutrition flow, eventually killing the tree (Bhattarai et al., 2020). *Ganoderma* spp. caused mortality of Acacia auriculiformis in Jahangirnagar University Campus, Bangladesh. The investigation on disease severity of the tree revealed that highest infection (52.2%) at site-1 of the study area. (Bhadra 2014) studied 11 hosts for their susceptibility towards *Ganoderma* spp. and observed that all of the tested wild Ganoderma spp., namely G. lucidum-1, G. lucidum-2, G. lucidum-3, and G. applanatum, preferred Mangifera indica saw dust, followed by Ceriops decandra, and the least preference was recorded for *Albizia procera* and *Dipterocarpus turbinatus* .

Trichoderma species are soil-borne fungi with strong antifungal activity and are well known biological control agents for variety of fungal phytopathogens. The antimycotic potential of *Trichoderma* spp. is also reported against *Ganoderma* boninense (Siddiquee et al., 2009), *Ganoderma lucidum* and *G. applanatum* (Srinivasulu et al., 2001; Palannaet al., 2017; Neeraja et al., 2018.). The secondary metabolites produced by Trichoderma spp. have been shown to be effective in suppressing the growth of harmful microbes while stimulating the plant growth (Kullnig et al., 2000; Kubicek et al., 2001; Contreras-Cornejo et al., 2015a, b). The growth inhibition of pathogen by antagonist in dual culture may be due to inhibitory chemicals secreted by one or both interacting organisms, competition, and hyperparasitism (Dennis and Webster, 1971a). *Trichoderma* species significantly suppress the growth of plant pathogenic microbes and regulate the rate of plant growth. Recent research has demonstrated that use of *Trichoderma* species can control the common plant disease like root rot, damping off, wilt, fruit rot etc. (Begum et al., 2010; El Komy et al., 2015;). Previous research on the phytochemicals of *Trichoderma harzianum* confirmed the presence of alkaloids, phenolics, tannins, flavonoids, carbohydrates, reducing sugar. The presence of these phytoconstituents suggests that it has the potential to be used as a precursor in the development new biofungicides (Omomowo et al., 2020). In this study, we followed dual culture techniques to assess the biocontrol efficacy of *Trichoderma harzianum* in the management of *Ganoderma lucidum*.

Methods

The pathogen (*Ganoderma lucidum*) and antagonist (*Trichoderma harzianum*), were procured from the Forest Protection Division, Himalayan Forest Research Institute, Shimla.

Growth inhibition of G. lucidum by *T. harzianum* in dual culture

On PDA Petri plates, 5 mm discs of actively growing antagonist and pathogen cultures were inoculated 4 cm apart. In control, only disc of pathogen was inoculated in a similar manner. On the fifth day of incubation at 25°C ± 1°C, the radial growth of the pathogen was measured and compared to the pathogen's growth in control plate (Dennis and Webster, 1971a). Percentage of inhibition was determined by following formula

$$\text{Percent growth inhibition} = \frac{\text{Colony diameter in control} - \text{Colony diameter in treatment}}{\text{Colony diameter in control}} \times 100$$

Growth inhibition by volatile compounds

5 mm discs of antagonist and pathogen were inoculated in different Petri plates. The lids of Petri plates were removed under laminar and two plates were sealed with adhesive tape, keeping the antagonist in lower and pathogen in the upper Petri plate and incubated at 25 °C ± 1°C. In control, Petri plates containing pathogen was inverted over the Petri plates containing the medium only (Dennis and Webster, 1971b). The colony diameter of pathogen was measured on fifth day of incubation and compared with control.

Growth inhibition by non-volatile compounds

The effect of non-volatile compounds released by the antagonist on the growth of pathogen was evaluated by using poisoned food technique (Nene and Thapliyal, 1993). Antagonist was cultivated in Potato Dextrose Broth (PDB) up to 4 weeks. At one week intervals, the culture of antagonist was filtered through Whatman -1 filter paper. Just before pouring, the filtrate was passed through 0.22-micron filter, and utilized to amend PDA @10%, 20%, 30%. The pathogen was inoculated in the Petri plates amended with culture filtrate and incubated at 25 °C ± 1°C. In control, the pathogen was inoculated on the same medium without culture filtrate. The colony diameter of pathogen was measured every day at same time and compared with control.

Detection of important phytochemical in *T. harzianum*

T. harzianum was cultivated in PBD for 14 days at 25 °C ± 1°C under shaking conditions. The content was filtered through Whatman No. 1 filter paper and mycelial biomass was collected and dried at 40°C in oven until it reaches constant weight.

The mycelial mat was crushed and homogenised with ethanol and distilled water and filtered. The filtrate was utilized for phytochemical test.

Test for Alkaloids: Few mL of filtrate was taken in test tube and 1-2 mL of Dragendroff's reagent was added (Dragendroff's reagent test). The presence of alkaloids was confirmed by the appearance of yellow-coloured precipitates.

Test for Phenolics and Tannins: The extract was dissolved in distilled water with a few drops of a 5% ferric chloride solution (Ferric chloride test). The presence of phenolics and tannins was indicated by a dark green colour.

Test for Flavonoids: A fraction of the extract was treated with 5 mL of dilute ammonia solution, followed by a few drops of strong sulphuric acid. The existence of flavonoids was confirmed by the appearance of a yellow tint.

Test for reducing sugars: After heating 1 mL of filtrate in water, 1 mL of Fehling solution was added (Fehling's solution test). The emergence of red coloured precipitate indicated the presence of reducing sugars.

Test for Terpenoids: 5 mL of extract was mixed with 2 mL of chloroform and 3 mL of sulphuric acid was added from the sides of the test tube (Salkowski test). Formation of reddish-brown coloration at interface indicated the presence of terpenoids.

Test for amino acids: 1-2 drops of phenolphthalein were added to the extract, followed by few drops of dilute sodium hydroxide solution. The pink colour confirmed the presence of amino acids.

Test for protein: A drop of 2 percent copper sulphate solution was added to 2 mL of filtrate, followed by addition of 1 mL ethanol (95%) and an excess of potassium hydroxide pellets (Biuret test). The presence of proteins was confirmed by a pink tint in the ethanolic layer.

Results and Discussion

Growth inhibition in dual culture: *T. harzianum* restricted the growth of G. *lucidum* and forming a zone of inhibition at the point of contact between antagonist and pathogen. On the fifth day of incubation, there was a growth inhibition of 20.86 ± 1.15 (Table-1). The radial growth inhibition of pathogen in dual cultures could be due to antagonist-released antimicrobial metabolites, competition, mechanical obstruction or *hyperparasitism* (Dennis and Webster, 1971a). (Srinivasulu et al. 2001) recorded suppression in the mycelial development of *G. applanatum* and *G. lucidum* by *T. harzianum* in dual cultures. (Siddiquee et al. 2009) screened 48 *T. harzianum* isolates for their antagonistic activity against *G. boninense* in dual cultures and recorded growth inhibition in the range of 47.86×72.06 percent. (Sangeeta et al. 2009) identified 12 Trichoderma species from banana plantations and found that they inhibited two pathogens that cause banana crown rot. (Muniroha et al.2019) evaluated *Trichoderma* species and Pseudomonas to see whether they could inhibit G. boninense-induced basal stem rot in oil palm. P. aeruginosa and *T. asperellum* were able to inhibit *G. boninense* by 71.42 percent and 76.85 percent respectively, in dual culture test. Although in our study, the growth inhibition of G. *lucidum* by *T. harzianum* was modest in dual culture test but growth inhibition by the volatile and non-volatile compounds was higher.

Growth inhibition by volatile compounds: The volatile compounds released by *T. harzianum* inhibited *G. lucidum* growth by 56.15% (Tab. 1). The antagonist growing in the bottom culture plate may have release gaseous secondary compounds that supressed the growth of *G. lucidum*. (*Dennis and Webster 1971b*) investigated the effect of *T. harzianum* volatile metabolites on the growth of *Rhizoctonia solani* and other fungi. *Trichoderma* species produces a variety of volatile secondary metabolites, including ethylene, hydrogen cyanide, aldehydes, and ketones, which play a significant role in growth inhibition of pathogens and plant disease management (Vey et al., 2001; Bhagat et al., 2014). (Gveroska and Ziberoski 2011) recorded restriction in the development of Alternaria alternata by the volatile chemicals released by Trichoderma species. (Nagamani et al.2017) observed that *Trichoderma harzianum* isolate (ATPP 6) was most effective in inhibiting mycelial growth of *R. bataticola, F. oxysporum ciceri,* and *S. rolfsii*. The major benefit of volatile chemicals in disease management is

that the toxic molecules released by the antagonists can spread via air-filled pores in soil and restrict the pathogen without actual physical contact. (Singh and Singh 2019) identified six Trichoderma isolates and evaluated their efficacy on the basis of non-volatile and volatile compounds for the management of pigeon pea wilt pathogen. They found that *Trichoderma harzianum* inhibited the most growth of *Fusarium udum*, followed by *Gliocladium virens*.

Table 1. Growth inhibition of *G. lucidum* by *T. harzianum* in dual culture and by volatile compounds

S. No.	Treatment	Dual Culture	Volatile Compounds
1	Pathogen growth in control (mm)	32.23 ± 6.50	20.36 ± 1.05
2	Pathogen growth in treatment (mm)	25.73 ± 3.15	8.93 ± 0.55
3	Growth Inhibition (%)	20.86 ± 1.15	56.15 ± 4.31

Growth inhibition by non-volatile compounds: The growth of *G. lucidum* was significantly reduced by *T. harzianum* culture filtrate, and the rate of growth inhibition increased as the concentration of culture filtrate in the media increased (Table-2). After one week of incubation, the plate amended with culture filtrate showed growth inhibition in the range of 4.73 ± 0.15 × 16.63 ± 0.05 at 10% concentration, 14.23 ± 0.28 × 22.13 ± 0.85 at 20% concentration, and 19.36 ± 0.11 × 34.13 ± 0.05 at 30% concentration of culture filtrate in media. After two weeks of incubation, the culture filtrate exhibited growth inhibition in the range of 3.23 ± 0.15 × 3.26 ± 0.20 at 10% concentration, 8.13 ± 0.05× 14.23 ± 0.15 at 20% concentration, and 18.06 ± 0.23 × 24.16 ± 0.20 at 30% concentration of culture filtrate in media. Similarly, the growth inhibition of culture filtrate obtained after three weeks of incubation was varied between 11.56 ± 0.05 × 8.83 ± 0.05 at 10% concentration, 6.63 ± 0.15 × 13.36 ± 0.11 at 20% concentration, and 16.56 ± 0.05 × 23.26 ± 0.15 at 30% concentration of culture filtrate. Similarly, the culture filtrate obtained after four weeks of incubation, recorded growth inhibition of *G. lucidum* in the range of 16.33 ± 0.15×3.26 ± 0.20 at 10%, 16.26 ± 0.25×29.03 ± 0.05 at 20% and 18.06 ± 0.15×31.36 ± 0.32 at 30% concentration of culture filtrate. The results revealed that the non-volatile compounds of *T. harzianum* have invariably restricted the mycelial growth of *G. lucidum*. Maximum growth inhibition recorded by the culture filtrate of four week old cultures. In similar study, (Nagamani et al. 2017) isolated 20 *Trichoderma* isolates from chickpea rhizosphere soil and tested their antimycotic efficacy against soil-borne plant diseases caused by *R. bataticola*, *F. oxysporumciceri*, and *S. rolfsii*. The results revealed that *Trichoderma harzianum* (KNO 9) exhibited 91.1 percent growth inhibition and *T. harzianum* (ATPP 6) with 93.8 percent growth inhibition were identified as most efficient antagonist in releasing of volatile and non-volatile metabolites. The antagonistic ability of non-volatile metabolites released by *Trichoderma viride*, *Trichoderma harzianum*, and *Trichoderma koningii*, against three isolates of *Fusarium oxysporum* f. sp. ciceri (FOC), was evaluated by (*Kumar et al. 2019*). *Trichoderma harzianum* inhibited the FOC strain of *Fusarium oxysporum* f. sp. ciceri the most (76.90 %), followed by *Trichoderma viride* (70.10 %). In vitro experiments revealed that volatile chemicals released by *Trichoderma harzianum* had significant inhibitory effect on the mycelial growth of all isolates of test pathogen, with the highest inhibitory effect against FOC2 isolate (79.25 %), followed by *Trichoderma viride* (64.16 %) against FOC1 isolate (Tab 2).

Table 2. Growth inhibition of G. *lucidum* by T. *harzianum* in dual culture and by volatile compounds

Age of *T. harzianum*	Concentration of culture filtrate in PDA (%)	Growth inhibition of *Ganoderma lucidum* (%)				
		Day 1	Day 2	Day 3	Day 4	Day 5
	0	– (63.1)*	– -69.12	– -75.13	– -83.1	– -90.09
One Week		4.73 ± 0.15	8.63 ± 0.11	12.1 ±	14.43 ±	16.63 ±

	10	(60.20 ± 0.26)	(63.16 ± 0.25)	0.25 (66.33 ± 0.11)	0.05 (71.03 ± 0.15)	0.05 (75.1 ± 0.36)
	20	14.23 ± 0.28 (54.23 ± 0.30)	15.93 ± 0.15 (58.2 ± 0.26)	17.33 ± 0.32 (62.03 ± 0.15)	14.43 ± 0.05 19.13 ± .20 (67.13 ± 0.30)	22.13 ± 0.85 (70.03 ± 0.20)
	30	19.36 ± 0.11 (50.06 ± 0.15)	30.83 ± 0.05 (56.1 ± 0.3)	32.26 ± 0.11 (61.16 ± 0.25)	33.33 ± 0.20 (70.33 ± 0.05)	34.13 ± 0.05 (77.23 ± 0.15)
	0	– -61.01	– -69.1	– -75.13	– -84.08	– -91.13
Two Week		3.23 ± 0.15	4.33 ± 0.05	4.06 ± 0.25	3.46 ± 0.05	3.26 ± 0.20
	10	(59.16 ± 0.05)	(66.13 ± 0.05)	(72.1 ± 0.26)	(81.16 ± 0.25)	(88.26 ± 0.11)
	20	8.13 ± 0.05 (56.06 ± 0.15)	13.06 ± 0.15 (60.06 ± 0.23)	12.03 ± 0.05 (66.03 ± 0.80)	16.63 ± 0.15 (70.2 ± 0.26)	14.23 ± 0.15 (78.2 ± 0.1)
	30	18.06 ± 0.23 (50.06 ± 0.15)	20.26 ± 0.05 (55.03 ± 0.15)	21.26 ± 0.05 (59.06 ± 0.49)	23.83 ± 0.15 (64.03 ± 0.11)	24.16 ± 0.20 (69.13 ±)
	0	-60.02	-68.08	-72.11	-80.12	-90.11
Three Week						
	10	11.56 ± 0.05 (53.03 ± 0.05)	10.23 ± 0.25 (61.06 ± 0.32)	4.2 ± 0.1 (69.03 ± 0.15)	5.06 ± 0.11 (76.03 ± 0.25)	8.83 ± 0.05 (82.06 ± 0.11)
	20	6.63 ± 0.15 (52.06 ± 0.15)	11.73 ± 0.20 (59.06 ± 0.15)	8.36 ± 0.30 (63.06 ± 0.15)	12.56 ± 0.28 (69.03 ± 0.05)	13.36 ± 0.11 (76.06 ± 0.15)
	30	16.56 ± 0.05 (50.33 ± 0.57)	19.16 ± 0.20 (53.3 ± 0.26)	18.06 ± 0.11 (57.03 ± 0.37)	20.06 ± 0.11 (62.16 ± 0.20)	23.26 ± 0.15 (69.03 ± 0.05)
Four Week	0	-61.02	-67.21	-71.01	-78.02	-86.11
		16.33 ± 0.15	22.33 ± 0.05	26.26 ± 0.15	28.23 ± 0.15	29.03 ± 0.05

10	(51.03 ± 0.05)	(52.06 ± 0.11)	(54.06 ± 0.15)	(56.03 ± 0.11)	(61.03 ± 0.15)
20	16.26 ± 0.25 (51.06 ± 0.15)	20.83 ± 0.11 (53.06 ± 0.11)	22.53 ± 0.15 (55.06 ± 0.20)	24.53 ± 0.20 (59.03 ± 0.20)	25.56 ± 0.15 (64.03 ± 0.20)
30	18.06 ± 0.15 (50.33 ± 0.66)	23.85 ± 0.07 (51.06 ± 0.15)	25.26 ± 0.47 (53.03 ± 0.11)	29.36 ± 0.05 (55.06 ± 0.15)	31.36 ± 0.32 (59.03 ± 0.28)

*Value given in the parenthesis indicates growth of *G. lucidum* in mm

Screening of Trichoderma harzianum for the presence of important phytochemicals

Preliminary phytochemical analysis revealed that Alkaloids, Phenolics, Tannins, Flavonoids, carbohydrates, reducing sugar and proteins were present while, terpenoids and amino acids were not recorded in *T. harzianum* (Fig.1a). (Tapwal et al. 2015) also detected the presence of alkaloids, phenolics and tannins, flavonoids, carbohydrates and glycosides, terpenoids, amino acids and proteins in the fungal endophytes (Fig. 1b). The presence of such phytoconstituents is an indication for its potential as a precursor for developing potential biofungicides (Omomowo et al., 2020), (Fig.1c).

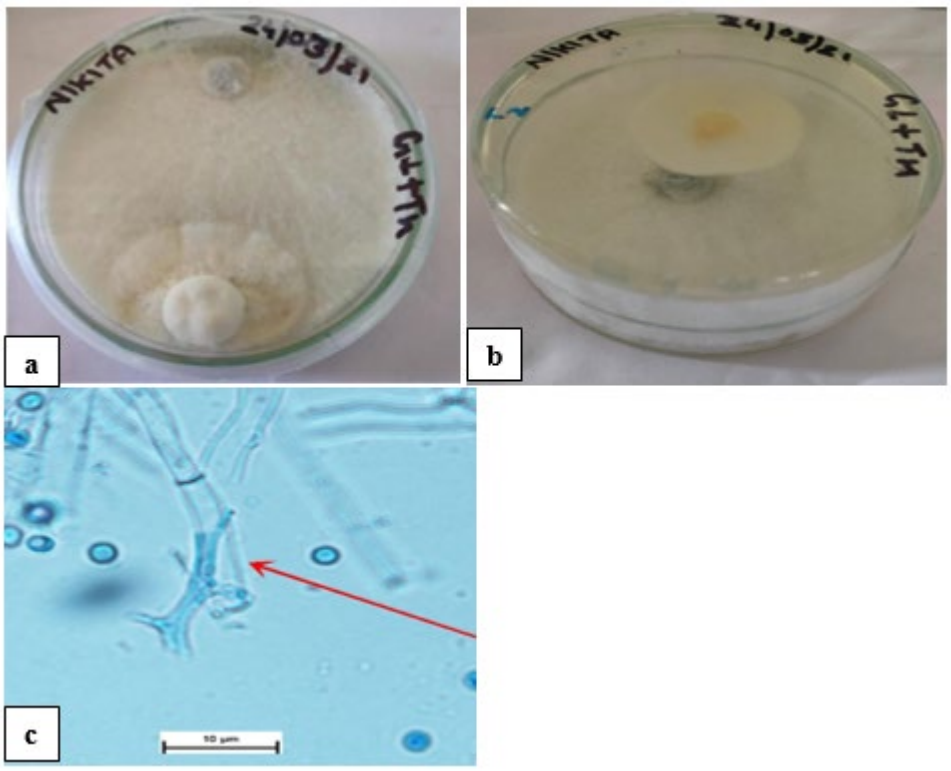

Figure 1. a. Trichoderma harzianum +Ganoderma lucidum in dual culture; b. Trichoderma harzianum +Ganoderma lucidum in volatile compounds experiments; c. Hyphal interaction between Trichoderma harzianum and Ganoderma lucidum

Conclusion

In the dual cultures, 20.18% growth inhibition was recorded on the fifth day of incubation. *Harzianum* have supressed the growth of G. The majorities of *Ganoderma* species are pathogenic, causing root and stem rot in a wide range of monocots, dicots, and gymnosperms, resulting in tree mortality. The investigation on disease severity of the tree revealed that highest infection (52.2%) at site-1 of the study area. The secondary metabolites produced by Trichoderma spp. Trichoderma species significantly suppress the growth of plant pathogenic microbes and regulate the rate of plant growth. Growth inhibition of G. On the fifth day of incubation at 25°C ± 1°C, the radial growth of the pathogen was measured and compared to the pathogen's growth in control plate. Percentage of inhibition was determined by following formula. The lids of Petri plates were removed under laminar and two plates were sealed with adhesive tape, keeping the antagonist in lower and pathogen in the upper Petri plate and incubated at 25 °C ± 1°C. The colony diameter of pathogen was measured on fifth day of incubation and compared with control. The effect of non-volatile compounds released by the antagonist on the growth of pathogen was evaluated by using poisoned food technique the pathogen was inoculated in the Petri plates amended with culture filtrate and incubated at 25°C ± 1°C. Detection of important phytochemical in *T. harzianum* was cultivated in PBD for 14 days at 25°C ± 1°C under shaking conditions. Test for Alkaloids: Few mL of filtrate was taken in test tube and 1 mL-2 mL of Dragendroff's reagent was added (Dragendroff's reagent test). The presence of alkaloids was confirmed by the appearance of yellow-coloured precipitates. Test for Phenolics and Tannins: The extract was dissolved in distilled water with a few drops of a 5% ferric chloride solution (Ferric chloride test). The presence of phenolics and tannins was indicated by a dark green colour. Test for Flavonoids: A fraction of the extract was treated with 5 mL of dilute ammonia solution, followed by a few drops of strong sulphuric acid. The existence of flavonoids was confirmed by the appearance of a yellow tint. Test for reducing sugars: After heating 1 mL of filtrate in water, 1 mL of Fehling solution was added (Fehling's solution test). The emergence of red coloured precipitate indicated the presence of reducing sugars. Test for Terpenoids: 5 mL of extract was mixed with 2 mL of chloroform and 3 mL of sulphuric acid was added from the sides of the test tube (Salkowski test). Formation of reddish-brown coloration at interface indicated the presence of terpenoids. Test for amino acids: 1 ml-2 ml drops of phenolphthalein were added to the extract, followed by few drops of dilute sodium hydroxide solution. The pink colour confirmed the presence of amino acids. Test for protein: A drop of 2 percent copper sulphate solution was added to 2 mL of filtrate, followed by addition of 1 mL ethanol (95%) and an excess of potassium hydroxide pellets (Biuret test). The presence of proteins was confirmed by a pink tint in the ethanolic layer. harzianum restricted the growth of G. On the fifth day of incubation, there was a growth inhibition of 20.86 ± 1.15. The antagonist growing in the bottom culture plate may have release gaseous secondary compounds that supressed the growth of G. investigated the effect of T. Growth inhibition of G. Growth inhibition by non-volatile compounds: The growth of G. The results revealed that the non-volatile compounds of T. Growth inhibition of G. Age of T. Value given in the parenthesis indicates growth of G. Preliminary phytochemical analysis revealed that Alkaloids, Phenolics, Tannins, Flavonoids, carbohydrates, reducing sugar and proteins were present while, terpenoids and amino acids were notre cordedin *T. harzianum*.

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