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Comprehensive *In Vitro* Evaluation of the Biocontrol Potential of *Trichoderma virens* Against Selected Phytopathogenic Fungi: Integration of Interaction Dynamics and Best-Fit Mathematical Modeling

Munasinghe J[1*], Poornima VA[2], Dissanayake T[3], Kannangara S[4*], Undugoda L[5] and Thambugala KM[6,7,8]

[1]*Department of Mathematics, Faculty of Science, University of Kelaniya, Kelaniya, Sri Lanka*
[2]*Department of Mathematics and Statistics, College of Art and Sciences, University of Texas Tech, Texas*
[3]*Department of Biology, College of Arts and Sciences, Eastern Michigan University, Michigan*
[4]*Department of Plant and Molecular Biology, University of Kelaniya, Kelaniya, Sri Lanka*
[5]*Department of Biosystems Technology, Faculty of Technology, University of Sri Jayewardenepura, Nugegoda, Sri Lanka*
[6]*Genetics and Molecular Biology Unit, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda 10250, Sri Lanka*
[7]*Center for Plant Materials and Herbal Product Research, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda, 10250, Sri Lanka*
[8]*Center for Biotechnology, Department of Zoology, University of Sri Jayewardenepura, Nugegoda, 10250, Sri Lanka.*

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Abstract

In the context of plant disease management, biocontrol is often used as an environmentally friendly alternative to synthetic chemical pesticides. The genus *Trichoderma* is known for its ability to suppress various plant diseases caused by pathogenic fungi through antagonism, competition, and induced resistance. This research attempt was to evaluate the mechanisms employed by *Trichoderma virens* isolate (KP985643.1) in controlling three plant pathogenic fungi (*Fusarium oxysporum*, *Colletotrichum gloeosporioides*, and *Lasiodiplodia theobromae*) under *in vitro* conditions, along with the selection of the best-fitting mathematical models for their interactions. The biocontrol potential of *T. virens* against the selected plant pathogenic fungi was evaluated using the dual culture method. Further biocontrol mechanism was identified using the analysis of volatile and non-volatile compounds and the hyphal growth patterns. In addition to that the results of the dual culture method were used to analyse the best-fitting growth models for each pathogenic fungus separately for two conditions which were in the absence of *T. virens* and in the presence of *T. virens*. These mathematical models of microbial growth serve as powerful tools for predicting the future performance of fungal biocontrol agents in managing plant diseases under diverse environmental conditions. Such predictive capabilities are crucial for optimizing field applications, improving consistency in biocontrol outcomes, and supporting the development of sustainable, environmentally friendly plant disease management strategies in agricultural systems. Results of the dual culture experiment demonstrated a significant growth control of *L. theobromae* and *F. oxysporum* and a moderate growth control of *C. gloeosporioides* by *T. virens*. Considering the growth models, the best fitted models were given

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Corresponding Authors: Munasinghe J & Kannangara S – e-mails – munasing@kln.ac.lk & sagarikadpk@kln.ac.lk

by Gompertz, Exponential, and Exponential, respectively, for *C. gloeosporioides*, *F. oxysporum*, and *L. theobromae* in the absent condition of *T. virens*. In the present condition of *T. virens*, the best-fitted models were given by Brody, Exponential, and Brody respectively for *L. theobromae*, *C. gloeosporioides*, and *F. oxysporum*. The increase in volatile production was not uniform across all pathogens. For instance, the highest VOC production showed against *F. oxysporum*, suggesting that *T. virens* may be more efficient at preventing *F. oxysporum* from producing volatile compounds. Among many VOCs, ethanol and 6-Pentyl-2H-6-Pentyl-2H-pyran-2-one production were the highest when *T. virens* controlled the growth of *F. oxysporum*. However, non-volatile compounds produced by *T. virens* significantly controlled the growth of *L. theobromae*, with a moderate inhibition of *F. oxysporum*. It was further observed the ability of *T. virens* to inhibit the growth of *L. theobromae* through the production of coiling structures. Results of the present study clearly indicated the potential of *T. virens* in controlling the tested phytopathogenic fungi by employing a combination of mechanisms including the production of volatile and non-volatile compounds and hyphal interactions.

Keywords – Biocontrol – Coefficient of determination – Dual culture – Mathematical growth models –Non-volatile – *Trichoderma virens* – Volatile

Introduction

Plant diseases caused by diverse groups of phytopathogenic fungi are considered as one of the most prominent threats to crop plants (Almeida et al. 2019) and have the potential to pose a significant threat to the world's food supply and security (Gomes et al. 2021). The impacts of fungal plant diseases include a decrease in plant quality or plant products and an increase in production cost because of the additional cost of disease monitoring and fungicide application (Palm 2001). Therefore, the successful control of fungal plant diseases is essential for ensuring continuous food production and ultimately for providing security and market stability (Thambugala et al. 2020).

The widespread use of chemical disease control methods stems from their capacity to deliver rapid results with minimal effort. However, the environmental damage caused by the persistence of these chemicals is often irreversible and generates several health issues (Palou et al. 2008). The extensive use of chemical fungicides has further led to the increase of fungicide-resistant fungal variants. As a result, much research has been conducted in recent years to discover more eco-friendly and health-conscious alternatives to chemical pesticides. Among these alternatives, the use of biofungicides is a more reliable alternative to conventional control methods because of their practicality, efficiency, inherently low cost, persistence in the field, and considerably less impact on human health and the environment (Thambugala et al. 2020, Gomes et al. 2021).

Biological control refers to the use of natural or genetically modified organisms, including microbes and their genes, to manage plant pathogens, through a complex network of interactions among the microbial biocontrol agents, the target pathogen, the local microbial community, and the host plant (Gomes et al. 2021, Singh et al. 2020). Among different fungal species, most *Trichoderma* species have been introduced as successful biocontrol agents of plant pathogens (Thambugala et al. 2020). They are commonly occurring soil fungi and can be easily isolated from a variety of substrates, including soil, decaying wood, leaf litter, and coir. As a biocontrol agent, *Trichoderma* species are highly effective against several common plant pathogenic fungi, including *Botrytis* spp., *Pythium* spp., *Sclerotinia sclerotiorum*, *Fusarium* spp., and *Rhizoctonia solani*. Out of many phytopathogens *F. oxysporum* is a soil-borne pathogen responsible for vascular wilt diseases in a wide range of economically important crops, often causing severe yield losses. *Lasiodiplodia theobromae* is another common postharvest and field pathogen associated with dieback, stem-end rot, and fruit rots in many perennial and horticultural crops, leading to substantial postharvest wastage. *Colletotrichum gloeosporioides* causes anthracnose in fruits, vegetables, and ornamental plants, reducing both market quality and yield. However, the ability to control these critical phytopathogens is associated with several *Trichoderma* spp. Furthermore, the most used *Trichoderma* species for the control of plant phytopathogens include *T. koningii*, *T. harzianum*, *T. viride*, and *T. virens* by employing a wide range of control mechanisms such as antibiosis, parasitism, inducing host-plant resistance, and

competition (Howell 2003, Sandle 2014, Thambugala et al. 2020). Among these, *T. virens* is one of the most common and widely reported soil fungi, capable of producing a range of lytic enzymes and antibiotics that effectively control numerous plant pathogens (Ao & Vi 2018). They are rapidly growing, conidia-forming fungi that produce bluish-green colonies (Howell 2003). Nelson et al. (1994) indicated the potential in *T. virens* to control many fungal pathogens, including *Rhizoctonia solani* and *Pythium* spp., by employing several biocontrolling mechanisms. Although numerous studies have reported the biocontrol potential of various *Trichoderma* species, translating these findings into consistent agricultural field applications remains challenging. Therefore, developing mathematical models to describe the growth dynamics of these biocontrol agents in suppressing pathogens is essential for predicting and optimizing their performance in agroecosystems under diverse environmental conditions.

Mathematical modeling is one of the important tools in the discipline of mathematics which is used to solve real-life situations or to convert problems into mathematical explanations, especially for understanding future conditions as well as to analyse the things which cannot be solved using analytical techniques. According to the literature, even in the field of biological sciences, many linear and nonlinear growth models have been used to describe the growth of various fungi and their interactions (Behzadi et al. 2014, Pla et al. 2015). The application of respective growth models clearly explains how biocontrol agents control the growth of respective plant pathogens. National reports estimate that fungal diseases can cause yield reductions of 20–40% annually, with certain localized outbreaks leading to complete crop failure in smallholder farms (Department of Agriculture Sri Lanka 2022). Even though Sri Lankan farmers have been facing this issue, there are no mathematical models developed to predict pathogen inhibition potential using biocontrol agents under different environmental conditions.

Based on this background information, *T. virens* was selected to investigate the biocontrol potential against three common plant diseases causing pathogenic fungi prevalent in agricultural fields, namely, *Fusarium oxysporum*, *Lasiodiplodia theobromae*, and *Colletotrichum gloeosporioides.* At the same time, this study aims to identify the best-fitting mathematical model that corresponds to the biocontrol potential of *T. virens* against the selected pathogenic fungi. Although several models can be used to optimize the biocontrol patterns of *T. virens* against selected pathogens, this study selected five nonlinear growth models, Exponential, Logistic, Brody, Von Bertalanffy, and Gompertz based on key numerical parameters, including the coefficient of determination (R^2), root mean square error (RMSE), sum of squares due to error (SSE), and residual plots, to effectively describe the growth inhibition of the aforementioned plant pathogens by *T. virens* as a potent biocontrol agent.

Materials & Methods

Obtaining *T. virens* and test pathogen cultures

Trichoderma virens (KP985643.1) and the test pathogenic fungi, *F. oxysporum* (isolated from banana), *L. theobromae* (isolated from mango cv. Karutha Colomban), and *C. gloeosporioides* (isolated from banana), were obtained from the Department of Plant and Molecular Biology, University of Kelaniya, Sri Lanka.

Dual culture method

Trichoderma virens was tested for antagonistic properties against the above-mentioned fungal pathogens using the dual culture method. A 5 mm diameter disc from a seven-day-old pure culture of *T. virens* and the corresponding pathogen were placed at the opposite sides of a newly prepared potato dextrose agar (PDA) plate, maintaining an equal distance from the periphery. Control plates contained sterile agar discs in place of *T. virens*. The plates were incubated at 25±1°C for 6 days. The experiment was followed using a completely randomized design with six replicates. The area of the fungal colony of the pathogens was measured at frequent intervals of 2, 4, and 6 days after the

inoculation (Hajieghrari et al. 2008). The percentage inhibition area of the fungal colony was calculated using the following equation.

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

where L is the percentage inhibition area of the fungal colony; C is area of the fungal pathogen in control; T is area of the fungal pathogen in the presence of *T. virens* (Bardin et al. 2015).

Model development

The area of the fungal colony results taken from the dual culture method was used to develop the mathematical model. Here, the data set, the area of the fungal colony and the time were identified as the two variables. The colony area of the fungus was considered as the dependent variable while time was considered as the independent variable. As the starting point, statistical analysis was carried out to identify the distribution of the data, characteristics of the data, and detect the outliers. Outliers, which are the data values that lie at an abnormal distance from other data values were identified using box plots. The one-way ANOVA test was then performed to check the interaction between the dependent variable (colony area of the fungus) and independent variable (time) using Minitab statistical software. Statistically significant level was considered at the level of $p < 0.05$. Then the Tukey's test was done to identify the differences between the group means.

Growth curves of fungi were analyzed using four growth models which are Exponential, Logistic, Brody, and Gompertz.

1. Exponential growth model: $y(t) = ae^{bt}$ where $y(t)$ is the area of the fungal colony at time t , b is the growth rate and a is the initial colony area.

2. Logistic growth model: $y(t) = \frac{a}{1+be^{-ct}}$, where $y(t)$ is the area of the fungal colony at time t, a is the carrying capacity, c is the growth rate, a_0 is the initial colony area and $b = (\frac{a}{a_0} - 1)$.

3. Brody growth model: $y(t) = a(1 - be^{-ct})$, where $y(t)$ is the area of the fungal colony, a is the upper asymptote, a_0 -initial colony area, $b = 1 - \frac{a_0}{a}$, c- growth rate, t – time.

4. Gompertz growth model: $y(t) = ae^{-be^{-ct}}$, where $y(t)$ is the area of fungal colony at time t, a is the asymptote, a_0 is the initial colony area, $b = \log(\frac{a}{a_0})$ and c is the growth rate.

Furthermore, the accuracy of the growth models was compared using the coefficient of determination value (R^2) and the residual plots. If the residual plot shows a high density of points near the origin and is symmetric about the origin, then the model provides the best fit. Then, using R^2 and residual plots, the most suitable mathematical model for each pathogenic fungus was identified. Furthermore, the parameter values for each model were calculated. Finally, the growth inhibition was analyzed by plotting graphs related to the first condition and the second condition in one figure using the formulated growth models.

Effect of volatile components secreted by *T. virens*

Trichoderma virens and the pathogen were cultured on Czapek-Dox Agar and incubated under optimal conditions (25±1°C) for six days to ensure sufficient VOC production. For VOC accumulation, *T. virens* and the pathogen were subsequently co-cultivated in a sealed system. 20 mL glass vials that were hermetically sealed were used for headspace collection. Using 50/30 µm DVB fibers, VOCs were extracted using Solid Phase Microextraction (SPME) after being exposed to the headspace for 60 minutes at 40°C. An Agilent 7890B gas chromatograph and an Agilent 5977A mass spectrometer were used for the Gas Chromatography-Mass Spectrometry (GC-MS) analysis. For the study, an HP-5MS column (30 m × 0.25 mm × 0.25 µm) was used.

Effect of non-volatile components produced by *T. virens*

In 250 mL conical flasks, 100 mL of sterilized potato dextrose broth (PDB) was inoculated with three mycelial plugs (5 mm) from a 7-day-old *T. virens* pure culture. For 14 days, the flasks were incubated at 25±1°C at 100 rpm on a rotary shaker. After removing mycelial mats from the broth cultures using Millipore filters, microbiological filters (0.2 µm pore size) were used to sterilize them. Melted PDA (at 40±3°C) was combined with the filtrate, which contained non-volatile compounds secreted by *T. virens*, to reach a final concentration of 10% (v/v). After filling sterile petri dishes with the prepared medium, each pathogen was individually inoculated with 5 mm mycelial plugs. The dishes were then incubated for seven days at 25±1°C. Sterile control plates were included. Control plates consisted of sterile agar discs instead of *T. virens*. There were four replicates from each treatment, and the experiment was completely randomized design (Hajieghrari et al. 2008). The colony diameter of the pathogen was recorded at 2-day intervals for six days and the percentage inhibition was calculated (Bardin et al. 2015).

Microscopic observations for the mycoparasitism of *T. virens* against the selected plant pathogenic fungi

The hyphal interactions between *T. virens* and the selected fungal pathogens were observed using the slide bi-culture method. An Agar disk with a diameter of 6 mm containing the pathogenic fungus was placed at the center of a glass slide coated with a thin film of sterilized PDA. A similar sized agar disk containing *T. virens* was placed 3 cm away from the pathogen. A moist sterile filter paper was placed inside a sterilized petri dish. The slide was then placed in the sterilized petri dish with the moist filter paper in an elevated manner. The petri dish was then covered with the respective lid creating a moist chamber. The slides were observed after seven days using a compound light microscope (Santos et al. 2018).

Statistical analysis

Data obtained from each experiment were subjected to two-way analysis of variance (ANOVA) and Tukey's pairwise comparison test using MINITAB 17 statistical software package.

Results and Discussion

Inhibition efficiency of *T. virens*

The results in Figure 1 depict that *T. virens* was capable of a rapid colonization in the medium, significantly inhibiting the growth of all three tested pathogenic fungi (*F. oxysporum, L. theobromae* and *C. gloeosporioides*). *Trichoderma virens* exhibited an inhibition efficiency of more than 50% against *F. oxysporum* and *L. theobromae* on the second day of their growth. However, on the 4th day, the inhibition efficiency against *F. oxysporum*, dropped considerably, while no significant change was observed for *L. theobromae* compared to the 2nd day. However, on the 6th day, again, there is a significant increase in the inhibition efficiency exceeding 60% against both *F. oxysporum,* and *L. theobromae*. Similarly, the findings of Girma (2022) revealed that *T. virens* exhibited potential antagonistic potential against *F. oxysporum,* which was isolated from hot pepper plants with *Fusarium* wilt disease. However, among the tested pathogens, the biocontrol effect against *C. gloeosporioides* was the least effective. Nevertheless, all the pathogenic fungi reported percentage inhibition levels greater than 40% after a 6-day incubation period *in vitro*, assuming that *T. virens* was capable of controlling the growth of mycelial in the selected plant pathogenic fungi successfully (Fig. 1).

Best-fitting interaction-based mathematical models

The statistical analysis results revealed that the growth of each pathogenic fungus was increased with time in both conditions except for *F. oxysporum*. Even though several models were examined exponential, Brody, Von Bertalanffy and Gompertz were identified as the best fitted models based on the coefficient of determination (R^2) value, root mean square error (RMSE) values, the sum of

squared due to error (SSE) values, and residual plots. The findings revealed that when *T. virens* was absent, the best-fitted growth models of *F. oxysporum*, *L. theobromae*, and *C. gloeosporioides* were Exponential, Exponential, and Gompertz, respectively (Table 1). The Exponential model fitting for *F. oxysporum* and *L. theobromae* is reasonable because, under favorable and unrestricted conditions, fungal mycelia typically undergo rapid colonization of the substrate without obvious growth limitations. In such early growth phases, nutrient availability, space, and environmental conditions are optimal, enabling unrestricted multiplication that reflects a continuous, accelerating growth rate consistent with exponential dynamics. By contrast, the growth of *C. gloeosporioides* was best described by the Gompertz model, which is sigmoidal in nature. This suggests that after an initial lag phase, growth accelerated rapidly but eventually slowed as the colony approached saturation due to self-inhibition, nutrient depletion, or spatial limitations.

When *T. virens* was present, the best-fitted growth models were Brody, Exponential, Von Bertalanffy, and Brody, respectively. These nonlinear models generally reflect constrained or decelerating growth patterns, implying that antagonistic interactions with *T. virens* imposed biological stress or competition that reduced the pathogens' capacity for unrestricted proliferation. Comparisons of growth patterns of the fungi with and without *T. virens* clearly showed the significant growth-controlling potential of *T. virens* against all the tested pathogenic fungi.

For *F. oxysporum*, the model that performs best under the first condition is the Exponential model. This model has a high R^2 value of 0.9923, indicating a strong fit to the data. Additionally, it has a relatively low RMSE of 0.7940 and SSE of 2.5220, both of which are lower than many of the other models for this fungus. This combination of metrics suggests that the Exponential model is the most accurate in describing the growth or behavior pattern of *F. oxysporum* under the given condition.

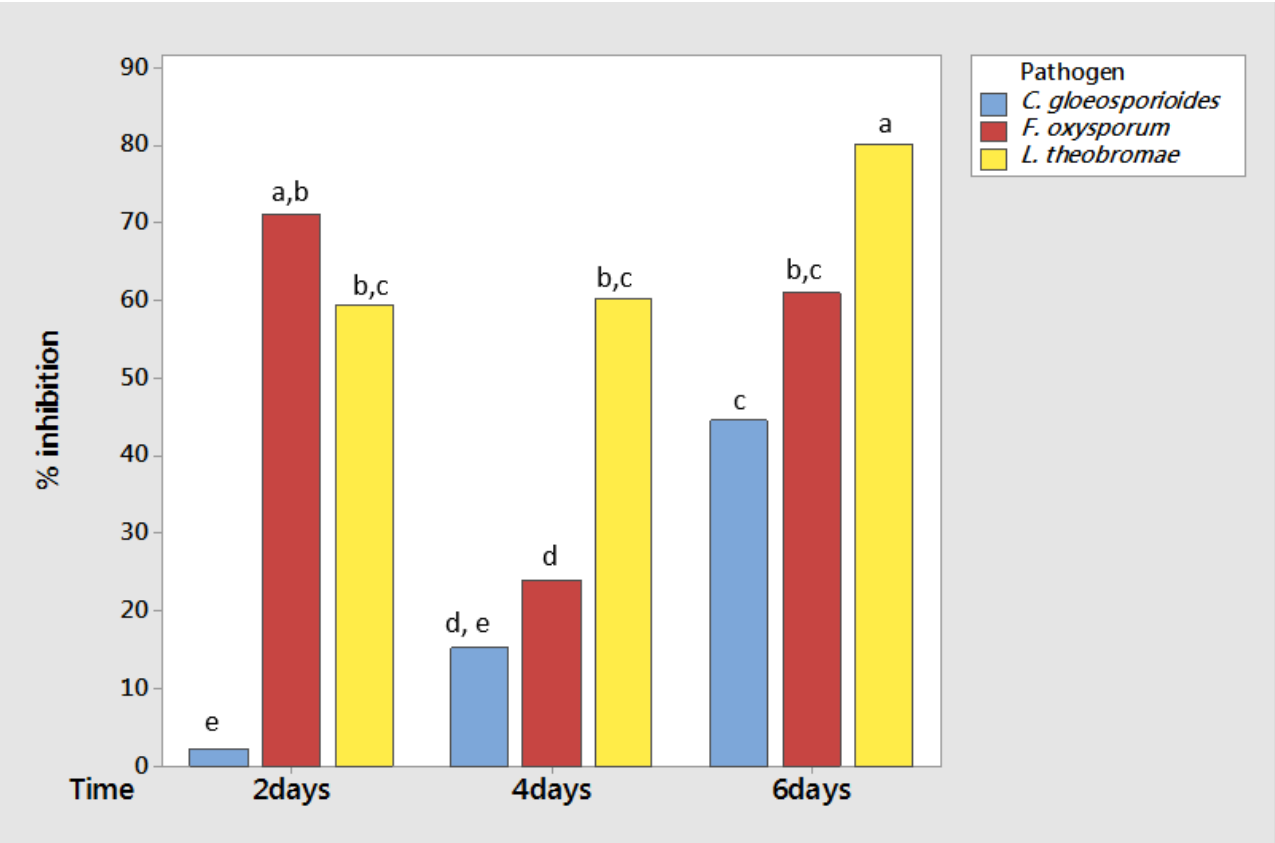

Fig. 1 – Inhibition efficiency of test pathogens according to the dual culture method. Each value represents the mean of 6 replicates. Means followed by the same letter are not significantly different by Tukey's pairwise comparison test at α=0.05.

Table 1 Growth condition of pathogenic fungi in the absence of *Trichoderma virens*.

Pathogenic fungus	Mathematical model	Parameter values	*R*2	RMSE	SSE
F. oxysporum	Exponential	a − 0.148 b − 0.834	0.9923	0.7940	2.5220
	Logistic	a − 3.693 × 10^4 b − 2.497 × 10^5 c − 0.834	0.9923	0.9174	2.5250
	Brody	a − 1.075 × 10^4 b − 1.001 c − 0.000	0.7296	5.4400	88.7700
	Von Bertalanffy	a − 1.207 × 10^5 b − 1.014 c − 0.0119	0.9677	1.8820	10.6200
	Gompertz	a − 2.661 × 10^6 b − 17.720 c − 0.069	0.9882	1.1360	3.8700
C. gloeosporioides	Exponential	a − 0.111 b − 0.590	0.9967	0.0905	0.0327
	Logistic	a − 10.080 b − 148.700 c − 0.748	0.9993	0.0490	0.0072
	Brody	a − 1948.000 b − 1.001 c − 0.000	0.8896	0.6050	1.0980
	Von Bertalanffy	a − 1.518 × 10^4 b − 0.995 c − 0.001	0.9980	0.0817	0.0200
	Gompertz	a − 186.600 b − 8.404 c − 0.128	0.9997	0.0292	0.0026
L. theobromae	Exponential	a − 0.483 b − 0.6191	0.9962	0.4935	0.9741
	Logistic	a − 7.734 × 10^4 b − 1.603 × 10^5 c − 0.6192	0.9962	0.5700	0.9746
	Brody	a − 1.147 × 10^4 b − 1.000 c − 0.000	0.8474	3.6280	39.4800
	Von Bertalanffy	a − 5.262 × 10^4 b − 0.996 c − 0.001	0.9810	1.2810	4.9200
	Gompertz	a − 1.006 × 10^6 b − 14.640 c − 0.050	0.9924	0.8086	1.9610

For *C. gloeosporioides*, the Gompertz model emerges as the most suitable model. It has the highest R^2 value at 0.9997, along with the lowest RMSE of 0.0292 and the lowest SSE of 0.0026. These values suggest an exceptional fit, meaning the Gompertz model closely captures the growth dynamics of *C. gloeosporioides*. The Gompertz model is widely known for modeling sigmoidal growth, which seems to match the data well for this fungus under these conditions.

In the case of *L. theobromae*, the Exponential model is the most fitting option. It has a high R^2 of 0.9962, with the lowest RMSE (0.4935) and SSE (0.9741) among all models considered for this fungus. These metrics indicate that the Exponential model effectively captures the growth pattern of

L. theobromae under the specified conditions. The model's high accuracy makes it a reliable choice for representing the behavior of this fungus.

For *F. oxysporum*, the Brody model proves to be the best-fitting model under this condition. It has the highest R^2 value at 0.9219, indicating a strong fit to the data. The RMSE and SSE for the Brody model are also low at 0.0300 and 0.0026, respectively. This combination of metrics suggests that the Brody model accurately captures the growth or behavior of *F. Oxysporum* under the given conditions, outperforming the other models in terms of fit and predictive power.

For *C. gloeosporioides*, the Exponential model stands out as the most suitable choice. With an R^2 value of 0.9681, it shows a strong fit to the data. The model also has a low RMSE of 0.0848 and an SSE of 0.0288, both of which are favorable compared to the other models. These metrics indicate that the Exponential model closely aligns with the data for *C. gloeosporioides*, making it the best model to describe its growth dynamics in this case.

For *L. theobromae*, the Brody model emerges as the best option under the second condition. It has the highest R^2 value among the models at 0.9566, suggesting a very good fit to the data. Additionally, the Brody model has the lowest RMSE of 0.2372 and SSE of 0.1688, further supporting its suitability. This combination of high R^2 and low error values indicates that the Brody model effectively represents the growth characteristics of *L. theobromae*.

Effect of volatile compounds produced by *T. virens*

Previous studies have determined that *Trichoderma* species are known producers of volatile compounds (VOCs) that have anti-fungal and anti-bacterial properties. Further, some of these volatile compounds are capable of inducing plant defenses in response to pathogen invasions. These compounds are secondary plant metabolites that have low molecular weight, low boiling point, high vapor pressure, and low polarity. These compounds are a diverse group of molecules, including aromatic hydrocarbons, amines, thiols, and terpenes (Li et al. 2018). Figure 2 provides a detailed analysis of the volatile compound production by *T. virens* over six days of incubation when grown individually with the pathogen. *Trichoderma virens* produced different volatile compounds such as 6-Pentyl-2H-6-Pentyl-2H-pyran-2-one (6-PP), Methyl Isothiocyanate, Acetaldehyde, Ethanol, Ethanol, Dimethyl Trisulfide (DMTS), 2-Methyl-1-butanol, 3-Methyl-1-butanol, Phenylethanol, and Benzyl Alcohol in their individual growth in the medium. However, when *T. virens* was co-cultured with the pathogens *F. oxysporum, L. theobromae,* and *C. gloeosporioides,* these metabolite production concentrations increased in the medium. This enhanced volatile compound concentration indicates a potential response of the antagonistic activity of *T. virens* to the presence of a pathogen as a biocontrol strategy. The results of the present study indicated that *T. virens* was indeed capable of producing volatile compounds that can affect the mycelial growth of the test pathogens. Nevertheless, the increase in volatile production was not uniform across all pathogens. For instance, the most significant increase in VOCs was observed in *T. virens* against *F. oxysporum,* and *L. theobromae.* This implies that *F. oxysporum* and *L. theobromae* may release certain signals or stress factors that *T. virens* may be able to identify, leading to a more robust production of bioactive chemicals. The highest VOC production showed against *F. oxysporum*, suggesting that *T. virens* may be more efficient at preventing *F. oxysporum* from producing volatile compounds. Among many VOCs, ethanol and 6-Pentyl-2H-6-Pentyl-2H-pyran-2-one production were the highest when *T. virens* controlled the growth of *F. oxysporum*. This finding was validated by the highest inhibition efficiency of *T. virens* against *F. oxysporum.* A similar test has been carried out by Lee et al. (2016), to evaluate the effects of the volatile compounds produced by *T. harzianum, T. asperellum, T. virens,* and *T. viride* against *F. oxysporum*. It was reported that the volatile compounds produced by all the selected *Trichoderma* species, including *T. virens*, have significantly inhibited the mycelial growth of *F. oxysporum*. Moreover, in that study, higher inhibition percentages were observed for the *F. oxysporum* colonies treated with the volatile components produced by *T. virens*. In that study, the percentage inhibition was recorded against the *F. oxysporum* strain 9605 (15.2±3.8%). However, the volatile compounds of *T. virens* caused an inhibitory effect on *F. oxysporum* strains used during the present is (10.50±0.00%). Regardless of that, the *C. gloeosporioides* colonies treated with the

volatile components produced by *T. virens* showed considerably low inhibition percentages after the 6-day incubation period. However, the effectiveness of the secondary metabolites of *Trichoderma* species against *Colletotrichum* species has been widely reported in previous studies (Lumsden et al. 1992, Joshi et al. 2016). The results of the present study support the assumption that the volatile compounds produced by *Trichoderma* species cannot inhibit the growth of *Colletotrichum* species up to significant levels.

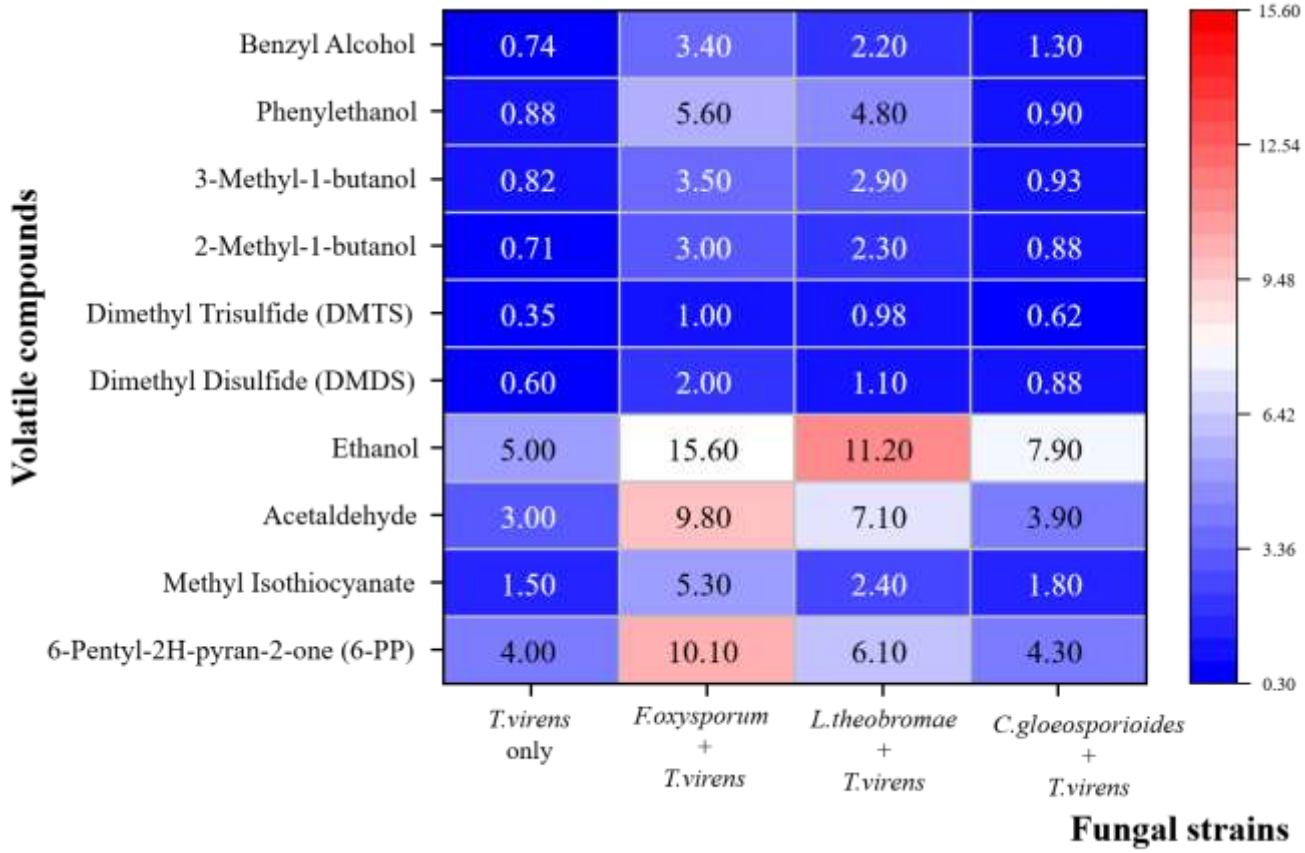

Fig. 2 – The synthesis of volatile organic compounds (VOCs) by *T. virens* during contact with several pathogens is depicted in a heatmap. The VOC concentration levels are represented by the color gradient, where red denotes higher concentrations and blue denotes lower concentrations.

Table 2 Growth condition of pathogenic fungi in the presence of *T. virens*.

Pathogenic fungus	Mathematical model	Parameter values	*R*2	RMSE	SSE
F. oxysporum	Exponential	*a* − 0.176 *b* − 0.125	0.6808	0.0520	0.0107
	Logistic	*a* − 0.324 *b* − 9.746 *c* − 1.845	0.9126	0.0313	0.0026
	Brody	*a* − 0.331 *b* − 1.722 *c* − 1.029	0.9219	0.0300	0.0026
	Von Bertalanffy	*a* − 0.328 *b* − 0.956 *c* − 1.264	0.9183	0.0300	0.0030

Table 2 Continued

Pathogenic fungus	Mathematical model	Parameter values	*R*2	RMSE	SSE
	Gompertz	*a* − 0.327 *b* − 3.811 *c* − 1.399	0.9167	0.0305	0.0028
C. gloeosporioides	Exponential	*a* − 0.062 *b* − 0.501	0.9681	0.0848	0.0288
	Logistic	*a* − 1648.000 *b* − 2.722×10^4 *c* − 0.502	0.9681	0.0980	0.0288
	Brody	*a* − 658.200 *b* − 1.000 *c* − 0.000	0.8127	0.2373	0.1689
	Von Bertalanffy	*a* − 9208.000 *b* − 0.987 *c* − 0.006	0.9308	0.1442	0.0624
	Gompertz	*a* − 3.365×10^5 *b* − 16.140 *c* − 0.034	0.9609	0.1084	0.0352
L. theobromae	Exponential	*a* − 0.873 *b* − 0.219	0.9044	0.3048	0.3717
	Logistic	*a* − 3.567 *b* − 5.426 *c* − 0.574	0.9566	0.2936	0.2585
	Brody	*a* − 4.988 *b* − 0.996 *c* − 0.156	0.9566	0.2372	0.1688
	Von Bertalanffy	*a* − 4.033 *b* − 0.571 *c* − 0.306	0.9485	0.2584	0.2003
	Gompertz	*a* − 3.838 *b* − 2.265 *c* − 0.377	0.9445	0.2683	0.2159

Effect of non-volatile components produced by *T. virens*

The effect of the non-volatile compounds produced by *T. virens* on the test pathogens is illustrated in Figure 3. These compounds exhibit various biological activities including cytotoxic, antifungal, antibacterial, antiviral, antibiotic, and plant defense-inducing properties (Raza et al. 2013). The results of the present study indicated that the non-volatile compounds produced by *T. virens* were capable of inhibiting the mycelial growth of the selected pathogens. Those compounds were highly effective in controlling the mycelial growth *of L. theobromae* (i.e. 61.05±3.20%). A study conducted by Bui et al. (2018) has reported somewhat similar percentage inhibitions by the non-volatile compounds produced by *T. harzianum* (KU904458) and *T. harzianum* (KU933474) against *L. theobromae* (i.e. 53.7% and 74.31%, respectively). Other *Trichoderma* species used during that study including *T. asperellum*, *T. koningiopsis*, *T. hypocrea*, *T. ovalisporum*, and *T. atroviride* have reported much higher inhibition percentages by the non-volatile compounds against *L. theobromae.*

Fusarium oxysporum reported moderate levels of growth inhibition compared to *L. theobromae* at the end of the 6-day incubation period (i.e., 41.45±0.84%). A similar study conducted by Meena et al. (2017) has reported similar levels of inhibition against *F. oxysporum* by the non-volatile compounds produced by both *T. harzianum* and *T. viride* (i.e. 43.08% and 40.00%, respectively).

Both *T. virens* and *T. harzianum* are known to produce the same non-volatile compound called dimethyllincisterol A3 (Li et al. 2019). So, the non-volatile compounds produced by *T. virens* and *T. harzianum* may have a similar inhibitory effect on *F. oxysporum*.

Unfortunately, the growth of *C. gloeosporioides* was not observed even in the control plates after repeated attempts.

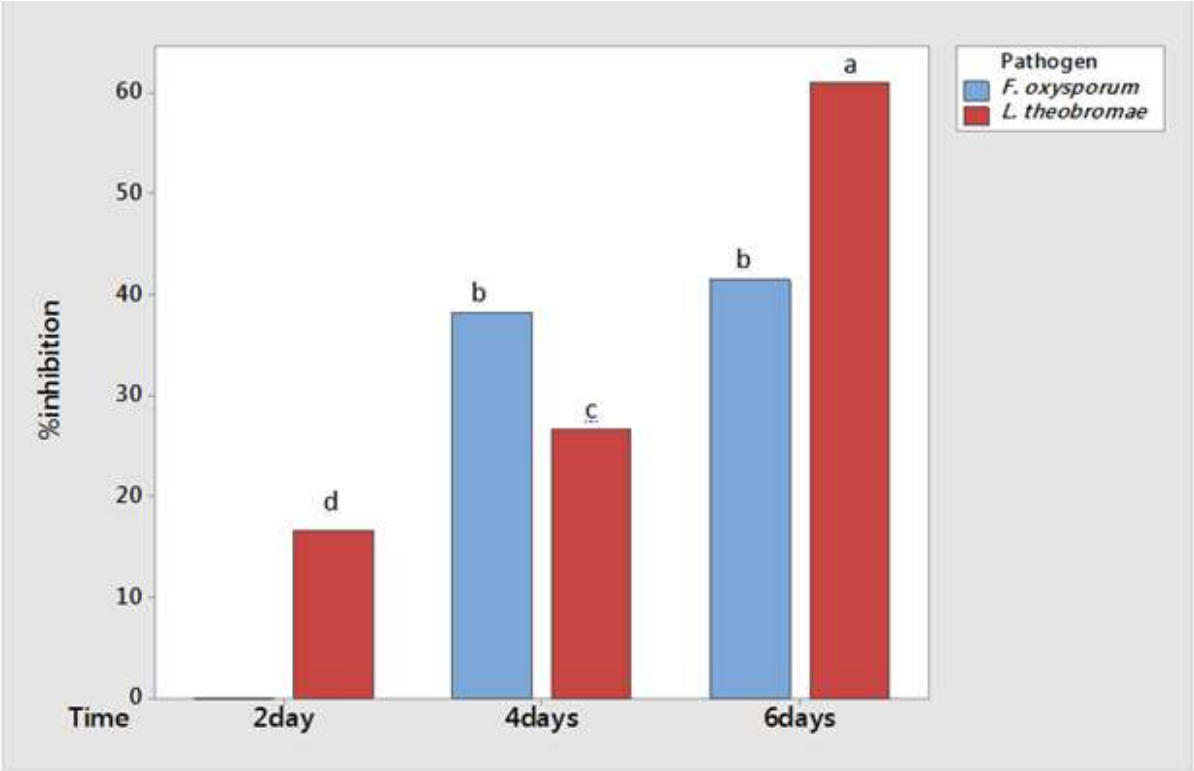

Fig. 3 – Inhibition percentages of test pathogens due to the effects of non-volatile components produced by *T. virens*. Each value represents the mean of 6 replicates. Means followed by the same letter are not significantly different by Tukey's pairwise comparison test at α=0.05.

Microscopic observations of mycoparasitism

The hyphal interactions used by the *Trichoderma* species in controlling the pathogenic fungal species include penetration, branching, sporulation, resting body production, and barrier formation (El-Debaiky 2017).

In the present study, hyphal interactions were observed using the slide culture method. In the slide bi-culture tests with *F. oxysporum*, clear zones were observed in all *Trichoderma*-pathogen interactions. However, light microscope observations did not reveal any physical interactions between pathogen and *Trichoderma* isolates.

Similarly, although clear zones were observed between *C. gloeosporioides* and *T. virens* isolates, hyphal interactions were not observed between the pathogen and *T. virens* isolates. A similar study was conducted by De la Cruz-Quiroz et al. (2018) using *T. harzianum, T. longibranchiatum, T. yunnanense, T. asperellum* against *C. gloeosporioides.* It was reported that the selected *Trichoderma* species, except *T. longibranchiatum,* could completely inhibit the growth of *C. gloeosporioides* by growing over those colonies. It was further observed that *Trichoderma* species could grow using the phytopathogen biomass as a carbon and energy source. Such observations could not be made in the present study, possibly because of the use of a different *Trichoderma* species.

In contrast, clear zones of inhibition were not observed between the *T. virens* and *L. theobromae* colonies. However, when the region of interaction between two colonies was examined under the microscope, coiling structures produced by *T. virens* against *L. theobromae* were observed.

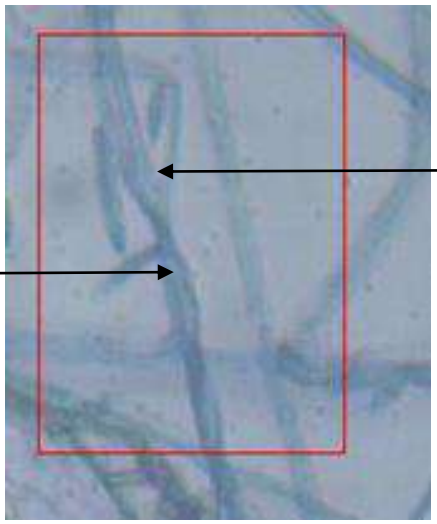

Fig. 4 – Coiling structures formed by *T. virens* against *L. theobromae*. *Trichoderma virens* hypha is visible in a darker color when compared to *L. theobromae* (10x10).

The selection of the bio-control agents and accurate identification of the bio-control mechanisms employed by them is essential for the designing and the employment of effective bio-control strategies for fungal plant diseases. The results of the present study indicate that *T. virens* (KP985643.1) is capable of inhibiting the growth of targeted test pathogens using a combination of mechanisms including hyphal interactions and through the production of volatile and non-volatile compounds. *Trichoderma virens* may be capable of controlling the selected fungal plant pathogens through other mechanisms as well. Apart from that, environmental conditions such as temperature, humidity, and soil pH might also affect the biological controlling capacity of the selected *Trichoderma* species. Therefore, tests under field conditions are required to determine the broad-spectrum bio-control efficacy of *T. virens* against the test pathogens. That will aid in the determination and implementation of safe and effective bio-controlling strategies.

Conclusion

Results of the dual culture test indicated that *T. virens* have the potential to control the growth of *F. oxysporum, C. gloeosporioides,* and *L. theobromae* successfully by implementing them under *in vitro* conditions. The volatile components produced by *T. virens* were moderately effective against *C. gloeosporioides*. Although the volatile components produced by *T. virens* were not effective in controlling *L. theobromae,* the non-volatile components produced by *T. virens* were highly effective against *L. theobromae*. *Trichoderma virens* successfully inhibited the growth of *L. theobromae* using hyphal interactions.

Statistical analysis revealed that the growth of each of the pathogenic fungi was increased with time in both conditions (presence and absence of *T. virens*) except for the *F. oxysporum*. After six days, *F. oxysporum* showed complete growth inhibition, as *T. virens* effectively outcompeted the pathogen, leading to its early senescence. This inhibition was supported by mathematical modeling, which demonstrated a plateau in colony growth in the presence of *T. virens*. ANOVA results confirmed that the presence of *T. virens* (independent variable) significantly affected pathogenic fungal growth (dependent variable), with a clear interaction between *T. virens* presence and growth rate over time. Among the five mathematical models considered (Exponential, Logistic, Brody, Von Bertalanffy, Gompertz), the best-fitted models were given by Exponential, Gompertz, and Exponential respectively for *F. oxysporum, C. gloeosporioides* and *L. theobromae* in the absence of *T. virens*. However, in the present condition of *T. virens*, the best fitted models were given by Brody, Exponential, and Brody respectively for *F. oxysporum, C. gloeosporioides,* and *L. theobromae*. Growth comparison graphs showed that the presence of *T. virens* reduced the growth of all tested pathogenic fungi by more than 60% after six days, underscoring its potential as an effective biocontrol agent.

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Conflict of Interest

None

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