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# *In vitro* bioefficacy of *Trichoderma* species against two *Botryosphaeriaceae* fungi causing *Eucalyptus* stem canker disease in Ethiopia

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ABSTRACT

Phytopathogenic fungi, which are leading to significant economic and production losses, pose a serious threat to global forestry. Field surveys were carried out on *Eucalyptus* trees in Ethiopia between 2019/20 and 2020/21. The *E. camaldulensis* predominantly grew in the surveyed fields and was severely affected by progressive stem canker disease. The diseased stems and branches exhibiting symptoms were collected, and the *Lasiodiplodia theobromae* and *Neofusicoccum parvum* fungal pathogens were identified *via* morphological and DNA sequence analyses of ITS1 and ITS2 from rDNA. The objective of this research was to assess the *in vitro* growth inhibition potential of *Trichoderma* species against *Lasiodiplodia theobromae* and *Neofusicoccum parvum* fungal species. Researchers are now exploring alternate eco-friendly management strategies due to the hazardous effects chemical fungicides have on human and environmental health. The use of biocontrol agents, such as *Trichoderma* species, is one of the potential approaches employed today. The antagonistic activities of *Trichoderma asperellum* (T4), *Trichoderma asperellum* (T5), *Trichoderma atroviride* (T1), *Trichoderma atroviride* (T11), *Trichoderma longibrachiatum* (T3), and *Trichoderma longibrachiatum* (T31) were tested *in vitro* against *Lasiodiplodia theobromae* and *Neofusicoccum parvum* that cause canker disease symptoms in *E. camaldulensis*. In all antagonism tests, *T. asperellum* and *T. longibrachiatum* effectively inhibited the growth of *L. theobromae* and *N. parvum*. This implies that the *Trichoderma* species could be useful to control *E. camaldulensis* canker disease.

## 1. Introduction

Plantation forests are developed by planting native or exotic tree species. In Ethiopia, forest plantations consist mainly of *Eucalyptus*, *Cupressus*, *Casuarina,* and *Pinus* (Yirdaw, 2002). *Eucalyptus* trees are grown for a variety of purposes, including paper, pulp, fuel wood, fiberboard, sawlogs, poles, timber, furniture, and building materials (Li et al., 2018). *Eucalyptus* plantations are now widely planted in state-owned and private enterprise plantations, as well as community woodlots and household compounds, providing an alternative income source and employment opportunities in Ethiopia(Gizachew, 2017). The widespread cultivation of *Eucalyptus* in Ethiopia is due to the rising demand in the construction industry resulting in increase in *Eucalyptus* wood prices, and its ability to grow with minimal labor and financial expenditure (Tesfaw et al., 2022). As a result, *Eucalyptus* is now the second most important source of income for rural households in Ethiopia, following crop farming (Asabeneh and Yoseph, 2022). Despite its significant economic importance, *Eucalyptus* faces numerous challenges, including diseases that have a detrimental effect on its production (Wingfield et al., 2008a, 2008b; Old et al., 2003).

Stemcanker disease is one of the most important diseases affecting the quality and quantity of *Eucalyptus* products (Alemu et al., 2003a; Slippers and Wingfield, 2007). Cankers on *Eucalyptus* are often caused by *Botryosphaeria* species, leading to substantial losses (Wingfield et al., 2008a, 2008b; Chungu et al., 2010). These species are asymptomatic latent, wound, and stress-associated plant pathogens (Pérez et al., 2008; Chungu et al., 2010). The most common *Botryosphaeria* species causing *Eucalyptus* stem canker disease presently are *Neofusicoccum parvum* and *Lasiodiplodia theobromae* (Alemu et al., 2004; Alemu et al., 2003, and Aylward et al., 2019). These opportunistic plant pathogens cause canker and dieback diseases in over 500 tree species, including *Eucalyptus* species (Yu et al., 2009; Chen et al., 2011; Sulaiman et al., 2012; Phillips et al., 2013; Soltaninejad and Massumi, 2017).

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The use of biopotential organisms or fungicides can control *Eucalyptus* diseases (Garrett et al., 2018). However, However, these methods must be evaluated against the specific fungal pathogens causing the diseases for effective management. Chemical control of plant pathogens is effective in improving crop productivity, but it is not recommended due to its significant adverse effects on environmental health, ecology, soil productivity, and water resources (Rai and Mamtha, 2005). Therefore, alternative safe management options such as biological control methods involving biopotential antagonistic microorganisms must be screened and implemented (Chanchaichaovivat et al., 2007; Evueh and Ogbebor, 2008). Mycorrhizal fungi, plant growth-promoting fungi, and plant growth-promoting rhizobacteria are the major groups of plant-beneficial microorganisms. These bacteria belong to the plant growth-promoting bacteria group, which improves plant health by enhancing nutrient acquisition, protecting against phytopathogenic agents, and promoting resistance to various abiotic stresses (Riseh et al., 2022). The application of cell free supernatant and crude lipopeptide extracts from *Bacillus subtilis* controls effectively the cucurbit powdery mildew disease under greenhouse and field conditions than the bacteria itself (Trupo et al., 2023). Cruz-Martín et al. (2022) also demonstrated the crucial role of the chitinases produced by *Bacillus pumilus* in the antifungal activity *in vitro* against *Pseudocercospora fijiensis*.

*Trichoderma* species are potential fungal bioagents for the management of various economically important plant pathogens. This species is usually preferred due to rapid growth, antagonistic effect, ability to increase nutrient availability, and uptake, secretion of cell wall-degrading enzymes, ability to produce antibiotics and volatile compounds responsible for antibiosis properties (Gerhardson, 2002; Harman et al., 2004; Sarrocco et al., 2006). Most *Trichoderma* spp. usually produce volatile compounds such as acetaldehyde, ethylene, acetone and carbon dioxide, as well as antibiotics like trichodermin, trichodermol, herzianolide, gliotoxin, viridin, and ergokonin, which are responsible for antibiosis properties against diseases causing fungal pathogens (Meena et al., 2017). *Trichoderma* strains such as *T. harzianum*, *T. hamatum*, *T. asperellum,* and *T. atroviride* are often used for plant diseases control (Korpi et al., 2009). Mohamed et al. (2022) proved that *Trichoderma viride* and *Trichoderma harzianum* strains had highest efficiency against *Alternaria alternata*, *B. sorokiniana*, *D. halodes* and *F. proliferatum.* Degani et al. (2023) also showed that *Trichoderma* spp. are considered potential eco-friendly biocontrol agents and growth-promoting fungi against the cotton charcoal rot disease caused by *Macrophomina phaseolina*.

Studies by Bhadra et al. (2015), Rusin et al. (2021), and Langa-Lomba et al. (2022) have shown that *Trichoderma* species have potential inhibitory activity against *N. parvum* and *L. theobromae* under *in vitro* and *in vivo* growth conditions. *T. harzianum* and *T. atroviride* spore suspensions are also effective to control *Botryosphaeria berengeriana* shoot and stem canker diseases (Kexiang et al., 2002). Mulatu et al. (2022a, 2022b) also investigated in their *in vitro* antagonistic potential test that *T. asperellum* and *T. longibrachiatum* are effective biocontrol agents against *Fusarium xylarioides*. In general, with global climate change causing serious impact on plant health by stressing the host and forcing fungal pathogens to become virulent in unusual ways, a study on safe alternative disease management options involving biopotential microbes like *Trichoderma* species will be essential for effective diseases prevention and control. This study aimed to assess the *in vitro* growth inhibition potential of *Trichoderma* species against *Botryosphaeria* (*Lasiodiplodia theobromae* and *Neofusicoccum parvum*) fungal species causing *Eucalyptus* stem canker disease in Ethiopia. The results of this study will pave the way for future research on preventing and controlling tree diseases involving biopotential microorganisms. The results of this study will pave the way for future research on preventing and controlling tree diseases involving biopotential microorganisms. The results of this study will pave the way for future research on preventing and controlling tree diseases involving biopotential microorganisms.

## 2. Materials and methods

### 2.1. Eucalyptus diseases survey and sampling

Field surveys of *Eucalyptus* diseases were conducted in the *Eucalyptus* growing areas of Ethiopia in 2019/20 and 2020/21. Surveys and sampling were performed at different locations as described by Wendu et al. (2022c).

### 2.2. Fungal pathogen isolation and identification

To isolate and identify the fungal pathogens, cankered samples were first sterilized using a solution of 90% ethanol and 1% sodium hypochlorite for one minute and two minutes, respectively. The samples were then rinsed twice in distilled water for 30 s, dried on sterile filter paper, and transferred to potato dextrose agar (PDA) medium using aseptic techniques, as specified by Wendu et al. (2022b). Additionally, conidia released from pycnidia that grew on diseased stem and branch samples were used for isolation and identification. Pure fungal isolates were obtained using a single hypha transfer method and were identified based on their macroscopic and microscopic morphology (Pillay et al., 2013; Silva et al., 2020; Wendu et al., 2022).

#### 2.2.1. Phylogenetic analysis, DNA extraction, PCR amplification, and DNA sequencing

In this study DNA extractions were conducted using the protocol described by Wendu et al. (2022c). In brief, the phenol-chloroform extraction method was utilized, and the fungal mycelia used for extraction were harvested from seven-day-old cultures of the fungal isolates. To extract DNA, one gram of frozen mycelium was ground into fine powder in liquid nitrogen and incubated in 5 ml of 2% extraction buffer (10 NaCl (2%), mercaptoethanol (0.1%), and PVP (0.2%)) at 65ºC for 15 min. An equal volume of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) was then added, mixed using a vortex, and centrifuged at 12,000 revolutions per minute (rpm) for 5 min.

For DNA precipitation, the supernatant was mixed with an equal volume of ice-cold isopropanol and incubated at 25ºC (Slippers et al., 2017). The precipitate was collected by centrifugation, washed with 0.1 M ammonium acetate in 70% ethanol, and incubated for 15 min. The pellet was then resuspended in 500 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and the DNA concentration was determined using a NanoDrop 2000 Spectrometer. PCR and sequencing were conducted using a BigDye Terminator version 3.1 cycle sequencing kit from Applied Biosystems (Life Technologies, UK). The internal transcribed spacer regions of the rDNA (5.8 S gene) of the fungal isolates were amplified using universal ITS1 and ITS2 primers in a reaction mixture consisting of 1.0 µL DNA template, 5 µL PCR buffer, 5 µL dNTP, 1.0 µL polymerase, and 36 µL PCR water using a thermocycler (White et al., 1990) and viewed under UV light on a 1% agarose gel. The average size of the amplicons of ITS was about 500 base pairs. The purified ITS products were sequenced using the BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) as described by Raja et al. (2017). The obtained ITS sequences were edited using BioEdit version 7.0.9.0 using the methods of Hall (1999).

#### 2.2.2. Pathogenicity test

The pathogenicity of the fungal isolates was assessed using 7-day fungal cultures. Mycelial plugs of each fungal species were cut to 5.0 mm and inoculated on five E. camaldulensis seedlings through small wounds on the stems, made with a corkscrew borer to expose the xylem, about 30 cm above the root collar (Badillo-Vargas et al., 2008). To prevent desiccation and contamination, mycelial plugs were placed upside down into the wounds and sealed with Parafilm (Pechiney Plastic Packaging, USA) (Ahmad and Ashraf, 2016). An additional set of five seedlings was used as controls, inoculated with sterile MEA. The inoculated seedlings were examined for symptoms of canker disease

after 3–4 weeks, and the length of the lesions was measured. Segments cut from the margins of necrotic lesions were sterilized, cultured in MEA at 25 ºC, and used for growth, reisolation, and identification of fungal species to fulfill Koch's postulate (Wendu et al., 2022c).

### 2.3. Evaluation of a bioagent for the management of fungal pathogens in vitro

*Trichoderma atroviride* species used in this study (*T. atroviride* strain (T1), *T. atroviride* strain (T11) was isolated from the diseased *Eucalyptus* stem and branch samples. These *Trichoderma* species were grown on the MEA, morphologically identified as species of the genus *Trichoderma,* and finally categorized into *T. atroviride* strain (T1) (E0000338001) and *T. atroviride* strain (T11) (E0000338002) species using ITS sequences at the Center for Agriculture and Bioscience International (CABI). The *T. atroviride* species were identified by ITS rDNA sequence analysis using the FASTA algorithm in the fungus database from NCBI. ITS results gave > 99% match to many sequences of this genus and predominantly to strains of *Trichoderma atroviride*, including 100% to *Trichoderma atroviride* strain CBS 126.65 [AF501329] and *Trichoderma atroviride* strain TUB F384 [AY857204] which has been cited in Druzhinina et al.(2006). In addition, *T. asperellum* strain (T4), *T. asperellum* strain (T5), *T. longibrachiatum* strain (T3), as well as *T. longibrachiatum* strain (T31) obtained from Addis Ababa University, were also used for the *in vitro* control study (Fig. 1).

#### 2.3.1. In vitro dual culture confrontation test using Trichoderma species against L. theobromae and N. parvum

For the dual culture confrontation experiment, a 5-mm actively growing mycelium of the fungal pathogen was placed 10 mm from the edge of the plate. A similar plug of *Trichoderma* spp. mycelium was placed 10 mm from the opposite edge of the plate in front of the pathogen with corresponding control plates for each pathogen (Martnez-lvarez et al., 2012). The plates containing dual cultures were kept in the lab for six days and the mycelial growth length of the fungal pathogen was measured along three axes as described by Martínez-Álvarez et al. (2012). The fungal cultures radial diameter were compared with the radial growth of the fungal pathogens in the corresponding control for *Lasiodiplodia theobromae,* and *Neofusicoccum parvum*, respectively, using the procedures of Mamza et al. (2010), Asman et al. (2021), Wendu et al. (2022b).

D = (d1 +d2)/2, where: D = Diameter of the grown fungi mycelia.

d1 = Vertical diameter of the grown fungi mycelia.

d2 = Horizontal diameter of the grown fungi mycelia.

The relative reduction in mycelial growth for each fungal pathogen compared to the control was determined using the formula (Meena et al., 2017; Wendu et al., 2022b):

Percent inhibition of fungal mycelia (PI) =(C − T)/C)×100, where:

(C) Colony diameter in control.

(T) Colony diameter in treatment (mm).

The degree of antagonistic activity was estimated as follows: 4 – very high antagonistic activity (PI > 75), 3 – high antagonistic activity (PI = 61–75), 2 – moderate antagonistic activity (PI = 51–60), 1 – low antagonistic activity (PI < 51) (Karličić et al., 2021).

Growth inhibition tests using nonvolatile and volatile metabolites were used to evaluate the effect of the *Trichoderma* strains against *Lasiodiplodia theobromae* and *Neofusicoccum parvum* identified in this study, as described by Negash and Tesfaye (2010) and Meena et al. (2017).

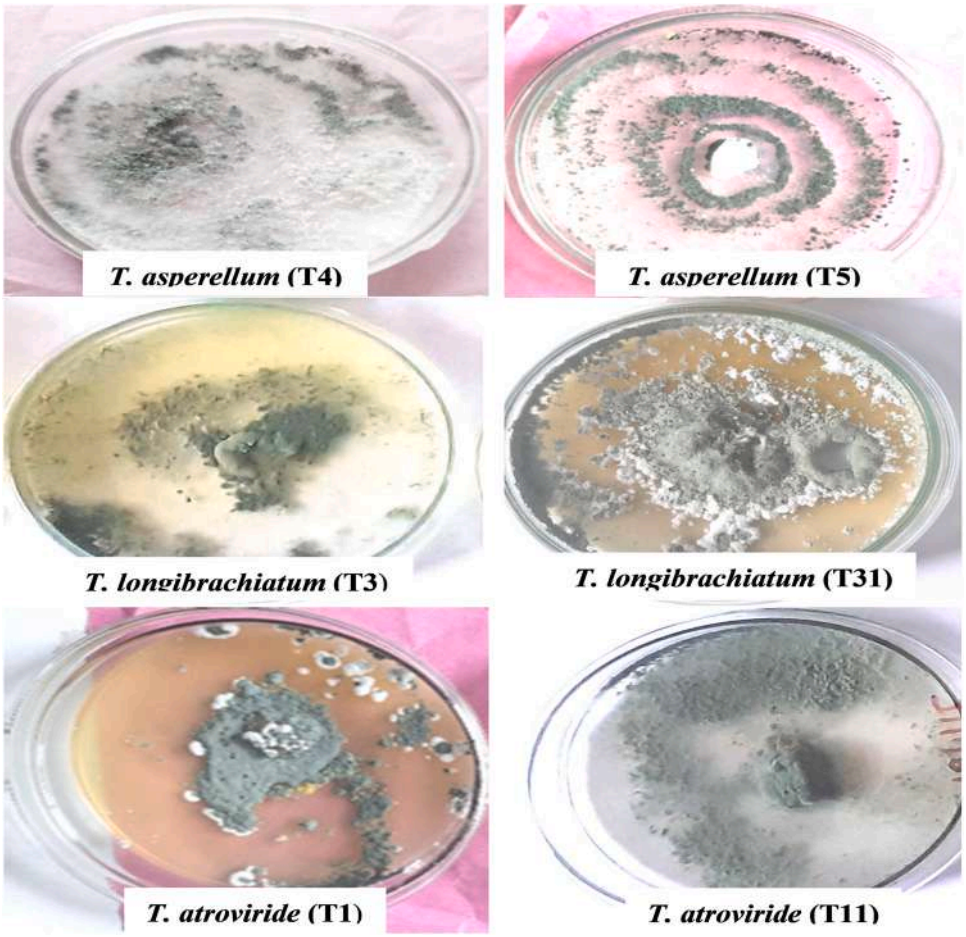


**Fig. 1.** *Trichoderma* species used for the *in vitro* management test against selected fungal pathogens.

**Table 1**
New and reference fungal sequences used for the phylogenetic study.

| GenBank accession number | Species | Isolate | Host | Origin |
|---|---|---|---|---|
| **E864005FR-001** | ***N. Parvum*** | **F02** | *E. camaldulensis* | Ethiopia |
| **E864005FR-004** | ***N. Parvum*** | **F04** | *E. camaldulensis* | Ethiopia |
| **E864005FR-003** | ***N. Parvum*** | **F03** | *E. camaldulensis* | Ethiopia |
| MT626612 | *N. Parvum* | MY4 | *Vaccinium ashei* | China |
| MN634051 | *N. Parvum* | IRNBS51 | Nut | China |
| MT495431 | *N. parvum* | EX2019-M7 | *Vitislabruscana* | China |
| MT261980 | *N. parvum* | CVG444 | *Vaccinium corymbosum* | Italy |
| MT229074 | *N. parvum* | BM_9 | Pear | China |
| MN607172 | *N. parvum* | 310453 | *Vitis labrusca* | Brazil |
| MK563984 | *N. parvum* | LY-J | *Kadsura coccinea* | n/a |
| MN583002 | *N. parvum* | JZZK | *Acer palmatum* | China |
| MN121536 | *N. parvum* | 3-bye-10 | *Phoebe bournei* | China |
| MG019916 | *N. parvum* | CS1–4 | *Camellia sasanqua* | n/a |
| MT355871 | *N. parvum* | YYcbCZ | *Zanthoxylum bungeanum* | China |
| KX464030 | *N. ribis* | FO 4837 | *Ribes rubrum* | n/a |
| EU821958 | *N. ribis* | CMW7772 | Ribes species | USA |
| MT592439 | *N. ribis* | CBS:122553 | *Theobroma cacao* | Panama |
| MN634050 | *N. parvum* | IRNBS30 | Almond | Iran |
| MT587330 | *B. dothidea* | CBS:110484 | *Vitis vinifera* | Argentina |
| MH133917 | *L. theobromae* | CFCC 52443 | n/a | China |
| MK530073 | *L. theobromae* | PHM350 | *Mangifera indica* | Malaysia |
| MK530050 | *L. theobromae* | PBBG184 | *Mangifera indica* | Malaysia |
| **Q203241039** | ***L. theobromae*** | **M09 035** | *E. camaldulensis* | Ethiopia |
| **Q203241034** | ***L. theobromae*** | **C09045** | *E. camaldulensis* | Ethiopia |
| EF622071 | *L. theobromae* | CBS 306.58 | n/a | Iran |
| GU97386 | *L. theobromae* | CBS 111530 | n/a | Iran |
| MK860754 | *L. theobromae* | PPS6 | n/a | Colombia |
| EF622075 | *L. theobromae* | CBS 112874 | *Vitis vinifera* | S. Africa |
| MK860753 | *L. theobromae* | LAK20 | *Vaccinium corymbosum* | Peru |
| MK530016 | *L. theobromae* | BPPCA117 | *Mangifera indica* | Malaysia |
| MN634044 | *L. theobromae* | IRNB573 | walnut | Iran |
| MK860751 | *L. theobromae* | LAK12 | *Vaccinium corymbosum* | Peru |
| KY657465 | *L. theobromae* | BTMA10 | *Mangifera indica* | Ghana |
| MT823467 | *L. theobromae* | BRM004720 | *Vaccinium corymbosum* | Brazil |
| KR709026 | *L. theobromae* | HGAJ-7 | Mulberry | Chania |

**Fig. 2.** Phylogram trees of the *Lasiodiplodia* species obtained from the ITS sequence data, including *B. dothidea* as an outgroup.

**Fig. 3.** Phylogram trees of the Neofusicoccum species obtained from the ITS sequence data, including *N. ribis* as an outgroup.

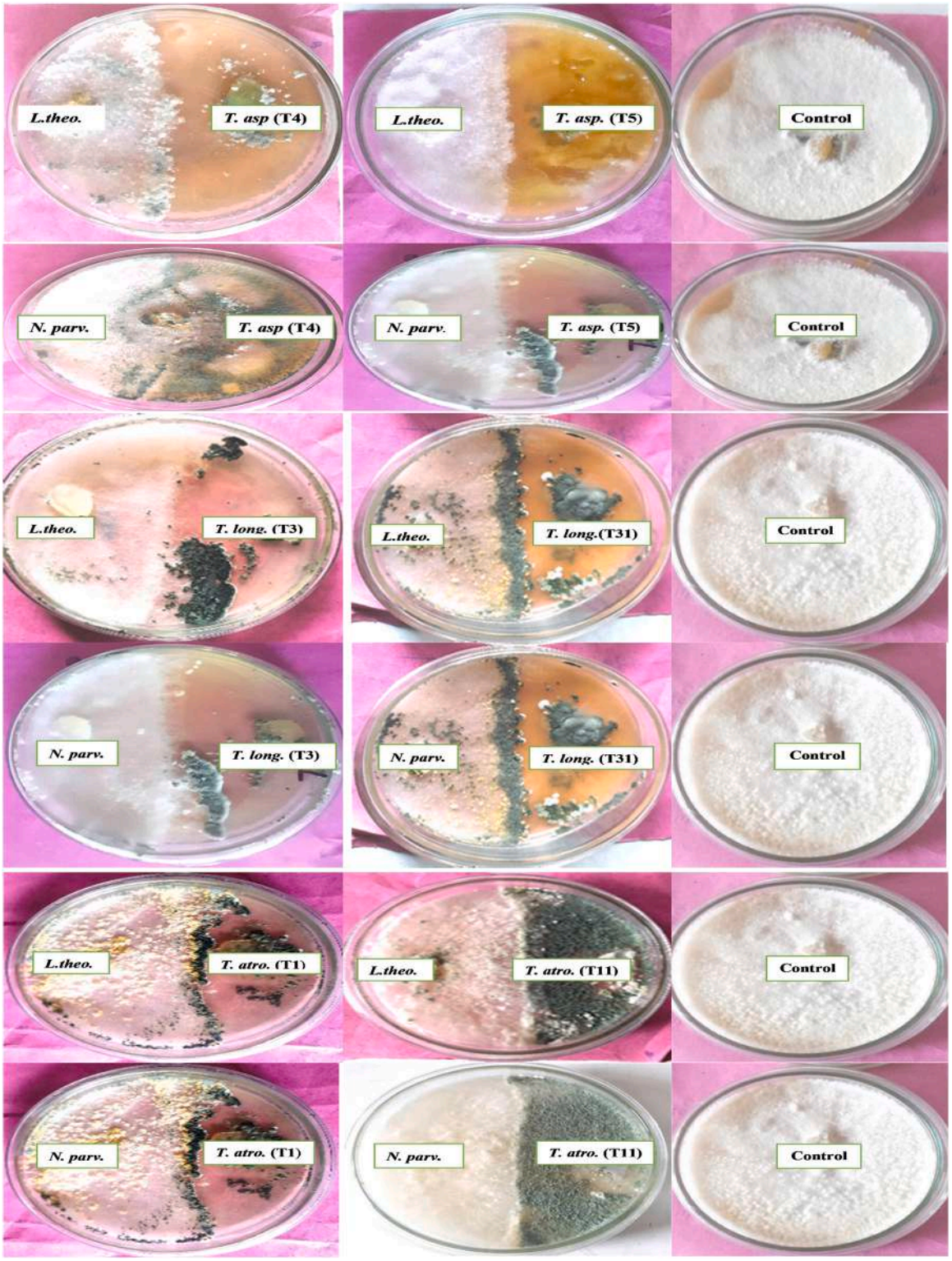


**Fig. 4.** Antagonistic effects of *Trichoderma* species against fungal pathogens in dual culture: *L. theo.* = *L. theobromae*, *N. pav.* = *N. parvum*, *T. asp.* = *T. asperellum*, *T. long.* = *T. longibrachiatum,* and *T. atro.* = *T. atroviride*.

*2.3.2. Effect of culture filtrates of Trichoderma species on mycelial growth of L. theobromae and N. parvum*

To investigate the effect of *Trichoderma* culture filtrate on the test pathogens, a mycelial disc (4 mm) from a 6-day culture was inoculated in 100 ml of potato dextrose broth (PDB) and incubated for fifteen days at 28 °C with constant shaking in an orbital shaker as described by Ghanbarzadeh et al. (2014) and Meena et al. (2014). The broth was filtered through Whatman number 1 filter paper and collected in a sterile conical Erlenmeyer flask. The filtrate of each isolate was centrifuged at 10,000 rpm for ten minutes to remove cells (2017). The culture filtrates of *T. asperellum* strain (T4), *T. asperellum* strain (T5), *T. atroviride* strain (T1), *T. atroviride* strain (T11), *T. longibrachiatum* strain (T3), and *T. longibrachiatum* strain (T31) were tested for growth inhibition against *L. theobromae* and *N. parvum*. To determine the growth

**Table 2**
*In vitro* inhibitory activity of *Trichoderma* strains against *Lasiodiplodia theobromae* growth compared to the control after six days.

| | Percent growth inhibition (PI) by *Trichoderma* species | | | | | |
|---|---|---|---|---|---|---|
| *Trichoderma* species | Dual culture[a] | A. act. | Non-volatile inhibitors[b] | A. act.[d] | Volatile inhibitors[c] | A. act. |
| *T. asperellum* (T4) | 86b | 4 | 56.8b | 1 | 44.3c | 1 |
| *T. asperellum* (T5) | 86b | 4 | 57.8a | 2 | 44.3c | 1 |
| *T. longibrachiatum* (T3) | 88a | 4 | 57.8a | 2 | 56.3a | 2 |
| *T. longibrachiatum* (T31) | 88a | 4 | 54.6c | 2 | 55.4b | 2 |
| *T. atroviride* (T1) | 58d | 2 | 26.5e | 1 | 32.5e | 1 |
| *T. atroviride* (T11) | 59c | 2 | 26.9d | 1 | 32.7d | 1 |
| P | < 0.01 | | < 0.01 | | < 0.01 | |
| LSD | 1.24 | | 0.09 | | 0.08 | |

A. activity = Antagonistic activity, where 1 = low antagonistic activity (PI < 51), 2 = moderate antagonistic activity (PI = 51–60), 3 = high antagonistic activity (PI = 61–75), 4 = very high antagonistic activity (PI > 75), LSD = Least significant difference. [abc]Values marked with the same letter do not differ significantly at P < 0.05 using Duncan's multiple range test.

**Table 3**
*In vitro* inhibitory effects of *Trichoderma* strains against *Neofusicoccum parvum* growth compared to the control after six days.

| | Percent growth inhibition (PI) by *Trichoderma* species | | | | | |
|---|---|---|---|---|---|---|
| *Trichoderma* species | Dual culture | A. act. | Non-volatile inhibitors | A. act. | Volatile inhibitors | A. act. |
| *T. asperellum* (T4) | 86b | 4 | 57.3b | 1 | 45.33b | 1 |
| *T. asperellum* (T5) | 86b | 4 | 57.8a | 2 | 44.3c | 1 |
| *T. longibrachiatum* (T3) | 88a | 4 | 57.6a | 2 | 56.3a | 2 |
| *T. longibrachiatum* (T31) | 88a | 4 | 54.6c | 2 | 56.4a | 2 |
| *T. atroviride* (T1) | 55.7d | 2 | 30.3d | 1 | 33.5d | 1 |
| *T. atroviride* (T11) | 58c | 2 | 27.6e | 1 | 31.7e | 1 |
| P | < 0.01 | | < 0.01 | | < 0.01 | |
| LSD | 1.20 | | 0.07 | | 0.06 | |

A. act. = Antagonistic activity, where 1 = low antagonistic activity (PI < 51), 2 = moderate antagonistic activity (PI = 51–60), 3 = high antagonistic activity (PI = 61–75), 4 = very high antagonistic activity (PI > 75), LSD = east significant difference. [abc]Values marked with the same letter do not differ significantly at P < 0.05 using Duncan's multiple range test.

reduction efficacy of the culture filtrates of *Trichoderma* strains against the test pathogens, the mycelial disc (5 mm) of each pathogen was inoculated in 20 ml of PDA medium at 45 °C containing 3 ml, 4 ml, and 5 ml of the filtrates in three replicates and incubated at 28 °C to allow the fungal pathogen growth (Tesfaye and Kapoor, 2004; Ghanbarzadeh et al., 2014). Plates with the tested fungal pathogen without culture filtrate were used as a control. After six days, the radial growth of the fungal pathogens with the filtrates and the control was evaluated (Ghanbarzadeh et al., 2014).

#### *2.3.3. The effect of volatile compounds of Trichoderma spp. on the growth of L. theobromae and N. parvum*

Growth inhibition of the fungal pathogens by volatile compounds released by *T. asperellum* strain (T4), *T. asperellum* strain (T5), *T. atroviride* strain (T1), *T. atroviride* strain (T11), *T. longibrachiatum* strain (T3), and *T. longibrachiatum* strain (T31) was evaluated using the inverted plate technique (Ghanbarzadeh et al., 2014; Meena et al., 2017). *Trichoderma* species and fungal pathogens were inoculated in a PDA medium to test the effects of volatile compounds released by *Trichoderma* species on the radial growth of fungal pathogens. The Petri plate inoculated with the pathogen was inverted over the plates containing antagonistic strain and sealed with adhesive tape (Parafilm; Sigma). The antagonist was kept in the lower Petri plate and the pathogen in the upper Petri plate. The experiment was performed in triplicate. In the control, plates containing the fungal pathogens were inverted onto a plate containing only PDA medium and incubated at 28 °C. After six days, the colony diameters of the pathogens were measured and compared with those of the control. Percent inhibition of mycelial growth of the tested pathogens was calculated.

## 3. Statistical data analysis

ANOVA was performed for the mean radial growth and mean percent inhibition data using SAS version 9. (SAS, 2002). Duncan's multiple range test was used to determine the mean separation at P = 0.05. The data in the figures represent the mean and standard deviation (SD).

## 4. Results

### *4.1. Eucalyptus diseases survey, symptoms, sampling, and identification*

According to the field assessment results, *E. camaldulensis* was commonly planted in the survey areas and was found severely affected with small and progressive canker disease symptoms on its trunk and branches. The ITS sequences of the fungal isolates were successfully amplified using the primers ITS1 and ITS2. The amplified ITS regions had an average size of 500 base pairs. The fungal isolates from the diseased samples were identified as *Neofusicoccum parvum* (E864005FR-001, E864005FR-004, and E864005FR-003) and *Lasiodiplodia theobromae* (C09–035 and C09–045) using the morphological and ITS sequence study (Wendu et al., 2022c)(Table 1).

#### *4.1.1. DNA Sequencing, phylogenetic analysis and Identification of the Fungi to species level*

In this study, the fungal sequences of the rDNA 5.8 S gene from MRC-Holland and CABI were compared to the sequence database in the World GenBank using the nucleotide BLAST search algorithm for each fungal isolate (Li et al., 2020). Comparisons of the ITS and 5.8 S gene sequences to sequences obtained from the NCBI databases showed that our *Lasiodiplodia sequences* (C09–035 and C09–045) (Table 1) were 90–99.8%

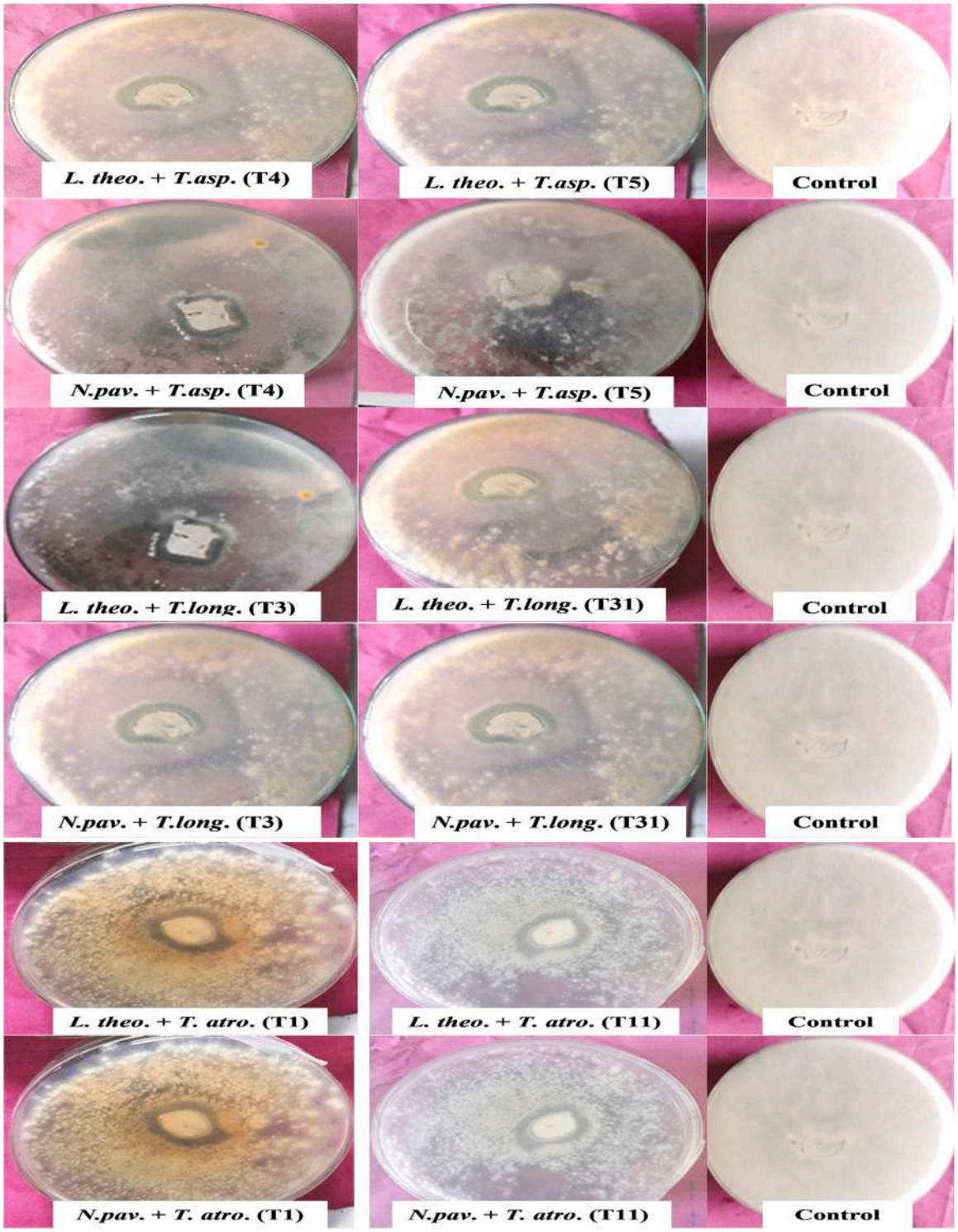


**Fig. 5.** *In vitro* growth inhibitory effects of volatile substances from *Trichoderma* species against fungal pathogens: *L. theo.* = *L. theobromae*, *N. pav.* = *N. parvum*, *T. asp.* = *T. asperellum*, *T. long.* = *T. longibrachiatum* and *T. atro.* = *T. atroviride*.

identical with the reference sequences of the *Lasiodiplodia theobromae* (Pat., Griffon and Maubl) isolates in GenBank, represented by accession numbers MT823467, MK530073, MK530016, KR709026 and KY657465 (Fig. 2). The phylogenetic tree from sequence analysis also revealed that *Lasiodiplodia* isolates in this study were strongly associated with a *Lasiodiplodia theobromae* (EF622075, CBS 112874) and *Lasiodiplodia theobromae* (EF622071, CBS 306.58) (Table 1) reference strain from GenBank, with supportive bootstrap values of 84% and 100% (Fig. 2). Based on the analysis of the ITS sequence data, the *Neofusicoccum* fungi in this study were found to have 100% sequence identity with the *Neofusicoccum parvum* (Pennycook and Samuels, Crous, Slippers and A.J.L. Phillips) strains identified by Sohrabi et al. (2020) and designated by the GenBank accession numbers MN634050 and MN634051 (Fig. 3). In the phylogenetic tree, the fungi were resided within *N. parvum* (MN634051, IRNBS51) *N. parvum* (MG019916, CS1–4) and *N. parvum* (MN121536, 3-bye-10) with bootstrap values of 52%, 81%, and 97% (Fig. 3).

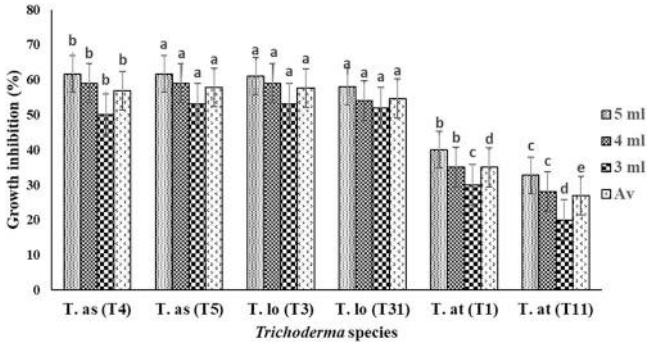


**Fig. 6.** *In vitro* growth inhibition activity of non-volatile culture filtrate of *Trichoderma* species at different concentrations on *L. theobromae*: T. as = *Trichoderma asperellum*, T. lo = *Trichoderma longibrachiatum*, T. at = *Trichoderma atroviride* and Av = Average. The results are the mean of three replicates. Different letters indicate that the values are significantly different from each other at $P < 0.05$ using Duncan's multiple range test.

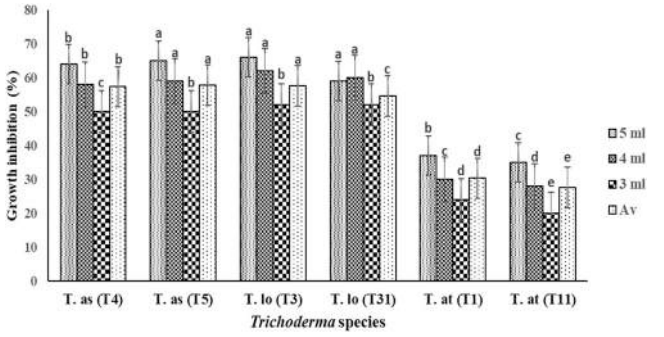


**Fig. 7.** *In vitro* growth inhibition of nonvolatile culture filtrate of *Trichoderma* species on *N. parvum* at different concentrations: T. as = *Trichoderma asperellum*, T. lo = *Trichoderma longibrachiatum*, T. at = *Trichoderma atroviride* and Av = Average . The results are the mean of three replicates. Different letters indicate that the values are significantly different from each other at $P < 0.05$ using Duncan's multiple range test.

In the Fig. 2 the best tree with the highest maximum likelihood is displayed. The phylogenetic tree of the sample sequences involved in this study consisting the isolates identified as Q203241039 and Q203241034 and the reference sequences obtained from the Genebank were used to generate phylogenetic tree. The tree was based on the 628 total characters, where 477 characters are constant, 87 characters are variable and 13 characters are parsimony informative. The average nucleotide composition in the tree was 522 base pairs (bp). The phylogenetic tree confirmed the morphologically identified *Lasiodiplodia* genus of Ethiopian isolates are closely resemble with *L. theobromae* that fell in two clades that have 80% and 100% Bootstrap support value. The phylogenetic tree displayed a consistency index (CI) value of 0.992 and retention index (RI) value of 0.983.

The phylogenetic tree of the Ethiopia isolates and those reference sequences obtained from GenBank demonstrated that the Ethiopian isolate are closely related with *Neofusicoccum* species. The tree was constructed using a total of 733 characters, where, 469 characters are constant, 61 characters are variable and 203 characters are parsimony informative. The average nucleotide composition in the tree was 542 base pairs (bp). The phylogenetic tree had CI value of 0.928 and RI value of 0.90.

#### *4.1.2. In vitro confrontation test using Trichoderma species against L. theobromae and N. parvum*

*In vitro* evaluation of the antagonistic activity of the *T. asperellum* strain (T4), the *T. asperellum* strain (T5), the *T. atroviride* strain (T1), the *T. atroviride* strain (T11), the *T. longibrachiatum* strain (T3) and the *T. longibrachiatum* strain (T31) showed that the species restricted the growth of the identified fungal pathogens (*L. theobromae* and *N. parvum*) and formed an inhibition zone at the point of contact (Fig. 4).

The results showed that *Trichoderma* species in this study significantly ($P < 0.01$) inhibited the mycelial growth of fungal pathogens after six days of incubation. *T. longibrachiatum* and *T. asperellum* had the highest inhibitory effects (88% and 86%) on both *L. theobromae* and *N. parvum* in dual culture. *T. atroviride* had the lowest inhibitory effect (58.5% and 56.85%) on both species (Tables 2 and 3).

#### *4.1.3. Effect of volatile compounds released by Trichoderma species on the growth of the fungal pathogen*

The results of the test for effect of volatile compounds released by *Trichoderma* species on the growth of fungal pathogens also showed a varied degree of inhibitory effect (Fig. 5). *T. asperellum* strain (T4), *T. asperellum* strain (T5), *T. atroviride* strain (T1), *T. atroviride* strain (T11), *T. longibrachiatum* strain (T3), *T. longibrachiatum* strain (T31) had the highest inhibitory effect on *L. theobromae* (44.3%, 55.85) and *N. parvum* (44.8%, 56.35) after six days, while *T. atroviride* had the lowest inhibitory effect (32.6%) (Tables 2 and 3).

#### *4.1.4. Effect of culture filtrates of Trichoderma species on mycelial growth of L. theobromae and N. parvum*

Figs. 6, 7, and 8 display the impact of culture filtrates from *T. asperellum* strain (T4), *T. asperellum* strain (T5), T. *atroviride* strain (T1), *T. atroviride* strain (T11), *T. longibrachiatum* strain (T3), and *T. longibrachiatum* strain (T31) on *L. theobromae* and *N. parvum*. The results demonstrate a significant inhibition in the growth of all fungal pathogens compared to the respective control. *T. asperellum* (T5) and *T. longibrachiatum* (T3) exhibited the greatest average growth inhibition (57.8% and 57.6%) against *L. theobromae* after six days of incubation, followed by *T. asperellum* strain (T4) (56.8%) and *T. longibrachiatum* strain (T31) (54.6%). The culture filtrates from *T. atroviride* strain (T11) and *T. atroviride* strain (T1) possessed the lowest average growth inhibition efficacy of 26% and 35%, respectively (Fig. 6). Similarly, the growth inhibition activity test of *Trichoderma* strains culture filtrate against *N. parvum* showed that *T. asperellum* (T4), *T. asperellum* (T5), and *T. longibrachiatum* (T3) strains exhibited the greatest average growth inhibition of 57.3%, 57.8%, and 57.6%, respectively, followed by *T. longibrachiatum* strains (T31) (54.6). *T. atroviride* (T1) and *T. atroviride* (T11) strains showed the lowest growth inhibition of 30.3% and 27.6%, respectively (Fig. 7).

## 5. Discussion

Based on the dual confrontation assay, *Trichoderma* species significantly inhibited the mycelial growth of *N. parvum* and *L. theobromae*, with inhibition potentials ranging from 59% to 88%. *Trichoderma* species showed varying degrees of inhibition in the dual culture antagonistic test, with *T. longibrachiatum*, showing the maximum growth inhibition followed by *T. asperellum,* while *Trichoderma viride* showed the lowest growth inhibition. According to Negash and Tesfaye (2010) and Meena et al. (2017), *T. viride* had the least inhibitory potential on mycelial growth of the fungal pathogens, ranging from 53.7% to 59% compared to *T. harzianum*, *T. longibrachiatum*, and *T. asperellum*. Ommati and Zaker (2014) and Mulatu et al. (2022a,2022b) also found that *T. longibrachiatum* and *T. asperellum* were effective in inhibiting the fungal growth under *in vitro* conditions, with varying degrees of efficacy ranging from 44.5% to 88.2%. In volatile metabolites inhibitory test, *T. longibrachiatum* and *T. asperellum* were the most effective in inhibiting the growth of *N. parvum* and *L. theobromae*, while *T. atroviride* was the least. *T. asperellum* demonstrated the highest inhibition potential in the non-volatile metabolites released followed by *T. longibrachiatum* and *T. atroviride*. Several studies involving volatile and nonvolatile metabolite tests using *Trichoderma* species have showed that *T. asperellum* and *T. longibrachiatum* differ in their ability to inhibit the growth of fungal

**Fig. 8.** *In vitro* growth inhibition effects of *Trichoderma* species culture filtrates against fungal pathogens: *L. theo.* = *L. theobromae*, *N. pav.* = *N. parvum*, *T. asp.* = *T. asperellum*, *T.long.*= *T. longibrachiatum* and *T. atro.* = *T. atroviride*.

pathogens, with volatile and nonvolatile substances released ranging from 30.67% to 54.22% and 29.97–58.67% respectively (Meena et al., 2017). Ommati and Zaker (2012) also found that *T. longibrachiatum* and *T. asperellum* were more effective in reducing the growth of *F. solani* using volatile and non-volatile inhibitor tests compared to the control. Ghanbarzadeh et al. (2014) also reported that T. *longibrachiatum*, *T. asperellum*, and *T. atroviride* are potential growth inhibitors against *Fusarium solani in vitro* using a non-volatile and volatile metabolite produced with efficacy ranging from 32.67% to 54.22% and 32.67–54.22%. Based on studies conducted by Bogumił et al. (2013), Ghanbarzadeh et al. (2014), and Karlii et al. (2021), *Trichoderma* species have the potential to act as bioagents against *Gibberella xylarioides*, *Fusarium solani*, and *Botrytis cinerea*, as demonstrated by antagonism, non-volatile, and volatile substance inhibition tests. Furthermore, Bhadra et al. (2015), Rusin et al. (2021) and Langa-Lomba et al. (2022) have also found that *Trichoderma* species can also be effective bioagents to control *Botryosphaeria* tree diseases caused by *N. parvum* and *L. theobromae* species. The growth inhibition in fungal pathogens in these

testes is due to the presence of inhibitory substances, such as enzymes and antimicrobial substances that are released by *Trichoderma* species and used for antagonism and mycoparasitism. As per the research conducted by Mohamed et al. (2022), *T. harzianum* produces 6-pentyl-α-pyrone, 1-hexadecanol, isopentyl acetate, dioctyl ester, hexanedioic acid, and 9-eicosane. On the other hand, *T. viride* produces cyclooctanol, propyl-benzene, cadinene, epizonaren, D-limonene, α-bisabolol, β-farnesene, and propanoic acid, which are active ingredients exhibiting antiphytopathogenic activity. Mulatu et al. (2022a, 2022b) demonstrated in their Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the extracts from *T. asperellum* that cis-13-Octadecenoic acid, 2,4-bis(1,1-dimethylethyl)-Phenol, n-hexadecanoic acid, hexadecanoic acid ethyl ester, and linoleic acid ethyl ester were present. They also detected cis-13-Octadecenoic acid, 2,4-bis (1,1-dimethylethyl)-Phenol, ethyl oleate, and n-hexadecanoic acid from *T. longibrachiatum*. Anita et al. (2012) also discovered the presence of several compounds in the secondary metabolites of *T. atroviride*, including N-phenylethylenediamine, phenol, 2-(2-benzoxazolyl), phthalic acid, diallylamin, 1,2,4,5-tetrazine, 3,6-bis(1-methylethyl), propanal, 2-methyl-oxime, N-(2-propynyl)− 2,2-dimethylaziridine, phthalic acid, diallylamine, and 1,2,4,5-tetrazine 3,6-bis (1-methylethyl). They also reported the presence of extracellular enzymes such as amylase, cellulase, polygalacturonase, protease, and chitinase in the secondary metabolites.

## 6. Conclusion

The study conducted with *Trichoderma* species revealed that *T. longibrachiatum* and *T. asperellum* have the potential to be utilized as biocontrol agents for managing stem canker disease caused by *N. parvum* and *L. theobromae* in Ethiopia. Additionally, this study provided evidence that *Trichoderma* species can effectively control *Eucalyptus* canker disease. This is the first study to use *Trichoderma* species from Ethiopia in the management of *Eucalyptus* stem canker disease.

## 7. Recommendation

The *T. longibrachiatum* and *T. asperellum* tested in this study were the most promising bioagents for the control of *Eucalyptus* canker diseases caused by *N. parvum* and *L. theobromae*. However, these *Trichoderma* species have only been tested under laboratory conditions. We recommend similar studies be conducted in the field (*in vivo)* to determine the effectiveness of these *Trichoderma* spp.

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## CRediT authorship contribution statement

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by [Wendu Admasu], [Zewdu Terefework], and [Alemu Gezahgne]. The first draft of the manuscript was written by [Wendu Admasu] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Declaration of Competing Interest

We declared that we do not have any conflict of interest.

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