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Research Article

IN VITRO EVALUATION OF FUNGICIDES AGAINST EUCALYPTUS STEM CANKER PATHOGENS IN ETHIOPIA

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ABSTRACT

Field surveys of *Eucalyptus camaldulensis* diseases were conducted during 2019-20 and 2020-21 in Ethiopia. This species was the most commonly planted in the surveyed fields and exhibited stem canker diseases. Sampling was performed from diseased stems and branches displaying clear symptoms. Morphological and PCR assays of the ITS1 and ITS2 loci were conducted to identify the fungal pathogens. *Lasiodiplodia theobromae* and *Didymella pinodella* were identified as the fungal pathogens causing *E. camaldulensis* stem canker diseases. Four nationally licensed fungicides viz. Vitra (copper hydroxide), Ridomil Gold (metalaxyl-M + mancozeb), Liveshow (pyraclostrobin + epoxiconazole), and Sabozeb (mancozeb) were evaluated for their *in vitro* control efficacy against the identified fungi at different concentrations (500, 1000, 1500, and 2000 ppm). Vitra and Liveshow were found to be the most effective fungicides at 2000 ppm for the control and treatment of *E. camaldulensis* stem canker diseases. The identification of *L. theobromae* and *D. pinodella* as causal agents of *E. camaldulensis* is crucial for targeted disease management strategies. The evaluation of four fungicides at different concentrations provided valuable information for selecting the most effective treatment options for practical management practices of the diseases. This research serves as the first report on the management of *Eucalyptus* disease in Ethiopia, laying the foundation for future studies and contributing to the development of sustainable disease management practices. The findings of this research can guide policymakers, researchers, and agricultural practitioners in implementing targeted fungicide applications to mitigate the impact of stem canker on *Eucalyptus* plantations and improve their productivity.

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INTRODUCTION

Eucalyptus is one of the most widely planted forestry species worldwide due to its fast growth, wood quality, economic value, and contributions to energy and ecosystem services (Li et al., 2018). The most commonly planted *Eucalyptus* species in Ethiopia include *E. globulus*, *E. camaldulensis*, *E. saligna*, *E. grandis*, and *E. citriodora* (Gessesse and Teklu, 2011). In Ethiopia, Eucalyptus trees are cultivated extensively for construction, industrial raw materials, agroforestry, fuel, and as a source of income (Lemenih and Kassa, 2014).

However, the cultivation and productivity of *Eucalyptus* plantations have declined due to the occurrence of fungal diseases causing stem canker and reduced wood quality, rendering it unsuitable for construction and industry (Cieslae et al., 2010). The primary fungal genera responsible for *Eucalyptus* stem canker disease are *Teratosphaeria*, *Botryosphaeria* (*Neofusicoccum* and *Lasiodiplodia*), and more recently, *Didymella* (Alemu et al., 2003; Alemu et al., 2005; Chen et al., 2017; Aylward et al., 2019).

Lasiodiplodia theobromae is an opportunistic pathogen causing canker and dieback diseases in more than 500 tree species in the tropics and subtropics (Sulaiman et al., 2012). It has been associated with stem canker, gummosis, and dieback diseases in *Eucalyptus*, *Prunus*, and *Pinus* species (Slippers and Wingfield, 2007; Yu et al., 2009; Chen et al., 2011; Phillips et al., 2013). In Ethiopia, *L. theobromae* has also been linked to stem canker diseases in *Boswellia papyrifera* and *Araucaria heterophylla* (Alemu et al., 2017; Wendu, 2017).

According to Chen et al. (2015) and Barilli et al. (2016), the genus *Didymella* is widely distributed in field and ornamental plants, occurring as a saprophyte, endophyte, or pathogen, causing blight, dieback, and necrosis. Aveskamp et al. (2009) also associated this fungal genus with leaf spot disease in *Eucalyptus* plants. In the Ethiopian context, *Didymella coffeae-arabicae* has been identified and reported in *Coffea arabica* by Vu et al. (2019).

Effective management of plant diseases typically involves the use of one or a combination of approaches, including effective quarantine measures, resistant cultivars, improved cultural practices, and biological control methods (Nelson, 2008; Mukhtar et al., 2023). Establishing new quarantine strategies, such as understanding the pathways by which pests and pathogens move globally, helps reduce the likelihood of accidental introductions into new areas (Wingfield et al., 2013).

According to Bruggen et al. (2015), Kumari et al. (2020), and McLaughlin et al. (2023), agronomic practices such as site-species matching, appropriate planting densities and spacing, sanitation, tillage, proper watering, fertilization, mulching, pruning, and thinning can significantly reduce the vulnerability of plants to fungal pathogens. These practices disrupt the pathogen life cycle, minimize plant stress, and improve the removal of inoculum sources from the field.

Biological management of tree diseases is an environmentally sound and effective method for reducing or mitigating pests and their effects using natural enemies instead of chemical treatments (Singh, 2014). This approach involves antagonistic microorganisms, such as *Trichoderma* species, mixed with other potential bioagents. These organisms exhibit active hyperparasitism, produce lytic enzymes, antimicrobial substances, and other secondary metabolites that inhibit the growth of disease-causing fungal pathogens (Chanchaichaovivat et al., 2007; Langa-Lomba et al., 2022).

Protective fungicides such as mancozeb, copper oxychloride, systemic fungicides, triadimenol, and triforine are recommended for managing *Eucalyptus* canker and leaf spot diseases (Masson et al., 2013; Iqbal and Mukhtar, 2020). Changes in the global climate are currently exacerbating serious plant diseases by selecting for fungal pathogens and inducing stress that makes host plants more susceptible to diseases. In Ethiopia, no research has been conducted on managing diseases that threaten the health and productivity of *Eucalyptus*.

The objective of this research was to evaluate the *in vitro* efficacy of commercially available fungicides against fungal species causing *Eucalyptus* stem canker diseases in Ethiopia. Assessing and monitoring *Eucalyptus* diseases and identifying the causal fungal pathogens are crucial for effective prevention, control, and the design of alternative disease management strategies. Evaluating and screening the most effective commercial fungicides is also important for selecting appropriate treatments and providing immediate control of *Eucalyptus* diseases. Research on fungicide evaluation against economically significant fungal diseases contributes to developing sustainable disease management practices in *Eucalyptus* plantations, supporting the long-term health and productivity of the species.

The current research serves as the first report on managing *Eucalyptus* disease in Ethiopia, filling a knowledge gap, laying the groundwork for future studies, and contributing to sustainable disease management practices. The findings of this research can guide policymakers, researchers, and agricultural practitioners by providing evidence-based recommendations for effective fungicides to mitigate the impact of stem canker on *Eucalyptus* plantations and enhance their productivity.

MATERIALS AND METHODS

Eucalyptus disease survey and sample collection

Field surveys on *Eucalyptus* diseases were conducted during the 2019-20 and 2020-21 seasons in the regional states of Amhara (35°46'E to 40°25'E longitude, 8°45'N to 13°45'N latitude), Oromia (34°E to 43°E longitude, 4⅔°N to 10⅔°N latitude), and the Southern Nations, Nationalities, and Peoples' Region (SNNP) (36°43'E to 38°28'E longitude, 6°03'N to 31°03'N latitude) in Ethiopia. In each region, three zones representing three agroecologies (highland, midland, and lowland) were purposely selected. These zones included the commonly growing *Eucalyptus* species in the districts of Eferatana Gidem, Dewa Cheffa, Guba Lafto, Bahir Dar Zuria, Habru, and Fogera in the Amhara region; Arba Minch Zuria, Abeshge, Humbo, Analemmo, and Masken in the SNNP region; and Omo Nada, Sekoru, Bedele Zuria, and Gumay in the Oromia region (Figure 1).

In each zone, at least one district was selected per agroecology, and within each district, at least one kebele representing a locality was chosen. From each locality, two plantation sites 3-5 km apart were randomly selected and assessed for disease incidence, severity, and sample collection. The plantation sites were selected based on their location, road access, production, and history of disease incidence and severity. Surveys and sampling were carried out at nine locations across the regions. For disease evaluation, ten *Eucalyptus* trees were randomly selected from each plantation site. The trees were assessed for disease symptoms, and samples showing symptoms of stem and branch cracking, cankers, and tip diebacks were collected from each *Eucalyptus* tree as described by Li et al. (2018).

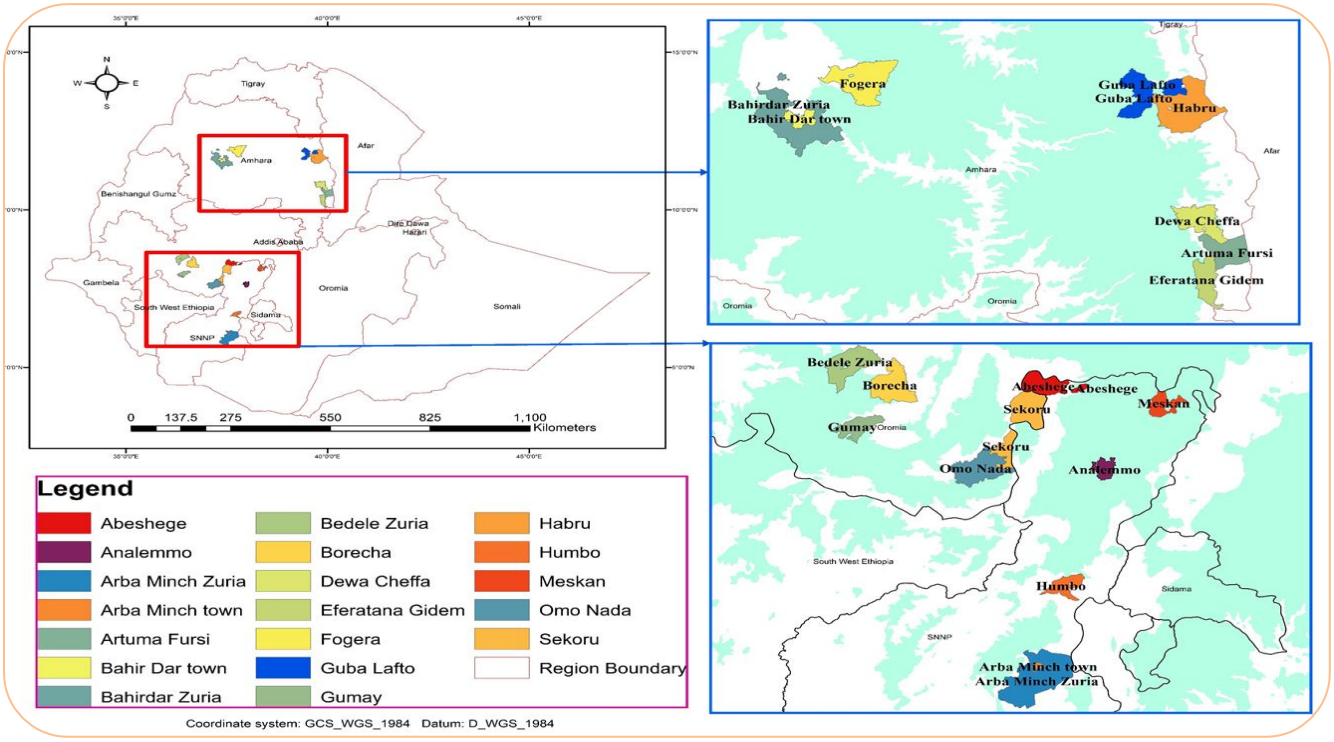

Figure 1. Map of Ethiopia showing study locations and nearby areas where *Eucalyptus* canker disease samples were collected.

Isolation, characterization, and identification of fungi used in the study

To isolate and purify fungal cultures, diseased segments from cankered stems and branches of *Eucalyptus* were sterilized in 70% ethyl alcohol for 1 minute and then in 2% sodium hypochlorite for 2 to 4 minutes. These segments were washed three times with sterile distilled water, dried on sterile filter paper, and aseptically transferred to malt extract agar (MEA), where they were kept at room temperature (25-27°C) for 5 to 15 days (Ahmadpour et al., 2017). Stems and branches with clear cankers were surface sterilized and incubated in moist

chambers to induce the growth of fruiting bodies. Once mature, fruiting body samples were crushed and inoculated in MEA for culture growth and mounted in distilled water to examine the structures of conidiomata, ascospores, and conidia under a microscope (Chen et al., 2015; Li et al., 2018). The hyphal tip transfer method was used to subculture and obtain pure isolates for morphological studies. In cultures, characteristics such as colony diameter, color, texture, concentric zones, fruiting body shape, conidia shape, septation, pigmentation, and size were studied (Alemu et al., 2003; Aveskamp et al., 2010; Silva et al., 2020). All pure fungal isolates were classified based on morphology and identified at the species level using ITS rDNA sequence analysis (Li et al., 2020).

DNA isolation and amplification

DNA extraction was performed on seven-to-ten-day-old fungal cultures as described by Li et al. (2020). The harvested fungal mycelia were ground in liquid nitrogen and lysed with a buffer containing phenol, chloroform, and isoamyl alcohol to obtain DNA fragments. The DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer. To assess the quality of the DNA, electrophoresis was performed on a 1% agarose gel (White et al., 1990). The internally transcribed spacer (ITS) region of the DNA was amplified using the ITS1 and ITS2 primers, as described by White et al. (1990) and Raja et al. (2017).

Sequencing and identification

The PCR product was sequenced at the Netherlands Microbiology Research Centre (MRC) and the Center for Agriculture and Bioscience International (CABI), United Kingdom, using forward and reverse primers as described by White et al. (1990). The ITS sequences obtained were trimmed using BioEdit v. 7.0.9.0 (Hall, 1999), and alignments for each sequence were determined by performing online searches using the Basic Local Alignment Search Tool (BLAST) in the NCBI database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA) for identification.

Evaluation of fungicides against mycelial growth of *Lasiodiplodia theobromae* and *Didymella pinodella*

Four nationally licensed and commercially available fungicides, Vitra (contact), Ridomil Gold (systemic), Liveshow (systemic), and Sabozeb (contact), were selected for the mycelial inhibition test against *L. theobromae* and *D. pinodella* fungal species under *in vitro* conditions (Table 1). The fungicides were tested against the pathogens using the poisoned food technique (Pitt et al., 2012).

Stock solutions of each fungicide were prepared using sterile distilled water to obtain final concentrations of 500, 1000, 1500, and 2000 ppm as described by Rai and Mamatha (2005) and Ahmad and Ashraf (2016). These solutions were added to the MEA medium, and 20 ml of fungicide-amended media was poured into separate Petri dishes (9 cm in diameter). All Petri dishes were inoculated with a 5 mm mycelial-agar disc taken from the margin of 10-day-old cultures of *L. theobromae* and *D. pinodella*, incubated at 25°C. MEA media without fungicides were used as a control. All treatments had three replications, and the experimental design was a completely randomized design (CRD) consisting of five treatments, including a control, repeated three times.

The colonies were incubated at 25°C for 10 to 14 days. The radial diameters of the fungal cultures were measured twice at right angles, and the mean values were calculated. These values were then compared with the radial growth of the fungus in the control plates at 10 and 14 days for *L. theobromae* and *D. pinodella*, respectively, using the procedures described by Mamza et al. (2010) and Asman and Rosmana (2021).

$$D = \frac{d1 + d}{2}$$

Where: D = Diameter of fungi mycelia grown

d1 = Vertical diameter of the mycelia grown

d2 = Horizontal diameter of mycelial grown

The relative reduction in mycelial growth for each fungicide was determined compared to the control using the following formula (Ahmad and Ashraf, 2016):

$$PI = \frac{C - T}{C} \times 100$$

Where: PI = Percent inhibition of fungal mycelia

C = Colony diameter in the control

T = Colony diameter in treatment (mm)

Statistical analysis

SAS 9.0 software (SAS Institute, 2002) was utilized to analyze the data from the study. The statistical analysis involved comparing the efficacy of various fungicides at different concentrations against fungal isolates using appropriate tests, such as analysis of variance (ANOVA), to determine the differences in efficacy among the fungicides *in vitro*. Duncan's multiple range tests were employed to separate means that differed significantly at the 0.05 probability level. All graphs were created using GraphPad Prism (version 8).

Table 1. Fungicides used for *in vitro* screening against the fungal isolates.

Trade name	Active ingredient	Formulation	Concentration (%)	Manufacturer
Vitra	Copper Hydroxide	WP	50	Industrias Quimicas, Barcelona-Spain
Ridomil gold	Metalaxyl-M + Mancozeb	WP	4: 64	Syngenta Crop Protection AG, Basle-Switzerland
Liveshow	Pyraclostrobin + Epoxiconazole	SE	27.4: 4.7	Eastchem co., Ltd, Jiangsu-China
Sabozeb	Mancozeb	WP	80	Coromandel International Ltd., Telangana-India

RESULTS

Eucalyptus disease survey: sample collection and identification

Based on morphological and electrophoretic analyses of ITS, the fungal isolates found in the *Eucalyptus camaldulensis* samples were identified as *Lasiodiplodia theobromae* and *Didymella pinodella*.

In vitro studies revealed that fungicides had inhibitory effects on the mycelial growth of *L. theobromae* and *D. pinodella*. The inhibitory effects increased with concentration, and the efficacy of the fungicides varied significantly against the fungal isolates.

Evaluation of fungicide effects on *L. theobromae*

Four fungicides were evaluated for their ability to inhibit *L. theobromae*. The effects of these fungicides on fungal mycelium growth are illustrated in Figures 2 and 3 Results indicated that all tested fungicides significantly inhibited the mycelial growth of *L. theobromae* ($P < 0.05$) compared to the control over a period of 10 days (Table 2). Among the four fungicides, Liveshow (Pyraclostrobin + Epoxiconazole) and Vitra (Copper Hydroxide) were particularly effective, showing significant inhibition at all treatment concentrations compared to the control. The treatment reduced mycelial growth to 0.3 mm with a maximum efficacy of 99.6% on MEA agar (Figure 3. Specifically, Liveshow and Vitra reduced the radial growth of *L. theobromae* to 15-0.3 mm and 20.4-0.3 mm, respectively, with efficacies ranging from 83% to 99.6% and 77% to 99.6%, respectively (Figure 3. The results confirm that Liveshow and Vitra are the most effective fungicides at concentrations of 1000, 1500, and 2000 ppm, with 2000 ppm being the optimal concentration for maximum fungal growth inhibition (Table 2).

Ridomil Gold and Sabozeb (Metalaxyl-M + Mancozeb) inhibited the mycelial growth of *L. theobromae* with efficacies ranging from 66% to 88% and 54.5% to 79.8%, respectively. The efficacy of Sabozeb (Mancozeb) in inhibiting the fungus was relatively lower compared to the other treatments, with a maximum efficacy of 79.8% at a concentration of 2000 ppm. Overall, the inhibition of mycelial growth by Ridomil Gold and Sabozeb was lower compared to other fungicides but improved with higher concentrations.

Table 2. Effect of fungicides on *L. theobromae* mycelial growth at different concentrations.

Fungicide	*Lasiodiplodia theobromae* mycelium growth (mm)			
	Fungicides concentration (ppm)			
	500	1000	1500	2000
Liveshow	15d	3d	1.3c	0.3c
Ridomil gold	30.4b	20.6b	10.8b	10.5b
Vitra	20.4c	10.6c	1.3c	0.3c
Sabozeb	40.6a	40.9a	30.7a	16.3a
Control	90	90	90	80.8
P	< 0.0001**	< 0.0001**	< 0.0001**	<0.0001**
LSD (P=0.05)	1.0226	1.1594	0.1719	0.1719

The mean values followed by different letters differ significantly at P=0.05.

** Highly significant.

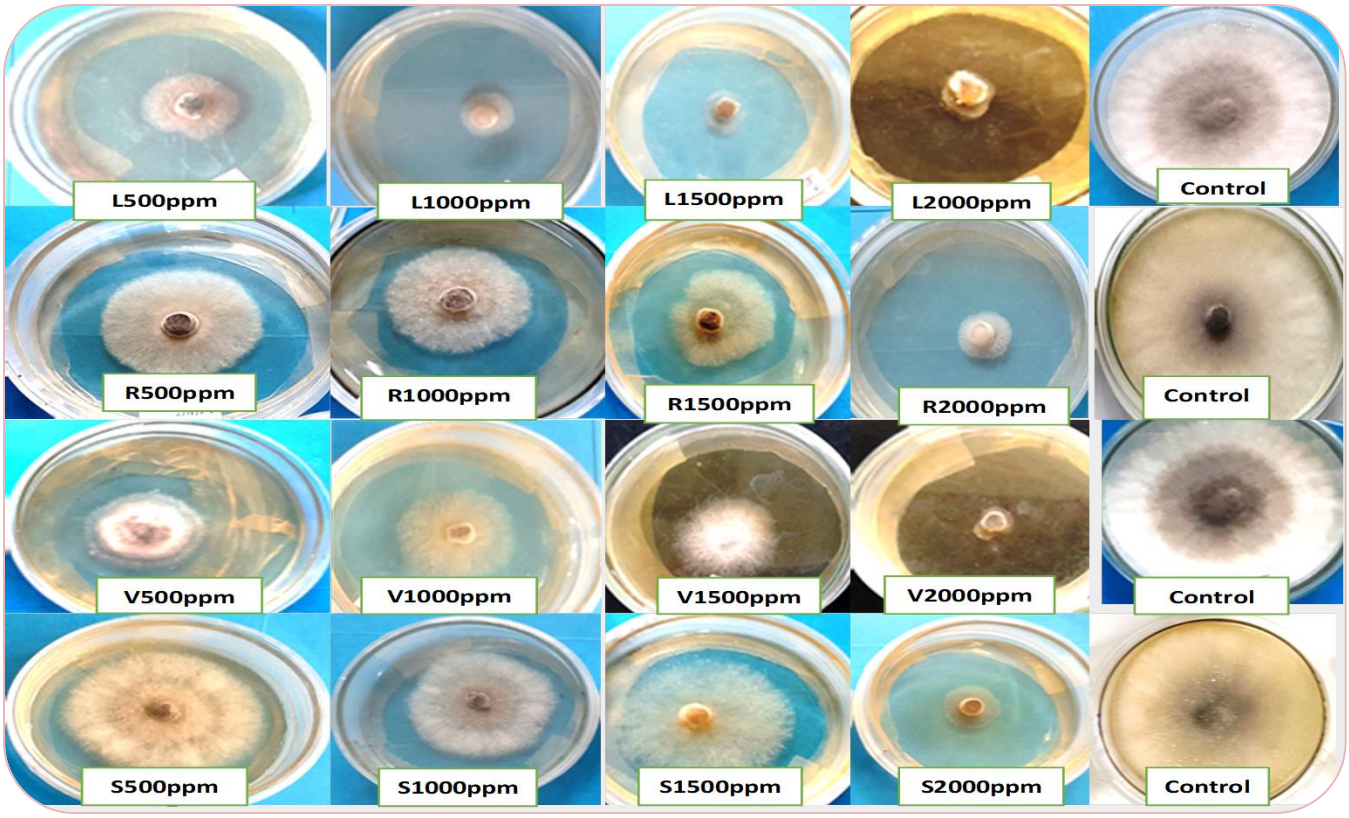

Figure 2. *In vitro* effect of fungicides at different concentrations on the mycelial growth of *L. theobromae*, where L = Liveshow, R = Ridomil gold, V = Vitra, and S = Sabozeb.

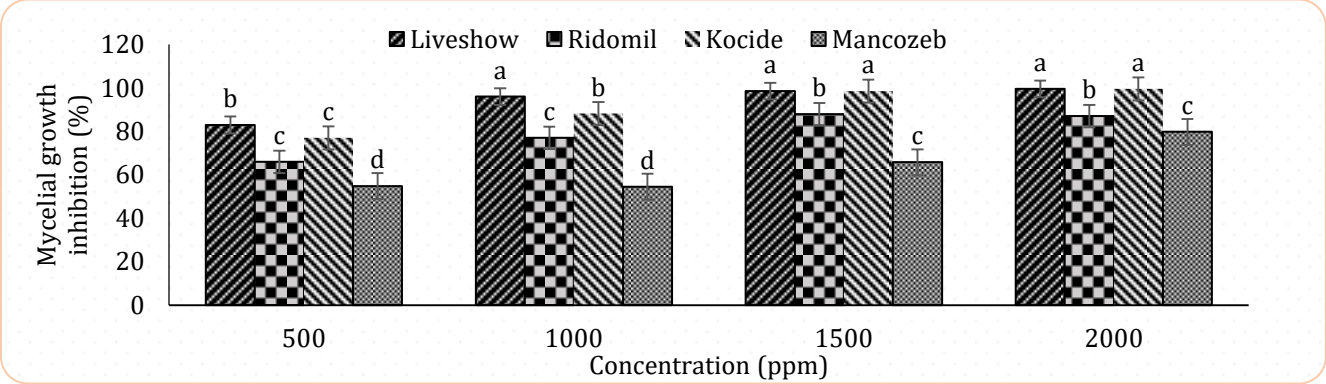

Figure 3. *In vitro* inhibitory efficacies of tested fungicides at different concentrations on mycelial growth of *L. theobromae*. The mean values on the graph followed by different letters differ significantly at P=0.05.

Evaluation of the effects of fungicides on the mycelial growth of *D. pinodella*

An experimental trial was conducted to assess the inhibitory effects of four fungicides on *D. pinodella* fungus *in vitro*. The mean inhibition zones of the fungicides after 14 days are presented in Table 3. The effectiveness of the fungicides on *D. pinodella* mycelial growth at various concentrations is illustrated in Figures 4 and 5 Among the four fungicides, Liveshow (Pyraclostrobin + Epoxiconazole) and Vitra (Copper Hydroxide) significantly ($P < 0.05$) inhibited the growth of fungal mycelium at all treatment concentrations compared with the control. Vitra exhibited the highest mycelial growth inhibition efficacy, achieving 99.6%, whereas Sabozeb showed the lowest efficacy at 82% at the 2000 ppm

concentration. The efficacy of Liveshow ranged from 92.5% to 97.1%, while the efficacy of Vitra ranged from 92.5% to 99.6%. Both Vitra and Liveshow achieved the maximum radial growth reduction (0.3 mm) at a 2000 ppm concentration, while Sabozeb showed the least reduction (10.33 mm) at the same concentration. These results indicate that while the fungicides are effective against the fungus at concentrations of 1000 and 1500 ppm, they are most effective at a concentration of 2000 ppm (Table 3). Ridomil Gold (Metalaxyl-M + Mancozeb) and Sabozeb restricted the radial growth of *D. pinodella* to an average maximum of 10.3 mm, achieving an efficacy of 82% at a concentration of 2000 ppm. This finding indicates that while Ridomil Gold and Sabozeb increase in growth inhibition with higher concentrations, they are less effective at all concentrations compared to Liveshow and Vitra.

Table 3. Effect of fungicides on the growth of *Didymella pinodella* mycelial growth at different concentrations.

Fungicides	*Didymella pinodella* mycelial growth (mm)			
	Fungicide Concentration (ppm)			
	500	1000	1500	2000
Liveshow	6c	3c	2.3c	0.3c
Ridomil gold	20.6b	18.3b	10.3b	10.4b
Vitra	6c	3.4c	1d	0.3c
Sabozeb	20b	19.6b	10.43b	10.33b
Control	60.5a	60.5a	60a	60.6a
P	<0.0001**	<0.0001**	<0.0001**	<0.0001**
LSD (P=0.05)	0.32	0.14	0.18	0.17

The mean values followed by different letters differ significantly at P=0.05.
** Highly significant.

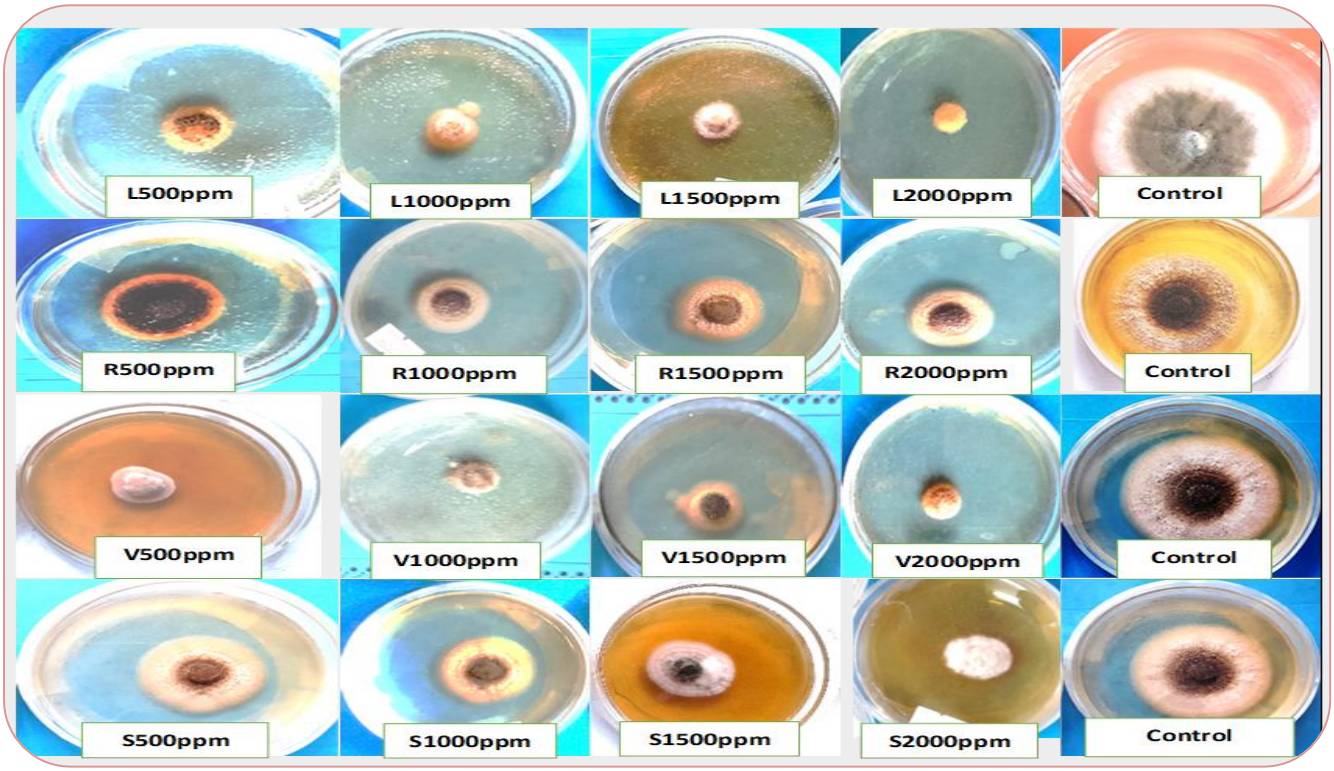

Figure 4. *In vitro* effect of fungicides at different concentrations on the mycelial growth of *D. pinodella*, where L = Liveshow, R = Ridomil gold, V = Vitra, and S = Sabozeb.

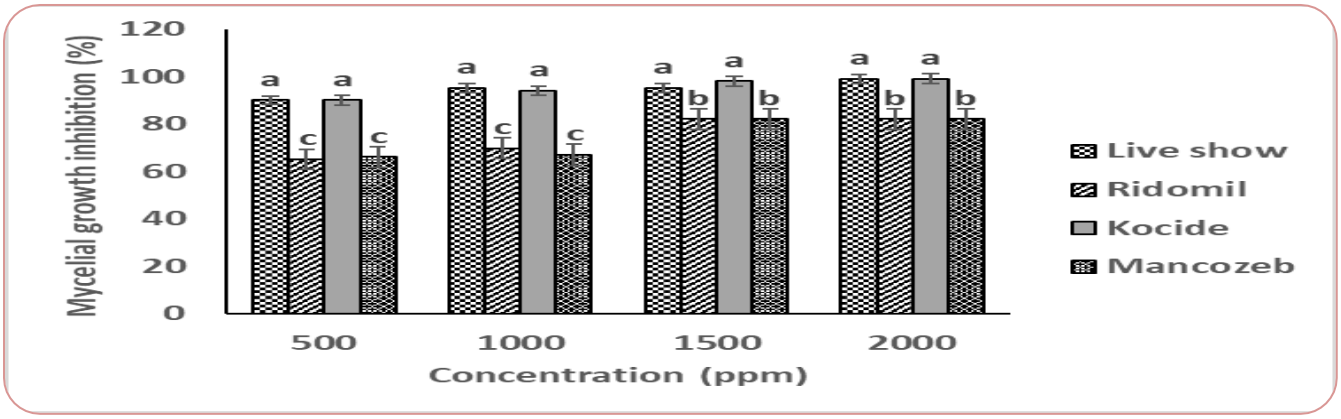

Figure 5. *In vitro* inhibitory efficacies of tested fungicides at different concentrations on mycelial growth of *D. pinodella*. The mean values on the graph followed by different letters differ significantly at P=0.05.

DISCUSSION

The results of the study indicated that the mycelial reductions caused by the fungicides vary with their concentrations. Among systemic fungicides, Liveshow (Pyraclostrobin + Epoxiconazole) was the most effective at a concentration of 2000 ppm, achieving 99.6% efficacy in reducing the mycelial growth of both studied fungi followed by Ridomil Gold (Metalaxyl-M + Mancozeb). For contact fungicides, Vitra (Copper Hydroxide) was found to be as effective as Liveshow at a concentration of 2000 ppm in inhibiting fungal mycelial growth, followed by Sabozeb (Mancozeb).

Studies show that factors such as fungicide concentration, overuse, and the genetics of fungal pathogens can lead to changes in the efficacy of mycelial inhibition by fungicides *in vitro* (Lurwanu et al., 2020). According to Dias (2012), contact fungicides are protectants with multisite inhibitors, while systemic fungicides can act as protectants, eradicants, or both.

Sarkar et al. (2010) found that contact fungicides such as copper oxychloride and Vitra (copper hydroxide) were effective in reducing fungal growth that causes tea black rot disease under *in vitro* conditions. Vitra is characterized by a high capacity for penetration and preventive action, inhibiting germination and blocking the growth and development of fungal spores in plants. Copper ions released from the compound are not easily washed away by rain, as they are relatively water-insoluble, and they inhibit fungal growth by disrupting enzymatic systems (Leta et al., 2023).

Pitt et al. (2012), Twizeyimana et al. (2013), and Olmo et al. (2017) considered systemic fungicides such as Pyraclostrobin to be the most effective in inhibiting the mycelium growth of fungal pathogens *in vitro*. Rehman et al. (2015) and Suresh et al. (2016) demonstrated that diethofencarb + thiophanate-methyl, carbendazim, difenoconazole, thiophanate-methyl, carbendazim + mancozeb, and propiconazole were the best fungicides against *L. theobromae in vitro*. According to Sahi et al. (2012), contact fungicides such as Mancozeb and copper oxychloride are the least effective in inhibiting the mycelial growth of fungal species such as *L. theobromae* compared to other fungicides at different concentrations. However, in this study, Vitra (Copper Hydroxide) was found to be the most effective. Fungicides such as tebuconazole, boscalid, iprodione, carbendazim, and fludioxonil are effective in controlling *D. pinodella* diseases (Liu et al., 2016). The differences in efficacy among the fungicides in this study can be attributed to variations in the concentrations of the active ingredients in the commercial fungicides.

These results suggest that the fungicides evaluated in this study can effectively manage *Eucalyptus* stem canker disease caused by *L. theobromae* and *D. pinodella*.

CONCLUSION

The present study demonstrates that the fungicides Liveshow (Pyraclostrobin + Epoxiconazole) and Vitra (Copper Hydroxide) are highly effective in inhibiting the mycelial growth of the fungi *Lasiodiplodia theobromae* and *Didymella pinodella*. Conversely, the fungicides Ridomil Gold (Metalaxyl-M + Mancozeb) and Sabozeb (Mancozeb) exhibited lower efficacy in reducing the mycelial growth of these fungal isolates *in vitro*. Screening fungicides *in vitro* allows for the identification of effective treatments for use in plant disease

management. To the best of our knowledge, this is the first study to address the management of *Eucalyptus* stem canker disease in Ethiopia.

RECOMMENDATION

Based on the findings, Liveshow (Pyraclostrobin + Epoxiconazole) and Vitra (Copper Hydroxide) were recommended for field trials, treatment, and control of *Eucalyptus* stem canker disease caused by *Lasiodiplodia theobromae* and *Didymella pinodella*. However, it is important to note that the tests were conducted under laboratory conditions. Similar research was suggested to be conducted under field conditions to further assess the effectiveness of these fungicides.

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AUTHORS' CONTRIBUTIONS

WA, AS, and AG designed, formulated and laid out the study; WA, AS, and AG conducted the experiments; WA collected, arranged and analyzed the data; AG provided technical assistance; AS supervised the work; WA and AG wrote the manuscript; AS proofread the paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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