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First report of inhibitory abilities of dark septate endophytic fungi against white root rot disease on *Hevea brasiliensis* seedlings in nursery conditions

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Abstract

Background The dark septate endophytes (DSE) are endophytic and non-mycorrhizal fungi with the ability to impact and control some plant pathogens and promote plant growth. The aim of this study was to investigate the effectiveness of five different DSE fungal isolates in controlling white root rot disease (WRRD) caused by the plant pathogen, *Rigidoporus microporus* in *Hevea brasiliensis* in a nursery system. There are no previous reports on the role of DSE in controlling WRRD. In this study, the efficacy of five DSE isolates, including *Acrocalymma vagum* SBTBMDS 1, *Clonostachys chloroleuca* TMDS 2.1, *Lasiodiplodia theobromae* APDS 3.2, *Penicillium oxalicum* TMDS 3.2, and *Fusarium falciforme* TBMDS 2.4b, was tested for their ability to reduce the severity of WRRD in vivo.

Results The results showed that all the DSE isolates were able to inhibit *R. microporus*, leading to a decrease in the disease severity of WRRD, with percentages ranging from 7.50 to 17.5% and percentage of disease inhibition from 57.67 to 83.33% than the controls, which had a severity percentage of 45%. The TMDS3.2 isolate showed high efficacy in increasing the girth (137%) and height (63.3%) of *H. brasiliensis* seedlings, while the isolates of *P. oxalicum* TMDS 3.2 and *F. falciforme* TMDS 2.4b enhanced the dry weight (123 and 122%, respectively) than the control. Two isolates of *P. oxacilum* TMDS 3.2 and *C. chloroleuca* TMDS 2.1 increased the root volume (120 and 107%, respectively) than the control. Gas chromatography–mass spectrometry (GC–MS) analysis showed the ability of three selected DSE isolates, *L. theobromae* APDS 3.2, *F. falciforme* TBMDS 2.4b, and *P. oxacilum* TMDS 3.2, to produce acetic acid butyl methyl-phosphinoylmethyl ester and ethanone, 1-(4-methyl-1H(imidazol-2-yl)—that previously reported as antimicrobials.

Conclusion This study demonstrated that DSE fungal isolates had the potential to act as a biocontrol agent against *R. microporus* in *H. Brasiliensis* seedlings. In the future, the findings of this study could be utilized to prevent WRRD, one of the most serious problems in rubber plantations, in an environmentally friendly way by reducing the usage of fungicides.

Keywords Metabolite compounds, Microbial biocontrol agent, Non-mycorrhizal fungi, Plant defence, White root rot disease

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Background

White root rot disease (WRRD) is a significant issue in tropical rubber plantations such as those in Indonesia, which has been extensively studied by researchers (Hardiyanti et al. 2017). *Rigidoporus microporus* (Sw.) Overeem (syn. *Rigidoporus lignosus* (Klotzsch) Imazeki) is the causative agent of this disease (Sakpetch et al. 2018), and it can infect rubber trees at any stage of growth, including the nursery, immature, and mature stages (Amaria et al. 2013). Symptoms of WRRD include yellowing leaves, inward folding of the edges or tips, falling off of leaves, and the death of branch tips (Kaewchai and Soytong 2010). Rhizomorphs, which are white and slightly thick mycelial threads, can be found on the roots of the infected plants, as well as on young leaves, flowers, and fruit (Berlian et al. 2013). This disease is a significant economic burden for smallholder plantations in Indonesia, with potential costs ranging from hundreds to thousands of dollars per hectare (Rahayu et al. 2017).

While hexaconazole, triadimenol, and triadimefon fungicide can control WRRD, they are expensive and environmentally hazard, which can negatively affect farmers and planters (Soytong et al. 2005). Raising awareness of this issue can promote the use of environmentally friendly methods and materials for long-term plant disease management (Ogbebor et al. 2014). *Trichoderma* spp. have long been used as a biological agent to control white root rot disease in Indonesian rubber plantation areas due to their bio-fungicidal potential (Fairuzah et al. 2014). However, there is a little information in Indonesia about the use of other fungi as biocontrol agents against *R. microporus*.

Biological control of white root rot disease (WRRD) is possible by using various biological agents that are effective in lowering costs and addressing environmental and health concerns (Kaewchai and Soytong 2010). However, research on the use of these agents is limited, with only a few studies investigating the effectiveness of antagonistic microbes against *R. microporus* (Nakaew et al. 2015). In Indonesia, *Trichoderma* spp. are one of the fungal biocontrol agents (FBAs) that have been used as bio fungicides in rubber plantations at the commercial level (Fairuzah et al. 2014), while endophytic fungi like *Eupenicillium javanicum*, *Penicillium simplicissimum*, *P. citrinum*, and *Hypocrea* sp. can also help for controlling WRRD (Amaria et al. 2013). Some fungi, such as *Aspergillus niger*, *Chaetomium bostrychodes*, *C. cupreum*, *T. hamatum*, and *T. harzianum*, have been shown to inhibit *R. microporus* development by over 50% (Kaewchai and Soytong 2010).

Interestingly, there is still a need to isolate and select endophytic fungi as biological agents from natural environments to discover new species that have not previously been reported (Rodriguez et al. 2009). Such studies could also investigate the production of bioactive compounds that have the potential to be used as biocontrol agents for pathogens (Sakpetch et al. 2018). Dark septate endophytes (DSE), a group of endophytic fungi, have been reported to promote plant growth under abiotic and biotic stress conditions (Surono and Narisawa 2018). DSE fungi have dark colonies on agar media, dark melanin hyphae, form microsclerotia, and can colonize both inter- and intracellular plant roots without causing disease symptoms in the host plant (Surono and Narisawa 2017). DSE have been found to have a symbiotic relationship with 600 plant species, including forestry and crop plants (Mahmoud and Narisawa 2013).

Many volatile organic compounds, including ethyl acetate, 3-methyl-1-butanol, 2-methyl-1-butanol, and styrene, are produced by DSE fungi, such as *Leptodontidium* sp. and *Cadophora* sp., and have been found to be effective in suppressing plant pathogens (Berthelot et al. 2016). DSE TKC 2.2a, as reported by Rahayu et al. (2021), could be a potential biocontrol agent for *G. boninense* in oil palm due to its production of secondary metabolites, such as 2H-1-Benzopyran-7-ol, 3,4-dihydro-5-methoxy-6-methyl-2-phenyl, 4-Allyl-2,6-dimethoxyphenol, and hexadecanoic acid, which exhibit antifungal properties. A promising new strategy for developing biopesticides based on volatile organic compounds and other bioactive compounds with antifungal activity has been proposed (Naik 2018). Our previous research has shown that DSE can be isolated from the roots and soil rhizosphere of *H. brasiliensis* (Dalimunthe et al. 2019). Results obtained from in vitro selection of five DSE isolates, including *Acrocalymma vagum* SBTBMDS 1, *Clonostachys* sp. TMDS 2.1, *Lasiodiplodia theobromae* APDS 3.2, *Penicillium oxalicum* TMDS 3.2, and *Fusarium falciforme* TBMDS 2.4b, suggested that they could potentially serve as biological agents against *R. microporus*-induced white root fungal diseases (Dalimunthe et al. 2019). However, the symbiotic relationship between DSE and *Hevea brasiliensis* in tropical regions, particularly in Indonesia, has not been reported, nor has the production of volatile organic and antimicrobial compounds by DSE in this relationship.

Our DSE isolates showed potentials in suppressing *R. microporus* in in vitro tests (Dalimunthe et al. 2019) and may also promote the growth of *H. brasiliensis*. The DSE fungus produces several metabolite compounds that have been found to inhibit *R. microporus* in vitro. Tienaho et al. (2019) identified 318 metabolites in DSE extraction, of which 220 were identified and 98 were unidentified. A majority of these metabolites were found to be amino acids and peptides. Therefore, the aim of this study was to investigate the effectiveness of

five different DSE fungal isolates in controlling WRRD caused by *Rigidoporus microporus* in *Hevea brasiliensis* in vivo, and to identify any volatile organic compounds produced by the selected DSE isolates. So far, the bulk of the active components in biopesticide formulae used as microbial WRRD biocontrol agents in Indonesia have been *Trichoderma* spp., therefore this study adds to the body of knowledge that other types of fungi, particularly DSE, have the capability to control *R. microporus*. This research will also contribute to environmentally friendly agriculture by minimizing the use of agrochemicals such as fungicides and inorganic fertilizers in rubber plantation, hence improving soil and plant health.

Methods

The study was conducted between October 2018 and May 2019 at two locations: the Laboratory of Soil Mycology, Indonesian Soil Research Institute, Cimanggu, Bogor, West Java, Indonesia (6°34′40.8″ S, 106°47′22.3″ E), and the Plant Laboratory and Greenhouse Facility of the Sungei Putih Rubber Research Center, Galang, Deli Serdang, North Sumatra, Indonesia (3°25′36.87″ N, 98°52′5.66″ E).

Dark septate endophytic and pathogenic fungal isolate materials

The study utilized five DSE isolates, namely *Acrocalymma vagum* SBTBMDS 1, *Clonostachys chloroleuca* TMDS 2.1, *Lasiodiplodia theobromae* APDS 3.2, *Penicillium oxalicum* TMDS 3.2, and *Fusarium falciforme* TBMDS 2.4b. These isolates were originally obtained from the root of *H. brasiliensis* and exhibited high antagonistic activity against *R. microporus* in vitro. Previous laboratory tests did not indicate their potential as plant pathogens (Dalimunthe et al. 2019). In Fig. 1, the phylogenetic tree of each isolate was illustrated. DSE isolates were identified using morphologically based on colony appearance and examined under light microscope (Fig. 2), which were then verified using molecular analysis and phyologenetic tree construction. The present study focused on the ecological role of these selected DSE isolates in controlling WRRD in *H. brasiliensis* in nursery systems. The *R. microporus* isolate and *H. brasiliensis* (PB 260 clone) seedlings of two months were obtained from the Sungei Putih Research Center, Galang, Deli Serdang, North Sumatra, Indonesia.

Preparation of dark septate endophytic fungal inoculum

The preparation of inoculum propagation for each DSE isolate was carried out according to Surono and Narisawa (2018). Each DSE isolate was cultured in 150 mL of liquid Potato Dextrose Broth (PDB) (HiMedia Laboratories LLC, USA) and incubated for 3–4 weeks at 100 rpm and 23–25 °C. The fresh mycelia were filtered and washed with sterile water to remove the liquid media. The collected mycelia were blended with sterilized distilled water for 1 min on low speed using a blender. A part of the mycellium from each DSE isolate was spread on Potato Dextrose Agar (PDA) (HiMedia Laboratories LLC, USA) media using the plating method to assess the viability of the DSE isolate. The concentration of DSE mycelia required for the subsequent application mentioned in Method Section of "Applying dark septate endophytic fungi and *R. microporus* on *H. brasiliensis* seedlings" bellow was 5% of mycelia DSE isolate added and mixed with the sterilized water samples in each treatment with a mycelial density of 10^5 mycelia mL^{-1}.

Preparation of *Rigidoporus microporus* inoculum

The preparation of pathogenic fungal (*R. microporus*) inoculum, followed the method of Kaewchai and Soytong (2010) with some modifications. The culture was firstly maintained on potato dextrose agar (PDA) medium and then, cultured on sterilized inoculum medium consisting of pieces of sterile rubber roots moistened with water. The plastic bags containing the inoculum medium were then, incubated at room temperature (28–30 °C) for 30 days, until all the pieces of rubber plant roots were covered by the pathogen's mycelia. The technical treatment of pathogenic inoculum on rubber plant seedlings was described in Method Section of "Applying dark septate endophytic fungi and *R. microporus* on H. *brasiliensis* seedlings" bellow.

Preparation of planting materials

PB 260 is often used as rootstocks for rubber seedlings in Indonesia, where its usage percentage exceeds 80% compared to other rubber seed clones in the field, and is susceptible to WRRD. These rubber seeds were treated with dark septate endophytic (DSE) fungi through seed treatment and nursery treatment. PB 260 clone rubber seeds that were germinated (star stage) were soaked with a suspension of selected DSE fungus isolates for ± 12 h. After that, it is planted in polybags until it is ± 2 months old for testing materials. Meanwhile, treatment with WRRD pathogens was carried out in the nursery together with the second stage of DSE treatment, as described in Method Section of "Applying dark septate endophytic fungi and *R. microporus* on *H. brasiliensis* seedlings" bellow.

Challenging dark septate endophytic fungi against *Rigidoporus microporus* in the nursery system

This experiment was set up to select isolates of the DSE fungi that could stimulate plant growth and inhibit the attack of *R. microporus* on *H. brasiliensis* plants in the

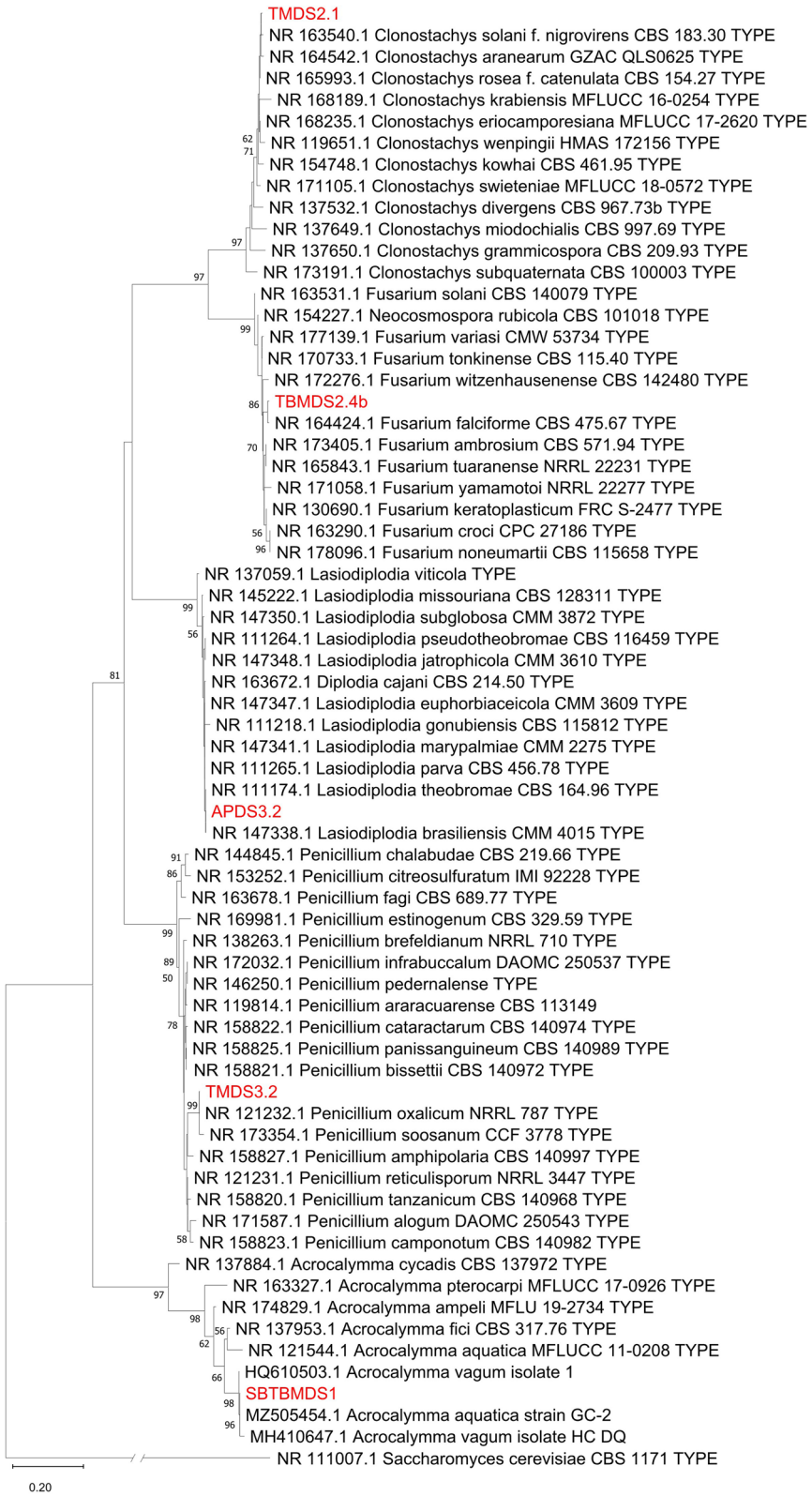

Fig. 1 Maximum likelihood phylogeny of the dark septate endophytes (DSE) isolates inferred from ITS sequences. Bootstrap was performed with 1000 replications and value higher than 50 are shown. The tree is rooted with *Saccharomyces cerevisiae* CBS 1171. DSE isolates are indicated in red type

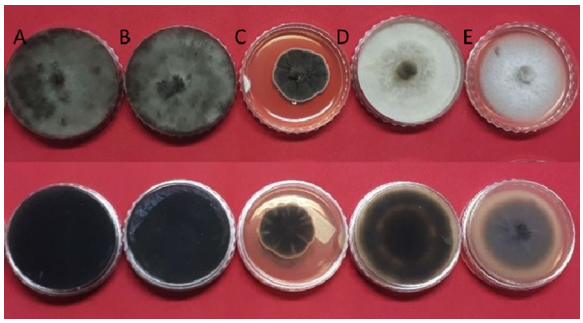

Fig. 2 The dark septate endophytes (DSE) fungal isolates colony appearance on CMMYA medium, **A** *Lasiodiplodia theobromae* APDS 3.2, **B** *Fusarium falciforme* TBMDS 2.4b, **C** *Penicillium oxalicum* TMDS 3.2, **D** *Clonostachys chloroleuca* TMDS 2.1, **E** *Acrocalymma vagum* SBTBMDS 1. Top and bottom side of Petri dish

nursery system (in vivo test). This experiment was conducted in a completely randomized design with six treatments and five replications, each replication consisted of 10 plants. The following treatments were administered to *H. brasiliensis* seedlings: 1) DSE isolate SBTBMDS 1 (*Acrocalymma vagum*), 2) DSE isolate TMDS 2.1 (*Clonostachys chloroleuca*), 3) DSE isolate APDS 3.2 (*Lasiodiplodia theobromae*), 4) DSE isolate TMDS 3.2 (*Penicillium oxalicum*), 5) DSE isolate TBMDS 2.4b (*Fusarium falciforme*), and 6) Control, which only involved treating the *H. brasiliensis* seedling with the pathogen (*R. microporus*) without administering DSE. The technical application of DSE and pathogenic fungal inoculum was explained in Method Section about "Applying dark septate endophytic fungi and *R. microporus* on *H. brasiliensis* seedlings" bellow.

Applying dark septate endophytic fungi and *Rigidoporus microporus* on *Hevea brasiliensis* seedlings

To apply DSE fungi, the following methods were used for disease prevention with a double application of DSE: The first application involved seed treatment, where a DSE, fungal mycelium solution was prepared as outlined in Method Section of "Preparation of dark septate endophytic fungal inoculum". Next, the planting material (the PB 260 rubber clone seed) that germinated in a sand media at the needle stage (21 days of age) was soaked in a suspension of DSE fungal isolate with a concentration of 5% (1 l suspension containing 950 ml of sterilized distillate water and 50 ml of DSE mycelial solution) for six hours. Following this, the germinated DSE-treated seed was planted in growth media in polybags, until it was two months-old and ready for the next treatment. Each polybag contained 2 kg of sterilized mixed soil (soil: compost; 2:1). The second application involved DSE treatment on two-month-old *Hevea brasiliensis* seedlings in polybags. The DSE fungal isolate solution used was 100 ml per polybag (5% concentration) for each treatment, mixed with soil media in polybags. After a week of incubation period for DSE in soil media in polybags, the pathogen was then infected to rubber plant seedlings.

To infect the pathogen (*R. microporus*) on *H. brasiliensis* seedlings, the pathogen inoculum was planted in the form of two rubber root pieces in the seedling root neck parallel to the taproot. The inoculum was firmly attached to the plant's roots at a depth of 5–10 cm before being covered with soil and litter to retain moisture (Fairuzah et al. 2014).

Control of WRRD can be achieved through a variety of methods, including prevention and curative treatment by combining several control components, such as technical culture, biology, and chemistry (Setyawan et al. 2013). In this study, we focused on prevention, because it has the potential to be used effectively and efficiently in nursery systems.

Assessment of *Rigidoporus microporus* infection in the treatments

In this study, the number of plants observed in each treatment was 10 polybags. Each treatment was repeated 5 times and in each replication consisted of 10 plants were taken as observation samples. To assess the extent of *R. microporus* infection in the various treatments, observations were made eight weeks after inoculation of the pathogen by carefully removing the soil around the roots of the rubber plant to evaluate the level of disease infection. The categories of attack were classified according to Rahayu et al. (2017) as follows:

Category 0: Healthy plant
Category 1: *R. microporus* mycelium attached to the root surface of *H. brasiliensis.*
Category 2: *R. microporus* mycelium started to penetrate the root tissues of *H. brasiliensis.*
Category 3: *R. microporus* mycelium penetrated the phloem tissue, resulting in root bark rotting and browning.
Category 4: Root rot and plant death.

Once the attack category value was determined, the intensity of *R. microporus* infection was calculated according to Fairuzah et al. (2014) using the following formula:

$$I = \frac{n \times v}{N \times Z} \times 100\%$$

where I = intensity of infection (severity of disease); n = number of diseased plant roots in each infection category; v = scale value of each infection category; Z = scale

value of the highest infection category; N = number of plants observed.

Evaluation of DSE-induced plant growth promotion in *H. brasiliensis* seedlings

To investigate the potential of selected DSE fungi to promote plant growth, *H. brasiliensis* seedlings were inoculated with *R. microporus* and evaluated after 4 and 8 weeks (Abraham et al. 2021). The assessment of plant vigor was based on various parameters, including vegetative growth such as plant height and stem diameter (girth), dry weight, and root volume. The effect of DSE fungi on *R. microporus*-induced biotic stress was also evaluated.

Determining the efficacy level or percentage of inhibition

The efficacy level (EL) of a DSE isolate is considered to be effective as a biocontrol agent of pathogenic fungi or diseases if it is at least 50% and the intensity of the treatment infection differed significantly from the control (Sari et al. 2021). The following formula was used to calculate the efficacy level (EL):

$$\mathrm{EL} = \frac{\mathrm{IDC} - \mathrm{IDT}}{\mathrm{IDC}} \times 100\%$$

where EL—efficacy level; IDC—intensity of disease infection in control treatment (without DSE); IDT—intensity of disease infection on DSE treatment.

Analysis of volatile organic compounds produced by dark septate endophytic fungal isolates

Detection of volatile organic compounds (VOCs) in three isolates of dark septate endophytic (DSE) fungi namely, *Lasiodiplodia theobromae* APDS 3.2, *Penicillium oxalicum* TMDS 3.2, and *Fusarium falciforme* TBMDS 2.4b, was accomplished using gas chromatography-mass spectrometry (GC–MS). These isolates have previously been shown to suppress *R. microporus* in both in vitro and in vivo trials. The bioactive compounds were extracted from the selected DSE fungal isolates using the method of Waruwu et al. (2016). The constituents were identified by comparing them with data available in the GC–MS library in the literature.

Statistical analysis

The data obtained from each experiment were subjected to analysis of variance (ANOVA) and Tukey's test at a 5% level using SAS 9.4 software with an IPB university license.

Results

Identification of selected DSE isolates

Five DSE isolates were classified as five different species based on the phylogenetic tree analysis using MEGA 11 software for Windows. Three isolates could be directly identified to the species level, namely isolates SBTBMDS1, TBMDS 2.4b, and TMDS as *Acrocalymma vagum*, *Fusarium falciforme*, and *Penicillium oxalicum*, respectively. The other two species, TMDS 2.1 and APDS 3.2, were identified as *Clonostachys* sp. and *Lasiodiplodia* sp. (Fig. 1). Furthermore, the DSE isolates were identified by comparing sequences from species within the genus in the NCBI database for confirmation. The comparison of sequences with other species within the genus of those isolates confirmed that SBTBMDS1, TBMDS 2.4b, and TMDS are the species mentioned above. As for TMDS 2.1 and APDS 3.2, they can be identified as *Clonostachys chloroleuca* and *Lasiodiplodia theobromae*, respectively.

Assessment of the efficacy of the selected DSE fungal isolates in enhancing the growth of rubber seedlings infected with *Rigidoporus microporus* under nursery conditions

In this study, DSE isolates were inoculated onto *H. brasiliensis* seedlings in a nursery system (in vivo test), and observed that this suppressed the plant pathogen *R. microporus* significantly, leading to low disease severity and healthy rubber plant performance compared to the control treatment after 8 weeks of *R. microporus* inoculum infection (Figs. 3, 4 and 5). Disease severity in *H. brasiliensis* seedlings treated with only the pathogenic fungus *R. microporus* (Control treatment, with pathogen, and without DSE) was 45%, compared to 7.5–17.5% in *H. brasiliensis* seedlings inoculated with each DSE fungal isolate (Fig. 3). The highest percentage of disease

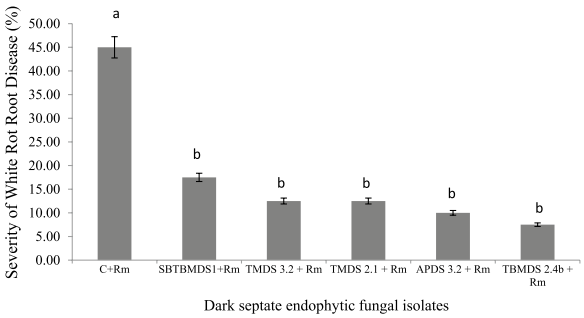

Fig. 3 The effect of dark septate endophyte (DSE) fungal inoculation on the severity of white root rot disease in *Hevea brasiliensis* seedlings after 8 weeks of *Rigidoporus microporus* inoculum infection. Values are means ± SD, $n = 5$. After Tukey's Honestly Significant Difference test, those with the same letter were not significantly different ($P < 0.05$). C = Control treatment. Rm = *Rigidoporus microporus*. SBTBMDS1, TMD 3.2, TMDS 2.1, APDS 3.2, TMDS 2.4b = DSE isolates

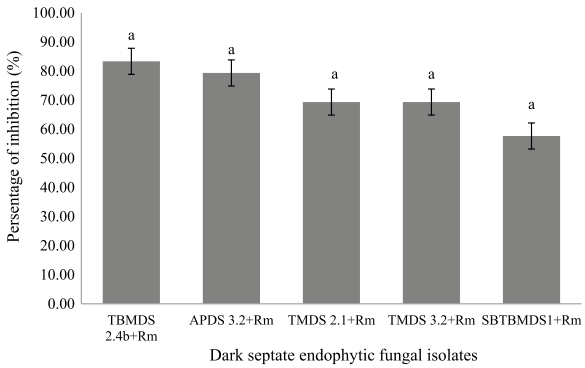

Fig. 4 Percentage of the dark septate endophytic (DSE) fungal isolates inhibiting *Rigidoporus microporus* development after 8 weeks of *R. microporus* inoculum infection. Values are means ± SD $n = 5$. After Tukey's Honestly Significant Difference test, those with the same letter were not significantly different ($P < 0.05$). Rm = *Rigidoporus microporus*. SBTBMDS1, TMD 3.2, TMDS 2.1, APDS 3.2, TMDS 2.4b = DSE isolates

Fig. 5 The conditions of *Hevea brasiliensis* seedlings infected with *Rigidoporus microporus* without the dark septate endophytic (DSE) fungal inoculation were more stressed and stunted growth (**A**) compared to *Hevea brasiliensis* seedlings inoculated with DSE isolates APDS 3.2 (**B**), TBMDS 2.4b (**C**), TMDS 3.2 (**D**), *Clonostachys* sp. TMDS 2.1 (**E**), and *Acrocalymma vagum* SBTBMDS1 (**F**) challenged with *R. microporus* after 8 weeks of *R. microporus* inoculum infection

inhibition was observed with TBMDS 2.4b (83.33%), followed by APDS 3.2, *Clonostachys* sp. TMDS 2.1, TMDS 3.2, and *A. vagum* SBTBMDS1, which inhibited at 78.33, 69.33, 69.33, and 57.67%, respectively (Fig. 4). *R. microporus* that did not develop after DSE fungi were applied to rubber seedlings, and *R. microporus* mycelium was only attached to the surface of the plant roots (scale 1). This was significantly different from the control, in which the plants inoculated with *R. microporus* were infected at a scale 2 infection level, namely the *R. microporus* mycelium was firmly attached to the roots and covered the entire root surface (Fig. 6).

After 8 weeks of infection with *R. microporus* inoculum, the seedling growth of *H. brasiliensis* was significantly improved in the DSE treatment than the control treatment, as evidenced by increased plant height, stem diameter and root volume (Fig. 4). In particular, the DSE APDS 3.2, TBMDS 2.4b, and TMDS 3.2 treatments demonstrated superior plant performance and vigor, even when challenged with *R. microporus* (Fig. 5). The roots of *H. brasiliensis* seedlings also exhibited better development in the DSE treatment than in the control treatment (Fig. 6). The DSE treatment resulted in a greater root volume than the control treatment (Fig. 8). The presence of DSE inoculation resulted in increases in root volume of *H. brasiliensis* plants, from 40 to 120%, despite the challenge from *R. microporus*, indicating that DSE provided protection to the plant roots and enabled them to develop optimally (Figs. 6 and 8). Healthy plant roots that thrive in the presence of DSE can improve nutrient uptake and plant growth even under pathogen infection conditions.

The roots of rubber plants were examined following an 8-week infection period with *R. microporus* to investigate the impact of DSE on the severity of WRRD and to identify differences in root morphology. The analysis revealed a significant contrast between the DSE and control treatments. The DSE fungus treatment resulted in firmer and more compact roots when compared to the control treatment with no DSE fungus (Fig. 6). Moreover, the DSE fungi could modify the root architecture, leading to an increase in root hairs (Fig. 6).

In this study, we observed a significant increases in the dry weight of *H. brasiliensis* seedlings inoculated with isolates TMDS 3.2 and TMDS 2.4b, with respective weights of 9.85 g and 9.80 g, representing an increase of 123 and 122%, compared to control plants weighing 4.42 g (Fig. 7). Furthermore, treatment with DSE led to an increase in plant height compared to control plants under pathogen infection, with the treatment of *H. brasiliensis* seedlings with TMDS 3.2 isolate resulting in a growth of 24.5 cm or an increase of 63.3%, compared to a height of 15.0 cm in control seedlings treated with pathogen (Table 1), after 8 weeks of *R. microporus* inoculum infection (Fig. 8).

The treatment of DSE also had an impact on the girth of *H. brasiliensis* seedlings although not significantly different than the control treatment, as evidenced by measurements taken at four and eight weeks after inoculation with the plant pathogen *R. microporus*. Four weeks after application of *R. microporus*, inoculation with the DSE TMDS 3.2 isolate caused an increase in stem diameter of 0.62 cm, while the control

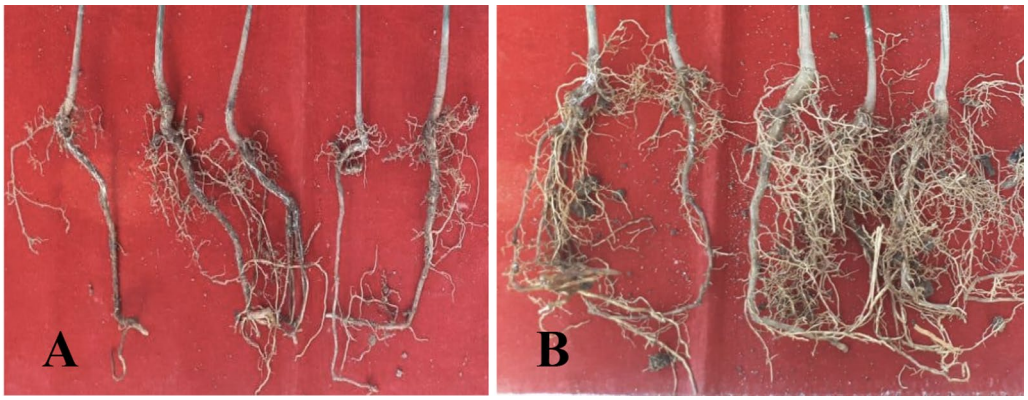

Fig. 6 The roots of *Hevea brasiliensis* infected with *Rigidoporus microporus* did not develop well and were damaged in the control treatment (**A**), whereas the roots of *Hevea brasiliensis* inoculated with the dark septate endophytic (DSE) isolate APDS 3.2 and challenged with *R. microporus* (**B**) developed well compared to the control plant roots (**A**) after 8 weeks of *R. microporus* inoculum infection

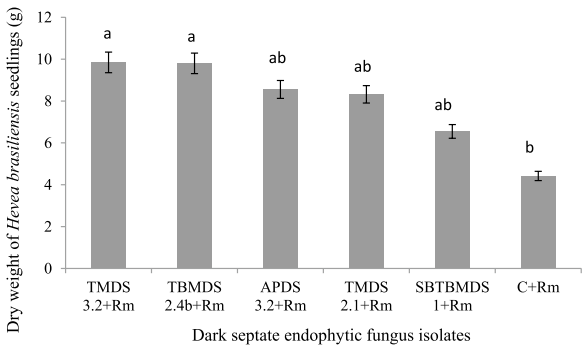

Fig. 7 The dry weight of *Hevea brasiliensis* seedlings inoculated with the dark septate endophytic (DSE) fungal isolates and challenged with *Rigidoporus microporus* after 8 weeks of *R. microporus* inoculum infection compared to the dry weight of *R. microporus* infected control plant. Values are means ± SD $n = 5$. Samples with the same letter were not significantly different ($P < 0.05$) after Tukey's Honestly Significant Difference test. C = Control treatment. Rm = *Rigidoporus microporus*. SBTBMDS1, TMD 3.2, TMDS 2.1, APDS 3.2, TMDS 2.4b = DSE isolates

treatment (*R. microporus* infection without DSE inoculation) resulted in a smaller increase in stem diameter of only 0.21 cm (Table 1). Measurements, taken 8 weeks after application of *R. microporus* revealed that treatment with the TMDS 3.2 isolate caused a greater enhancement in growth of stem girth of 1.02 cm, followed by other DSE fungi, APDS3.2, SBTBMDS1, TBMDS2.4b, and TMDS2.1, which increased the growth of girth by 0.49 cm, 0.44 cm, 0.55 cm, and 0.46 cm, respectively (Table 1). An increase in the diameter of rubbers plant stems was important as it determines the future production of rubber plants.

Table 1 The effect of the dark septate endophytes (DSE) fungi application on the growth of *Hevea brasiliensis* seedlings clone PB 260

Treatments	Girth increment (cm/week)		Plant height (cm)	
	4 WAI	8 WAI	4 WAI	8 WAI
TMDS3.2 + Rm	0.62 ± 0.13 a	1.02 ± 0.37 a	13.65 ± 3.45 a	24.45 ± 1.24 a
APDS3.2 + Rm	0.49 ± 0.23 a	0.83 ± 0.36 a	11.70 ± 2.55 a	19.85 ± 1.01 ab
SBTBMDS1 + Rm	0.44 ± 0.07 a	0.76 ± 0.21 a	11.75 ± 1.60 a	17.05 ± 3.11 ab
TBMDS2.4b + Rm	0.55 ± 0.25 a	1.01 ± 0.29 a	11.60 ± 3.71 a	20.00 ± 5.10 ab
TMDS2.1 + Rm	0.46 ± 0.32 a	0.85 ± 0.33 a	11.80 ± 5.41 a	17.10 ± 5.76 ab
Control + Rm	0.21 ± 0.17 a	0.43 ± 0.24 a	6.60 ± 1.68 a	14.95 ± 2.18 b
F-value	2.157	1.988	2.029	3.474
CV	0.501	0.454	0.362	0.252

WAI: week after infection, Rm: *Rigidoporus microporus*. Numbers are average from five biological replications ± standard deviation. Numbers followed by same letters in the same column indicates not significantly different according to Tukey Multiple Comparison Test at α: 0.05

Volatile organic compounds produced by the selected dark septate endophytic fungal isolates

Based on GC–MS analysis, *L. theobromae* APDS 3.2, *P. oxalicum* TMDS 3.2, and *F. falciforme* TBMDS 2.4b produced acetic acid butyl methyl-phosphinoylmethyl ester (with more than 25% compound content) and also ethanone, 1-(4-methyl-1H-imidazol-2-yl). These compounds were previously reported to have antimicrobial activity (Table 2).

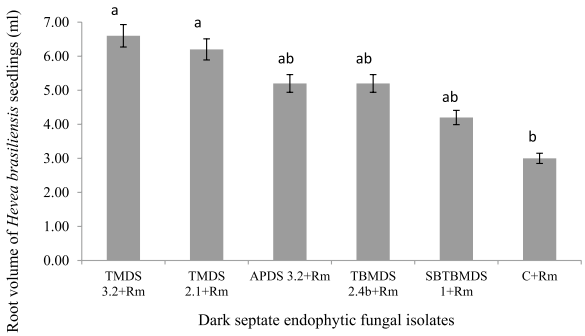

Fig. 8 The root volume of *Hevea brasiliensis* seedlings inoculated with dark septate endophytic fungal (DSE) isolates and challenged with *Rigidoporus microporus* was compared to the root volume of control plants with *R. microporus* infection after 8 weeks of *R. microporus* inoculum infection. Values are means ± SD $n = 5$. Standards with the same letter were not significantly different ($P < 0.05$) after Tukey's Honestly Significant Difference test. C = Control treatment. Rm = *Rigidoporus microporus*. SBTBMDS1, TMD 3.2, TMDS 2.1, APDS 3.2, TMDS 2.4b = DSE isolates

Discussion

In previous in vitro studies, we demonstrated that DSE fungal isolates, i.e. *A.vagum* SBTBMDS 1, *C. chloroleuca* TMDS 2.1, *L. theobromae* APDS 3.2, *P. oxalicum* TMDS 3.2, and *F. falciforme* TBMDS 2.4b could suppress *R. microporus* growth by over 50% in dual culture testing, as well as the activity of organic volatile compounds produced by those DSEs (Dalimunthe et al. 2019). Those isolates of DSE also that shown were consistently able to suppress the growth and infection of *R. microporus* in vivo testing in the nursery conditions in this study. Therefore, this is the first report of DSE's ability to inhibit *R. microporus* growth, development, and infection activity, as well as the existence of a DSE-induced defense system in *H. brasiliensis* seedlings. This suggested that these DSE isolates could potentially be used on a large scale in rubber plantations through effective and efficient inoculation at the nursery stage of *H. brasiliensis*.

Table 2 Antimicrobial compounds produced by the selected dark septate endophytic (DSE) fungal isolates APDS 3.2, TBMDS 2.4b, and TMDS 3.2

No	Name of the compound	Molecular formula	Origin compound	Compound content (%)			Ref
				APDS 3.2	TBMDS 2.4b	TMDS 3.2	
1	Acetic acid butyl methyl-phosphinoylmethyl ester	$C_8H_{17}O_3P$	Ester	30.76	25.06	29.14	Siddiquee (2014)
2	Dimethyl sulfoxide	C_2H_6OS	Organosulfur compounds	11.38	–	10.06	Sanmartín-Suárez et al. (2011)
3	Benzenemethanol, 3,5-dimethyl-	$C_9H_{12}O$	Alcohol	5.13	–	–	Ma et al. (2012)
4	1,5,6,7-Tetramethylbicyclo |3.2.0| hepta-2,6-diene	$C_{11}H_{16}$	Alcohol	1.40	–	1.37	Wang et al. (2018)
5	Ethanone, 1-(4-methyl-1H-imidazol-2-yl)-	$C_6H_8N_2O$	Azol	3.55	2.87	3.30	Akter et al. (2018)
6	Ethanimidic acid, 2-cyclohexylidene-*N*-phenyl-methyl ester	$C_{15}H_{19}NO$	Carboxylic acid	1.57	–	1.39	Saladino et al. (2011)
7	Boric Acid	H_3BO_3	DMSO	1.16	1.16	–	Rolshausen & Gubler (2005)
8	Triethylsilanol	$C_6H_{16}OSi$	Organic silanol	–	1.30	–	Yun-mi et al. (2006)
9	L-Norvaline	$C_5H_{11}NO_2$	Amino acids	–	1.04	1.30	Samardzic and Rodgers (2019)
10	Palmitic acid	$C_{16}H_{32}O_2$	Carboxylic acid	–	1.94	–	Liu et al. (2008)
11	11-Octadecenoic acid	$C_{18}H_{34}O_2$	methyl ester	–	2.35	–	Reis et al. (2019)
12	i-Propyl 9-octadecenoate	$C_{21}H_{40}O_2$	Propyl Ester	–	1.74	–	Reis et al. (2019)
13	Tricosane	$C_{23}H_{48}$	Alkane	–	2.32	–	Tao et al. (2018)
14	i-Propyl 11,12-methylene octadecenoate	$C_{22}H_{42}O_2$	Ester	–	2.22	–	Tang et al. (2016)
15	2-Carene	$C_{10}H_{16}$	Monoterpene hydrocarbon	–	–	7.55	Teixeira et al. (2013)

All the DSE isolates used in this study had morphological characteristics of black septate colonies and did not cause disease symptoms when tested on plants, similar to the typical characteristics of DSE. Molecular analysis using ITS 4 and ITS 5 primers revealed that three isolates, which are SBTBMDS1, TMDS 3.2, and TBMDS 2.4b, had bootstrap support values above 80% and, when compared to sequences of other species within the genus, solidified their positions as *A. vagum*, *P. oxalicum* and *F. falciforme*, respectively, with bootstrap support values of 99, 99, and 81%. The other two isolates, TMDS 2.1 and APDS 3.2, were grouped as *C. chloroleuca* and *L. theobromae*, respectively, after comparing their sequences with the others within the genus. For APDS 3.2, with a bootstrap value of 86% and a position among other *L. theobromae* sequences, APDS 3.2 was sufficiently strong and could be identified as *L. theobromae*. Ojha et al. (2022) suggested that isolates with bootstrap support values below 50% should not be considered in constructing the phylogenetic tree. However, the phylogenetic tree analysis with 1000× bootstrap on the TMDS 2.1 isolate showed that it clustered among the other *C. chloroleuca* sequences, indicating a close relationship. Therefore, the TMDS 2.1 isolate can be provisionally identified as *Clonostachys* cf. *chloroleuca*, suggesting a close relationship with *C. chloroleuca*.

Interestingly, we found that DSE isolates TMDS 3.2 and TBMDS 2.4b, identified as *P. oxalicum* and *F. falciforme*, respectively, had a dark colony on medium. Although the typical colony colors for these species are dark green (*Penicillium*) and white to brown (*Fusarium*), it is possible that these fungi undergo melanization, resulting in a darker colony color than the usual green or brown. This possibility is supported by reports on melanization in the same genus of fungi (Chiewchanvit et al. 2017), indicating that such melanization can occur in these species (*P. oxalicum* and *F. falciforme*) as well.

As DSE is a root endophyte, it holds great potential as a tool for protecting host plants against soil-borne diseases such as *R. microporus*. Under biotic stress conditions, DSE on plant roots can increase yields and enhance plant resistance (Yuliani et al. 2020). Mandyam and Jumpponen (2005) suggested that DSE fungi could improve plant performance by increasing nutrition and enabling plants to withstand unfavorable environmental conditions and plant pathogen infection. Visual observations in this study indicated that there were differences in root architecture between the DSE treatments and the control. Root hairs play a crucial role in the ability of roots to absorb water and nutrients from the soil. Plants colonized by DSE generally showed enhanced root length and number, which enabled them to absorb more nutrients from the soil (Giehl and von Wirén 2014). Although *R. microporus* posed a challenge, in this study, there was an interaction between the DSE fungus and the roots of the rubber plant, so that the performance of the plants could still grow healthy and well, including the roots inoculated with DSE grew well compared to the roots of the control plants.

As a plant growth promoter, dark septate endophytes (DSE) have been demonstrated to increase the height and dry weight of their host plants in previous studies (Vergara et al. 2018). These results suggested that nutrient transfer was facilitated and protected by DSE, resulting in enhanced plant growth and development even under pathogen infection. These findings are consistent with previous studies that have shown the ability of DSE-inoculated plants to promote growth in the presence of pathogenic fungi (Surono and Narisawa 2018). Plant-endophytic fungal interactions can regulate plant growth, particularly under biotic stresses such as plant diseases (Waqas et al. 2015). Dark septate endophytic (DSE) fungi have been shown to increase plant growth in response to biotic stress or plant disease infection (Surono and Narisawa 2018). This study supported the use of DSE fungi to protect *H. brasiliensis* seedlings from infection by the plant pathogen, *R. microporus*. By inoculating DSE into rubber plants in a nursery stage, healthy seedlings can be produced that are protected by DSE, thus reducing or eliminating the use of fungicides in the *H. brasiliensis* nursery phase through an environmentally friendly approach.

Three DSE isolates, *L. theobromae* APDS 3.2, *P. oxalicum* TMDS 3.2, and *F. falciforme* TBMDS 2.4b had the potential to inhibit the growth of the plant pathogen, *R. microporus* based on in vitro (Dalimunthe et al. 2019) and in vivo testing in this study. The bioactive compounds produced by these isolates were believed to be responsible for this activity. Notably, when compared to other DSE fungal isolates (*C. chloroleuca* TMDS 2.1 and *A. vagum* SBTBMDS 1), these three isolates showed low activity of *R. microporus* infections in *H. brasiliensis* nurseries. In this study, it was proposed that *F. falciforme* TBMDS 2.4b isolates produced antimicrobial compounds such as 11-octadecenoic acid, which could inhibit *R. microporus* growth. This compound has been previously reported to be produced by *Trichoderma asperellum* and can inhibit *R. microporus* growth (Sakpetch et al. 2018). To the best of our knowledge, there has been no information on DSE's ability to produce volatile organic compounds. This information represented the first report of the volatile organic compounds produced by DSE fungi. Consequently, it could be concluded that the volatile organic compounds produced by DSE fungi exhibited antimicrobial properties that could inhibit the growth of *R. microporus* in rubber plants.

Conclusion

Based on the results of this study, *L. theobromae* APDS 3.2, *P. oxalicum* TMDS 3.2, and *F. falciforme* TBMDS 2.4b. which had demonstrated in vitro control of *R. microporus* growth, also possessed the potential to reduce disease severity on *H. brasiliensis* seedlings in nursery systems. In addition, these isolates have been found to produce volatile organic compounds with antimicrobial properties, making them potential biofumigation agents. Further investigation is needed to confirm the efficacy of these isolates in promoting *H. brasiliensis* growth and suppressing *R. microporus* infestation in large-scale field tests, in order to develop them as biological fertilizers and biopesticides. These findings will contribute to the development of eco-friendly and sustainable plant disease management practices, which can reduce the use of hazard chemicals in agriculture and promote the use of natural products.

Abbreviations

WRRD	White root rot disease
DSE	Dark septate endophytes
FBAs	Fungal biocontrol agents
VOCs	Volatile organic compounds
GC-MS	Gas chromatography-mass spectrometry
ANOVA	Analysis of variance

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Author contributions

CID, S, BPWS, and AM designed the research. S, BPWS, AM: supervision and funding acquisition. CID, S, BPWS, AM, ND analyzed and interpreted the data. CID and S performed the experiments and provided data. CID, S, BPWS, AM, LKTA, CS, ND wrote the manuscript. All authors reviewed, edited, and contributed the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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