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The first report of dark septate endophytes from Indonesian *Pinus merkusii* and its symbiosis role as a plant growth promoter in nursery condition

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Abstract. *Akhir J, Herliyana EN, Surono, Budi SW. 2024. The first report of dark septate endophytes from Indonesian* Pinus merkusii *and its symbiosis role as a plant growth promoter in nursery condition. Biodiversitas 25: 312-321.* To encourage the healthy growth of *Pinus merkusii* Jungh et de Vriese seedlings at the seedling stage, Dark Septate Endophytic (DSE) fungi were used as growth promoters. In this study, four DSE strains, i.e., Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c isolated from *P. merkusii* roots, were tested for their ability to stimulate the growth of *P. merkusii* seedlings. After 14 weeks of incubation in nursery conditions, *P. merkusii* seedlings inoculated with four DSE strains experienced a higher increase in height and stem diameter when compared to the control treatment. Overall, the biomass results of *P. merkusii* seedlings treated with the DSE strains performed better than those of the control. Root colonization of *P. merkusii* seedlings showed that the 4 DSE strains significantly outperformed the control. The Hs14.6c strain outperformed the other strains and controls regarding nutrient (nitrogen) uptake. The findings of this study are the first to show that the DSE Apls 1.5.3 strain is similar to *Cylindroccarpon*, the Hs14.6a and Hs14.6c strains are similar to *Cladophialophora* sp., and the Pls 32.1 strain is similar to *Oncopodiella trigonella* based on identification results and phylogenetic trees and can be in symbiosis with seeds of *P. merkusii*, a pine species native to Indonesia.

Keywords: *Cladophialophora* sp., *Cylindroccarpon* sp., nutrient uptake, *Oncopodiella trigonella*, seedlings

INTRODUCTION

Pinus merkusii Jungh. & de Vriese is an indigenous Indonesian pine species (Hadiyane et al. 2015; Imanuddin et al. 2020). *Pinus merkusii* is a pioneer plant with needle-like leaves that belongs to the Pineceae family (Richardson et al. 2007; Farjon 2021). It grows in podzolic soil between 800 and 2000 meters above sea level (Hartiningtias et al. 2020). *Pinus merkusii* offers various advantages over other forestry plants. Stems, for example, can be used as a source of wood for building materials and raw materials for paper, and pine stands can be used for tapping sap to produce resin (Imanuddin et al. 2020; Purba et al. 2022), restoration and rehabilitation of forests in the lowlands and highlands (Imanuddin et al. 2020), and provide economic benefit (Stambaugh et al. 2011; Hadiyane et al. 2015).

Because of the enormous potential of *P. merkusii*, it is being considered for the development of *P. merkusii* forest areas for conservation and industrial uses broadly. The problem is that there aren't enough healthy seedlings available in the nursery. While the health of the seedlings will determine the rate of growth in the field. The availability of nutrients will be critical during the seedling growth phase in the nursery which determines whether the seedlings are healthy or not (Etesami and Adl 2020). If inorganic fertilizers are used continuously during the seedling growth phase in the nursery even in the field when transplanting, soil fertility and the community and diversity of soil microorganisms tend to change (Beltran-Garcia et al. 2021); additionally, this practice is not good for the environment (Miransari 2013; Etesami and Adl 2020; Poveda et al. 2021). Using biological agents to mitigate the negative effects of inorganic fertilizers is an appropriate alternative. The use of biological agents is expected to increase seedling growth for *P. merkusii* seedlings to be more resistant to biotic and abiotic challenges while in the nursery before and after being transferred to the field. One group of endophytic fungi that has been identified and functions as a biological agent is the Dark Septate Endophytic fungi (DSE) group which functions as a promoter of plant growth and induction of resistance (Schulz and Boyle 2006; Backman and Sikora 2008; Fontana et al. 2021), and respond to biotic and abiotic stress conditions (Liu et al. 2017; Surono and Narisawa 2018; Santos et al. 2021). DSE fungi have dark-colored melanin hyphae, dark-colored colonies on agar media, and the capacity to colonize host plant roots without damaging plants or causing disease symptoms (Surono and Narisawa 2018; Sharma et al. 2021). Fungal hyphae that produce melanin structures (intercellular hyphae, intracellular hyphae, and microscleria)

in plant roots, indicate DSE fungi (Surono and Narisawa 2017; Wang et al. 2022).

Studies of DSE fungal symbiosis in pine plants have been widely reported such as a study on the positive impact of DSE colonization on *Pinus roxburghii* growth (Dhyani et al. 2019), profiles of metabolic compounds in three species of DSE from Scottish pine roots (*Pinus sylvestris*) that have impact on increasing that pine growth (Tienaho et al. 2019), the effect of DSE on growth and nutrient uptake on *Pinus sylvestris* seedlings under limited N and CO_2 conditions (Alberton et al. 2010), effect of DSE colonization patterns on increasing *Pinus bankiana*, *Pinus strobus* and *Pinus contorta* seedlings growth (Wagg et al. 2008), DSE strain *Phialocephala bamuru* A024 isolated from the roots of *Pinus sylvestris* var Mongoliaca that increase that pine species (Deng et al. 2020). However, study on DSE fungi derived from *P. merkusii* roots to support the growth of *P. merkusii* seedlings in nurseries has not been reported so far.

The aim of this study was to investigate if four DSE strains isolated from *P. merkusii* roots, Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c, could assist increase in *P. merkusii* seedling growth in nursery conditions. In the future, this research is predicted to promote the availability of healthy *P. merkusii* seedlings following DSE inoculation and to increase the growth and resistance of *P. merkusii* seedlings when planted in the field. We hypothesized that inoculating *P. merkusii* seedling with DSE strains would support symbiosis and increase *P. merkusii* seedling growth in the nursery conditions.

MATERIALS AND METHODS

Testing the DSE strain on *Pinus merkusii* seedlings in the nursery condition

DSE inoculant preparation

DSE was isolated from the roots of *Pinus merkusii*, originating from two locations, namely the IPB University Dramaga Bogor and the Mount Halimun Salak National Park. The DSE inoculant was prepared by the Surono and Narisawa (2018). The DSE strain used consisted of four strains i.e., Apls 1.5.3, Pls 32.1, Hs14.6a and Hs14.6c. Propagation of DSE strains used Potato Dextrose Broth (PDB) media, with the composition (w/v, PDB 2.4%, chloramphenicol 0.05%). A total of 5 DSE plates were taken using a cork borer (0.5 cm in diameter) from the PDA media that had been grown, and put into 200 mL culture bottles. The DSE strain obtained grew slowly, required 30 days of incubation, and was placed in an incubator shaker at 150 rpm at 28°C. At the end of the incubation period, the culture filtrate was separated from the mycelium using filter paper Whatman No 1. All operations were carried out in aseptic chamber to prevent contamination. Preparation of 5% mycelium suspension, of 5 g of mycelium that has been filtered using filter paper Whatman No 1, and then let stand until no more water drips and squeeze while pressing the mycelium using a stirring spoon. The mycelium that has been filtered was then weighed until it reached 5 g and 100 mL of sterile distilled water was added to the blender using a portable mini chopper.

Preparation of Pinus merkusii *seedlings*

The *Pinus merkusii* seedlings used in this study were seven months old after being selected in the Perum Perhutani KPH Probolinggo nursery, East Java Province, Indonesia (-7°96'31.91"S, 113°49'02.26"E). *Pinus merkusii* seedlings were planted in polybags measuring 10 cm × 12 cm, with a media composition consisting of 15% sand, 16% silt and 69% clay with an average polybag weight of 332.14 g. The chemical characteristics of growing media are as follows; pH 5.8; C-organic 1.86%, N 0.17%, C/N 11, Ca 9.19 $cmol_c$/kg, 1.62 $cmol_c$/kg; Cation Exchange Capacity (CEC) 12.58 $cmol_c$/kg and base saturation 74%.

Experimental design

This study used a Completely Randomized Design (CRD) and was conducted in greenhouse of Department of Silviculture, Faculty of Forestry and Environment, IPB University, Bogor, Indonesia (-6° 33’ 23.3” S and 106° 43’ 43.9” E), with average temperature condition at the day 37.50°C for 12 h and at night 25.30°C for 12 h and with relative humidity 83%-45%. Treatments consisted of 1) *P. merkusii* seedlings inoculated with DSE strain Apsl 1.5.3, 2) *P. merkusii* seedlings inoculated with DSE strain Pls 32.1, 3) *P. merkusii* seedlings inoculated with DSE strain Hs14.6a, 4) *P. merkusii* seedlings inoculated with DSE strain Hs 14.6c, and 5) *P. merkusii* seedlings not inoculated with DSE (control treatment). Each treatment was performed three times, including the control treatment, for a total of 15 experimental pots. *Pinus merkusii* seedlings treated with and without the DSE strain were placed in the house greenhouse at random growing for 14 weeks to observe growth and collect data.

Inoculation of DSE strain on Pinus merkusii *seedlings*

Pinus merkusii seedlings were placed in the nursery condition for 2 weeks before being treated with DSE strains, with the aim that the seedlings could adapt to environmental conditions. During the placement of the *P. merkusii* seedlings in the nursery condition, they were watered once a day in the morning. After 2 weeks the *P. merkusii* seedlings were placed in the nursery condition, the next step was preparing to make holes around the seedling stems. However, before that, watering is done until it is saturated to make it easier to make holes. Make 3 triangular holes 2 cm from the base of the seedling, 5 cm deep and 0.7 cm in diameter. Holes were made for the application of DSE strain mycelium obliquely towards the center of the root, with the aim that when the DSE strain mycelium was applied to *P. merkusii* seedlings it would go straight to the plant roots.

The DSE strain treatment was given in the form of mycelium based on the weight of the *P. merkusii* seedlings growth media. The amount of DSE mycelium given was 5% of the weight of the media with a polybag size of 10 cm × 12 cm and the average weight of the media in water-saturated conditions with an average weight of 332.14 g. Giving the amount of mycelium in each treatment of *P.*

merkusii seedlings was done after preliminary experiments to obtain the amount of mycelium in the form of liquid media. In each treatment of *P. merkusii* seedlings were given 17 mL of DSE mycelium which had been mixed with sterile distilled water. After being given DSE mycelium, the holes in the seedling media were closed at the top and then watered on the second day and the next day once a day in the morning.

Observation of the growth of Pinus merkusii *seedlings*

Growth data were collected at one week interval for 14 weeks in the nursery conditions. for increase in height, stem diameter and root length. The height of the *P. merkusii* seedling was measured using a ruler, which starts from the base of the seedling to the shoot or growing point. The diameter of the *P. merkusii* seedlings was measured using a caliper, starting from the base of the seedling which was 0.5 cm from the soil surface. Measurement of the length of the roots of *P. merkusii* seedlings was done by disassembling the plant and measuring the longest root from the growing point.

Biomass of Pinus merkusii *seedlings*

The biomass data of *P. merkusii* seedlings were in the form of fresh weight of the roots and shoots. The samples were then placed in an oven for 48 h at 80°C (Chu et al. 2019). Then the dry weight samples of roots and shoots were weighed using a digital balance.

Root colonization of Pinus merkusii *seedlings*

Root colonization data on *P. merkusii* seedlings were obtained by taking *P. merkusii* seedlings each in each DSE strain treatment after 14 weeks of incubation in the nursery. Root staining method by compositing the roots according to the DSE strain treatment of *P. merkusii* seedlings was used which has been modified by adding a cleaning solution (Kormanik et al. 1980). Roots were cleaned from the soil and cut into 1 cm lengths. The root pieces were put into a test tube containing 10% KOH until submerged, heated at 70°C for 1 h, then the KOH solution was discarded and rinsed with running water, soaked with a cleaning solution (HN_4OH 25% and mixed with H_2O_2 30% for 600 mL of distilled water) for 1 day until the roots turned pale white, discard the cleaning solution (HN_4OH 25%, H_2O_2 30%) and rinse with running tap water, then soak with HCl 1% for 30 min, then discard the HCl 1% solution, add fushin acid dye and leave for 30 min, heat for 30 min at 70°C and leave for 24 h (store at room temperature).

The number of root segments of the *P. merkusii* seedlings used for observing root colonization was 30 root segments for each treatment, so that a total of 150 root segments of the pine seedlings were used including the control treatment. Observation of root colonization using a Novel brand trinocular microscope with serial number XSZ-107E. Percent colonization of DSE strains i.e, Apls 1.5.3, Pls 32.1, Hs 14.6a and Hs 14.6c on the roots of *P. merkusii* seedlings was calculated based on the method (Ban et al. 2017).

Carbon and nitrogen uptake in Pinus merkusii *seedlings*

Samples of *P. merkusii* seedling biomass in the form of stems and leaves were analyzed for organic C and N nutrient content and uptake. The analysis was carried out in the Animal Feed Technology and Nutrition Science laboratory, Faculty of Animal Husbandry, IPB University, Indonesia. C-organic analysis used the Walkey and Black method and nitrogen analysis used the wet destruction method (Kjeldahl). Nutrient uptake analysis was calculated by multiplying nutrient content (%) by dry weight (g).

Characteristics of the morphology of the DSE strain from *Pinus merkusii*

Characteristics of microscopical morphology

Microscopic identification was carried out by growing the DSE strain on glass slides by taking a piece of the DSE strain using a cork borer (diameter 0.5 cm). The DSE strain in the form of 0.5 cm pieces was placed in the middle of the slide and covered with a cover glass. The next step was incubated for 2 weeks and placed in an incubation room with a temperature of 26°C-28°C. After 2 weeks, the hyphae growth was observed on the cover glass and then transferred to a new glass preparation which had previously been given with a drop of lactophenol cotton blue solution. Observations using the Axioscope Carl Zeiss Serial No microscope 3366000185 were carried out by looking at the shape of hyphae, conidiophores, conidia and septa.

Molecular identification of DSE strains

DNA isolation, PCR and sequencing activities on DSE strains Apls 1.5.3, Hs14.6a, Hs14.6c and Pls 32.1 were carried out at the Genetika Science Indonesia laboratory, Tangerang, Indonesia. Molecular identification of DSE strains was carried out by analyzing the ITS rDNA sequence using universal primer pairs ITS1 (forward) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse) (5'-TCCCTCCGCTTATTGATATGC-3'). DNA sequences were analyzed using the BioEdit ver.7 program and aligned using the cluster W program. Sequence suitability was determined using functional DNA sequences available in MycoBank and GenBank (BLAST). Phylogenic tree analysis was performed using the Neighbor-Joining (NJ) method in MEGA11 (Tamura et al. 2013). The Neighbor-joining tree was built using the Jukes-Cantor model which was previously analyzed with the BIC (Bayesian Information Criterion) where the model with the lowest BIC score was considered the best in describing the pattern of substitution and was analyzed using bootstrap of 1000 replications (Kumar et al. 2017).

Data analysis

All growth parameters such as height increase, stem diameter, root length, biomass, root colonization, C-organic and total N content of *P. merkusii* seedlings were processed and analyzed using Microsoft Office Excel and Statistical Analysis System (SAS) version 9.0 software. The effect of DSE strain treatment on *P. merkusii* seedlings was statistically analyzed using ANOVA (Analysis of Variance) at a 95% confidence level. If there is a significant effect on the

experimental variable, it will be followed by Duncan's test with a 95% confidence level.

RESULTS AND DISCUSSION

Pinus merkusii seedling growth in the nursery condition

Pinus merkusii seedlings inoculated with strains Hs14.6a, Apls 1.5.3, and Hs14.6c showed an increase in height of 3.10 cm, 2.47 cm, and 2.33 cm respectively or an increase of 52.46%, 21.31% and 14.75% compared to the control treatment (not inoculated DSE) and Pls 32.1 (inoculated DSE) of 2.03 cm and 2.03 cm respectively. In addition, *P. merkusii* seedlings inoculated with the DSE strains Hs14.6a, Apls 1.5.3, Hs14.6c, Pls 32.1 showed an increase in stem diameter of 0.057 cm, 0.063 cm, 0.057 cm, and 0.057 cm respectively or an increase of 42.50%, 57.50%, 40.00%, and 42.50% compared to the control treatment (not inoculated DSE), namely 0.04 cm. In measuring the root length of *P. merkusii* seedlings inoculated with the DSE strain, Hs14.6a, Apls 1.5.3, Pls 32.1 showed an increase in root length of 26.30 cm, 24.63 cm and 24.07 cm respectively or an increase of 12.39%, 5.26% and 2.86% compared to the control treatment (not inoculated DSE). Compared to the other three strains, the DSE Hs14.6c strain showed a lower root length of *P. merkusii* which was 21.30 cm compared to the root length of the control plants (Table 1).

Biomass of *Pinus merkusii* seedlings

Biomass of *P. merkusii* seedlings inoculated with DSE strains Apls 1.5.3, Hs14.6a, and Hs14.6c were 0.85 g, 0.89 g and 1.11 g respectively or an increase of 10.45%, 16.28%, and 44.39% when compared to the control treatment (not inoculated DSE) of 0.77 g after 14 weeks of incubation in the nursery condition. Compared to the other three strains, DSE strain Pls 32.1, showed a lower biomass yield of 0.74 g if compared to the control plant biomass (Table 1 and Figure 1).

Colonization of the roots of *Pinus merkusii* seedlings

The results of the root colonization analysis of the *P. merkusii* seedlings are presented in Table 1 and Figure 2. The root colonization of the *P. merkusii* seedlings showed that all DSE strains Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c had a significant effect, each by 93.33%, 86.67%, 93.33% and 96.67% when compared to the control treatment (not inoculated DSE) of 33.33% after 14 weeks of incubation in the nursery condition.

Table 1. Effect of DSE strains treatment on height increase, stem diameter, root length, biomass, and root colonization of *P. merkusii* seedlings after 14 weeks of incubation in the nursery

DSE strains	Parameter				
	Height increase (cm)	Stem diameter increase (cm)	Root length (cm)	Biomass (g)	Root colonization (%)
Apls 1.5.3	2.47±0.68	0.063±0.01	24.63±2.12	0.85±0.16 ab	93.33±11.55 a
Pls 32.1	2.03±0.57	0.057±0.02	24.07±0.51	0.74±0.14 b	86.67±11.55 a
Hs14.6a	3.10±0.9	0.057±0.02	26.30±9.06	0.89±0.30 ab	93.33±5.77 a
Hs14.6c	2.33±0.21	0.056±0.01	21.30±4.11	1.11±0.12 a	96.67±5.77 a
Control	2.03±0.47	0.04±0.01	23.40±3.95	0.77±0.09 ab	33.33±5.77 b

Description: mean±SD, numbers followed by the same letter is not significant at the 0.05 level

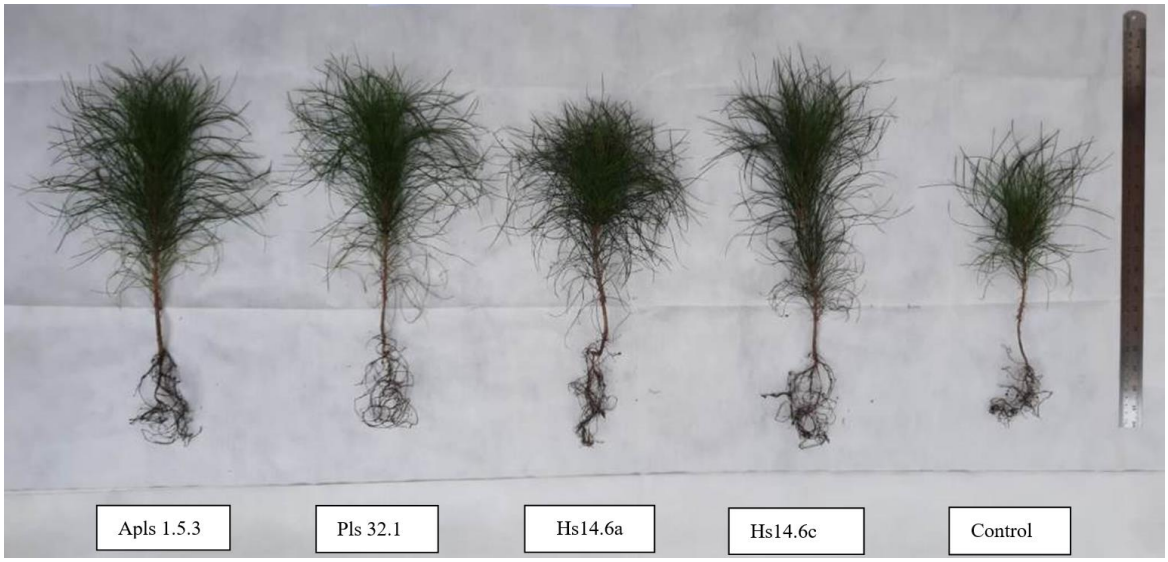

Figure 1. Height increment, stem diameter, root length and biomass and root colonization of *P. merkusii* seedlings for 14 weeks after incubation under nursery conditions due to DSE strains treatment

Carbon and nitrogen uptake by *Pinus merkusii* seedlings

The results of the C-organic content analysis of *P. merkusii* seedlings inoculated with DSE strains Hs14.6a and Hs14.6c showed a significant difference of 39.88% and 39.63%, respectively, when compared to the Apls 1.5.3, Pls 32.1 strain and the control treatment (not inoculated) 37.61%, 32.19% and 31.23% respectively after 14 weeks of incubation period in the nursery condition. The results of the N total content analysis of *P merkusii* seedlings inoculated with DSE strains Pls 32.1, Hs14.6a, Apls 1.5.3 and Hs14.6c showed a significant difference of 2.99%, 2.74%, 2.58% and 2.50%, respectively, when compared to the control treatment (not inoculated) 2.26% after 14 weeks of incubation period in the nursery condition (Table 2).

The C-organic uptake analysis of *P merkusii* seedlings inoculated with DSE strain Hs14.6c revealed a significant difference of 0.440 mg/plant when compared to DSE strains Apls 1.5.3, Pls 32.1, and control 0.274 mg/plant, 0.230 mg/plant, and 0.289 mg/plant, respectively, after 14 weeks of incubation in the nursery condition. The result of the N uptake analysis of *P. merkusii* seedlings inoculated with DSE strain Hs 14.6c showed a significant difference, when compared to the control treatment (no inoculated) of 0.028 mg/plant and 0.017 mg/plant, respectively, but was not significantly different from the DSE strains Apls 1.5.3, Pls32.1 and Hs14.6a of 0.022 mg/plant, 0.022 mg/plant, and 0.024 mg/plant respectively, after 14 weeks of incubation period in the nursery condition (Table 2).

Morphological identification of DSE strains

Characteristics of microscopical morphology

Microscopic identification of DSE strains is shown in Figure 3. DSE strain Apls 1.5.3 has microconidia and macroconidia, simple conidiospores, branched or rarely branched, form on hyphal septa. Microconidia were oval in shape, appeared small as single-celled and were different from macroconidia. Macroconidia consist of 3 septate with elongated flat shape. The hyphae look like aerial hyphae and elongated. Dark septa were the boundaries of each branch of hyphae. DSE strain Hs14.6a consists of conidiospores with a branching pattern like solitary phialides, and the branches appear to form conidia. The conidia was small, like an oval shape. Hyphae with an elongated shape, with denser septum and look like a chain. Dark septa were visible between the hyphal partitions. Microscopically, the DSE strain Hs14.6c was not much different from the DSE strain Hs14.6a. The conidiophores formed and conidia were not visible. Hyphae appear elongated and insulated to form long chains. Septa on hyphae visible. Furthermore, DSE strain Pls 32.1 consists of unbranched, macronematous-like conidiospores. The shape of the conidia is like a triangle with pointed ends and consists of 3 septate. Hyphae form elongated, branched and insulated. Septa are visible on each branching hyphae. For detailed genetic relationship information of DSE Apls 153, Hs 14.6a, Hs 14.6c and Pls 32.1 strains, we displayed the phylogenic tree in Figure 4.

Molecular identification of DSE strains

The results of identification using BLAST at NCBI GenBank and phylogenic trees using MEGA 11 are shown in Table 3 and Figure 4. DSE strain Apls 1.5.3 has a similarity of 98.32% based on BLAST results at NCBI with *Cylindroccarpon* sp. KO-2013, this is supported by the results of phylogenic tree analysis showing that the DSE strain Apls 1.5.3 was in the same branch as *Cylindroccarpon* sp. KO-2013 with accession number AB846995.1 (Obase and Matsuda 2014), DSE strains Hs14.6a and Hs14.6c have the same resemblance to *Cladophialophora* sp. KO-2013 with accession number AB847068.1 (Obase and Matsuda 2014) respectively by 99.82% and 99.83% based on the BLAST results at NCBI and likewise the DSE stran Pls 32.1 has a resemblance to *Oncopodiella trigonella* species FMR_10788 of 84.44% with an accession number KY853455.1 (Hernández-Restrepo et al. 2017). Based on the BLAST results at NCBI by looking at the taxonomic description of the strains *Cylindroccarpon* sp. KO-2013; *Cladophialophora* sp. KO-2013 and *Oncopodiella trigonella* FMR_10788 are included in the ascomycetes class of fungi.

Discussion

This study is the first report that revealed the DSE association with *P. merkusii*, a pine species native to Indonesia. Several studies have reported that DSE is capable of symbiosis with various species of pine plants, but until now there has been no report on symbiotic relationship between DSE and *P. merkusii*. Based on our study, we have proven the dual function of DSE as a growth promoter of *P. merkusii* seedlings and supporting nutrient uptake from the soil to the *P. merkusii* especially C and N uptake. Generally, the positive effect and function of DSE as a growth promoter are almost similar to that of mycorrhizal fungi (Kageyama et al. 2008; Hiruma et al. 2016; Almario et al. 2017). Like mycorrhizal fungi, DSE in plant tissues helps plants absorb nutrients effectively and efficiently (Alberton et al. 2010; Xie et al. 2021; Wang et al. 2022).

Table 2. Effect of DSE strain treatment on the growth of *P. merkusii* seedlings on C-organic and N-Total nutrient content

DSE Strains	Nutrient content (%)	
	C-organic (%)	N-total (%)
Apls 1.5.3	32.19±0.10 c	2.58±0.12 bc
Pls 32.1	31.23±0.36 d	2.99±0.06 a
Hs14.6a	39.88±0.15 a	2.74±0.06 b
Hs14.6c	39.63±0.09 a	2.50±0.12 c
Control	37.61±0.11 b	2.26±0.06 d
	Nutrient uptake (NU) (mg/plant)	
Apls 1.5.3	0.274±0.05 b	0.022±0.00 ab
Pls 32.1	0.230±0.05 b	0.022±0.00 ab
Hs14.6a	0.357±0.12 ab	0.024±0.01 ab
Hs14.6c	0.440±0.05 a	0.028±0.00 a
Control	0.289±0.03 b	0.017±0.00 b

Description: mean±SD, numbers followed by the same letter are not significantly different based on Duncan's test at 95% confidence interval. n=3

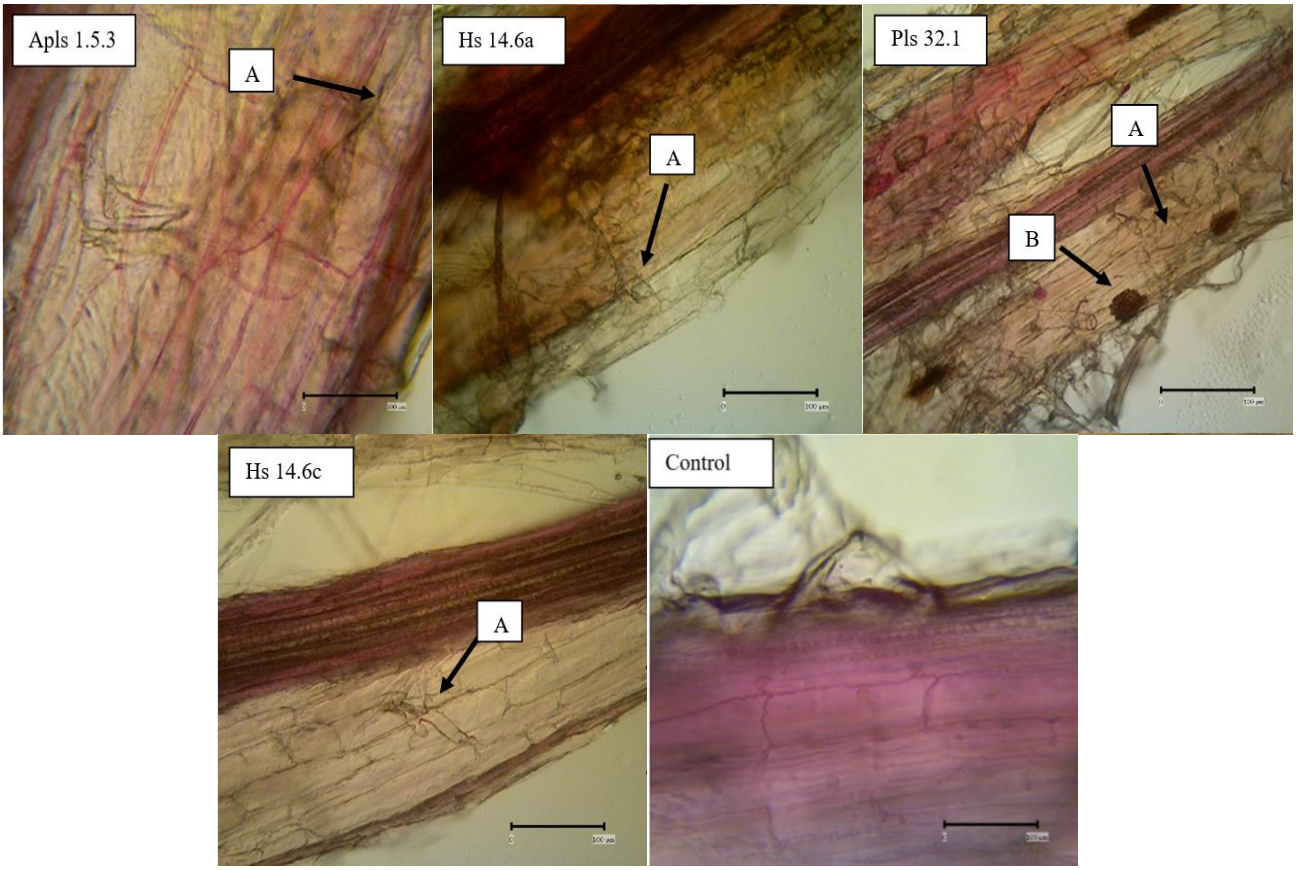

Figure 2. DSE strains Apls 1.5.3, Pls 32.1, Hs 14.6a, Hs 16.6c and control treatment on root colonization of *P. merkusii* seedlings for 14 weeks after incubation in a nursery, arrows: A. Indicate hyphae, B. Microsclerotia, with a scale bar of 100 µm

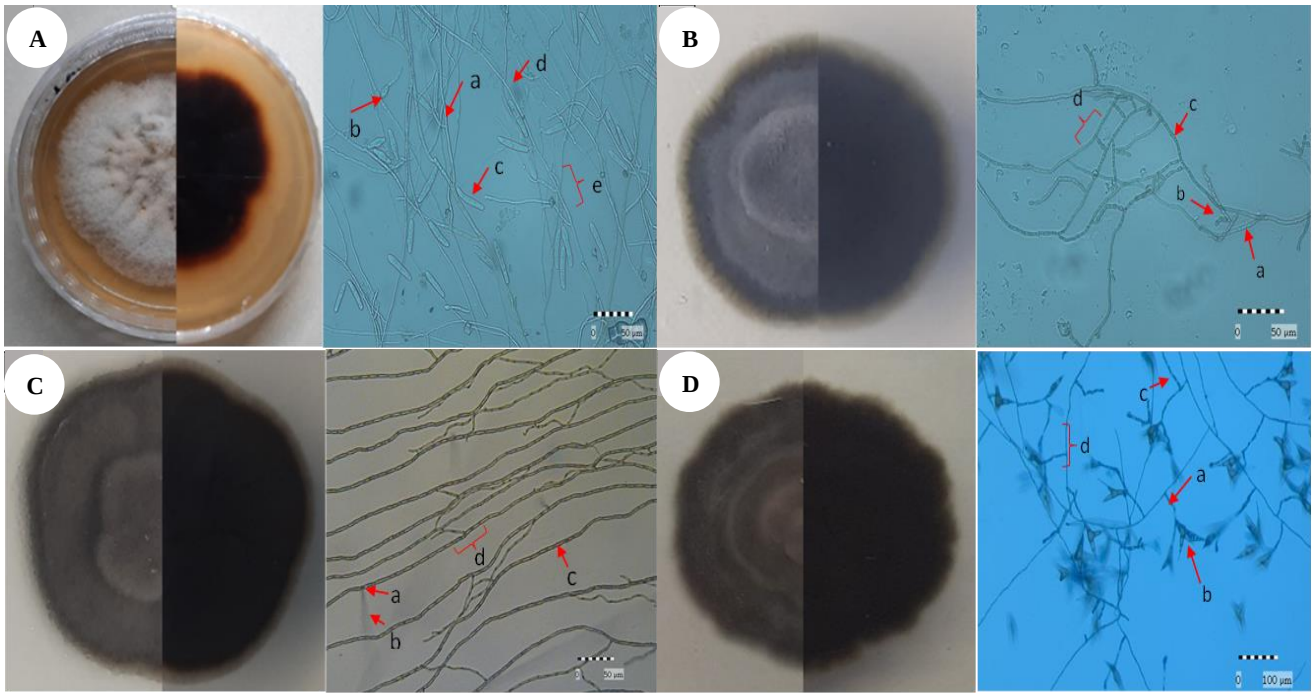

Figure 3. Morphology of DSE strains macroscopically and microscopically, A. Apls 1.5.3 strain (a. Conidiophore, b. Microconidium, c. Macroconidium, d. Hyphae and e. Septa, B. Hs14.6a strain (a. Conidiophore, b. Conidium, c. Hyphae, d. Septa), C. Strain Hs14.6c (a. Conidiophore, b. Conidium, c. Hyphae, d. Septa) with hyphae size scale bar 50µm and D. Strain Pls 32.1 (a. Conidiophore, b. Conidium, c. Hyphae, d. Septa) with a scale bar hyphae size of 100µm. Conidiophore is the part that grows at the base of the hypha. Conidium is the part that contains spores

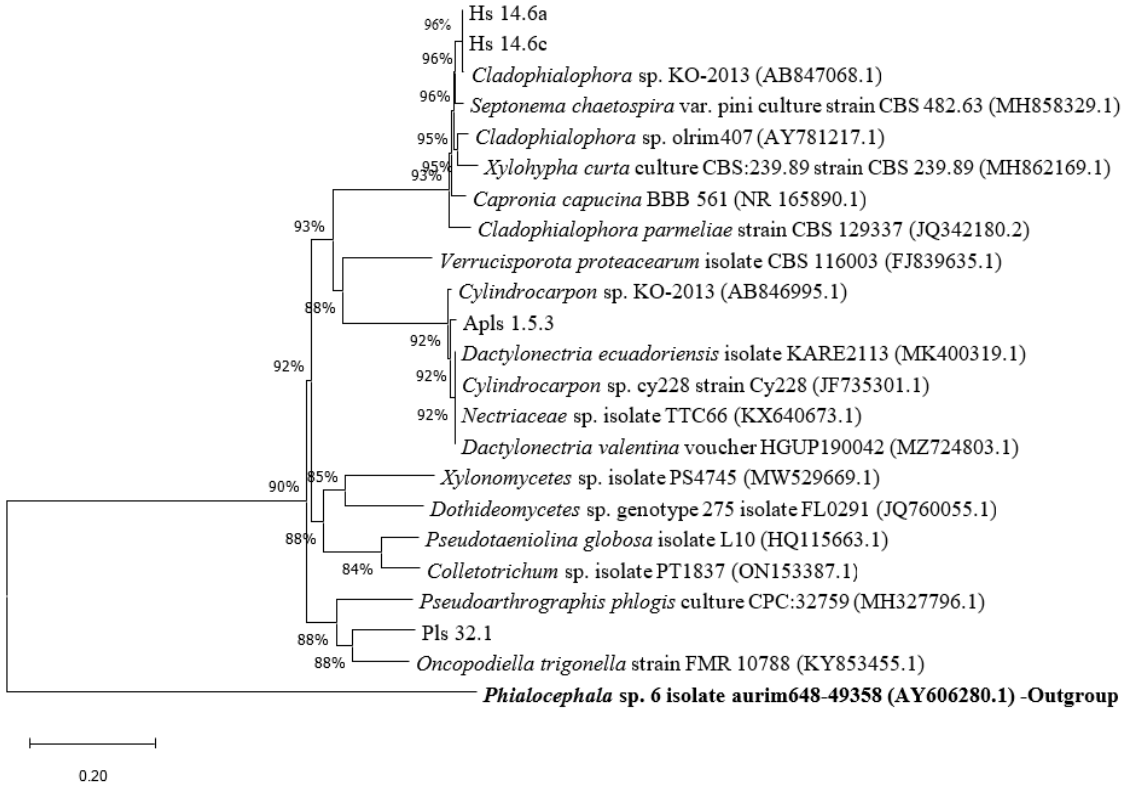

Figure 4. Phylogenetic tree of DSE strains Apls 1.5.3, Hs 14.6a, Hs 14.6c and Pls 32.1 based on the Internal Transcribed Spacer (ITS) sequences

Table 3. The fungal strains used in the construction of the phylogenic tree in this study

Species name	Strain number	ITS sequencing Acc. number
Cylindrocarpon sp. KO-2013	Sa0306	AB846995.1
Dactylonectria ecuadoriensis	KARE2113	MK400319.1
Nectriaceae sp.	TTC66	KX640673.1
Ilyonectria vitis	CBS 129082	JF735303.1
Dactylonectria valentina	HGUP190042	MZ724803.1
Oncopodiella trigonella	FMR 10788	KY853455.1
Pseudoarthrographis phlogis culture	CPC 32759	MH327796.1
Xylonomycetes sp.	PS4745	MW529669.1
Dothideomycetes sp.	FL0291	JQ760055.1
Pseudotaeniolina globosa	L10	HQ115663.1
Verrucisporota proteacearum	CBS 116003	FJ839635.1
Colletotrichum sp.	PT1837	ON153387.1
Cladophialophora parmeliae	CBS 129337	JQ342180.2
Cladophialophora sp.	olrim407	AY781217.1
Cladophialophora sp. KO-2013	Sa0153	AB847068.1
[Septonema] chaetospira var. *pini*	CBS 482.63	MH858329.1
Xylohypha curta	CBS 239.89	MH862169.1
Capronia capucina	BBB 561	NR_165890.1
Phialocephala sp.	aurim648-49358	AY606280.1

As plant growth promoters, DSE strains Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c used in this study increased the *P. merkusii* growth in height, stem diameter, biomass and root length similar to other DSEs have been reported promote the growth of other pine species (Ban et al. 2017; Hou et al. 2020). DSE strains Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c can accelerate plant growth and increase plant survival, including several kinds of pine species (Alberton et al. 2010). According to (Doty 2011), inoculation of a dark septate endophytic fungus on Scottish pine (*Pinus sylvestris* L.) had a positive effect, with a significant increase in root length in the first few months after infection. The symbiotic process of DSE in roots will play an important role for plants in supporting growth and absorption of nutrients (Li et al. 2019; Wang et al. 2022) and affects the biomass of *Pinus sylvestris* seedlings (Alberton et al. 2010).

DSE strains Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c have ability to colonize *P. merkusii* seedling roots during the 14 weeks inoculation period in the nursery condition. The DSE colonization inside *P. merkusii* roots have positive effect of the growth of *P. merkusii* seedlings and its performance was better than the control plant (not inoculated). Based on colonization morphology, DSE strains Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c can be categorized as DSE because the penetration of hyphae from each of these DSE strains does not cause disease symptoms in *P. merkusii* seedlings and even improves their growth and health (Surono and Narisawa 2018). Hyphae that penetrate inside the root area will then come into keep contact with the root

surface and carry out host recognition, penetration, and colonization of the epidermis or root hairs (Carpenter et al. 2021). The next stage is the formation of inter and intracellular hyphae (Santos et al. 2021) in the root cortex area (Carpenter et al. 2021), and transfer of nutrients occurs between the DSE fungus and the host plant. Another study found that DSE inoculation on *P. tabuliformis* seedlings caused the formation of microsclerotia in the root tissues (Chu et al. 2019), which is similar to our recent finding that DSE strain Pls 32.1 inoculated on *P. merkusii* seedlings similarly caused the formation of microsclerotia inside root tissues.

DSE strains Apls 1.5.3, Pls 32.1, Hs14.6a, and Hs14.6c have affected C and N uptake in *P. merkusii* seedlings. Other report stated that inoculation of DSE species, *Phialocephala fortinii*, had a positive effect on N availability in *Pinus contorta* seedlings (Alberton et al. 2010). Other studies have shown that inoculation of two DSE species such as *Acrocalymma vagum* and *Paraboeremia putaminum* also has an effect on the availability of C and N in the growth of *Glycyrrhiza uralensis* plants (He et al. 2019), and increases the efficiency of nitrogen use (Lavallee et al. 2020).

DSE strain Apls 1.5.3 had a positive effect on the growth of *P. merkusii* seedlings during the 14 weeks incubation period in the nursery and this strain is similar to *Cylindroccarpon* sp. based on morphological and molecular identification. This paper is the first report the species as a DSE. Inoculation of DSE strains in *P. merkusii* seedlings showed a positive effect on increasing agronomic parameters such height, stem diameter, root length, and biomass. This DSE strain is able to colonise the roots of *P. merkusii* roots without causing disease symptoms, even making the seedlings healthy so that they perform better. Some reports stated that *Cylindroccarpon* sp. are more likely to become plant pathogens, such as; root disease in almonds (Capote et al. 2022); and stem diseases (Punja et al. 2021); from roots of vines affected by black foot disease (Lawrence et al. 2019); from rootstock of vines affected by black foot disease (Aigoun-Mouhous et al. 2019); from plant disease *Sapium ellipticum* (Kamdem et al. 2018); from driftwood disease (Suzuki et al. 2018; Yoshida et al. 2018); soilborne pathogens causing rot on ginseng roots (Han et al. 2017); necrotic symptoms of strawberry roots (Adhikari et al. 2013); shoot death symptoms on vines (Schroers et al. 2008). In contrast to these reports, *Cylindroccarpon* sp. Apls 1.5.3 used in this study does not act as a pathogen of *P. merkusii*, which has the characteristics of DSE.

Similar to *Cylindroccarpon* sp. Apls 1.5.3, the DSE strains Hs14.6a and Hs14.6c have positive impact on *P. merkusii* growth in nursery conditions. Those strains were similar to *Cladophialophora* sp. based on morphological and molecular identification. Generally, several species of *Cladophialophora* are known as DSEs and promote various plant growth such as a DSE species *Cladophialophora chaetospira* SK51 has ability to promote strawberry plant growth and positively suppress strawberry disease development (Harsonowati et al. 2020). Generally *Cladophialophora* sp. obtained from healthy plants such as *Tillandsia catimbauensis* leaves (Nascimento et al. 2021); healthy orchid roots of *Spiranthes sinensis* and *Cyrtosia septentrionalis* (Harsonowati et al. 2020); *Persicaria fauriei* leaves (Park and Shin 2011); and *Babassu coconut* (da Silva et al. 2023). No reports regarding on *Cladophialophora* sp. strains found in other pine plants, therefore our findings in this study are the first report that *Cladophialophora* sp. has mutualistic symbiosis with *P. merkusii* and promotes the seedling under nursery conditions and also acts as a DSE with colonization activity inside those pine roots.

This study also used other DSE strain such as Pls 32.1 which has similarities with *Oncopodiella trigonella*. DSE *Oncopodiella trigonella* Pls 32.1, functions similar to DSE *Cylindroccarpon* sp. and *Cladophialophora* sp. Inoculation of DSE strain Pls 32.1 on *P. merkusii* seedlings showed healthy growth, increased in height, diameter, root length, biomass, and root colonization. The DSE strain Pls 32.1 had a significant effect on root colonization of *P. merkusii* seedlings, fulfilling the characteristics of DSE. However, there are no reports on *Oncopodiella trigonella* species obtained from *P. merkusii* roots. On the other hand, information on *Oncopodiella trigonella* is still limited, and includes *Oncopodiella trigonella* isolated from pieces of tree bark (Magyar and Révay 2009). Furthermore, the species *Oncopodiella trigonella* has not been widely published as an endophyte, let alone as a DSE, so this study reports for the first time that *Oncopodiella trigonella* has the characteristics and abilities as DSE, especially in symbiosis with *P. merkusii*.

This study adds new information that pines living in the tropics of Indonesia has a symbiotic mutualism relationship with DSE in pine in subtropical regions. In addition, the genus or species of DSE isolated from *P. merkusii* are DSE species that have not previously been reported as DSE on pine trees such as *Cylindroccarpon* sp., *Cladophialophora* sp. and *Oncopodiella trigonella.* The role of DSE will be very important in the current and future conditions as a biological microbial agent and plays an important role in supporting plant growth, especially in *P. merkusii* seedlings starting from symbiotic relationship in the nursery through inoculation techniques to planting in the field. *Pinus merkusii* seedlings that have been inoculated with DSE in the nursery phase are expected to survive when transferred to the field and can adapt to biotic and abiotic stress conditions.

In conclusion, this study is the first to report that DSE is capable of symbiotic relationship with *P. merkusii*, a pine species native to Indonesia. All DSE strains i.e., Apls 1.5.3, Hs14.6a, Hs14.6c and Pls 32.1 used in this study had a positive effect on the increase in height, stem diameter, root length, biomass, root colonization, carbon and nitrogen content, carbon and nitrogen uptake of *P. merkusii* seedlings for 14 weeks incubation in the nursery condition. The results of morphological observations and molecular analysis of DSE strains showed that the Apls 1.5.3 strain had similarities to the *Cylindroccarpon* sp., the Hs14.6a and Hs 14.6c strains had the same resemblance to *Cladophialophora* sp., and the Pls 32.1 strain belonged to the *Oncopodiella trigonella*. The use of DSE in the future as a biological microbial agent will greatly support nursery

activities in nursery condition so that healthy seedlings will be obtained and resistant to biotic and abiotic stresses.

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