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**STUDY ON ECTOMYCORRHIZAL COMMUNITIES
ASSOCIATED WITH *Pinus kesiya* IN LAM DONG
PROVINCE**

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INTRODUCTION

Ectomycorrhizal fungi (EMF) play a fundamental role in forest ecosystems, particularly in association with tree species belonging to families such as Pinaceae, Fagaceae, Betulaceae, and Dipterocarpaceae. Approximately 6,000 plant species and 20,000–25,000 fungal species are known to form ectomycorrhizal associations, which are essential for seedling establishment, forest regeneration, and the long-term maintenance of forest communities. EMF enhance nutrient and water uptake by host plants, improving temperature stress, and pathogenic microorganisms. Many EMF species also was important food resources for both humans and animals.

The family Pinaceae, comprising 11 genera and approximately 215 species, represents the largest group of coniferous trees worldwide. Their obligate symbiosis with EMF has significantly contributed to their ecological dominance and broad distribution in regions characterized by seasonal climates and nutrient-poor soils. In Vietnam, *Pinus kesiya* is the dominant pine species in the southern and Central Highlands regions and has the high adaptability, strong regenerative capacity, and considerable economic value, *P. kesiya* is the only conifer species widely utilized in reforestation programs in Vietnam. During seedling establishment and plantation development, ectomycorrhizal fungi are played an important role in seedling survival, and increasing adaptability after outplanting into plantation. Despite their ecological and economic importance, information regarding the diversity and composition of ectomycorrhizal fungi in Vietnam remains extremely limited. Therefore, this thesis “Study on ectomycorrhizal communities associated with *Pinus kesiya* in Lam Dong Province” was conducted to establish a baseline dataset on the diversity and composition of EMF associated with *P. kesiya* forests in Lam Dong, Vietnam.

Aims of this study:

To establish a dataset on ectomycorrhizal communities associated with different aged stages of *Pinus kesiya* and to apply the obtained findings to the restoration and rehabilitation of natural pine forests in Lam Dong.

Research activities

- Investigating the ectomycorrhizal fungal (EMF) communities associated with different aged stages of *Pinus kesiya*.
- Evaluating the effects of EMF communities on the growth of *P. kesiya* seedlings under *in situ*, *ex situ*, and *in vitro* conditions.
- Examining the formation of EMF sporocarps in 3-year-old *P. kesiya* plantations.

Key findings and novel contributions

1. This was the first study focusing on EMF communities in Vietnam, specifically with *Pinus kesiya* host.
2. The study revealed differences in EMF species composition across different developmental stages of *P. kesiya*.
3. This was the first record of *Rhizopogon boninensis* in Southeast Asia, successfully isolated this species, and evaluated its potential application in the restoration and reforestation.
4. This was the first record of *Nothojafnea* in the Northern Hemisphere.
5. Edible ectomycorrhizal fungal species occurring in 3-year-old *P. kesiya* forests were studied, providing an addition information for sustainable forest management and supporting livelihood improvement for local communities living near forest areas.

Chapter 1. LITERATURE REVIEW

1.1 ECTOMYCORRHIZA

1.1.1. Mycorrhiza

The concept of mycorrhiza was first introduced by A.B Frank in 1885 to describe the symbiotic association between fungi (mykes) and plant roots (rhiza). In 1969, Peyronel et al. classified mycorrhizae into ectomycorrhiza (ECM) and endomycorrhiza (Vesicular-arbuscular mycorrhiza, Arbutoid mycorrhiza, Ericoid mycorrhiza, Orchid mycorrhiza, Monotropoid mycorrhiza) based on fungal colonization patterns.

1.1.2. Ectomycorrhizae

Ectomycorrhizae (ECM) are among the most widespread and ecologically significant symbiotic associations in forest ecosystems, commonly occurring in tree families Pinaceae, Salicaceae, Betulaceae, and Dipterocarpaceae... ECM structures are formed through the mutualistic interaction between specific soil fungi and plant fine roots, resulting in characteristic swollen, dichotomously branched root tips lacking root hairs.

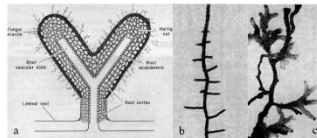


Fig 1. Typical ectomycorrhiza

- (a) Typical ECM with Hartig net, mantle and external hyphae; (b) Non-mycorrhizal and (c) mycorrhizal root

1.2 ECTOMYCORRHIZAL FUNGI (EMF)

1.2.1. EMF community composition

Most EMF belong to the phylum Basidiomycota and Ascomycota and are widely distributed worldwide. They form visible fruiting bodies above

ground and a hidden mycelium network below ground, near host plants. Fruiting body formation of EMF depends on specific environmental conditions, host characteristics and human activities such as forest harvesting and plantation establishment. However, some EMF species produce inconspicuous or no visible fruiting bodies, such as *Cenococcum geophilum*.

1.2.2. Ectomycorrhizal host plants

ECM host plants represent less than 5% of vascular plant species but are ecologically important in many forest ecosystems. This symbiosis may be obligate or flexible, and plants' survival and growth were greater in ECM seedlings compared to non-ECM seedlings. In natural forests, a single plant species can associate with multiple EMF species, while one fungal species may associate with several host plants. These interactions form complex, diverse symbiotic networks within forest ecosystems.

1.2.3. Roles of EMF and ECM

EMF enhance plant water and nutrient uptake, improve soil structure and carbon storage, and protect roots from pathogens and environmental stress. EMF communities can also serve as biological indicators of forest ecosystem quality and stability. EMF also contribute to ecological restoration by improving plant establishment in degraded soils and provide economic and cultural value as food, medicinal resources, and dyes.

1.3 PINE (*Pinus* spp.) FORESTS AND EMF WORLDWIDE

EMF are important for the diversification, distribution, and adaptation of *Pinus* species, particularly in harsh environments. EMF enhance water and nutrient uptake, plant growth, and stress tolerance. EMF inoculation at the nursery stage promotes seedling growth and enhances seedling vigor after transplantation to the planting site. The components and diversity of EMF in

forest soil are strongly influenced by climate, temperature, forestry practices, and natural disturbances. Climate change and forest harvesting can alter EMF communities, potentially affecting pine regeneration and ecosystem recovery. Therefore, understanding EMF diversity is essential for sustainable management and conservation of pine forests.

1.4. RESEARCH ON EMF AND PINE SPECIES IN SOUTHEAST ASIA

Natural pine forests in Southeast Asia include *Pinus kesiya*, *P. latteri*, and *P. merkusii*, as well as the endemic Vietnamese species *P. dalatensis* and *P. krempfii*. Among them, *P. kesiya* is widely distributed in mountainous regions and is commonly used in reforestation and ecosystem restoration due to its adaptability to drought and nutrient-poor soils. However, studies on EMF associations with subtropical pines, particularly *P. kesiya*, remain limited, with only a few studies conducted in India, Thailand, and the Philippines. In Vietnam, EMF research on *P. kesiya* is still scarce, highlighting the need to investigate the dominant EMF taxa in specific regions and at different stages of plant development to support selecting EMF species for a commercial biological product and enhance the effectiveness of afforestation

Chapter 2. RESEARCH SUBJECTS AND METHODS

2.1. RESEARCH SUBJECTS

This study focused on EMF species associated with *Pinus kesiya* in Lam Dong Province across three developmental stages: 6-month-old seedlings, 3-year-old trees, and mature trees. EMF species capable of producing fruiting bodies were also surveyed in 3-year-old *P. kesiya* plantations.

2.2. RESEARCH METHODS

2.2.1. Contents

- Experiment 1: Investigation of EMF species composition across different developmental stages of *P. kesiya*: 6-month-old seedlings, 3-year-old trees, and mature trees.
- Experiment 2: Evaluation of the effects of EMF on *P. kesiya* seedling growth under *in situ*, *ex situ*, and *in vitro* conditions.
- Experiment 3: Survey of EMF fruiting body formation in 3-year-old *P. kesiya* plantations.

2.2.2. Research Sites

- Soil samples were collected from mature *Pinus kesiya* forests managed by the Da Nhim Protection Forest Management Board, Lac Duong District, and from the Institute of Life Sciences (ILS), Da Lat City, Lam Dong Province. The study area covered 4 ha at each site.
- Seedlings used for reforestation and experiment 1–2 were germinated and maintained at the nursery of the Da Nhim Protection Forest Management Board.

- The reforestation site (2 ha) was established adjacent to the mature *Pinus kesiya* forests where soil was sampled.
- ECM root-tip collecting, morphological and molecular identification of ECM and EMF fruiting bodies, and *ex situ* and *in vitro* trials were conducted at the laboratory of Applied Biotechnology Institute, Ho Chi Minh City.

2.2.3. Method of experiment 1

At two forest sites, Da Nhim and ILS, two 4-ha sampling plots were established for soil and ECM root tip sampling. A total of 100 soil cores (15 × 15 × 10 cm) were collected beneath mature pine canopies along sampling transects, with a minimum distance of 20 m between sampling points. ECM root tips were separated from the soil, cleaned, and preliminarily classified using a stereomicroscope. ECM root tips for morphological observation were preserved in lactoglycerol, while those for molecular analysis were preserved in CTAB. The remaining soil was used to identify fungal communities using next-generation sequencing (NGS).

ECM root-tip morphology was characterized based on color, shape, branching pattern, and the presence of rhizomorphs and extramatrical hyphae under a stereomicroscope (SZM-2, Optika, Italy), following the detailed descriptions of Agerer (1987–2012). EMF taxa were identified using molecular analysis of the rDNA ITS region. Total DNA was extracted using the CTAB method, and PCR products were Sanger-sequenced. Results were compared with GenBank database using the BLAST algorithm. The chloroplast *TrnC-D* region was also amplified to confirm the host plant species. Besides, ten DNA samples isolated from soil using PrimeWay Soil DNA Extraction Kit (1st Base, Malaysia) were stored at -20 °C and sent to 1st Base for ITS amplicon sequencing with the ITS5F and ITS2 primers. Sequencing was performed on Illumina MiSeq platform (Illumina, USA).

Pine seeds were sown in biodegradable bags containing soil collected from a mature *Pinus kesiya* forest adjacent to the nursery. After six months, healthy seedlings were transplanted to the plantation site at a density of 2,200 trees/ha and maintained following standard silvicultural practices. Thirty 6-month-old seedlings in the nursery and thirty 3-year-old trees in the plantation were randomly selected for ECM investigation. The entire root systems were collected, and the number and morphology of ECM root tips were recorded, described and molecularly identified following the methods described above. The data were processed using Microsoft Excel and the Amplicon Analysis Platform provided by Nova Lifetech (Singapore).

2.2.4. Method of experiment 2

2.2.4.1. Evaluation of the effects of EMF on *P. kesiya* seedling growth under in situ conditions.

The 6-month-old *Pinus kesiya* seedlings used in this experiment were obtained from the nursery described in Section 2.2.3. ECM root tips were collected and processed following the methods described in Section 2.2.3. Seedling growth was evaluated based on plant height, the proportion of seedlings colonized by EMF, and the number of ECM root tips per seedling root system. Data were analyzed and graphed using Microsoft Excel.

2.2.4.2. Sterile pine seedling preparation

Pine seeds were surface-sterilized with 70% ethanol for 1 min, followed by 7% H₂O₂ for 15 min, and rinsed several times with sterile distilled water. Sterilized seeds were germinated on water agar for one week. Germinated seeds without contamination were transferred to test tubes containing Murashige–Skoog (MS) medium. After two weeks, uncontaminated healthy seedlings were selected for subsequent experiments.

2.2.4.3. Evaluation of the effects of EMF on *P. kesiya* seedling growth under ex situ conditions.

Soil was collected from a mature *Pinus kesiya* forest adjacent to the nursery of the Da Nhim Protection Forest Management Board. The soil substrate was distributed into PP plastic planting cups (500 g soil per cup). Thirty sterile seedlings prepared as described in Section 2.2.4.2 were transferred to the cups, with one seedling per cup. The seedlings were grown and maintained for six months at 20 °C under an average light intensity of 3,000–5,000 lux with a 12 h light/12 h dark photoperiod.

After six months, the seedlings were carefully removed from the soil. Stem height was measured, and the root systems were collected for EMF analysis. ECM root tips were identified following the methods described in Section 2.2.3. Seedling growth was evaluated based on plant height, the proportion of seedlings colonized by EMF, and the number of ECM root tips per seedling root system. Data were analyzed and graphed using Microsoft Excel.

2.2.4.4. Evaluation of the effects of EMF on *P. kesiya* seedling growth under in vitro conditions.

Clay balls (6–8 mm in diameter) were washed, adjusted to pH 5.0–5.5, and dried at 105 °C to constant weight. A total of 100 g of clay balls and 100 mL of sugar-free liquid MMN medium (pH 5.0) were added to glass bottles and sterilized at 121 °C for 15 min. After cooling for 24 h, sterile seedlings were transferred into the bottles (one seedling per bottle) and stabilized for four weeks at 25 °C under a 12 h light/12 h dark photoperiod before co-cultivation.

The EMF strain was activated on MMN medium at 25 °C in the dark. After four weeks, the mycelia were transferred to 50 mL of liquid MMN

medium in Erlenmeyer flasks and cultured for an additional 3–4 weeks for biomass production.

To perform the co-cultured experiment, the EMF biomass was transferred to culture bottles containing sterile seedlings following Ho. The experiment included 15 seedlings inoculated with EMF and 15 uninoculated seedlings as the control. The bottles were wrapped in aluminum foil and incubated at 25 °C under a light intensity of 3,000–5,000 lux with a 12 h light/12 h dark photoperiod for 12 weeks. The colonization of EMF with *P. kesiya* roots was evaluated based on the proportion of seedlings colonized by EMF and the number of ECM root tips per seedling. Seedling growth was assessed based on plant height, root length, fresh biomass, and dry biomass. Data were analysed and graphed using Microsoft Excel and Statgraphics Plus 3.0.

2.2.4. Method of experiment 3

The 3-year-old *Pinus kesiya* plantation established as described in Section 2.2.3 was selected as the sampling site. All EMF fruiting bodies occurring within the plantation were collected from April 2022 to May 2023, comprising a total of 24 sampling events.

Macroscopic characteristics of EMF fruiting bodies were described following Largent et al., including the size, shape, and color of the pileus, lamellae or spore-bearing surface, stipe, context, and other relevant morphological features. Molecular identification was based on the ITS and LSU regions following the methods described in Section 2.2.3.

Based on morphological and molecular identification, together with comparisons with literature and edible mushroom databases from China, Thailand, and Vietnam, particularly studies of *P. kesiya* and *P. merkusii*

forests in Da Lat, Lam Dong Province, the potential of edible EMF species occurring in the 3-year-old *P. kesiya* plantation was assessed.

Chapter 3. RESULTS AND DISCUSSION

3.1 EMF COMPOSITION IN DIFFERENT DEVELOPMENTAL STAGES OF *Pinus kesiya*

3.1.1. EMF species associated with the mature *Pinus kesiya* root systems

3.1.1.1. EMF composition in soil

A total of 1,700 ASVs were detected. Basidiomycota and Ascomycota were the two dominant fungal phyla, while Mucoromycota and Mortierellomycota occurred at lower proportions. Basidiomycota predominated at both sites, accounting for 61.6% at the Institute of Life Sciences and 52.6% at Da Nhim, with a statistically significant difference between sites ($p = 0.016$). Ascomycota was the second most abundant phylum, but its relative abundance did not differ significantly between sites. Both the Shannon diversity and Pielou's evenness indices were significantly higher at Da Nhim than at the Institute of Life Sciences ($p = 0.012$).

30 EMF genera were detected across the two study sites, all belonging to Basidiomycota. Shannon diversity was significantly higher at Da Nhim ($p = 0.012$), indicating greater EMF diversity than at the Institute of Life Sciences. In contrast, Pielou's J did not differ significantly between sites, suggesting similar patterns of relative species distribution. Twelve EMF genera, including *Amanita*, *Astrosporina*, *Ceratobasidium*, *Cortinarius*, *Hydnum*, *Hygrocybe*, *Inocybe*, *Rhizopogon*, *Russula*, *Sebacina*, *Thelephora*, and *Tomentella*, were recorded at both sites. Overall, 18 EMF genera showed higher relative abundance at Da Nhim, with 12 exhibiting significant differences ($p \leq 0.05$). Conversely, at the Institute of Life Sciences, 8 of 13 EMF genera showed significant differences compared with Da Nhim.

3.1.1.2. EMF composition in root tips

Three main ECM root-tip morphotypes were recorded: unbranched, dichotomous, and coralloid. More than one morphotype could occur within a single soil sample. At Da Nhim, 10% of soil samples contained no ECM root tips, while 14% contained old and desiccated root tips that could not be identified. A total of 17,324 ECM root tips representing 56 morphotypes were recorded. Each soil sample contained 1–5 morphotypes, with 50% of samples containing two or more. The number of ECM root tips ranged from 5 to 331 per soil sample, with several samples showing exceptionally high abundances. Molecular identification revealed 18 EMF genera associated with mature *P. kesiya* root tips: *Amanita*, *Boletus*, *Cenococcum*, *Coltricia*, *Hydnum*, *Inocybe*, *Laccaria*, *Lactarius*, *Lactifluus*, *Macowanites*, *Pulveroboletus*, *Rhizopogon*, *Russula*, *Sebacina*, *Thelephora*, *Tomentella*, *Tylospora*, *Zangia*, and one species belonging to the order Cantharellales. The dominant genera included *Lactifluus*, *Tomentella*, *Russula*, *Rhizopogon*, *Lactarius*, *Sebacina*, *Zangia*, *Cenococcum*, and *Amanita*. *Lactifluus* and *Tomentella* accounted for the highest numbers of root tips, representing 44.2% and 24.7%, respectively, whereas *Russula* and *Tomentella* were among the most frequently occurring genera.

At the Institute of Life Sciences, 10% of soil samples contained no ECM root tips and 10% contained old, desiccated root tips that could not be identified. A total of 34,096 ECM root tips representing 42 morphotypes were recorded. Each soil sample contained 1–5 morphotypes, with 52.5% containing two or more. Approximately 50% of samples yielded more than 180 ECM root tips, with two samples showing exceptionally high abundances. Nineteen EMF genera were identified: *Amanita*, *Austroboletus*, *Boletus*, *Coltriciella*, *Gomphidius*, *Hourangia*, *Inocybe*, *Lactarius*, *Multifurca*, *Phylloporus*, *Pulveroboletus*, *Retiboletus*, *Rhizopogon*, *Sebacina*, *Suillus*, *Thelephora*, *Tomentella*, *Tylopilus*, and *Zangia*. *Amanita* (35.7%)

and *Lactarius* (20.7%) accounted for the largest proportions of ECM root tips, followed by *Zangia*, *Rhizopogon*, *Tomentella*, *Retiboletus*, *Coltriciella*, *Pulveroboletus*, and *Suillus*. *Tomentella*, *Zangia*, and *Coltriciella* were among the most frequently occurring genera.

Ten genera occurred commonly at both sites: *Amanita*, *Boletus*, *Inocybe*, *Lactarius*, *Pulveroboletus*, *Rhizopogon*, *Sebacina*, *Thelephora*, *Tomentella*, and *Zangia*. Although the number of ECM root tips of the Institute of Life Sciences was 1.97 times higher than at Da Nhim, the EMF compositions differed markedly. *Lactifluus* sp1 and *Tomentella* sp14 dominated at Da Nhim, whereas *Amanita* sp1, *Lactarius subbrevipes*, and *Zangia* sp. were predominant at the Institute of Life Sciences. The species similarity between the two sites was only 3.2%. The Shannon–Wiener index also indicated higher EMF diversity at Da Nhim than at the Institute of Life Sciences.

Based on comparisons of EMF communities detected from ECM root tips and soil at the two sites, subsequent investigations were focused on the natural *P. kesiya* forest at Da Nhim.

3.1.2. EMF species associated with 6-month-old *P. kesiya* root systems

ECM root tips were present in 100% of the seedlings. A total of 11,499 ECM root tips were collected from 30 seedlings, with each seedling exhibiting 1–4 morphotypes. Of these, 53.33% of the seedlings had only one morphotype, with an average of 398.18 ECM root tips per seedling. Seedlings with two or more morphotypes had 21–1,016 ECM root tips per seedling, and only one seedling exhibited four distinct morphotypes.

The EMF community of nursery seedlings was relatively low in diversity, with five genera identified: *Cenococcum*, *Nothojafnea*, *Rhizopogon*, *Sebacina*, and *Tylospora*. Only *Cenococcum* has two species, while the remaining genera contained one species. *Nothojafnea* sp. was dominant,

accounting for 9,766 ECM root tips (84.93%) and occurring in all 30 seedlings. *Sebacina* and *Tylospora* had the lowest occurrence, being detected in only 1 and 2 seedlings, respectively, with 74 and 95 ECM root tips.

3.1.3. EMF species associated with 3-year-old *P. kesiya* root systems

The results showed that all seedlings had ECM root tips, which exhibited distinct morphological characteristics compared with non-mycorrhizal fine roots. A total of 37,361 ECM root tips were recorded, with each tree exhibiting 1–5 morphotypes. Among these, 43.33% had two morphotypes, while 26.67% and 23.33% had one and three morphotypes, respectively. Only one tree exhibited four morphotypes and another exhibited five.

A total of 16 EMF genera were identified from the root systems, including *Amanita*, *Cenococcum*, *Coltricia*, *Cortinarius*, *Cyanoboletus*, *Lactarius*, *Phylloporus*, *Pulveroboletus*, *Rhizopogon*, *Russula*, *Scleroderma*, *Sebacina*, *Suillus*, *Tomentella*, *Tylophilus*, and *Tylospora*, together with one sample belonging to the order Cantharellales. *Russula* and *Tomentella* showed the highest species diversity, with 9 and 7 species, respectively, occurring in 14 and 13 trees. They also accounted for the highest numbers of ECM root tips, with 11,235 and 11,594 tips, respectively. In contrast, *Cortinarius* was detected in only one tree, with 7 ECM root tips.

3.1.4. Comparison of EMF composition associated with *Pinus kesiya* across different stand ages

A total of 70 ECM root tip morphotype samples from the Da Nhim forest, 75 from the Institute of Life Sciences, 64 from 3-year-old *Pinus kesiya* trees, and 51 from nursery seedlings were molecularly identified. Overall, 32 EMF genera and 116 EMF species were recorded, including 30 genera of Basidiomycota and two genera of Ascomycota, *Cenococcum* and

Nothojafnea. TrnC-D analysis confirmed that all ECM root-tip samples belonged to *P. kesiya*.

Sebacina and *Rhizopogon* occurred across all three developmental stages. Previous studies have also identified *Sebacina*, *Rhizopogon*, *Cenococcum*, *Suillus*, and *Tomentella* as widespread EMF with broad host ranges. In contrast, EMF communities showed low similarity among nursery seedlings, 3-year-old trees, and mature forests, supporting the view that each host developmental stage has a characteristic fungal community. In 6-month-old nursery seedlings, *Nothojafnea* sp. was strongly dominant, occurring in 100% of samples with a relative abundance of 84.93%. The dominance of a few EMF taxa in young seedlings has also been reported in previous studies. After young seedlings were transplanted to the forest, EMF symbionts belonging to the Basidiomycota phylum increased rapidly and EMF composition changed substantially.

The fungal communities of 6-month-old and 3-year-old trees were comparable to those reported from recently restored or young plantation forests. In contrast, mature trees at Da Nhim were dominated by *Tomentella*, *Russula*, *Lactarius*, and *Amanita*, together with taxa belonging to Cantharellales and Boletales, consistent with patterns reported from older forests. The Shannon–Wiener index indicated substantially higher EMF diversity in 3-year-old and mature trees than in 6-month-old seedlings. The low evenness value in 6-month-old seedlings (0.322) reflected an uneven distribution of fungal taxa. Previous studies have similarly shown that EMF richness generally increases with host plant age, although differences among EMF communities become less pronounced in mature forests aged approximately 60–120 years or older.

For *P. kesiya*, previous studies in India and the Philippines have reported *Boletus*, *Scleroderma*, *Lactarius*, *Amanita*, *Russula*, *Suillus*, and *Cenococcum* as common EMF groups, with fungal diversity increasing with forest age. In Vietnam, however, this is the first study to investigate EMF diversity associated with *P. kesiya*. The results are consistent with previous studies of this host species and additionally provide new records of *Nothojafnea* sp. and *Rhizopogon boninensis*. Notably, *Nothojafnea* had previously been reported only in association with *Nothofagus* in Chile and Argentina and had not been recorded from Pinaceae or the Northern Hemisphere. *Rhizopogon boninensis*, previously considered endemic to Japan, was detected in soil from the mature Da Nhim forest. *Rhizopogon* is an important ectomycorrhizal genus supporting pine regeneration and growth and is commonly used as an inoculant in pine nurseries. These findings therefore demonstrate considerable scientific significance and promising applications for pine forest restoration.

3.2. EVALUATION OF THE EFFECTS OF EMF ON *P. kesiya* SEEDLING GROWTH UNDER IN situ, ex situ AND in vitro CONDITIONS.

3.2.1. Evaluation of the effects of EMF on P. kesiya seedling growth under in situ conditions.

Under nursery conditions, all seedlings formed ectomycorrhizal associations with at least one EMF species; however, the number of ECM root tips per seedling varied considerably. No clear correlation was observed between the number of ECM root tips and the number of ECM morphotypes. Many seedlings with only one morphotype had a high number of ECM root tips, except for a few cases. This suggests that an increase in the number of EMF taxa does not correspond to a proportional increase in ECM root tips,

reflecting competition and dominance among EMF species during symbiosis. EMF composition and abundance are influenced not only by species diversity but also by fungal adaptability, symbiotic efficiency, and physiological compatibility with the host. Furthermore, seedling height showed no clear relationship with either morphotype richness or ECM root-tip abundance. Seedlings with multiple morphotypes were all shorter than 16 cm, possibly due to interspecific EMF competition, which may reduce overall symbiotic efficiency and increase host carbon costs at the 6-month seedling stage. Although EMF did not clearly promote shoot height growth, their most notable effect was the 100% survival rate of seedlings in the nursery, together with their successful adaptation and development after transplantation to the plantation site.

3.2.2. Evaluation of the effects of EMF on P. kesiya seedling growth under ex situ conditions.

After six months of cultivation, 100% of the seedlings under *ex situ* conditions formed ECM root tips. Among them, 76.7% had one morphotype, 16.7% had two morphotypes, and 6.7% had three morphotypes within the same root system. However, similar to the *in situ* experiment, no clear relationship was observed among EMF diversity, ECM root-tip abundance, and seedling height. Molecular identification revealed marked differences in EMF composition between the two experimental conditions. The total number of ECM root tips under *in situ* conditions was approximately 6.7 times higher than that under *ex situ* conditions, indicating substantially greater colonization under natural conditions. Under *ex situ* conditions, *Rhizopogon* was dominant (86.3%), followed by *Cenococcum* (5%), whereas these genera accounted for only 10.1% and 3.5%, respectively, under *in situ* conditions. Conversely, *Nothofajfnea* accounted for 84.9% of the EMF community under *in situ* conditions but was not detected under *ex situ*

conditions. The occurrence of *Rhizopogon* and *Cenococcum* under both conditions suggests their strong competitive ability, adaptability, and potential as inoculum sources for nursery seedlings. The dominance of *Nothofajnea* under *in situ* conditions may be associated with seed sterilization.

Pinus kesiya seedlings readily formed ectomycorrhizal associations when suitable EMF inoculum was available in the environment. Although the six-month experimental period did not clearly demonstrate effects on seedling growth, the EMF community may play an important role in maintaining seedling survival and facilitating adaptation after transplantation to the plantation site.

3.2.2. Evaluation of the effects of EMF on P. kesiya seedling growth under in vitro conditions.

Rhizopogon was one of only two fungal genera detected across all three developmental stages of *Pinus kesiya* and occurred at relatively high abundance in soil samples. Numerous studies have demonstrated the important role of this genus in pine regeneration and seedling survival. Consequently, *Rhizopogon* is commonly used as a mycorrhizal inoculant in nurseries before seedlings are transplanted to field conditions. To evaluate its application potential in Vietnam, the study isolated *R. boninensis* (VNM00071130) and conducted co-cultivation with *P. kesiya* seedlings under *in vitro* conditions. After 12 weeks, 100% of the inoculated seedlings formed ECM structures, whereas no ECM structures were observed in the control group.

Growth data showed that seedlings inoculated with *R. boninensis* performed substantially better than the controls. Plant height increased 1.7 fold (14.25 ± 1.07 cm vs. 8.46 ± 0.80 cm), while root length increased 2.9

fold (10.08 ± 3.44 cm vs. 3.53 ± 0.72 cm). Fresh and dry biomass were also markedly higher in the inoculated seedlings. Although both treatments had a 100% survival rate, the differences in growth and biomass accumulation demonstrated the effectiveness of *Rhizopogon* in promoting *P. kesiya* seedling development. These findings are consistent with previous studies on other pine species, showing that *Rhizopogon* species can effectively colonize roots, persist in the rhizosphere, and enhance seedling growth, biomass production, and drought tolerance. Therefore, ectomycorrhizal inoculation, particularly with *Rhizopogon*, represents a promising approach for improving seedling quality and the adaptability of pine plantations under drought-prone or climate-change conditions.

3.3. SURVEY OF EMF FRUITING BODY FORMATION IN 3-YEAR-OLD *P. kesiya* PLANTATIONS

EMF fruiting body sampling was conducted from April 2022 to May 2023 over 24 survey rounds, when the plantation was three years old, yielding a total of 103 EMF samples. Sample abundance varied considerably among months, with the highest numbers recorded in April, May, and July 2022 and May 2023, whereas no specimens were recorded in August and October 2022 or from February to April 2023. Weather data from the Da Lat area showed relatively stable temperatures but marked monthly variation in rainfall. Fruiting bodies occurred mainly during the rainy season; however, months with prolonged and heavy rainfall yielded fewer EMF fruiting bodies.

Preliminary classification based on macroscopic morphology revealed three major morphological groups: gill mushroom, bolete mushroom, and truffle-like fungi. Molecular identification revealed 49 EMF species belonging to 20 genera. The most frequently recorded genera included *Amanita*, *Russula*, *Scleroderma*, *Zangia*, *Tylopilus*, *Laccaria*, and

Pulveroboletus, *Amanita*, *Russula*, and *Laccaria* had the highest species richness, whereas *Scleroderma* was represented by only one species. Some genera, such as *Amanita*, *Scleroderma*, and *Tylopilus*, occurred throughout much of the year, while *Russula*, *Zangia*, and *Pulveroboletus* showed pronounced occurrence during particular months.

Of the 49 fungal species recorded, approximately 31% were considered edible, including species commonly collected by local communities such as *Amanita sinensis*, *Cantharellus* sp., *Lactarius hatsudake*, *Russula virescens*, and *Suillus bovinus*. Approximately 28% of the species were potentially toxic, mainly belonging to *Cortinarius*, *Inocybe*, *Multifurca*, *Pulveroboletus*, and *Scleroderma*, while the edibility or potential uses of the remaining 41% could not be clearly determined. Information on fungal species composition, potential uses, and seasonal fruiting patterns is important for sustainable forest management and conservation, as well as for developing livelihood opportunities for local communities.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

- Total 100 soil samples from two mature *Pinus kesiya* forest sites at Da Nhim and the Institute of Life Sciences were collected. NGS analysis of soil fungal communities showed higher fungal diversity at Da Nhim than at the Institute of Life Sciences.
- A total of 70 ECM root-tip morphotypes from the mature Da Nhim forest, 75 from the mature forest at the Institute of Life Sciences, 64 from 3-year-old *P. kesiya* trees, and 51 from nursery seedlings were successfully collected, morphologically characterized, and molecularly identified. Overall, 32 EMF genera and 116 species were recorded, including 30 genera of Basidiomycota and two genera of Ascomycota. *Sebacina* and *Rhizopogon* were detected across all three developmental stages of *P. kesiya*.
- EMF communities associated with mature trees and 3-year-old seedlings were substantially more diverse and evenly distributed than those associated with 6-month-old nursery seedlings.
- *Rhizopogon boninensis* was recorded for the first time in Vietnam and demonstrated its potential to enhance the vigor and growth of *P. kesiya* seedlings when co-cultured under *in vitro* conditions.
- *Nothojafnea* sp. was recorded for the first time in the Northern Hemisphere.
- No clear relationship was observed between EMF species composition or the number of ECM root tips per seedling and *P. kesiya* seedling growth, as assessed by plant height under *in situ* and *ex situ* conditions.
- A total of 15 out of 49 EMF species recorded in the 3-year-old *P. kesiya* plantation were identified as potentially edible species.

RECOMMENDATIONS

- Increase the number of soil samples for NGS analysis to improve the detection of low-abundance fungal taxa. The use of multiple primer pairs, including EMF-specific primers, should also be considered to increase taxonomic coverage and improve the assessment of overall fungal and EMF community composition.
- Extend the experimental period by 1–2 years and incorporate additional growth parameters, such as root length, fresh and dry biomass, and number of leaves..., to better evaluate the effects of EMF communities on *P. kesiya* growth under *in situ* and *ex situ* conditions.
- Conduct analyses of soil physicochemical properties, including pH, organic matter, total nitrogen, phosphorus, potassium, soil moisture, and soil texture (e.g., clay and sand content), to clarify the effects of environmental conditions on EMF species composition and explain differences between the Da Nhim and Institute of Life Sciences sites.
- Expand the study area and establish additional sampling plots in 3–5-year-old *P. kesiya* plantations to monitor fruiting body formation and estimate EMF yields. These data would provide a basis for evaluating the potential of EMF as valuable non-timber forest products (NTFPs), contributing to increased income from plantations and more sustainable forest resource management and utilization.
- Further investigate the cultivation potential of selected edible EMF species with desirable flavor and high economic value.

LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

1. Ngo, T.T., Le, N.T, Dang Q.H., Nguyen, D.H., Duong, M.T.T., Ho, Q.B.T, Cai, L., Pham, H.N.D. Extended distribution of the ectomycorrhizal fungus *Rhizopogon boninensis* in tropical Asia and its verified growth-promoting effect on *Pinus kesiya*. Mycol Progress 25, 32 (2026)
2. Tram, N. T., Quyen, H. D., Nhan, L. T., Hoang, N. D. P., Phung, H. P. P., Mai, T. T. D., Quyen, B. T. H. and Dung, H. N. (2026). Using the baked clay balls for the *in vitro* model of ectomycorrhization. International Journal of Agricultural Technology xx(x): XX-XX
3. Tram T. Ngo, Quyen H. Dang, Nhan T. Le, Jaturong Kumla, Dung H. Nguyen, Hoang N. D. Pham. New record of ectomycorrhizal association of the genus *Nothofagfnea* in the Northern Hemisphere. Mycorrhiza 36, 74 (2026)