JJBS

Volume 16, Number 1,March 2023
ISSN 1995-6673
Pages 125 – 130
https://doi.org/10.54319/jjbs/160115

Jordan Journal of Biological Sciences

Cultivation of Edible Ectomycorrhizal Mushrooms (*Phelobopus portentosus*) Associated with *Sesbania javanica* Miq

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Received: July 16, 2022; Revised: September 28, 2022; Accepted: October 27, 2022

Abstract

Phlebopus portentosus is a popular wild edible mushroom that is found growing on both forest trees and fruit trees. Because it can grow either with various trees or without a host tree, it is reported that it is not a mycorrhizal fungus and may not be an obligate ectomycorrhizae. Furthermore, it was recognized that *P. portentosus* can be found in association with leguminous plants, such as *Sesbania javanica* Miq. The aims of this study were to cultivate *P. portentosus* with bioinoculants in solid and liquid forms and track the ectomycorrhizal association with *Sesbania javanica* Miq. A pure mycelium culture of this fungus was isolated, and the growth parameters, temperature, medium pH, and types of medium were examined. The mycelium grew very well on synthetic media such as MPDA and YM. The optimum pH and temperature were pH 4 and 30^{o}C, respectively. Bioinoculants of *P. portentosus* were inoculated on seedbeds of *Sesbania javanica* Miq. After inoculation, ectomycorrhizal-like roots were found at 6 months, and sporocarps were found at 8.5 months. To prove this, the ITS rDNA sequences were aligned, and the results revealed that all sequences of the ectomycorrhizal-like root tips and sporocarps that developed on seedbeds of S. *javanica* Miq. and black bolete used as a bioinoculant were 100% identical to those of *P. portentosus* MN962534 (GenBank database). This finding indicates that *P. portentosus* can form ectomycorrhizae with *S. javanica* Miq.

Keywords: ectomycorrhiza, edible mushroom, symbiosis, cultivation, ITS region, *Phelebopus portentosus*, *Sesbania javannica* Miq.

1. Introduction

Worldwide, approximately 2.2-3.8 million species are included in the fungus kingdom (Howksworth and Lücking, 2017), while only 35,000 species produce macroscopic fruiting bodies (He et al., 2019). Mycelium fungi that can form fruiting bodies are called “mushrooms.” Mushrooms consist of various types with different shapes, colors, and sizes. In addition to their varied morphology, mushrooms also have various habitats: belowground, aboveground, and saprophytes. Most below-ground and above-ground mushrooms are ectomycorrhiza (ECM). Ectomycorrhizal mushrooms are mostly symbiotic with woody plants. In temperate regions, ECM are associated with many species including birch, spruce, beech, willow, pine, fir, poplar, and oak. The Dipterocarpaceae species in Southeast Asia are mostly mutualistic with ECM. Both the plant and fungus benefit from each other. In addition to protecting against plant root diseases and providing drought resistance, fungi help plants to absorb more nutrients and water. In turn, the plants provide carbohydrates and growth factors to the fungi (Smith and Read, 2008).

Edible mushrooms have been used as food for a long time because they are rich in protein, vitamins, and minerals. Essential amino acids that humans cannot synthesize are found in mushrooms such as *Pleurotus ostreatus* (Chirinang and Intarapichet, 2009), *Agaricus bisporus*, *Boletus edulis* (Jaworska and Bernaś, 2013), and in wild edible mushrooms (Yuwa-amornpitak et al., 2020). Aside from their nutritional value, mushrooms are a source of bioactive compounds with antimicrobial (Titilawo et al., 2022; Muhsin et al., 2011) and anticancer (Oyetayo et al., 2013) properties.

P. portentosus is placed in the Boletinellaceae family and is distributed in tropical regions in Asia (Watling R.,2001). It is found in the central, northern, and northeastern parts of Thailand and is also found in China (Zhang et al., 2017), Laos (Mortimer et al., 2012), Myanmar (Zhang et al., 2015), and Sri Lanka (Berkeley and Broome, 1873). The fruiting bodies of *P. portentosus* mostly form on the ground under trees during the rainy season. The fungus can form associations with various tree species, such as *Mangifera indica*, *Dimocarpus longan*, *Elaeocarpus hygrophilus*, *Syzygium cumini*, Quercus spp., and *Minosa pigra*. With a good texture and rich nutritional value (Zhang, 2010), *P. portentosus* is a tasty wild edible mushroom that appeals to local people and mushroom hunters. Furthermore, *P. portentosus* contains high concentrations of bioactive compounds, such as phenolic compounds (Kaewnarin et al., 2016); pyrrole alkaloids (phlebopines); 2-[2-forml-5-(methoxy methyl)-1H-pyrrole-1-yl] propanoate; inotopyrrole B; and 1-isopentyl-2-formyl-5-hydroy-methylpyrrole (Hou et al., 2014). Sun et al. (2018) also found that inotopyrrole B exhibits

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neuroprotective effects against hydrogen peroxide-induced neuronal-cell damage in human neuroblastoma SH-Sy5Y cells.

In contrast to other wild edible ectomycorrhizal mushrooms, *P. portentosus* is associated with various tree species, not only with forest trees but also with fruit trees. Kumla et al. (2016) also reported that *P. portentosus* has the ability to form ECM-like structures in *Pinus kesiya* after 1 year of inoculation with fungal mycelium. Furthermore, it can be produced as basidiomes without a host plant, and this method shows an ability to produce basidiomes 2 years after original isolation from tissues (Kumla et al., 2012). Zhang et al. (2017) reported that *P. portentosus* creates a tripartite association with mealy bugs and roots. The fungus has a symbiotic association with soil mealy bugs and creates special fungus-insect galls. The galls grow on plant roots with parasitic habits. Fang et al. (2020) reported that mealy bugs excreted honeydew inside or outside the galls. Honeydew is rich in amino acids and sugars that promote mycelial growth. An annual plant such as *Sesbania javannica* Miq., is also found on the seedbedalong with *P. portentosus*. However, there are no reports on the association between them.

The objectives of this study were to cultivate and examine the association of black bolete with *Sesbania javannica* Miq. by using two types of bioinoculants (solid and liquid bioinoculants). The mycelium growth parameters were studied and used as growth conditions to propagate solid bioinoculants in sorghum grains. To prove the association of the fungus with the root tree, the sequences of the ITS region from black bolete, ECM from *Sesbania javannica* Miq. root, and sporocarps on the seedbed were aligned.

2. Materials and methods

2.1. Pure mycelium isolation

Basidiocarps of *P. portentosus*(black bolete) were purchased from a local market in Maha Sarakham Province in the rainy season (May-October 2019-2020). A pure mycelium culture was isolated from fresh sporocarps using aseptic techniques by cutting a small piece and transferring it onto a modified potato dextrose agar plate, MPDA (potato juice extract from 200 g boiling in water 1 liter for 30 min, glucose 20 gl^{-1}, yeast extract 2 gl^{-1}, peptone 2 gl^{-1}, malt extract 3 gl^{-1}, agar 20 gl^{-1}). The plates were incubated at 30^{o}C until the mycelia growth fully covered the plate. The pure culture was transferred onto a new medium plate after 3-4 weeks of age for further study.

2.2. Growth study

The temperature, initial pH of the medium, and types of media were the important parameters for fungal growth. In this study, temperatures ranging from 25^{o}C to 35^{o}C, pH levels ranging from 4 to 9, and three medium types were investigated: MPDA, YM (yeast malt agar; glucose 10 gl^{-1}, yeast extract 3 gl^{-1}, peptone 5 gl^{-1}, malt extract 3 gl^{-1}, and agar 20 gl^{-1}), and starch medium (starch 10 $gl^{-1}$and agar 20 gl^{-1}). One piece of fungus mycelium (diameter 0.5 cm) was transferred from the inoculum plate (mycelium on YM agar plate) to the center of a new plate and incubated at 30^{o}C. Each studied parameter had three replicates. The growth diameters of the fungi were measured and analyzed.

2.3. Solid bioinoculant preparation

A solid bioinoculant was prepared from the mycelium growth on sorghum grains. Sterilized sorghum grains were prepared by soaking in water for 3-5 h or overnight, decanting and cleaning. Sorghum grains were boiled until the grains were softened, and the water was decanted. The grains were used to fill containers (plastic bags or bottles) after they had cooled down. After that, they were sterilized at 121^{o}C for 15 min in an autoclave sterilizer. Then, 1-2 pieces of fungus mycelium were aseptically transferred (Figure 1-A) to a container with sorghum grains and incubated at 30^{o}C until the mycelium covered the sorghum grains (3-4 weeks, Figure 1-B).

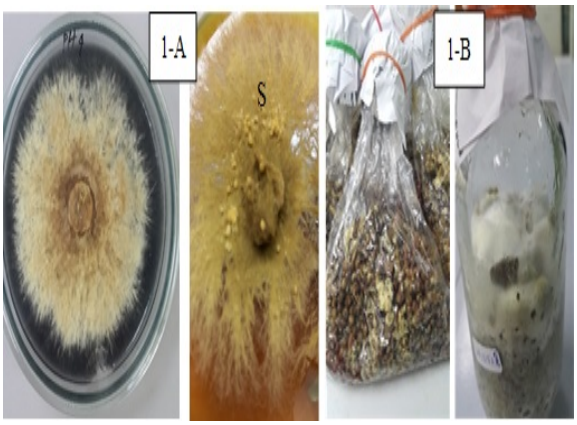


Figure 1. Bioinoculant of *P. portentosus* (black bolete) on sorghum grains**:**1-A shows mycelium growth on an agar plate with and without activated charcoal;1-B shows mycelium growth on sorghum grains used as a bioinoculant.S in Fig. 1-A represents sclerotia

2.4. Liquid bioinoculant preparation

Fresh and mature *Phebopus portentosus* sporocarps were used as spore suspension inocula (liquid bioinoculants). The sporocarps were pulverized with clean water. Mushroom debris was separated by sieving through a strainer for a long period. The spore suspension was placed in a bottle and kept at room temperature. If the liquid bioinoculant was immediately used, there was no need to remove the debris. The sporocarps could be separated by pulverizing only the tubes (spores are present inside these parts) to provide a high spore density.

2.5. Plantation and inoculation with bioinoculants

The planting area was prepared by tilling, removing weeds, constructing three seedbeds (1 m x 6 m), and adding organic matter such as compost. *S. javannica* Miq. seeds were immersed in water overnight, decanted and cleaned before sowing. The seeds were sown on the seedbeds, covered with rice stalks and watered twice a day. When the plants were at least 50 cm tall, the soil around the roots was carefully removed (plant roots were not disturbed) to reveal the plant roots, and *P. portentosus* bioinoculants on sorghum grains and liquid bioinoculants were inoculated on the roots of *S. javannica* Miq., then covered with soil, compost, rice stalks, and watered. For the spores to germinate and mycelia to grow, the seedbeds were not watered after 2 or 4 days of inoculation. The seedbeds were still watered twice each day, and compost and rice stalks were regularly added.

2.6. ECM on plant roots and fruiting bodies on seedbeds

Plant roots of *S. javannica* Miq. were sampled from the seedbeds by using four randomly selected samples per seedbed after inoculation for 4, 5 and 6 months. Samples were kept in plastic bags and stored in a refrigerator until examination under a stereomicroscope (Olympus SZ61, Japan). Soil and debris were removed from the samples by cleaning with water. The ECM-root-like structures at the fine root tips were kept in absolute ethanol and stored in a refrigerator.

Fruiting bodies on the seedbeds, including plant roots near or connecting to the fruiting bodies, were collected. Small pieces of mushroom were cut and kept in absolute ethanol and stored in a refrigerator. Plant roots were kept in plastic bags and stored in a refrigerator. Plant root tips were examined for ectomycorrhizal-root-like interactions under a stereomicroscope. The ECM-root-like morphological descriptions followed Agerer (1987-1998).

2.7. DNA extraction and sequencing

DNA was extracted from mushroom (*P. portentosus*) samples obtained from the local market, fruiting bodies from seedbeds, and ECM-root-like structures that were kept in absolute ethanol. Ethanol was removed from the samples until they dried. DNA was ground with a micropestle and extracted with a DNA preparation kit (Vivantis, GF1). The internal transcribed spacer of the nuclear ribosomal repeats (ITS1 and ITS2) was amplified from the genomic DNA using the forward primer, ITS5 (‘5-GGAAGTAAAAGTCGTAACAAGG-3’) and reverse primer ITS4 (‘5’-TCCTCCGCTTATTGATATGC-3’) (White et al., 1990). The PCR mixture contained 1 µl of DNA template, 1 µl of each primer at a concentration of 20 pmol/µl, 12 µl of master mix with Taq DNA polymerase (Vivantis), and deionized water in a total volume of 25 µl. The PCR conditions were performed by using a DNA thermal cycler (FINEMOULD PRECISION IND.CO, South Korea) as follows: initial denaturation at 94^{o}C for 3 min, followed by 30 cycles of denaturation at 94^{o}C for 40 s, annealing at 55^{o}C for 40 s, and extension at 72^{o}C for 1 min. A final extension to complete the unfinished strands was performed at 72^{o}C for 7 min. The PCR products of the ITS region were sequenced by the Sanger Coulson’s method (Sanger et al., 1980) using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.8. Molecular identification

To identify the ECM-root-like structures (at the fine root tips of *S. javannica* Miq.) that developed from the *P. portentosus* bioinoculant, the sequences of the ECM-root-like, mushrooms collected from seedbeds and sporocarps of *P. portentosus* samples obtained from the local market were searched for the most similar sequences by using the BLASTn tool in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast). After that, their sequences were aligned again with the most similar sequences retrieved from the GenBank database by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.9. Statistical analysis

One-way analysis of variance (ANOVA) in SPSS version 23 for Windows (SPSS Inc., Chicago, Illinois) was used to analyze the data. Duncan’s multiple range test was used to determine the significant differences ($p \leq 0.05$) between treatments from three replicates of each experiment.

3. Results

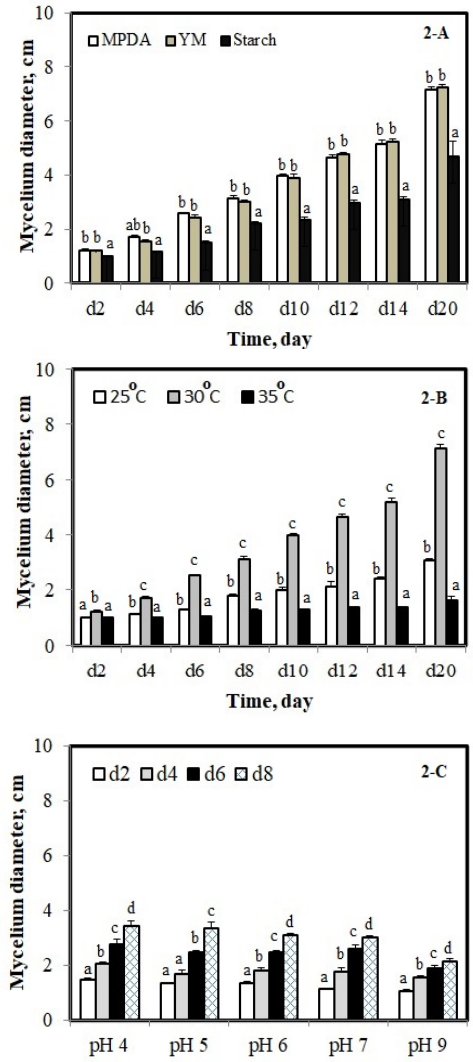
3.1. Effects of medium type, temperature, and initial pH on mycelial growth

The growth of pure mushroom mycelia on three media types (MPDA, YM, and starch medium) at 30^{o}C was studied. It was found that mycelia could use these media well. However, MPDA and YM produced the best growth, and the mycelia grew throughout the plate at 20 days (Figure 2-A). Furthermore, *P. portentosus* can be grown on starch medium at approximately 60% of the growth on MPDA and YM. The results indicate that all three media can be used as C-sources. However, MPDA and YM contained glucose that the fungus could use directly. Because fungi in the starch medium must synthesize and secrete enzymes to hydrolyze starch into glucose, the growth in the starch medium was slower than that in MPDA and YM. Furthermore, the growth of the fungus in MPDA and YM was nearly the same. Then, YM was chosen for pH and temperature growth studies because it is a synthetic culture medium that is commonly used for microbial growth and is easy to prepare.

Temperatures of 25, 30, and 35^{o}C were used for the mycelium growth study. Growth was assessed as the mycelium diameter on YM agar plates (Figure 2-B). The results indicated that the highest growth occurred at 30^{o}C with a nearly full plate at Day 20. The fungal growth amounts at temperatures of 25°C and 35°C were lower than that at 30^{o}C. Furthermore, mycelial growth at 25^{o}C was faster than that at 35^{o}C. At Day 20, the mycelial growth amounts at 25^{o}C, and 35°C were approximately 48% and 24% of the growth at 30°C, respectively. These results indicated that 30^{o}C was the optimum temperature for mycelium growth of *P. portentosus*.

The effects of the initial medium pH on black bolete growth were examined on YM medium at 30^{o}C. The mycelium growth diameters at various pH values are shown in Figure 2-C. The results indicated that the mycelia of the fungus grew well for a wide range of pH values from 4-9. However, mycelia slowly grew at pH 5-9, and growth clearly declined at pH 9. This study concluded that a medium at pH 4 was optimum for mycelium growth of the fungus.

Figure 2. Effects of *P. portentosus* growth on various media (2-A), various temperatures (2-B), and various pH values (2-C). Data are presented as the means, and the error bar for each graph indicates the ± standard deviation based on three replicates. Each graph's different letters for the same factors indicate significant differences ($p \leq 0.05$).



3.2 Plant–fungus colonization

Root tips of *S. javannica* Miq. were examined for ectomycorrhizal root-like structures under a stereomicroscope by obtaining samples from the seedbeds after inoculation for 4, 5, and 6 months. It was discovered that at month 6, the morphologies of some plant root tips developed ectomycorrhizal root-like structures. After that, the plant's root system was not disturbed when adding compost and covering it with rice stalks. The soil should contain some moisture all day and should not be flooded for a long period. Finally, sporocarps appeared at month 8.5 (Figure 3-B). In the morphology study, the sporocarps were bolete, and the caps and stalks were colored plain brown. Tube trama (hymenophoral) or pores where spores were produced were present under the caps. The mushroom color may be dark brown depending on the color of the soil and the environmental stage of the mushrooms (the mature stage has a darker-brown color than the early stage). Clamp connections were commonly found in basidiomycetes and were also found in this mushroom mycelium. Its spores were oval in shape. The caps of the sporocarps were soft. Ectomycorrhizae that colonized at the root tips and were connected to the mushrooms are shown in Figure 3-A. The ECM root tips were not branched, and their surface aggregates formed loose mycelial strands, as shown in Figures 3-C and 3-D. The root tip colors of the ECM were brown and dark brown.

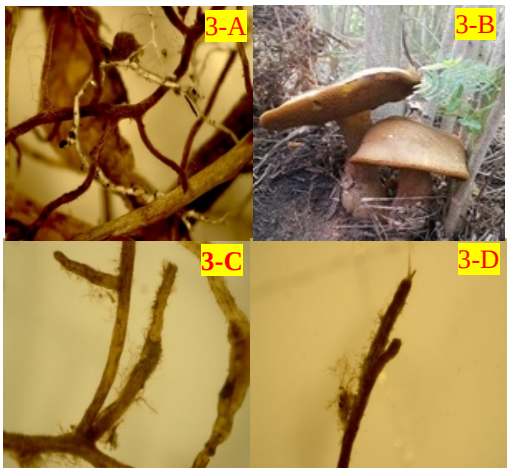


Figure 3. Ectomycorrhizae colonization associated with the plant root tips of *S. javannica* Miq. (3-A), fruiting bodies on the seedbed (3-B) at 8.5 months after inoculation, and ectomycorrhizal-like root tips (3-C and 3-D) from plant roots

3.2 Molecular Identification

To confirm that the ectomycorrhizal root tips and sporocarps on the seedbeds of *S. javannica* Miq. were the same species as the fruiting bodies of *P. portentosus* (black bolete), their ITS rDNA sequences were aligned together with sequences retrieved from GenBank using Clustal Omega. The results showed that the ITS sequences of the fruiting bodies that developed on the seedbeds and the ectomycorrhizal root tips connected to the sporocarps were 100% identical to those of *P. portentosus* MN962534 (GenBank database), as shown in Figure 4, including the black bolete sequence with 730 base pairs that was used as a bioinoculant. This finding indicated that the *P. portentosus* bioinoculant can associate with the root tips of *S. javannica* Miq. and develop sporocarps. Even though this method takes a long time (8.5 months) for fruiting body development, it is the most sustainable method to cultivate edible ectomycorrhizal mushrooms. This technique can be used in mass production and can control mushroom production by adding biofertilizer and watering.

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P._portentosus(MN962534)    cattatcgaaagcacaaagtccgaaggggggaaaaaaaagagggtgatgctaggtgggac  60
Fruiting_body               cattatcgaaagcacaaagtccgaaggggggaaaaaaaagagggtgatgctaggtgggac  60
ECMlike                     cattatcgaaagcacaaagtccgaaggggggaaaaaaaagagggtgatgctaggtgggac  60
Black_bolete                cattatcgAaagcacaaagtccgaaggggggaaaaaaaagagggtgatgctaggtgggac  60
                            ************************************************************

P._portentosus(MN962534)    gactgtcgctggcatatatcgtatgcatgtgcacgtcgaaaccttggctcgccccttctc  120
Fruiting_body               gactgtcgctggcatatatcgtatgcatgtgcacgtcgaaaccttggctcgccccttctc  120
ECMlike                     gactgtcgctggcatatatcgtatgcatgtgcacgtcgaaaccttggctcgccccttctc  120
Black_bolete                gactgtcgctggcatatatcgtatgcatgtgcacgtcgaaaccttggctcgccccttctc  120
                            ************************************************************

P._portentosus(MN962534)    ctttcggcgtaatgcttaatacacctgtgaacctgttgtaggttgttcccctacgagcag  180
Fruiting_body               ctttcggcgtaatgcttaatacacctgtgaacctgttgtaggttgttcccctacgagcag  180
ECMlike                     ctttcggcgtaatgcttaatacacctgtgaacctgttgtaggttgttcccctacgagcag  180
Black_bolete                ctttcggcgtaatgcttaatacacctgtgaacctgttgtaggttgttcccctacgagcag  180
                            ************************************************************

P._portentosus(MN962534)    taggaggacgatctatgtctttccatcacactacatgtatgtctacagaacgttgaaagt  240
Fruiting_body               taggaggacgatctatgtctttccatcacactacatgtatgtctacagaacgttgaaagt  240
ECMlike                     taggaggacgatctatgtctttccatcacactacatgtatgtctacagaacgttgaaagt  240
Black_bolete                taggaggacgatctatgtctttccatcacactacatgtatgtctacagaacgttgaaagt  240
                            ************************************************************

P._portentosus(MN962534)    cgtctcgacccttgacggggttggacgcaaaactataatacaacttttcagcaacggatct  300
Fruiting_body               cgtctcgacccttgacggggttggacgcaaaactataatacaacttttcagcaacggatct  300
ECMlike                     cgtctcgacccttgacggggttggacgcaaaactataatacaacttttcagcaacggatct  300
Black_bolete                cgtctcgacccttgacggggttggacgcaaaactataatacaacttttcagcaacggatct  300
                            ************************************************************

P._portentosus(MN962534)    cttggctctcgcatcgatgaagaacgcagcgaattgcgataagtaatgtgaattgcagat  360
Fruiting_body               cttggctctcgcatcgatgaagaacgcagcgaattgcgataagtaatgtgaattgcagat  360
ECMlike                     cttggctctcgcatcgatgaagaacgcagcgaattgcgataagtaatgtgaattgcagat  360
Black_bolete                cttggctctcgcatcgatgaagaacgcagcgaattgcgataagtaatgtgaattgcagat  360
                            ************************************************************

P._portentosus(MN962534)    tttcagtgaatcatcgaatctttgaacgcaccttgcgctccttggtattccgaggagcat  420
Fruiting_body               tttcagtgaatcatcgaatctttgaacgcaccttgcgctccttggtattccgaggagcat  420
ECMlike                     tttcagtgaatcatcgaatctttgaacgcaccttgcgctccttggtattccgaggagcat  420
Black_bolete                tttcagtgaatcatcgaatctttgaacgcaccttgcgctccttggtattccgaggagcat  420
                            ************************************************************

P._portentosus(MN962534)    gcctgtttgagtgtcatcgaattctcaaccatgtcttgatgttaactttcgaggcatggc  480
Fruiting_body               gcctgtttgagtgtcatcgaattctcaaccatgtcttgatgttaactttcgaggcatggc  480
ECMlike                     gcctgtttgagtgtcatcgaattctcaaccatgtcttgatgttaactttcgaggcatggc  480
Black_bolete                gcctgtttgagtgtcatcgaattctcaaccatgtcttgatgttaactttcgaggcatggc  480
                            ************************************************************

P._portentosus(MN962534)    ttggacttgggagctttgctggttggaccccccctctcttcgaggggggaatgtcagctct  540
Fruiting_body               ttggacttgggagctttgctggttggaccccccctctcttcgaggggggaatgtcagctct  540
ECMlike                     ttggacttgggagctttgctggttggaccccccctctcttcgaggggggaatgtcagctct  540
Black_bolete                ttggacttgggagctttgctggttggaccccccctctcttcgaggggggaatgtcagctct  540
                            ************************************************************

P._portentosus(MN962534)    cctcaaaagcattagcaaaagggacgtgttcgtcgcatgaactgacggccttcgacgtga  600
Fruiting_body               cctcaaaagcattagcaaaagggacgtgttcgtcgcatgaactgacggccttcgacgtga  600
ECMlike                     cctcaaaagcattagcaaaagggacgtgttcgtcgcatgaactgacggccttcgacgtga  600
Black_bolete                cctcaaaagcattagcaaaagggacgtgttcgtcgcatgaactgacggccttcgacgtga  600
                            ************************************************************

P._portentosus(MN962534)    taatgatcgtcgtggctggaggggaaaagtgtgatggcgaaggtctgtcctagcttctaat  660
Fruiting_body               taatgatcgtcgtggctggaggggaaaagtgtgatggcgaaggtctgtcctagcttctaat  660
ECMlike                     taatgatcgtcgtggctggaggggaaaagtgtgatggcgaaggtctgtcctagcttctaat  660
Black_bolete                taatgatcgtcgtggctggaggggaaaagtgtgatggcgaaggtctgtcctagcttctaat  660
                            ************************************************************

P._portentosus(MN962534)    caaaggcgaggggcttgtgtcacgcatgccctctgtcttatcgaaccttgacctcaaatc  720
Fruiting_body               caaaggcgaggggcttgtgtcacgcatgccctctgtcttatcgaaccttgacctcaaatc  720
ECMlike                     caaaggcgaggggcttgtgtcacgcatgccctctgtcttatcgaaccttgacctcaaatc  720
Black_bolete                caaaggcgaggggcttgtgtcacgcatgccctctgtcttatcgaaccttgacctcaaatc  720
                            ************************************************************

P. portentosus (MN962534)   aggtaggact                                                  730
Fruiting_body               aggtaggact                                                  730
ECMlike                     aggtaggact                                                  730
Black_bolete                aggtaggact                                                  730
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Figure 4. Sequence alignments of ITS rDNA of ectomycorrhizal-like root tips, sporocarps from the seedbeds of *S. javannica* Miq. (fruiting body), and black bolete (bioinoculum) with 100% identity to *P. portentosus* (MN962534), as determined by Clustal Omega

4. Discussion

Kumla et al. (2011) reported that *P. portentosus* mycelia were able to grow between pH 3-9 and grew well at pH 4. The optimal temperature was 30^{o}C, and there was no growth at 40^{o}C. They also found that modified Murashige & Skoog (MMS) and fungal host media were best for mycelium growth. Sanmee et al. (2010) found that *P. portentosus* mycelia grew well on modified Gamborg, modified Melin Norkans and MS media at 30^{o}C and pH 4. Kumla et al. (2020) found that *P. spongiosus* mycelia grew well on L-modified Melin-Norkans and Murashige and Skoog agar at 30^{o}C at an initial pH of 5. Siri-in et al. (2014) studied the growth conditions of pure *Scleroderma sinnamariense* mycelia. The optimal temperature and pH were 30^{o}C and 5, respectively. The results of our study on temperature and pH agree with the studies of Kumla et al. (2011) and Sanmee et al. (2010). According to Kumla et al. (2011), Sanmee et al. (2010) and this study, *P. portentosus* could grow well in various types of media. However, this study found that *P. portentosus* mycelia could grow well on common media, such as MPDA and YM. Thus, YM was chosen for the growth study and used as the inoculum media to prepare bioinoculants from sorghum grains. This was done because this approach is not complicated, and the medium is easy to prepare.

The features of the ECM root tips examined in this study are similar to those of the ECM root tips of *P. portentosus* on *Pinus kesiya* (Sanmee, et al., 2010). Further study by the same group reported by Kumla et al. (2016) confirmed the mutualistic relationship between *P. portentosus* and Pinus trees by using carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes measurements. Based on carbon assimilation studies, ECM fungi derive simple sugars from plant photosynthesis, while saprotrophic fungi obtain sugars from wood decay. Nitrogen from soil was absorbed by ECM fungi and transferred to their host plants. In contrast, saprotrophic fungi obtained nitrogen from decaying substrates (Hobbie et al., 1999). Although the lifestyle of *P. portentosus* is also found in saprophytic habits, it was proven that it was cultivated without a host plant (Sanmee et al. 2010, Ji et al. 2011). However, the ITS region of the rDNA sequence is a popular tool used for fungal identification. In this study, sporocarps that developed in seedbeds were of the same species as both the bioinoculants and ECM-root-like structures. These results confirmed that *P. portentosus* can be associated with an annual plant and that it is an ectomycorrhizal fungus. Because it can develop ECM-root structures, *P. portentosus* is a special species because it can adapt its lifestyle to be a saprophyte or ectomycorrhiza depending on the environment.

5. Conclusions

Attempts to overcome the difficulties associated with ECM mushroom cultivation are very challenging endeavors. This study demonstrated that bioinoculants of sorghum (pure mycelia) and liquid-formed (spores from mature sporocarps) have the potential to colonize the fine root tips of *S. javannica* Miq. Furthermore, the findings also prove that *P. portentosus* is an ectomycorrhizal mushroom and has a symbiotic association with the fine roots of *S. javannica* Miq. *P. portentosus* is a boletus

mushroom that may not be an obligate ECM. The host plants of the fungus are not only woody plants and fruit trees but also annual plants such as *S. javannica* Miq. However, to succeed in cultivating ectomycorrhizal mushrooms, biofertilizer and watering are needed.

Acknowledgments

This study was supported by grants from the Biodiversity-based Economy Development Office (Public Organization, BEDO) based on the plant genetic conservation project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn**.** Mahasarakham University and the Faculty of Technology are thanked for providing facility support.

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