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Mycelial Growth of Edible Ectomycorrhizal Fungi According to Nitrogen Sources

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ABSTRACT : Ectomycorrhizal fungi are associated with plants roots and acquire significant amounts of nitrogen sources from the soil. For artificial cultivation, mass production of ectomycorrhizal fungi in liquid media is required. We studied the edible ectomycorrhizal mushrooms *Hygrophorus russula*, *Ramaria fumigata*, *Sarcodon aspratus*, and *Tricholoma matsutake*. All strains except *S. aspratus* NIFoS 2031 grew generally well on modified Melin-Norkran's (MMN) medium compared to on other media. All strains analyzed in this study showed significantly higher growth on organic nitrogen. Specifically, two strains of *H. russula* significantly responded to both tryptone and neopeptone media. Among different species and strains, there were clear differences in the capacity to grow on animal-based organic nitrogen sources.

KEYWORDS : Ectomycorrhizal fungi, Edible mushroom, Liquid culture, Nitrogen source

Introduction

Ectomycorrhizal fungi are associated with plants roots [1]. The number of ectomycorrhizal species has been estimated to be 20,000 and are primarily associated with 6,000 plant species [2, 3]. The hyphae of ectomycorrhizal fungi grow inside the cortical cells of plant roots on the surface of the root and form a hartig net, which is the hyphal network between the fungus and host plant. Major nutrients such as carbon (C), nitrogen (N), and phosphorus (P) are exchanged via the hartig net. Ectomycorrhizal symbiosis influences plant growth, diversity, and fitness and is essential for the cycling of C, N, and P in ecosystems [4].

During plant growth, the utilization of soil N is restricted because of the slow accumulation of fixed N and space restriction [5]. In contrast, ectomycorrhizal fungi can acquire significant amounts of organic N in soil and provide plants with greater access to organic N bound in chitin, proteins, and tannins [6]. Ectomycorrhizal fungi can very efficiently absorb inorganic N sources such as ammonium (NH_4^+) or nitrate (NO_3^-) from soils. After uptake from the soil, NO_3^- is transformed into $NH4^+$ by nitrate and nitrite reductases in ectomycorrhizal fungi. NH_4^+ is utilized in the production of amino acids. The amino acids produced intracellularly will be transferred across the symbiotic interface such as ectomycorrhizal plasma membrane through plant membrane amino acid transporters [7, 8]. Ectomycorrhizal fungi use organic nitrogen as well as inorganic nitrogen. All species of ectomycorrhizal fungi have the ability to utilize a number of amino acids in culture or symbiosis with plants [9].

For artificial cultivation, the mass production of ectomycorrhizal fungi in liquid media broth is important. Factors involved in mycelium growth directly affect inoculum production. In this study, we evaluated different organic and inorganic nitrogen sources to optimize the culture conditions in the production of mycelium. We examined the impact of nitrogen sources that affect the growth of the four edible ectomycorrhizal mushrooms *Hygrophorus russula*, *Ramaria fumigata*, *Sarcodon aspratus*, and *Tricholoma matsutake*, which are important in industrial applications.

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Materials and Methods

Fungal strains

All strains were obtained from the National Institute of Forest Science (NIFoS). The 8 strains used this study were *H. russula* (NIFoS 1987, 2003), *R. fumigata* (NIFoS 2370, 2371), *S. aspratus* (NIFoS 1677, 2031), and *T. matsutake* (NIFoS 1256, 1266). They were grown on potato dextrose agar (Difco, Detroit, MI, USA) at 25°C for 60 days and used as the inoculum.

DNA extraction, PCR amplification, and sequencing

DNA was isolated from mycelium using the Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's recommendations. To amplify the internal transcribed spacer (ITS) regions of ribosomal DNA, the ITS1F/ITS4 primer set was used [10, 11]. PCR was performed in 20 mL volumes containing 10~100 ng of gDNA, 10 pmol of each primer, and PCR PreMix solution (Bioneer). The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles at 95°C for 30 sec, 55C for 30 sec, and 72°C for 60 sec, and a final elongation step at 72°C for 5 min. PCR products were purified and sequenced by Macrogen (Seoul, Korea). All sequence data were deposited in the GenBank of National Center for Biotechnology Information.

Culture conditions

The media used for this study were based on modified Melin-Norkran's medium (MMN), consisting of 10 g glucose, 0.5 g KH_2PO_4, 0.15 g $MgSO_4.7H_2O$, 1.2 mL 1% $FeCl_3$, 0.05 g $CaCl_2$, 0.025 g NaCl, 3 g malt extract, 0.25 g $(NH_4)_2HPO_4$, and 100 mg thiamin×HCl in 1 L water (pH 5.5). First, the 3 media (M0, M1, and M2) were used as liquid media. M is MMN medium containing 10 g/L of glucose rather than sucrose as a carbon source. M0 does not contain a nitrogen source on MMN. M1 contains ammonium nitrogen on MMN. M2 contains nitrate nitrogen on MMN [12]. Based on the animal-based organic nitrogen source, the media contained yeast extract, select soytone, peptone, tryptone, neopeptone, or casamino acid rather than malt extract in the MMN medium.

Preparation of culture filtrates of strains

The inoculum (6 × 6 mm) was cut out from the media, inoculated in 20 mL of sterile broth in a 100 mL Erlenmeyer flask, and incubated at 25°C for 60 days. The culture fluid was harvested by filtration through Whatman No.1 filter paper (Whatman, Piscataway, NJ, USA). The filter papers were dehydrated in a dry oven at 70°C for 2 days. Next, the weight and pH were measured.

Statistical analysis

One-way analysis of variance followed by Duncan's multiple range test ($p < 0.05$) was used to analyze the results. This analysis was performed using SPSS version 10.0 programs (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Mycelial growth of ectomycorrhizal fungi using inorganic nitrogen sources

The growth of eight strains of ectomycorrhizal fungi was measured in liquid media containing different organic and inorganic nitrogen sources. All liquid media were based on MMN medium because ectomycorrhizal fungi

Table 1. Information on the eight strains used in this study

Species	NIFoS strain No.	GenBank accession No.	Collection year
Hygrophorus russula	1987	KX814449	2012
Hygrophorus russula	2003	KX814450	2012
Ramaria fumigata	2370	KX814451	2013
Ramaria fumigata	2371		2013
Sarcodon aspratus[a]	1677	KX814452	2011
Sarcodon aspratus	2031	KX814453	2012
Tricholoma matsutake	1256	KX814454	2009
Tricholoma matsutake	1266	KX814455	2009

NIFoS, National Institute of Forest Science.

[a]*Sarcodon aspratus* is the same species as *S. imbricatus* according to internal transcribed spacer analysis. We showed *S. aspratus* rather than *S. imbricatus* in this paper because they are used by different host plants. *S. aspratus* always lives in oak trees in Korea, while *S. imbricatus* typically grows in needleleaf trees in other countries.

are typically grown on solid MMN at pH values ranging from 5.8 to 6.2 [13]. As shown in Fig. 1, all strains except *R. fumigata* NIFoS 2370 and *S. aspratus* NIFoS 2031 generally grew well on MMN medium compared to on other media. The final dry weight mycelia of the *Hygrophorus* strains were similar on ammonium and nitrate media. *Ramaria* strains showed much better growth on nitrate than on ammonium, but *R. fumigata* NIFoS 2371 showed the highest dry weight on MMN. Thus, inorganic nitrogen did not enhance the mycelial growth of two strains of *R. fumigata*. The growth of two *S. aspratus* strains showed considerable variation. *S. aspratus* NIFoS 1677 showed the highest growth on MMN and preferred ammonium to nitrate medium. In contrast, *S. aspratus* NIFoS 2031 grew well on ammonium. For *T. matsutake*, the dry weights following growth on ammonium medium were approximately 1.8- and 1.4-fold higher than on nitrate for NIFoS 1256 and 1266, respectively. It was previously reported that *T. matsutake* prefers ammonium to nitrate as a nitrogen source [12]. These results showed that all strains except *R. fumigata* NIFoS 2370 and *S. aspratus* NIFoS 2031 could use organic and inorganic nitrogen sources such as malt extract, ammonium, and nitrate and grew well. Generally, strains on organic media containing ammonium phosphate [$(NH_4)_2HPO_4$] showed higher growth than on inorganic media, and the growth of ectomycorrhizal fungi depends on the organic nitrogen sources used.

Mycelial growth of ectomycorrhizal fungi using organic nitrogen sources

To evaluate the utilization of organic nitrogen from ectomycorrhizal fungi, different animal-based organic nitrogen sources (yeast extract, select soytone, peptone, tryptone, neopeptone, casamino acid) were supplemented in the MMN media. Organic N is the most widely used source of nitrogen in microorganism media [9]. All strains analyzed in this study showed significant growth on organic N. The growth of two strains of *H. russula* significantly increased on both tryptone and neopeptone media. *Hygrophorus russula* NIFoS 1987 showed reduced growth on peptone and NIFoS 2003 exhibited low growth on yeast extract and peptone (Fig. 2A). For *R. fumigata*, NIFoS 2370 showed approximately 2.6-fold lower growth than NIFoS 2371 than on peptone media. NIFoS 2370 showed significant growth on select soytone, while the growth of NIFoS 2371 significantly increased on neopeptone media. In contrast, *R. fumigata* NIFoS 2370 and 2371 grew poorly on peptone and casamino acid, respectively (Fig. 2B). *Sarcodon aspratus*, NIFoS 1677 grew better than NIFoS 2031 in all tested media. Both strains preferred different nitrogen sources, except peptone and tryptone (Fig. 2C). *Tricholoma matsutake* strains grew well on select soytone. NIFoS 1256 showed the highest growth on select soytone, tryptone, and casamino acid. The lowest growth of NIFoS 1256 was on peptone. In contrast, NIFoS 1266 showed the lowest growth on neopeptone and casamino acid (Fig. 2D). Min et al. [14] studied the effect of nitrogen source of *T. matsutake* and found that yeast extract and soytone were good sources of organic nitrogen, while the growth of *T. matsutake* was low on malt extract medium. Yeast extract was a superior nitrogen source compared to ammonium nitrate, ammonium phosphate, and amino acids [15]. There were distinct differences in the capacity of different species and strains to utilize the animal-based media

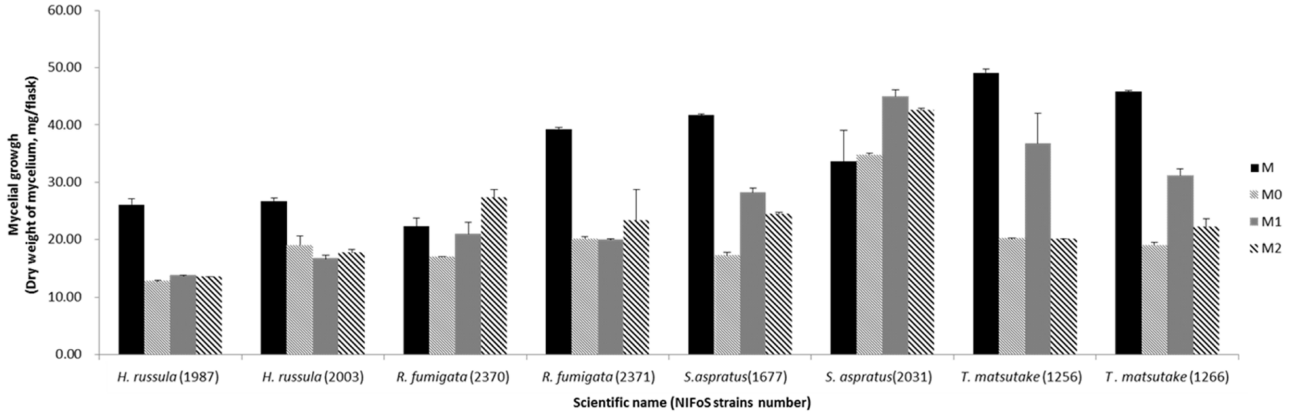

Fig. 1. Mycelial growth of ectomycorrhizal fungi in liquid media containing organic and inorganic nitrogen sources. M is a modified Melin-Norkran's (MMN) medium containing 10 g/L of glucose rather than sucrose as a carbon source. M0 does not contain a nitrogen source on MMN. M1 contains ammonium nitrogen on MMN. M2 contains nitrate nitrogen on MMN [12].

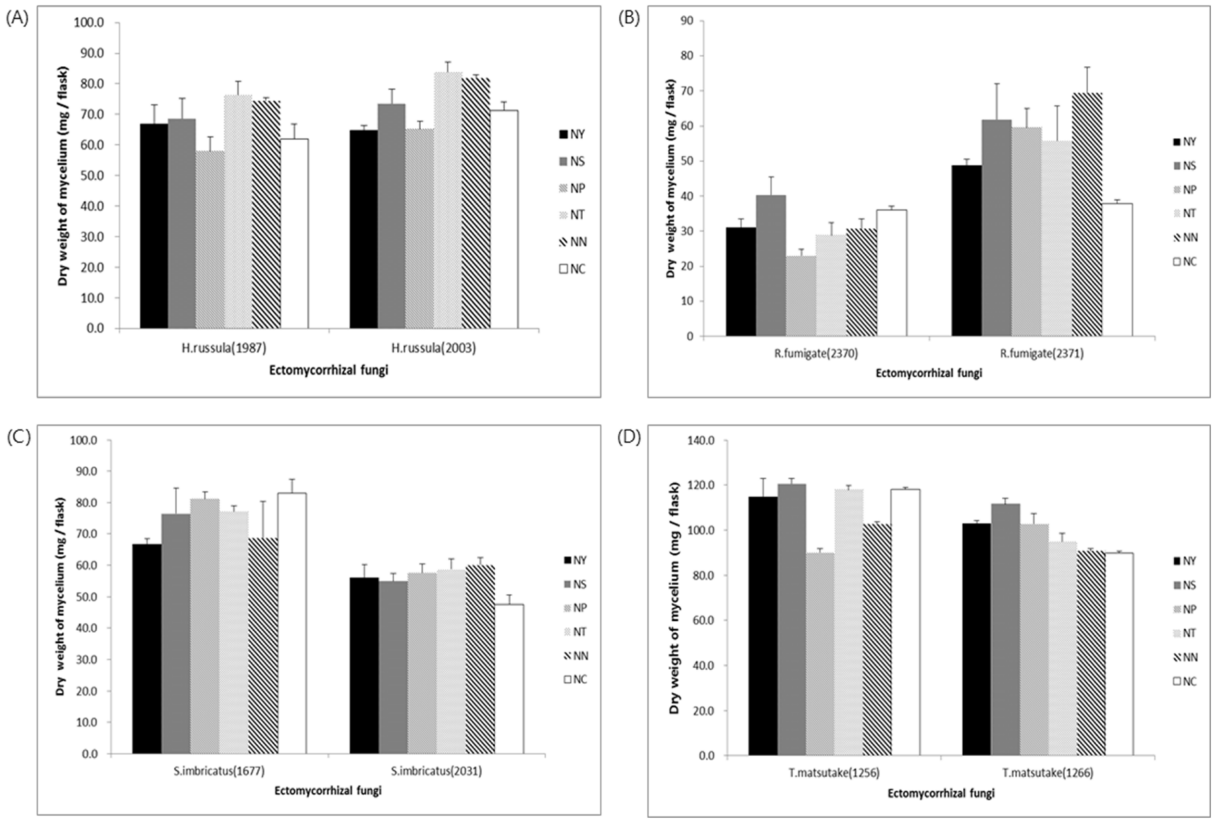

Fig. 2. Mycelium growth of eight strains (*Hygrophorus russula*, *Ramaria fumigata*, *Sarcodon aspratus*, *Tricholoma matsutake*) depending on the organic nitrogen sources in the growth media. NY, yeast extract; NS, select soytone; NP, peptone; NT, tryptone; NN, neopeptone; NC, casamino acid rather than malt extract in modified Melin-Norkran's medium.

Table 2. Final pH of liquid media containing different organic nitrogen sources

Culture medium[1,2]	*Hygrophorus russula*		*Ramaria fumigata*		*Sarcodon aspratus*		*Tricholoma matsutake*	
	1987	2003	2370	2371	1677	2031	1256	1266
NY	3.8 ± 0.2^{b,c3}	6.5 ± 0.1^{a}	4.0 ± 1.2^{b}	5.8 ± 0.1^{a}	7.8 ± 0.1^{a}	6.3 ± 0.1^{a}	3.7 ± 0.1^{b}	4.6 ± 0.7^{b}
NS	4.3 ± 0.2^{a}	5.9 ± 0.8^{a}	5.0 ± 1.2^{a}	5.9 ± 0.2^{a,b}	6.2 ± 1.5^{c}	6.3 ± 0.1^{a}	4.1 ± 0.1^{a}	4.2 ± 0.1^{b,c}
NP	3.7 ± 0.3^{b,c}	5.7 ± 1.1^{a}	5.6 ± 0.0^{a}	5.4 ± 0.1^{b}	4.9 ± 0.1^{d}	6.3 ± 0.1^{a}	3.5 ± 0.1^{c}	3.1 ± 0.0^{d}
NT	3.9 ± 0.5^{b}	4.2 ± 0.8^{b}	3.5 ± 0.4^{b}	5.0 ± 0.4^{c}	7.3 ± 0.1^{a,b}	5.8 ± 0.1^{c}	3.5 ± 0.0^{c}	3.3 ± 0.0^{d}
NN	3.6 ± 0.1^{b,c}	4.3 ± 0.6^{b}	5.4 ± 0.2^{a}	5.1 ± 0.3^{c}	4.7 ± 0.3^{d}	5.9 ± 0.1^{b}	3.5 ± 0.0^{c}	3.9 ± 0.5^{c}
NC	3.5 ± 0.2^{c}	4.7 ± 1.0^{b}	5.6 ± 0.2^{a}	5.4 ± 0.0^{b}	6.6 ± 1.1^{b,c}	5.6 ± 0.0^{d}	3.5 ± 0.0^{c}	5.2 ± 0.5^{a}

NY, yeast extract; NS, select soytone; NP, peptone; NT, tryptone; NN, neopeptone; NC, casamino acid rather than malt extract in modified Melin-Norkran's medium medium.

[1]Culture medium indicates the culture medium containing different nitrogen source (3 g/L).

[2]All liquid media before inoculation of test strains were adjusted to pH 5.5.

[3]Values with the same uppercase letters in each column were not significantly different (Duncan's multiple rage test, $p < 0.05$). Value is mean ± SD (n=7).

as organic nitrogen sources.

Final pH of mycelial growth on organic nitrogen

The final pH values of all species analyzed in this study are shown in Table 2. *Hygrophorus russula* NIFoS 1987 and *T. matsutake* NIFoS 1256 and 1266 showed an overall decrease in pH in all organic nitrogen media. The pH of *H. russula* NIFoS 2003 decreased on tryptone, neopeptone, and casamino acid. Two strains of *R. fumigata* showed a decrease in pH, except NIFoS 2370 on casamino

acid and peptone and NIFoS 2371 on select soytone and yeast extract. The pH of *S. aspratus* strains increased, except NIFoS 1677 on peptone and neopeptone. We detected no association between organic nitrogen media and pH. Ectomycorrhizal fungi commonly showed acidic pH because of the secretion of organic acids such as oxalic acid [16]. The fungi may secrete metabolites except for organic acid, increasing pH. To explain the differences between initial pH and final pH, specific metabolites secreted into media must be evaluated.

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