Regeneration of Ectomycorrhizal Fungal Isolates Following Deep Freezer Storage

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Mycelial growth and survival ratio of ectomycorrhizal fungi were determined after storage at −70°C for 1, 3, or 6 mon. Seventeen of 23 ectomycorrhizal fungi did not survive after storage for more than 6 mon, whereas Cenococcum geophilum, Lepista nuda, and some species of Rhizopogon and Suillus did survive.

KEYWORDS: Cenococcum geophilum, Cryopreservation, Deep freezer, Non-sporulate, stock culture

Ectomycorrhizal (EcM) fungi are important symbionts that associate with roots of woody plants, such as Betulaceae, Fagaceae, Salicaceae, and Pinaceae, in boreal and temperate regions [1]. In total, 20,000–25,000 fungal species that belong to Basidiomycota, Ascomycota, and Zygomycota are estimated to be involved in EcM associations [2]. EcM associations play significant roles in plant establishment by promoting nutrient and water uptake of host plants and enhancing tolerance towards stressful situations encountered by hosts [1]. Consequently, EcM fungi are an effective biological resource and are used for reforestation in degraded areas [3].

A pure, viable, and genetically stable inoculum source is essential for practical use of EcM fungi. However, frequent subculturing is necessary when stock cultures are stored at room temperatures. Other than the time and labor involved in making transfers, care must be exercised to prevent mutations. Various preservation methods have been developed to reliably store stock cultures for fungal collections [4]. Freeze-drying is definitely the best way, because stock cultures stored in ampoules can be stored without any special requirements. The products are light, inactive, and dry and have excellent longevity. This method can be applied successfully to conidia, spores, or sporulating fungi [5-9]; however, filamentous non-sporulating fungi are highly sensitive to freeze-drying [4, 10], except for some successes reported for Claviceps [11], some EcM fungi [12], and some edible mushrooms [13]. EcM fungi are highly sensitive to freeze drying due to their non-sporulating nature under in vitro conditions. Indeed, our preliminarily studies revealed that 34 EcM fungal isolates of Amanita, Cenococcum, Laccaria, Lactarius, Lepista, Paxillus, Pisolithus, Rhizopogon, Russula, Scleroderma, Suillus, and Tomentella did not survive after freeze-drying procedure. Deep freezing methods produce high survival rates for some fungal isolates [10, 14-18] and are applicable rather than freeze drying [4, 5]. Methods using nitrogen, that is, storing at ultra-low temperature (−196°C), yield good results [10, 16] but are rather expensive and troublesome because they require a regular supply of liquid nitrogen. Methods using electric deep freezers (−70 to −85°C) are relatively cheap, labor-saving, and reliable alternatives [17, 18]. However, whether EcM fungal isolates can be maintained for extended periods under deep freezing has not been well studied.

To understand how long EcM fungal isolates can be stored in a deep freezer (−70°C) and to understand whether growth characteristics change or not after freeze storage, EcM fungal isolates were stored in a deep freezer at −70°C for 1, 3, or 6 mon and their mycelial growth and survival ratios were determined.

Twenty-three EcM fungal isolates, including 22 species of Basidiomycota and 1 species of Ascomycota, were collected from several coastal pine forests and artificial forests in inland areas of Korea in 2008 and 2009 (Table 1). Each fungal isolate was identified by observation of vegetative structures and macroscopic characteristics and by observation of culture characteristics and microscopic features of conidia, spores, and spore mass. PCR amplification with primers targeting the 28S rDNA region was performed as described previously [19]. DNA extraction, PCR, and sequencing were performed as previously [19]. All isolates were deposited in the Laboratory of Tree Pathology and Mycology (TPML) at Kangwon College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 200-701, Korea.

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National University, Korea. The stock cultures for deep freezing were stored on modified Melin-Norkrans (MMN) agar medium [20] under dark conditions at 25°C. Precultured fungal colonies of each isolate, which were incubated for 30–45 days on agar plate medium, were bored around their peripheries with a 5 mm cork borer to make agar discs with fungal mycelia. Skimmed milk was used as a cryoprotectant, because it produced a higher survival ratio than that of dimethyl sulfoxide or glycerin after 7 days of storage in a deep freezer in a preliminary study. Each mycelial disk was transferred to a 1.5 mL cryotube that the cooling rate from 4 to −70°C, which is preferable for successful pre-freezing of fungi [4, 12]. Cryotubes were withdrawn from the −70°C freezer at 1, 3, and 6 mon after storage and immediately soaked in a water bath (37°C) until completely thawed. Five replicates were used for each treatment. Mycelial disks were then transferred onto fresh MMN agar plates, and their radial growth and survival rates were determined after 1 mon incubation. Mycelial disks that were not subjected to freezer storage were directly transferred onto MMN agar media as controls.

Comparisons of mycelial weight for the different durations of freezer storage were made using the nonparametric Kruskal-Wallis test. Data were analyzed by the Steel-Dwass test to determine the differences among treatments (p<0.05). The statistical tests were performed using R ver. 2.10.0 [21].

Table 1. Ectomycorrhizal (EcM) fungal isolates with their isolate numbers, collection areas, putative host trees, and forest types

| EcM fungal taxa | Isolate No. | Area    | Host                | Forest type        |
|----------------|-------------|---------|---------------------|--------------------|
| Amanita ibotengutake | 9-20        | Taean  | Pinus thunbergii   | Coastal pine forest|
| Amanita sp. 1      | 95-8        | Incheon| P. thunbergii      | Coastal pine forest|
| Amanita sp. 2      | 94-7        | Daejeon| P. densiflora       | Artificial pine forest|
| Cenococcum geophilum | G-11      | Gumi   | P. densiflora       | Artificial pine forest|
| Laccaria amethystina  | 08-19     | Hongcheon| P. thunbergii     | Artificial pine forest|
| Lactarius sp. 1    | 94-3        | Haenam | P. thunbergii      | Coastal pine forest|
| Lactarius sp. 2    | 95-1        | Gumi   | P. densiflora       | Artificial pine forest|
| Lepista nuda       | 08-31       | Samcheok| P. thunbergii      | Coastal pine forest|
| Paxillus involutus  | 9-57        | Chuncheon| Quercus spp.      | Isolated pine tree |
| Pisolithus tinctorius | Pt4       | Japan  | Unknown             | Unknown             |
| Rhizopogon sp. 1   | 93-2        | Hongcheon| P. densiflora     | Artificial pine forest|
| Rhizopogon sp. 2   | 93-3        | Hongcheon| P. densiflora      | Artificial pine forest|
| Rhizopogon sp. 3   | 08-21       | Hongcheon| P. densiflora       | Artificial pine forest|
| Rhizopogon sp. 4   | 9-17        | Gangneung| P. densiflora       | Artificial pine forest|
| Russula sp. 1      | 96-6        | Chuncheon| P. densiflora      | Isolated pine tree |
| Russula sp. 2      | 95-6        | Chuncheon| P. densiflora       | Isolated pine tree |
| Scleroderma sp.     | 08-08       | Samcheok| P. thunbergii      | Coastal pine forest|
| Suillus granulatus  | 08-16       | Gangneung| P. thunbergii       | Coastal pine forest|
| Suillus luteus      | 9-22        | Chuncheon| P. densiflora       | Isolated pine tree |
| Suillus placidus    | 08-27       | Hongcheon| P. korearensis      | Artificial pine forest|
| Tomentella sp. 1    | 9-60        | Taean   | P. thunbergii      | Artificial pine forest|
| Tomentella sp. 2    | 08-13       | Samcheok| P. thunbergii      | Coastal pine forest|

After the 1-mon freezer storage, only three isolates (A. ibotengutake, Russula sp. 2 and Scleroderma sp.) showed complete inhibition of mycelial growth, and two isolates (Amanita sp. 1 and Suillus pictus) had a low survival ratio; only one of five replicates were viable. These isolates also showed complete inhibition in mycelial growth or a low survival ratio after freezer storage for 3 mon. Eighteen isolates showed a high survival ratio; more than four of five replicates were viable after 1 and/or 3 mon of freezer storage. However, the survival ratio of fungal isolates that survived after 3 mon of storage decreased dramatically when the storage duration reached 6 mon. Nine isolates (Laccaria amethystina, two species of Lactarius, Paxillus involutus, Pisolithus tinctorius, Russula sp. 1, Suillus luteus, and two species of Tomentella), with a high survival ratio after 3 mon of storage, showed complete inhibition in mycelial growth, and two isolates (Rhizopogon sp. 1 and 4) showed a low survival ratio; two of five replicates were viable. Six isolates (C. geophilum, L. nuda, Rhizopogon sp. 2 and 3, Suillus granulatus, and Suillus placidus) had a high survival ratio; more than four of five replicates were viable after 6 mon of storage.

Among 12 isolates with a low survival ratio or complete inhibition in mycelial growth when the storage duration reached 6 mon, six isolates (P. involutus, P. tinctorius, Rhizopogon sp. 1 and 4, Russula sp. 1, and S. luteus) showed significantly lower mycelial growth after freezer storage for 1 and 3 mon compared to that of the control.
Numbers of replicates surviving (N; maximum of five) is also indicated. Averages and standard deviations are presented.

*\( p < 0.05 \), **\( p < 0.01 \) (Kruskal-Wallis test).

Different letters indicate significant differences at \( p < 0.05 \) (Steel-Dwass test).

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