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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 the isolates sources, i.e., sporocarps, except for *Amanita ibotengutake*, *Cenococcum geophilum*, *Lepista nuda*, and species of *Rhizopogon*, *Suillus* and *Tomentella*, which were identified based on sequencing of internal transcribed spacer regions, including the 5.8S rDNA region. DNA extraction, PCR, and sequencing were performed as described previously [19]. All isolates were deposited in the Laboratory of Tree Pathology and Mycology (TPML) at Kangwon

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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 pictus	08-27	Hongcheon	*P. korearensis*	Artificial pine forest
Suillus placidus	08-28	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

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 containing 1.0 mL of 10% sterilized skimmed milk. The cryotubes were sealed with caps and incubated for 1 hr at 4°C, stored in a freezer for 3 hr at −20°C, and then transferred immediately to −70°C. A preliminary study revealed that the cooling rate from 4 to −20°C was approximately 1°C/min, 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].

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

Table 2. Diameter of mycelial colonies of each ectomycorrhizal fungal isolate, after 1 mon incubation, that were stored in a deep freezer (−70°C) for 1, 3, or 6 mon

Fungal isolate	Isolate No.	Control		1 mon		3 mon		6 mon	
		Diameter of colonies (mm)	N	Diameter of colonies (mm)	N	Diameter of colonies (mm)	N	Diameter of colonies (mm)	N
Amanita ibotengutake	9-20	26.7 ± 3.6	5	-	0	-	0	-	0
Amanita sp. 1	95-8	25.8 ± 1.4	5	17.5	1	46.0	1	-	0
Amanita sp. 2*	94-7	49.4 ± 0.7^{a}	5	30.2 ± 4.5^{b}	5	40.0 ± 10.6^{ab}	5	28.2 ± 1.5	3
*Cenococcum geophilum***	G-11	22.2 ± 0.9^{a}	5	36.3 ± 2.2^{b}	5	19.5 ± 2.0^{a}	5	18.9 ± 2.7^{a}	5
*Laccaria amethystina**	08-19	46.6 ± 0.7^{a}	5	45.8 ± 2.0^{ab}	5	38.6 ± 5.3^{b}	4	-	0
Lactarius sp. 1*	94-3	12.4 ± 1.1^{a}	5	12.1 ± 4.2^{ab}	5	7.5 ± 1.9^{b}	5	-	0
Lactarius sp. 2*	95-1	8.2 ± 0.9^{a}	5	11.4 ± 1.6^{b}	5	16.6 ± 6.2^{ab}	5	-	0
*Lepista nuda**	08-31	41.9 ± 2.8^{a}	5	50.0 ± 1.4^{b}	5	40.4 ± 7.8^{ab}	5	34.8 ± 3.5^{ac}	5
*Paxillus involutus**	9-57	34.1 ± 1.5^{a}	5	20.9 ± 6.6^{b}	4	17.3 ± 1.7^{b}	4	-	0
*Pisolithus tinctorius***	Pt4	54.5 ± 2.7^{a}	5	27.4 ± 10.2^{b}	5	28.4 ± 13.0^{b}	5	-	0
Rhizopogon sp. 1*	93-2	49.3 ± 1.7^{a}	5	36.3 ± 4.4^{b}	5	32.0 ± 4.5^{b}	4	22.3 ± 8.1	2
Rhizopogon sp. 2*	93-3	41.5 ± 4.1^{a}	5	32.9 ± 3.1^{b}	5	44.0 ± 4.9^{a}	5	29.2 ± 1.0^{b}	5
Rhizopogon sp. 3	08-21	61.9 ± 7.0^{a}	5	53.4 ± 5.9^{a}	5	54.5 ± 10.2^{a}	5	49.7 ± 13.2^{a}	5
Rhizopogon sp. 4**	9-17	53.1 ± 2.3^{a}	5	47.4 ± .2^{b}	5	44.4 ± 1.9^{b}	5	31.8 ± 3.2	2
Russula sp. 1*	96-6	22.6 ± 1.4^{a}	5	11.4 ± 5.4^{b}	5	7.1 ± 1.2^{b}	5	-	0
Russula sp. 2	95-6	12.3 ± 0.7	5	-	0	-	0	-	0
Scleroderma sp.	08-08	34.5 ± 4.2	5	-	0	-	0	-	0
Suillus granulatus	08-16	36.5 ± 4.0^{a}	5	31.3 ± 2.8^{a}	5	34.8 ± 1.3^{a}	5	22.4 ± 7.1^{a}	4
Suillus pictus	08-27	40.1 ± 2.4	5	22.5	1	-	0	-	0
*Suillus luteus**	9-22	49.7 ± 1.5^{a}	5	28.8 ± 10.1^{b}	4	21.8 ± 14.3^{b}	4	-	0
*Suillus placidus***	08-28	25.9 ± 2.6^{a}	5	30.4 ± 0.4^{b}	5	24.7 ± 1.4^{a}	5	16.3 ± 4.4^{ab}	4
Tomentella sp. 1*	9-60	40.5 ± 5.5^{a}	5	43.4 ± 1.6^{a}	5	12.5 ± 7.3^{b}	4	-	0
Tomentella sp. 2	08-13	33.4 ± 1.2^{a}	5	28.8 ± 6.9^{a}	5	27.3 ± 7.1^{a}	4	-	0

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).

treatment. Three isolates (*L. amethystina*, *Lactarius* sp. 1, and *Tomentella* sp. 1) had significantly lower mycelial growth after 3 mon of storage. The remaining three isolates showed variable growth after 1 mon of storage and were not significantly different from the control treatment after 3 mon of storage. Among the six isolates that showed a high survival ratio after 6 mon of storage, *Rhizopogon* sp. 2 had significantly lower mycelial growth after 1 mon and 6 mon of storage. Five isolates (*C. geophilum*, *L. nuda*, *Rhizopogon* sp. 2 and 3, *S. granulatus*, and *S. placidus*) were not significantly different from the control treatment after 3 and 6 mon storage. Three of five isolates had significantly higher mycelial growth after 1 mon storage when compared to that of the control treatment.

Our results indicated that most of the EcM fungal isolates, representing 17 out of 23 fungal species, did not survive well after deep freezer storage at −70°C for more than 6 mon, even though deep freezer storage might be practical for *C. geophilum*, *L. nuda*, and some species of *Rhizopogon* and *Suillus* (Table 2). Our results are more or less contradictory to previous studies. Kramer and Mix [15] demonstrated that 55 of 451 isolates were unable to survive freezer storage for 1 yr. Carmichael [14] showed that only 17 of 400 isolates were unable to survive by freezer storage for 9 mon. Kitamoto *et al*. [18] demonstrated that all isolates, including some EcM fungi such as *Entoloma*, *Hebeloma*, *Lepista*, and *Paxillus*, survived for at least 1 yr. Ito and Nakagiri [17] reported that 88% of Agaricales isolates were able to survive for 1 yr. Further improvements in freezer storage such as cryoprotectant selection and pre-freezing conditions are needed to successfully store EcM fungi by deep freezer storage.

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