SHORT COMMUNICATION

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Recovery of mycorrhizas of a fungus, *Cenococcum geophilum*, after urea treatment in warm temperate forests in Japan

Abstract We studied recovery of the ectomycorrhizal species *Cenococcum geophilum* (Cg) after urea treatment in two types of vegetation. The recovery was as quick as 6 months after the treatment and overlapped the fruiting period of ectomycorrhizal ammonia fungi. Ectomycorrhizas investigated as Cg belonged to a single species irrespective of treatment and vegetation type. Cg ectomycorrhizas were significantly more abundant in density in nontreated soil than in treated soil. Between vegetation types, Cg ectomycorrhizas were significantly more in density in broad-leaved deciduous forest than in broad-leaved evergreen forest. Moreover, Cg was dominant in the nontreated soils of the former type of forest.

Key words *Castanopsis cuspidata* · *Cenococcum geophilum* · Ectomycorrhizal fungus · *Quercus serrata* · Urea treatment

"Ammonia fungi" have been recognized as one of the fungal communities that appear after N application. Sagara (1975; p 270) defined the ammonia fungi as "a chemoecological group of fungi which sequentially develop reproductive structures exclusively or relatively luxuriantly on the soil after a sudden addition of ammonia, some other nitrogenous materials which react as bases by themselves or on decomposition, or alkalis." This definition has referred solely to the fungal species reproducing on soil. Sagara (1995) also proposed the concept of succession of the ammonia fungi for sequential fruit body development and two successional phases: i.e., early phase (EP) and late phase (LP). Species in the EP are considered as saprotrophic and those in the LP as ectomycorrhizal (ECM) fungi, respectively (Sagara 1975, 1995).

Large numbers of fruit bodies in the ammonia fungi can be induced in small plots by addition of nitrogenous compounds such as urea. Furthermore, ECM ammonia fungi fruit abundantly (about $50\,g/m^2$) in almost all the treated plots (Sagara 1975, 1976; Fukiharu 1991). Although the number of ECM ammonia fungal species is quite limited, e.g., *Hebeloma* and *Laccaria* species (Sagara 1975; Fukiharu 1991), many ECM species other than ammonia fungi should be detected in urea-treated soils.

This study focused on *Cenococcum geophilum* Fr. (Cg). The fungus has very distinctive morphology and is recognized as a cosmopolitan, frequently detected, sometimes dominant, and sometimes pioneer (so-called multi-stage) fungus (LoBuglio 1999). Thus, Cg may be detected highly frequently in both nontreated soils and urea-treated soils. The present study aimed at the following two points: (1) discovering the recovery of ECM tips of Cg in soils treated with urea; and (2) definitely identifying Cg by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis among different treatments and vegetation types.

Experimental sites were established in two types of vegetation in warm temperate Japan, one in an evergreen broad-leaved forest dominated by *Castanopsis cuspidata* (Thunberg) Schottky (site C) and another in a deciduous forest dominated by *Quercus serrata* Murray (site Q). Site C was located at Kamitakano-saimyoujiyama, Kyoto city, Kyoto Prefecture, Japan (35° 4′N, 135° 48′E, 150 m alt.) and Site Q was located at Iwakura-hanazono-cho, Kyoto city, Kyoto Prefecture (35°4′N, 135°48′E, 154m alt.). Both dominant plant species belong to the family Fagaceae and are ectomycorrhizal (Imazeki and Hongo 1987). The former is a dominant species of the climax forest and the latter is dominant in the secondary forest in this area.

In the subcanopy layer of both sites were found *Quercus glauca* Thunberg, *Cleyera japonica* Sieb. et Zucc., *Eurya*

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japonica Thunberg, and *Camellia japonica* L. Some artificially planted trees of *Cryptomeria japonica* (L.f) D. Don. and *Chamaecyparis obtusa* (Sieb. et Zucc.) Endlicher also reached the canopy layer. Other than the dominant species, only *Q. glauca* is recognized as ectomycorrhizal (Imazeki and Hongo 1987). Thus, we established the study sites without *Q. glauca* as canopy trees.

The unit of urea treatment is 0.5 m long in the horizontal direction and 1 m along the vertical slope direction in the undulate topography. Remarkably abundant fruit body production has been induced by the large amount of chemical materials such as urea onto the small plots around the present site (Sagara 1975, 1976; Imamura 2001). Sites C and Q consisted of ten units to reduce the edge effects: one unit in the horizontal and ten units along the vertical slope direction, i.e., 0.5 m (horizontal) × 10 m (vertical). We divided the sites into five subplots of 2 m long in the sloping direction.

To induce fruiting of the ammonia fungi, 686 g fertilizer urea in granular form (nitrogen 46%: Kumiai-Nyouso, National Federation of Agricultural Co-operative Associations, Tokyo, Japan) was scattered for 1 m² by hand onto the experimental sites on June 15, 2000. This was the optimum for the study of the ammonia fungi to induce a large amount of fruit bodies (Sagara 1976; Fukiharu 1991; Yamanaka 1995) but was about 100 times higher in N content at one occasion than general N fertilization experiments (Alexander and Fairley 1983; Kåren and Nylund 1997; Jonsson et al. 2000; Peter et al. 2001).

The experimental sites were visited every 10 days. Collection of fruit bodies was continued for 15 months until the end of August 2001. The fruiting period of the ECM ammonia fungal species generally continues for about 15–18 months after the treatments in *Castanopsis* forests around Kyoto City (Fukiharu 1991; Imamura 2001). All the fungal fruit bodies of each ECM species occurring inside the sites were recorded and harvested for each subplot, as the occurrence is the only indicator of the succession of the ammonia fungi according to the original definition of Sagara (1975). The dry weight of the fruit bodies was measured after 1 week of drying at 55°C, and cumulatively totaled over the duration of soil sampling, i.e., 1 month.

Soil containing ECM tips was collected as a soil block with a surface of 5 × 5 cm and with a depth of 5–10 cm including O and A layers. One soil block from each subplot, i.e., five blocks in total per experimental site, was collected from the intact points where no soil block had been collected previously. Each soil block was taken at a point 10 cm away from either of the two edges in the vertical direction and more than 10 cm away from the horizontal edges of the plot at intervals of more than 2 m from the sampling points in the adjacent subplots. Soils were sampled monthly from both experimental sites. Soil sampling was not conducted in November 2001.

To detect the soil conditions in summer without treatment, five soil blocks were also collected for three times from each site: on the day when urea treatment was conducted (June 15, 2000) and on the days of the last stage of fruit body production of the ammonia fungi (July 31 and August 24, 2001). The nontreated soil blocks were collected apart at least 10 m from the edges of the urea-treated site to avoid the effects of the added urea; that was considered to be enough distance that the effect of urea was observed only inside the treated plots (Sagara 1976). All the soil blocks were brought back in polyethylene bags and stored at 4°C.

Each soil block was washed with tap water on sieves (2 mm and 0.5 mm in diameter) and fine roots (≤2 mm in diameter) were picked out. Thereafter, the roots were sorted into three categories: living ECM tips, living fine roots excluding ECM tips, and dead fine roots. Judgment of root senescence was made by "visual estimation" following Bauhus and Bartsch (1996): "live roots were intact, tough, and flexible, while dead roots were brittle and fractured easily."

On living ECM tips, morphological typing and picking out of Cg was made under a binocular microscope (×20) according to Ingleby et al. (1990) and LoBuglio (1999). The number of Cg ECM tips and the total ECM tips were also counted and calculated the density (per cm³) for each subplot. Observation in detail followed Ingleby et al. (1990), although typical Cg tips were mounted on glass slides stained in trypan blue (Nacalai Tesque, Kyoto, Japan) instead of cotton blue or toluidine blue. The rest of the tips were stored at −20°C for DNA extraction.

Specific identification of Cg ECM tips by PCR-RFLP was made using the internal transcribed spacer (ITS) region within rDNA following Gardes and Bruns (1993) and LoBuglio (1999). The DNA of the mycorrhizal tips was extracted and amplified following Gardes and Bruns (1993) and Yamada et al. (2001), with modification as follows: 1.25 units of Taq DNA polymerase (Toyobo, Osaka, Japan) per 50 µl reaction and 30 or 35 amplification cycles (95°C for 30 s, 55°C for 30 s, 72°C for 90 s) after 95°C for 3 min.

The Perkin Elmer GeneAMP PCR System 2400 was used in DNA amplification. Based on several studies (Gardes et al. 1991; Gardes and Bruns 1993) and after preliminary experiments, nested PCR amplifications with two primer pairs, ITS1F/ITS4 in the first and ITS1/ITS4 in the second, were executed for all the DNA extracts. This procedure was guaranteed to be fungus specific and produce a sufficient amount of amplification of DNA from a few milligrams of ECM tips.

Four enzymes were used in the RFLP procedures: *Afa*I, *Alu*I, *Hae*III, and *Hin*fI (Takara Shuzo, Shiga, Japan), and aliquots of 2–4 µl amplified DNA were digested at 37°C for more than 5 h. The digested DNA was electrophoresed on a 3%–3.2% agarose gel (#011-53; Nacalai Tesque) with a 100-bp DNA ladder (Bexel Biotechnology, Union City, CA, USA) in 0.5% Tris-borate + EDTA (TBE) buffer. The gel was stained with 1 µg/ml ethidium bromide for 15 min, and the length of DNA fragments was calculated with 1D Image Analysis Software, v3.0 (Kodak Digital Science).

Twelve samples were used for PCR-RFLPs: 3 replications for each condition of soil, i.e., treated or nontreated and two vegetation types. Two-way ANOVA (StatView J-5.0, SAS Institute, Cary, NC, USA) was used for the significance of the effects of urea treatment and of vegetation

Table 1. Mean density (no/cm^3) and frequency (in bold) of *Cenococcum geophilum* (Cg) ectomycorrhizal (ECM) tips among five soil blocks in each sampling and fruit body production (g/m^2) of *Alnicola lactariolens* and *Hebeloma vinosophyllum* in urea-treated plots

Site and treatment	Detection of Cg	Sampling date								
		June 2000	Jan. 2001	Feb. 2001	Mar. 2001	Apr. 2001	May 2001	June 2001	July 2001	Aug. 2001
CU	Total ECM	–	0.595	0.734	0.920	0.313	0.975	0.714	0.761	0.848
	Cg	–	0.008	0.000	0.000	0.000	0.000	0.024	0.030	0.029
	Frequency	–	**1**	**0**	**0**	**0**	**0**	**1**	**3**	**3**
	Al	0.000	0.000	0.000	0.000	0.000	0.000	24.010	3.449	0.000
CN	Total ECM	1.98	–	–	–	–	–	–	0.93	1.42
	Cg	0.02	–	–	–	–	–	–	0.08	0.26
	Frequency	**3**	–	–	–	–	–	–	**3**	**5**
QU	Total ECM	–	0.484	0.360	0.202	0.170	0.734	0.857	0.619	0.492
	Cg	–	0.016	0.003	0.041	0.002	0.056	0.046	0.014	0.113
	Frequency	–	**3**	**1**	**3**	**1**	**1**	**3**	**3**	**5**
	Hv	0.000	0.000	0.000	0.000	0.000	0.000	11.351	2.856	0.000
QN	Total ECM	1.55	–	–	–	–	–	–	2.44	0.91
	Cg	0.70	–	–	–	–	–	–	0.37	0.52
	Frequency	**5**	–	–	–	–	–	–	**5**	**5**

C and Q, site names; U and N, urea- and nontreated; Al, *Aclnicola lactariolens*; Hv, *Hebeloma vinosophyllum*. Although *A. lactariolens* and *H. vinosophyllum* fruited in September to October 2000 for about 1 g/m^2, Cg was not detected from July to December 2000
–, no sampling was conducted

Table 2. Mean density of Cg in each treatment and each site

Treatment	Vegetation type	Mean density of Cg tips ±SD (no/cm^3)
Urea	C	0.029 ± 0.033 (3.68)
Non	C	0.171 ± 0.143 (12.6)
Urea	Q	0.064 ± 0.121 (11.4)
Non	Q	0.445 ± 0.387 (20.5)
Urea treatment (A)		***
Vegetation type (B)		*
A × B		ns

Significant effects by urea treatment (A) and vegetation type (B) were analyzed by two-way ANOVA
The proportion of Cg ECM tips to total ECM tips is listed in the parentheses. Only the values in July and August 2001 are given because data for nontreated soils were limited
C, *Castanopsis cuspidata*; Q, *Quercus serrata*; ns, not significant
*Significant effect at $P < 0.05$; ***significant effect at $P < 0.001$

type, on the density of Cg ECM tips in July and August 2001.

In site C and site Q, fruit bodies of *Alnicola lactariolens* Clemençon et Hongo and *Hebeloma vinosophyllum* Hongo were very abundant and dominant in dry weight, respectively (Table 1). In urea-treated soils, ECM tips of Cg were detected in January 2001 for the first time in the both sites. Afterward, in site C, Cg was not detected again until June 2001. The duration of detection of Cg ECM tips in urea-treated soil overlapped partly the maximum fruiting period of the ECM ammonia fungi (Table 1). Frequency of Cg detection in the treated soils was less than that in the nontreated soils. In nontreated soils, ECM tips of Cg were detected in all the sampling occasions (no sampling January

to June 2001) (Table 1). The density of Cg ECM tips was significantly affected by the urea treatment and the vegetation type. However, the interaction of the two factors was not significant (two-way ANOVA; Table 2).

Comparing between the treated and the nontreated soils, the occurrence of ECM tips of Cg was more frequent and the density of ECM tips of Cg was significantly higher in nontreated soils than in treated ones (see Table 1). The proportion of Cg to total ECM tips was also higher in the urea-treated soils than that in the nontreated soils (Table 2). Comparing between vegetation types, the occurrence of ECM tips of Cg was significantly more frequent and the density of ECM tips of Cg was higher in site Q than in site C (Tables 1, 2). The proportion of Cg to total ECM tips were also higher in site Q. In nontreated soils in site Q especially, Cg occupied as much as 20% of the total ECM tips (Table 2).

PCR products were 560 bp for all the samples, and all the products gave the same restriction pattern with one exception for ECM 274 (Table 3). The products were uncut by *Afa*I, and digested into 390 and 170 bp by *Alu*I and into 170 and 120 bp and other shorter fragments by *Hin*fI. Although the PCR products were digested into 460 and 100 bp by *Hae*III, except for ECM 274, the shorter fragment was considered to be digested into shorter fragments in ECM 274.

We checked GenBank for the sequencing data including ITS1 and -2 of Cg (AY112935) and confirmed the identity of our PCR-RFLP. The only disagreement among 12 samples was considered as an intraspecific variation, as LoBuglio et al. (1991) reported on nuclear ribosomal DNA of Cg. Thus, all the ECM tips investigated as Cg in this study can be considered as the identical species.

The quantity of Cg ECM is smaller in urea-treated soil than in nontreated soil. The conditions after a large amount of urea addition, especially high-N content (ammonium-N

Table 3. ECM samples analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Serial number of ECM samples	Site and treatment	Month and year of collection	Cg ECM	
			Tip number	Proportion among total ECM tips (%)
175	CU	July 2001	9	7.7
197	QU	July 2001	7	4.7
207	QU	May 2001	18	15
274	CU	July 2001	8	8.6
294	CU	Aug. 2001	4	12
301	QU	Aug. 2001	8	22
321	CN	Aug. 2001	32	32
329	CN	Aug. 2001	25	16
337	QN	Aug. 2001	89	52
363	CN	July 2001	18	19
376	QN	July 2001	56	37
380	QN	July 2001	111	26

Number of Cg ECM tips and the proportion among total ECM tips of each soil are also listed
C and Q, *Castanopsis* and *Quercus* forests; U and N, urea-treated and nontreated

and nitrate-N) in soil as reported by Yamanaka (1995), might be unfavorable for Cg. However, in the urea-treated soils, the first detection of Cg in the sites C and Q was 6 months after the treatments, when preceded by the maximum fruit body production of the ECM ammonia fungi. We think that this is due to the tolerance of Cg against damaging effects of ammonia (Sagara 1975), with sclerotia and thick cell walled hyphae, and the ability of quick invasion as a pioneer species (LoBuglio 1999).

The relationship between Cg and two ECM trees (*C. cuspidata* and *Q. serrata*) was an shown for the first time. Although *Q. glauca* (evergreen) was an ECM plant species common to sites C and Q, almost all ECM tips were considered to be connected to the dominant species, i.e., *C. cuspidata* and *Q. serrata* in each site according to observation on plant roots (data not shown). Between vegetation types, Cg was more abundant in the number of the ECM tips in site Q (deciduous broad-leaved forest) than in site C (evergreen broad-leaved forest). Cg ECM was thought to be dominant in the nontreated soils in site Q, where it occupied about 20% of total ECM tips in the non-treated soils.

Cg is a unique fungus bearing various characteristics as a dominant, tolerant, and pioneer species (LoBuglio 1999); therefore, a longer-term investigation with more replications on Cg after urea treatment in warm temperate forests is needed.

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