Enzyme Activity of *Cenococcum geophilum* Isolates on Enzyme-specific Solid Media

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Enzyme activities of *Cenococcum geophilum* isolates were examined on enzyme- specific solid media. Deoxyribonuclease, phosphatase, and urease were detected in all isolates, whereas cellulase was not detected in any of the isolates. Variations in enzyme activities of amylase, caseinolysis, gelatinase, lipase, and ribonuclease were observed among isolates.

KEYWORDS : Ectomycorrhizal fungi, Extracellular enzyme, Intraspecific variation, Nutrient mobilization

Ectomycorrhizal (EcM) symbiosis is a mutualistic association between EcM fungi and certain woody plants such as species of Pinaceae, Fagaceae, Betulaceae, and Salicaceae [1]. EcM fungi colonize root tips of host plants and extend massive hyphae and rhizomorphs into the soil [2]. These fungi benefit host plants in a number of ways, although the most important function may be enhancing soil nutrient uptake, particularly for elements with a low mobility in the soil such as nitrogen (N) [3] and phosphorus (P) [4]. EcM fungi produce a wide range of extracellular enzymes that degrade N- and P-compounds contained in soil organic matter [5, 6]. Such assimilated nutrients are absorbed by the hyphal network, and then some of the nutrients are transferred to the host plant in exchange for carbon, i.e., photosynthate [1, 3]. It is likely that the ability to produce a range of extracellular enzymes by EcM fungi plays significant roles in nutrient acquisition of host plants from soils.

Cenococcum geophilum Fr. (= *C. graniforme*) is one of the most common and widespread EcM fungi [7, 8]. EcM roots of this fungus have been observed, often as a dominant species, from diverse ranges of habitats such as arctic forests and temperate and subtropical environments [7]. *C. geophilum* is well known for its pioneering capability and often invades dominantly in weakly developed soils on which EcM hosts have established, such as coastal pine forests on maritime sand dunes [9]. Due to its potential ecological importance as an EcM symbiont, many studies have been conducted on this fungus to understand its physiology [10]. Several studies have revealed protease [5] and phosphatase [11] activities of *C. geophilum*;

however, information about extracellular enzyme production by *C. geophilum* is still scarce. In particular, the variation in enzyme activities among isolates remains uncertain.

We investigated amylase, caseinolysis, cellulase, deoxyribonuclease (DNAse), gelatinase, lipase, pectinase, phosphatase, ribonuclease (RNAse), and urease activities in 29 isolates of *C. geophilum*. We used enzyme-specific solid media, because it permits an easy and rapid detection of specific enzyme activities from a large number of samples.

Small soil samples (500~1,000 mL) were collected from 13 locations of *Pinus thunbergii* Parl. coastal forests or *Pinus densiflora* Sieb. et Zucc. artificial forests in inland areas of Korea from 2008 to 2009 (Table 1). Trappe [12] revealed that the sclerotia of *C. geophilum* produced in soils varies from 0.05 to 4 or more mm in diameter. Thus, soil samples were put through a 0.25-mm pore size sieve with tap water to remove soil particles and to trap the *C. geophilum* sclerotia. The trapped soils were spread on a glass dish, and sclerotia were picked up under a dissecting microscope. The sclerotia were surface sterilized in 30% H_2O_2 for 5 min, rinsed once in sterilized distilled water, and then transferred to modified Melin-Norkans (MMN) agar medium [13] containing 300 μg/mL streptomycin sulfate. In total, 29 *C. geophilum* isolates were collected and used in this study (Table 1). Each isolate was identified to species by sequencing the internal transcribed spacer regions, including the 5.8S rDNA region, and by comparing the sequences to those at the GenBank database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). DNA extraction, PCR, and sequencing were performed as previously described [9]. The iso-
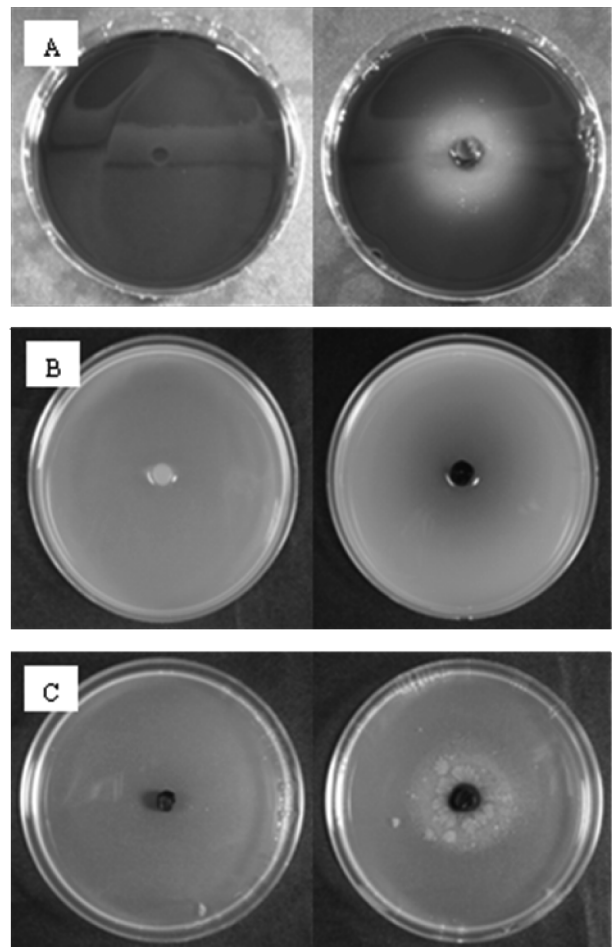
Table 1. Isolates of *Cenococcum geophilum* with their isolate numbers, collection areas, putative host trees, and forest types where they were collected

Isolate No.	Area	Host	Forest type
G-8	Buan	*Pinus densiflora*	Coastal pine forest
G-9	Buan	*P. densiflora*	Coastal pine forest
G-10	Buan	*P. densiflora*	Coastal pine forest
G-28	Busan	*P. thunbergii*	Coastal pine forest
9-2	Gangneung	*P. thunbergii*	Coastal pine forest
9-4	Gangneung	*P. thunbergii*	Coastal pine forest
9-7	Gangneung	*P. densiflora*	Artificial pine forest
9-8	Gangneung	*P. densiflora*	Artificial pine forest
9-50	Gangneung	*P. densiflora*	Artificial pine forest
9-51	Gangneung	*P. densiflora*	Artificial pine forest
99-1	Gangneung	*P. thunbergii*	Coastal pine forest
9-44	Gongju	*P. densiflora*	Artificial pine forest
G-11	Gumi	*P. densiflora*	Artificial pine forest
G-14	Gumi	*P. densiflora*	Artificial pine forest
G-38	Gumi	*P. densiflora*	Artificial pine forest
9-52	Hadong	*P. thunbergii*	Artificial pine forest
G-2	Haenam	*P. thunbergii*	Coastal pine forest
G-40	Haenam	*P. thunbergii*	Coastal pine forest
9-49	Incheon	*P. thunbergii*	Coastal pine forest
G-18	Namwon	*P. densiflora*	Artificial pine forest
G-20	Namwon	*P. densiflora*	Artificial pine forest
G-21	Namwon	*P. densiflora*	Artificial pine forest
08-02	Samcheok	*P. thunbergii*	Coastal pine forest
08-03	Samcheok	*P. thunbergii*	Coastal pine forest
9-9	Taean	*P. thunbergii*	Coastal pine forest
9-10	Taean	*P. thunbergii*	Coastal pine forest
G-23	Tongyeong	*P. thunbergii*	Coastal pine forest
F-2	Uljin	*P. thunbergii*	Coastal pine forest
G-35	Yeosu	*P. thunbergii*	Coastal pine forest

Fig. 1. Representative *Cenococcum geophilum* reactions on enzyme-specific solid media. A, Yellow zone around a colony (right photo) indicated amylolytic activity; B, Clear zone around a colony in an otherwise opaque medium (right photo) indicated DNAase production; C, A visible precipitate due to crystal formation of calcium salts around a colony (right photo) indicated lipase production.

lates were incubated on MMN agar media in the dark at 25°C until the next procedure. All isolates were deposited in the Laboratory of Tree Pathology and Mycology (TPML) at Kangwon National University, Korea.

MMN agar discs containing growing mycelia were cut from the edge of fungal cultures of each isolate, which were incubated on MMN agar plate medium for 2 mon, with a 5 mm cork borer. Each disc was placed individually on each enzyme-specific media. Agar media described by Hankin and Anagnostakis [14] were used for detecting the production of amylase, DNAase, gelatinase, lipase, RNAase, pectate trans-eliminase, and phosphatase. Agar media described by Paterson and Bridge [15], Pointing (cellulose agar) [16], and Vuye and Pijck (Christensen urea agar) [17] were used for detecting caseinolysis activity, cellulose, and urease production, respectively. After 1 mon incubation in the dark at 25°C, the production of each enzyme was determined as described previously [14-17]. Five replicates were used for each treatment. Agar discs without fungal mycelia were placed on each enzyme-specific media as a control treatment.

Production of DNAase, phosphatase, and urease was detected in all isolates (Table 2, Fig. 1). RNAase was detected in 28 of 29 isolates, indicating that it is common for *C. geophilum* isolates to produce DNAase, phosphatase, RNAase, and urease, irrespective of their location. The roles of DNAase and RNAase produced by *C. geophilum* are unknown. They may partly contribute to degrading intracellular nucleic acids in dead microorganisms such as bacteria [18] in soil. They may also be important sources for *C. geophilum* autolysis [19]. Phosphatase production is commonly observed in various EcM fungi [20], including *C. geophilum* [11, 21]. Although seedlings inoculated with *C. geophilum* could take up more P from organic matter than seedlings not inoculated with EcM [22], phosphatase activity was relatively lower than that of other EcM fungi [21]. Urea is a nitrogen source for the growth of many organisms and has been used as an

Table 2. Absence (−) or presence (+) of extracellular enzymatic activity[a] in each *Cenococcum geophilum* isolate

Isolate No.	Amy	Cas	Cel	DNA	Gel	Lip	Pec	Pho	RNA	Ure
G-8	+	+	−	+	+/−	+	−	+	+	+
G-9	+	+	−	+	−	+/−	−	+	+	+
G-10	+	−	−	+	+	+/−	−	+	+	+
G-28	+	−	−	+	−	−	−	+	+	+
9-2	−	−	−	+	+	−	−	+	+	+
9-4	+	−	−	+	−	−	−	+	+	+
9-7	+/−	−	−	+	−	−	−	+	+	+
9-8	−	−	−	+	−	+	−	+	+	+
9-50	+	−	−	+	−	+/−	−	+	+	+
9-51	+	−	−	+	−	−	?	+	+	+
99-1	+/−	−	−	+	−	−	?	+/−	+	+
9-44	−	−	−	+	+	+	?	+	+	+
G-11	+	−	−	+	−	−	−	+	+	+
G-14	+	−	−	+	−	+	?	+	+	+
G-38	−	−	−	+	+	+/−	−	+	+	+
9-52	+	−	−	+	+	−	−	+	+	+
G-2	+/−	−	−	+	+	−	−	+	+	+
G-40	+/−	−	−	+	+	+	−	+	+	+
9-49	−	−	−	+	−	−	−	+	−	+
G-18	+	−	−	+	−	+	−	+	+	+
G-20	+	−	−	+	−	+/−	−	+	+	+
G-21	−	−	−	+	−	−	?	+	+	+
08-02	+/−	−	−	+	+	−	?	+	+	+
08-03	+/−	−	−	+	−	−	?	+	+	+
9-9	+	−	−	+	+	+/−	−	+	+	+
9-10	+	−	−	+	−	+	−	+	+	+
G-23	+	−	−	+	−	−	−	+	+	+
F-2	+	−	−	+	−	+/−	−	+	+	+
G-35	+/−	−	−	+	+	+/−	?	+	+	+

+/− indicates that enzyme production was detected in some plates but not in others. ? indicates that it was doubtful whether the reaction was derived from enzyme production.
[a]Amy, amylase; Cas, caseinolysis; Cel, cellulase; DNA, DNAase; Gel, gelatinase; Lip, lipase; Pec, pectate trans-eliminase; Pho, phosphatase; RNA, RNAase; Ure, urease.

important fertilizer in forest soils. Several EcM fungi can use urea as their sole nitrogen source [23, 24]. Imamura and Yumoto [25] reported that *C. geophilum* EcMs are significantly more abundant in urea-nontreated soil than in treated soil, suggesting that external urea can be used by *C. geophilum* but that it is not likely a preferred nitrogen source.

Production of amylase, gelatinase, and lipase were variable among isolates; each enzyme activity was detected in 23, 11, and 15 isolates, respectively (Table 2, Fig. 1). Amylase production by EcM fungal species has been reported in species of *Amanita* and *Cortinarius* [23]. Bae and Barton [11] detected α-amylase binding on the cell surface of *C. geophilum*. Gelatinase production has been reported in various species of EcM fungi such as *Amanita* [23], and lipase activity has been reported in *Amanita*, *Cortinarius*, *Lactarius*, *Piloderma*, and *Thelephora* [23, 26], El-Badaoui and Botton [27] detected protease activity, including gelatinase, in *C. geophilum*, whereas Nygren *et al*. [5] did not detect protease activity. Caldwell *et al*. [26] did not detect lipase from a *C. geophilum* isolate.

The discrepancy among these studies and the present study could be partly attributed to variations in enzyme activity among *C. geophilum* isolates, as seen in the present study.

A reaction was observed for pectate trans-eliminase on enzyme-specific media in eight isolates; however, it was unlikely that the reaction was derived from the production of this enzyme. Hankin and Anagnostakis [14] stated that clear zones around a colony in an otherwise opaque medium indicate pectin degradation. However, in the present study, the color of all media changed to whitish from a very pale brown original color in eight isolates, and no clear zones were observed around the colonies. Several studies have found that pectinase activity was very low or absent in EcM fungi [23, 28]. A more accurate determination should be performed to detect the activity of this enzyme. Only two *C. geophilum* isolates had the ability to hydrolyze casein (skim milk). El-Badaoui and Botton [27] detected caseinolysis activity in *C. geophilum*, whereas Nygren *et al*. [5] did not. It is unlikely that *C. geophilum* has caseinolysis activity. Production of cellulase was not detected in any isolates. Previous studies also revealed

that cellulase activity is very low or absent in EcM fungi [23].

Our results revealed variations in enzyme activities among the isolates of *C. geophilum* obtained from various parts of Korea. These variations were also detected among isolates obtained from soil samples collected at the same location (e.g., isolate 9-7, 9-8, 9-50, and 9-51). Thus, further studies are needed to understand the significance of *C. geophilum* enzyme activities and the variability among isolates.

Acknowledgements

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