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# Variation in Sodium Chloride Resistance of *Cenococcum geophilum* and *Suillus granulatus* Isolates in Liquid Culture

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**We studied the resistance of *Cenococcum geophilum* and *Suillus granulatus* isolates to NaCl during growth under axenic culture conditions. *C. geophilum* isolates displayed variations in NaCl resistance; mycelial growth of most isolates was inhibited above 200 mM. All isolates of *S. granulatus* were tolerant to high NaCl content.**

**KEYWORDS : Coastal forest, Ectomycorrhizal fungi, NaCl, Salinity, Salt tolerance**

*Pinus thunbergii* Parl. is a major constituent of coastal forests in Korea and is used to alleviate damage by salt and sand dispersion to inland areas as well as for recreation. In recent years, however, pine wilt disease, caused by the pine wood nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, has seriously damaged coastal pine forests, especially in southern Korea. Further, the recent occurrence of severe fires has destroyed much of the coastal pine forests in the east. Therefore, several studies and projects such as afforestation have been conducted with the goal of recovery and proper maintenance of coastal pine forests in Korea [1, 2].

Soil salinity is a characteristic factor of coastal areas that can inhibit the establishment of plant seedlings. Several reports have shown that soil salinity can reduce the absorption of certain mineral nutrients such as phosphorus, nitrogen and potassium by plants [3]. In this case, mycorrhizal association appears to be one of the important factors for successful plant establishment. Mycorrhizal associations play a significant role in plant establishment, including nutrient promotion, water uptake by host plants and also tolerance towards stresses encountered [4]. A recent study revealed that inoculation of ectomycorrhizal (EcM) fungi alleviates salt stress in host plant seedlings [5]. Therefore, it is possible that practical use of EcM fungi can help establish coastal pine forests.

In a primary study, we investigated the EcM fungal community in mature *P. thunbergii* coastal forests in Korea and revealed that *Cenococcum geophilum* Fr. was one of the dominant species in both mature trees and naturally regenerating seedlings [6]. Other reports also showed that this fungus is one of the equivalent and dominant species in *P. thunbergii* coastal forests in Japan [7, 8]. Further, we identified EcM fungi associated with *P. thunbergii* seedlings in a young plantation and revealed that *Suillus granulatus* (L.) Roussel naturally invaded and dominated [9]. Sporocarps of *S. granulatus* were often observed in *P. thunbergii* coastal forests in Korea and Japan. Thus, both fungi appear to be important symbionts of *P. thunbergii* in coastal areas. To apply EcM fungi to the recovery of coastal pine forests, it is important to understand the degree of salt resistance of both fungi. Several studies have reported the growth of various EcM fungi under salt stress conditions and demonstrated interspecific and also intraspecific variation in NaCl resistance, especially in *Pisolithus* spp. [10, 11]. However, the degree of NaCl resistance and its variation among both *C. geophilum* and *S. granulatus* isolates remains uncertain. The purpose of this study was to understand the variation in NaCl resistance among isolates of *C. geophilum* and *S. granulatus*. Isolates of each EcM fungus were incubated in axenic liquid nutrient media containing different NaCl concentrations, and their dry mycelial weights were determined.

*C. geophilum* isolates were obtained from sclerotia obtained from soil samples. Sclerotia were surface sterilized in 30% $H_2O_2$ for 5 min, rinsed once in sterilized distilled water and then transferred to modified Melin-Norkrans (MMN) agar medium [12] containing 300 ppm of streptomycin sulfate. *S. granulatus* isolates were obtained from sporocarps or EcMs. EcMs of *S. granulatus* were surface sterilized in 15% $H_2O_2$ for 1 min, rinsed in sterilized distilled water and then incubated on MMN agar medium containing 300 ppm of streptomycin sulfate. Species identification of each fungal isolate was conducted by sequencing of internal transcribed spacer (ITS) regions, including the 5.8S rDNA region, and comparison of the obtained sequences with the GenBank database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). DNA extraction, PCR and sequencing were performed as previously described [6], except for the PCR conditions. PCR

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**Table 1.** Ectomycorrhizal fungal isolates used in this study, and their isolate numbers, collected areas, putative host trees, origins and forest types where they were collected

| Ectomycorrhizal fungi | Isolate no. | Area | Putative host | Origin | Forest type |
|---|---|---|---|---|---|
| *Cenococcum geophilum* | 9-2 | Gangneung | *Pinus thunbergii* | Sclerotium | Coastal forest |
| | 9-4 | Gangneung | *P. thunbergii* | Sclerotium | Coastal forest |
| | 9-7 | Gangneung | *P. densiflora* | Sclerotium | Artificial forest (inland area) |
| | 9-51 | Gangneung | *P. densiflora* | Sclerotium | Artificial forest (inland area) |
| | 9-44 | Gongju | *P. densiflora* | Sclerotium | Artificial forest (inland area) |
| | 9-45 | Gongju | *P. densiflora* | Sclerotium | Artificial forest (inland area) |
| | 9-49 | Incheon | *P. thunbergii* | Sclerotium | Coastal forest |
| | 08-02 | Samcheok | *P. thunbergii* | Sclerotium | Coastal forest |
| | 08-05 | Samcheok | *P. thunbergii* | Sclerotium | Coastal forest |
| | 9-12 | Taean | *P. thunbergii* | Sclerotium | Coastal forest |
| | 9-42 | Taean | *P. thunbergii* | Sclerotium | Coastal forest |
| *Suillus granulatus* | 08-16 | Gangneung | *P. thunbergii* | Sporocarp | Coastal forest |
| | 08-24 | Hongcheon | *P. densiflora* | Sporocarp | Artificial forest (inland area) |
| | 9-34 | Incheon | *P. thunbergii* | Ectomycorrhiza | Coastal forest |
| | 08-29 | Samcheok | *P. thunbergii* | Sporocarp | Coastal forest |
| | 08-23 | Chuncheon | *P. densiflora* | Sporocarp | Artificial forest (inland area) |

amplification was conducted with the primers ITS1f [13] and ITS4 [14] under the following conditions; 94°C (3 min), followed by 30 cycles at 94°C (30 sec), 50°C (30 sec) and 72°C (2 min), and a final hold at 72°C (10 min). As a result, eleven and five isolates of *C. geophilum* and *S. granulatus*, respectively, were obtained from several locations of *P. thunbergii* coastal forests and *Pinus densiflora* Sieb. et Zucc. artificial forests in inland areas of Korea in 2008 and 2009 (Table 1). All isolates were deposited in the laboratory of "Tree Pathology and Virology" at Kangwon National University, Korea. The isolates were incubated on MMN agar media under dark conditions at 25°C. Precultured fungal colonies of each isolate were bored around their peripheries with a 5 mm cork borer to make agar discs with fungal mycelium. Each disk was then transferred to a 50 mL falcon tube containing 30 mL of liquid MMN media with five different NaCl concentrations (0, 50, 100, 200 and 300 mM). The tubes were sealed with caps and incubated under dark conditions for 60 days at 25°C. There were five replicates for each treatment. Mycelial dry weights were determined after filtration using filter paper, rinsed by distilled water and dried at 40°C for 5 days.

Comparisons of mycelial weight for the different NaCl concentrations were conducted by nonparametric Kruskal-Wallis test. After evaluation, the data were analyzed by Steel's test in order to compare the NaCl concentrations of 0 mM and 50 to 300 mM ($p < 0.05$). Two-way ANOVA was used to test the effect of fungal isolate and NaCl concentration on mycelial weight. Kruskal-Wallis test and two-way ANOVA was performed using SPSS ver. 13.0 (SPSS Inc., Chicago, IL, USA), and Steel's test was performed using R ver. 2.10.0 [15].

Six out of 11 *C. geophilum* isolates (isolate 08-02, 9-2, 9-12, 9-44, 9-45 and 9-49) showed no significant differences in mycelial weight between 0 mM and 50 and 100 mM; however, they did show complete inhibition in mycelial growth above 200 or 300 mM (Table 2). Mycelial weight of isolate 9-42 was significantly higher in 50 mM than 0 mM ($p < 0.05$, Steel's test); however, it also showed complete inhibition in mycelial growth above 200 mM. Mycelial weight of isolate 08-05 was significantly lower in both 50 mM and 200 mM than 0 mM, and its mycelial growth was inhibited completely at 300 mM. Isolate 08-05 did not show a significant difference in mycelial weight between 0 mM and 100 mM, possibly due to the low number of replications and high variation in response to 0 mM. The remaining three isolates (isolate 9-4, 9-7 and 9-51) were not significantly different in mycelial weight between 0 mM and 50~300 mM ($p < 0.05$, Steel's test), even though isolate 9-51 tended to decline at a higher NaCl concentration. Analysis of variance revealed significant effects of fungal isolate ($F = 40.02$, $p < 0.001$), NaCl concentration ($F = 13.80$, $p < 0.001$) and their interaction ($F = 6.05$, $p < 0.001$) on the mycelial weights of *C. geophilum*. Saleh-Rastin [16] showed the maximum NaCl tolerance of *Cenococcum graniforme* (Sow.) Ferd (= *C. geophilum*) isolate was about 188 mM. The present study also indicated that the maximum NaCl tolerance for some isolates was within 100~200 mM; however, that of the other isolates was above 200 mM. These results indicate that there were variations in NaCl resistance among the isolates of *C. geophilum*. Previous studies also demonstrated intraspecific variations in NaCl resistance for the mycelial growth of other EcM fungal species. Chen *et al.* [10] showed variations among 18 isolates of *Pisolithus* spp. in a range of up to 200 mM of NaCl. Kernaghan *et al.* [17] showed low variation among 7 isolates of *Laccaria bicolor*. Matsuda *et al.* [11] showed variations among 9 isolates of *Pisolithus tinctorius* (Pers.) Coker and Couch

**Table 2.** Mycelial dry weights of each ectomycorrhizal fungal isolate incubated in modified Melin Norkrans (MMN) liquid media with different NaCl concentration[a,b]

| Ectomycorrhizal fungi | Isolate no. | NaCl concentration in MMN liquid media (mM) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 50 | | 100 | | 200 | | 300 | |
| | | Weight (mg) | n | Weight (mg) | n | Weight (mg) | n | Weight (mg) | n | Weight (mg) | n |
| *Cenococcum geophilum* | 9-2 | 25.6 ± 3.9 | 5 | 28.4 ± 5.3 | 5 | 31.2 ± 8.7 | 5 | – | 0 | – | 0 |
| | 9-4 | 25.4 ± 3.4 | 5 | 19.8 ± 5.8 | 5 | 18.2 ± 3.3 | 5 | 24.4 ± 4.9 | 5 | 22.0 ± 1.2 | 5 |
| | 9-7 | 20.8 ± 6.8 | 5 | 14.0 ± 4.7 | 5 | 15.0 ± 3.8 | 5 | 11.8 ± 1.3 | 5 | 13.6 ± 2.2 | 5 |
| | 9-51 | 33.8 ± 7.6 | 5 | 8.0 ± 4.6 | 5 | 9.4 ± 3.7 | 5 | 10.0 ± 2.3 | 5 | 12.6 ± 3.0 | 5 |
| | 9-44 | 11.4 ± 4.2 | 5 | 10.6 ± 4.0 | 5 | 9.6 ± 3.3 | 5 | 7.0 | 1 | 6.0 | 1 |
| | 9-45 | 11.4 ± 3.9 | 5 | 7.0 ± 1.9 | 5 | 6.8 ± 3.1 | 4 | 7.0 ± 1.4 | 2 | 3.0 | 1 |
| | 9-49 | 9.2 ± 3.6 | 5 | 6.6 ± 3.3 | 5 | 8.6 ± 2.9 | 5 | – | 0 | – | 0 |
| | 08-02 | 17.2 ± 2.4 | 5 | 17.8 ± 3.0 | 5 | 20.6 ± 2.6 | 5 | – | 0 | – | 0 |
| | 08-05 | 16.6 ± 7.8 | 5 | 3.6 ± 3.3* | 5 | 8.8 ± 4.7 | 5 | 3.2 ± 1.8* | 5 | – | 0 |
| | 9-12 | 18.2 ± 4.8 | 5 | 14.0 ± 4.8 | 5 | 15.6 ± 2.4 | 5 | – | 0 | – | 0 |
| | 9-42 | 7.0 ± 1.2 | 5 | 11.8 ± 1.9* | 5 | 12.2 ± 5.4 | 5 | – | 0 | – | 0 |
| *Suillus granulatus* | 08-16 | 18.4 ± 5.5 | 5 | 16.6 ± 3.2 | 5 | 14.4 ± 3.2 | 5 | 15.8 ± 1.8 | 5 | 13.4 ± 2.1 | 5 |
| | 08-24 | 19.8 ± 7.4 | 5 | 29.2 ± 10.3 | 5 | 24.2 ± 7.6 | 5 | 27.4 ± 1.5 | 5 | 27.0 ± 5.3 | 5 |
| | 9-34 | 13.8 ± 7.4 | 5 | 13.4 ± 4.8 | 5 | 9.2 ± 5.6 | 5 | 15.8 ± 5.9 | 5 | 12.4 ± 2.9 | 5 |
| | 08-29 | 13.0 ± 3.2 | 5 | 10.4 ± 7.1 | 5 | 11.8 ± 4.1 | 5 | 9.0 ± 1.6 | 5 | 11.0 ± 3.3 | 5 |
| | 08-23 | 17.2 ± 1.8 | 5 | 13.8 ± 1.6 | 5 | 16.2 ± 2.3 | 5 | 15.2 ± 2.9 | 5 | 14.4 ± 3.9 | 5 |

[a]Mean values ± SD.
[b]n indicated the numbers of isolates which could grow in MMN liquid media.
*Indicated significant differences between 0 mM and different NaCl concentration at $p < 0.05$ (Steel's test).

in a range of up to 500 mM.

All isolates of *S. granulatus* did not show significant differences in mycelial weight between 0 mM and 50~300 mM ($p < 0.05$, Steel's test) (Table 2). Analysis of variance revealed significant effects of fungal isolate ($F = 33.39$, $p < 0.001$) on the mycelial weights of *S. granulatus*. Although this study employed a low number of isolates of *S. granulatus* (n = 5), the results suggest that there were no obvious variations in NaCl resistance in a range of up to 300 mM and that all isolates were tolerant to high salinity. There are no studies that have directly measured the NaCl resistance of *S. granulatus*, although several studies have investigated that of other *Suillus* spp. Tang *et al*. [18] revealed that biomass production in response to NaCl stress differs between *Suillus bovinus* (L. ex Fr.) O. Kuntze and *Suillus luteus* (L. ex Fr.) Gray and that the maximum NaCl tolerances of both fungi are above 800 mM. Bois *et al*. [19] investigated the growth characteristics of five EcM fungi in response to increasing concentrations of NaCl and found that the biomass production of *Suillus tomentosus* (Kauff.) Sing. declines with increasing NaCl concentration and that the maximum NaCl tolerance for both is above 300 mM. It was also found that an isolate of *S. tomentosus* exhibits greater Na and Cl filtering capacities compared to other EcM fungi. Further, when subjected to higher NaCl concentrations, this isolate increase production of mannitol and proline, which possibly alleviate osmotic stress induced by high salinity. It appears that *Suillus* spp., involving *S. granulatus*, has the ability to grown in high salinity by alleviating salt stress, even though there is interspecific variation in NaCl resistance. Further tests that use more *S. granulatus* isolates with higher NaCl concentrations (above 300 mM) are required to ascertain the maximum NaCl tolerance and also the intraspecific variation.

In conclusion, we demonstrated that *C. geophilum* had variations in NaCl resistance among its isolates. Further, the mycelial growth of most isolates was inhibited above 200 mM, whereas *S. granulatus* had no obvious variation and was tolerant to high NaCl concentration.

Soil salinity in coastal areas varies spatiotemporally. For example, Ishikawa *et al*. [20] revealed that the NaCl concentration at sites within 40 m of the shoreline at Kadoori, Japan exceeds 400 mM temporarily due to typhoon conditions. However, the NaCl concentrations at sites beyond 40 m of the shoreline were lower than 30 mM, even after a typhoon. Salt tolerance of pine species often varies widely even among closely related species [21]. Townsend [21] showed high NaCl resistance of *P. thunbergii* seedlings compared to other pine species when 2% NaCl solution (about 342 mM) was sprayed. Indeed, *P. thunbergii* often colonizes not only sand dunes but also rocky cliffs where seawater (about 500 mM of NaCl concentration) sprays directly onto the trees. Thus, it appears that *P. thunbergii* can resist soil salinity at seaward sites. On the other hand, it seems that both *C. geophilum* and *S. granulatus* could resist soil salinity at relatively inland sites where soil salinity is low and stable; however, it was unclear whether or not *C. geophilum* can survive at seaward sites where soil salinity is temporally increased

higher than 200 mM.

The establishment of *P. thunbergii* seedlings at seaward sites should require association with salt-resistant EcM fungi, since the trees depend mostly on EcM fungi for nutrient acquisition. Further studies are required to understand whether or not salt-resistant EcM fungi can associate with *P. thunbergii* seedlings and also enhance the establishment of hosts under saline conditions. This information might be fundamental for the development of an effective inoculation program for the recovery of coastal pine forests.

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