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Effect of Sodium Chloride (NaCl) on the Mycelial Growth and Ascospore Germination of *Rhizina undulata,* the Root Rot Fungus of Coniferous Trees

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Abstract

Rhizina undulata, the causal agent of Rhizina root rot, is a soil-borne fungus occurring on coniferous trees. The destruction of coastal forests caused by *R. undulata* infection has been mainly associated with bonfires at camping sites. However, Rhizina root rot was observed in the western coastal forests without fire. It was hypothesized that Rhizina root rot in this area might be closely related to the soil salinity, which can facilitate the growth and survival of *R. undulata*. So, the variation in sodium chloride (NaCl) resistance among isolates of *Rhizina undulata* was compared using liquid media containing different concentrations of NaCl ranging from 0 mM to 300 mM. Our results showed that, albeit of no growth at a higher concentration of NaCl (300 mM), most of *R. undulata* isolates were capable of germinating and grew at up to 100 mM, indicating that NaCl resistance varies among *R. undulata* isolates. It was further found that isolates from coastal areas seemed to be more tolerant to NaCl than those further away the coast. We demonstrated that *R. undulata* could be possible to survive in coastal areas, but was lower NaCl tolerance than other fungi.

Key Words: *Rhizina undulata,* NaCl resistance, mycelial growth, ascospore germination

Introduction

Rhizina undulata Fr ex Fr. (Syn. *Rhizina inflata* (Schaeff) Karst), the causal agent of Rhizina root rot, is a soil-borne fungus occurring on coniferous trees (Germmen 1971). *R. undulata* has been reported to cause severe damage to costal pine forests in Korea (Lee and Kim 1990; Lee et al. 2005). The destruction of coastal forests caused by *R. undulata* infection has been associated with bonfires at camping sites and the incineration of plant material in proximity to coastal forests, implying that fires are a main source of the disease (Ginns 1968; Gibson 1970). Specifically, spore germination is dependent on high temperatures and the duration of exposure to heat; conditions ranging from 35°C for 24 hr to 45°C for 17 hr, are generally referred to as the 'heat shock' effect (Jalaluddin 1967; Germmen 1971; Lee and Kim 1990).

In June 2002, Rhizina root rot was identified in a 20 hectare Japanese black pine (*Pinus thunbergii*) forest located in

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Taean on the western coast of South Korea (Lee et al. 2005). Subsequently, *R. undulata* infected *P. thunbergii* were observed in the national park of Seocheon in 2005 (Korea Forest Service: unpublished data). However, disease incidence at both sites was not found to be associated with fire; it was hypothesized that those cases of Rhizina root rot might be closely related to other environmental factors, facilitating the growth and survival of *R. undulata*.

Salinity is one of the main features of coastal environments. Although sodium chloride (NaCl) may have significant impacts on coastal environments, little is known regarding the effect that the salinity could have on the growth and survival of microorganisms in the coastal environment. It was thus hypothesized that levels of salinity might have an effect on the growth, spore germination, and survival of *R. undulata*, and thus, its potential impact was determined *in vitro*.

Materials and Methods

Collections of *R. undulata* isolates were made from *P. thunbergii* and *P. densiflora* across the country between 2002 and 2009 (Fig. 1, Table 1). Fungal materials collected in the field were placed separately for each tree in paper bags, and then transported to the laboratory for further isolations.

Fig. 1. Schematic representation of *R. undulata* collection site.

Table 1. Mycelial dry weights of *R. undulata* isolates incubated in PDB with different NaCl concentrations

Collected locations	Distance from coast (km)	Host	Descriptions[a]	NaCl concentration of sampling location (mM)[b]	NaCl concentrations in PDB (mM)			
					0	50	100	300
Buan	0.3	*Pinus thunbergii*	Coast, Sand, Camping site	0.342	18.84±5.83[c]	7.34±2.99*	6.06±2.78*	0*
Gangneung	1.4			0.855	13.62±6.58	9.00±2.30*	7.18±2.50*	
Incheon	0.7			0.513	14.68±3.93	5.50±0.73*	4.00±1.98*	
Uljin	2.2			0.342	18.84±5.83	7.34±2.99*	6.06±2.78*	
Taean	1.1			0.684	10.10±6.56	7.42±4.01*	5.80±4.59*	
Yangyang	0.6			0.513	8.38±0.79	4.04±1.10*	4.16±1.62*	
Andong	60.0	*Pinus densiflora*	Mountain, Loam, Burned area	-	9.18±4.23	8.06±1.48*	5.48±1.52*	0*
Gimhae	20.9				11.62±5.07	5.02±3.52*	4.16±2.17*	
Gumi	95.7				14.06±2.74	8.78±2.59*	5.30±5.24*	
Gyeongju	21.6				8.02±3.83	3.56±2.15*	3.68±2.63*	
Haenam	3.8				15.08±2.33	8.52±3.84*	4.78±2.52*	
Hongcheon	76.4				14.26±2.73	9.70±1.29*	2.60±1.89*	
Namwon	60.0				14.82±4.64	8.76±2.03*	4.84±2.30*	
Okcheon	85.6				16.40±6.64	11.50±5.51*	7.68±2.10*	
Suncheon	8.5				13.24±4.48	6.28±2.67*	7.54±3.55*	
Ulsan	15.2				12.08±2.36	5.34±2.98*	1.22±1.70*	

[a]geographical condition, soil texture and descriptions of collection site; [b]were referred from the research report of National Institute of Forest Science (2012); [c]Dry weight (mg): Mean values±SD(Standard deviation) of 5 replicates.
*indicates significant differences between 0 mM and different NaCl concentrations based on one-way ANOVA tests at p=0.05 (Tukey's test).

Single ascospore isolations were made according to the method described by Jalaluddin (1967), and subsequently the isolates were cultured on PDA (Difco™ potato dextrose agar; Becton, Dickinson Co., USA) at 25°C for one week under the dark condition prior to use. In total, sixteen *R. undulata* isolates were obtained and all isolates used in this study were deposited with the Tree Pathology and Mycology laboratory (TPML), Kangwon National University, South Korea. Genomic DNA for all isolates included in this study was extracted using DNeasy Mini Plant Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. To ensure the correct identity of isolates obtained in this study, internal transcribed spacer (ITS) regions, including the 5.8S rDNA region were sequenced, following the technique described by Lee et al. (2007) (White et al. 1990). The resulting sequences were then blasted against the nucleotide database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). All sequences obtained in this study were deposited with GenBank under accession numbers, EU346951 to EU346953.

To determine the effect of different concentrations of NaCl on the growth of *R. undulata* isolates, five-millimeter of agar disks containing fungal mycelium from the pre-cultured fungal colonies were taken from the edges of actively growing isolates. Each agar disk was then transferred into a 50 mL Falcon tube containing 30 mL of PDB (Difco™ potato dextrose broth; Becton, Dickinson Co., USA) with four different NaCl concentrations (0, 50, 100, and 300 mM). The tubes were sealed with caps and incubated under the dark condition for 7 days at 25°C. Five replicates were prepared for each treatment (3 isolation by location). Dried mycelia were then harvested by filtration of the culture media using filter paper, rinsed with distilled water and then dried at 40°C for 7 days.

In addition, ascospores were harvested from sixteen ascocarps of *R. undulata*, and they were subsequently suspended in sterile water. The PDA with four different NaCl concentrations (0, 50, 100, and 300 mM) was smeared on surface with 1 mL of the ascospore suspension (approximately 100 ascospores/mL) to determine the possible impact that the salinity could have on the germination of *R. undulata* ascospore. The ascospore suspension was incubated at 40°C for 4 h before the smear in media. The media were sealed and incubated under the dark condition for 7 days at 25°C. The rate at which *R. undulata* ascospores were germinated was measured using the optical microscope (Carl Zeiss Axio Imager A1; Zeiss Co., Göttingen, Germany). Five replicates were prepared for each treatment. Each measured value was used in the following formula to calculate the germination rate: Germination rate (%)=(Total number of the germinated ascospores)/(Total number of ascospores)x100.

One-way analysis of variance (ANOVA) and Tukey's method were used to determine the significance of the differences in NaCl resistance measured for concentrations ranging from 0 mM to 300 mM. All statistical tests were performed using the SPSS program (IBM SPSS Statistics Version 19, USA).

Results and Discussion

Soil salinity varies spatiotemporally in coastal areas. NaCl concentrations at a site within 40 m of the shoreline in Kadoori, Japan exceeded 400 mM under typhoon conditions, but were lower than 30 mM post-typhoon (Ishikawa et al. 1995). *P. thunbergii* appears to be highly resistant to high levels of NaCl, since it is capable of colonizing sand dunes and rocky cliffs where seawater (with a NaCl concentration of approximately 500 mM) is sprayed directly onto the trees. The ectomycorrhizal fungus, *Suillus granulatus* is tolerant against NaCl concentrations exceeding 300 mM, whereas the mycelial growth of the dominant isolate, *Cenococcum geophilum* is inhibited at 200 mM NaCl. In addition, variations in NaCl resistance were apparent among *C. geophilum* isolates (Obase et al. 2010). In this study, results showed that the mycelial dry weight of sixteen *R. undulata* isolates decreased with increasing NaCl concentration except for three cases from Gyeongju, Suncheon and Yangyang (no growth observed at a NaCl concentration of 300 mM) (Table 1). Twelve out of the sixteen *R. undulata* isolates (Buan, Gangneung, Gimhae, Gyeongju, Haenam, Hongcheon, Incheon, Namwon, Suncheon, Uljin, Ulsan, Yangyang) demonstrated there were significant differences in their mycelial dry weight at all NaCl concentrations tested ($p<0.05$) (Table 1). However, the mycelial dry weight of four *R. undulata* isolates from Andong, Gumi, Okcheon and Taean did not exhibit sig-

nificant differences ($p>0.05$) (Table 1); this is most likely due to the fact that there are high variations in NaCl resistance for mycelial growth among *R. undulata* isolates. The fact that there are variations in NaCl resistance among fungal pathogens has been reported in numerous studies (Uraih and Chipley 1975; Hutchison 1990). In a previous study, the growth of ten soil and pathogenic fungi (*Alternaria alternata, Aspergillus flavus, Aspergillus wentii, Cunninghamella echinulata, Fusarium oxysporum, Fusarium moniliforme, Fusarium graminearum, Penicillium chrysogenum, Rhizoctonia solani*, and *Trichoderma viride*) cultured in Czapek's sucrose liquid medium supplemented with different concentrations of NaCl was compared. All tested fungi exhibited growth in up to 2.5% NaCl (approximately 427mM). *Aspergillus*, *Fusarium*, and *P. chrysogenum* were able to grow in >10% NaCl (approximately 1.7M) (Attaby 2001). Thus, we concluded that *R. undulata* possesses lower NaCl tolerance than other fungi.

The NaCl concentrations of coastal soil were 0.541 mM (the mean of 6 sampling locations) (Table 1). In land soils, the sodium ions in unburned-area were higher than burned-area in Kosung forest, and its concentration in burned- and unburned-areas were 0.013 and 0.023 me 100 g^{-1} (approximately 5.655 and 10.004 mM), respectively (Lee et al. 1997). Thus, we concluded that *R. undulata* could be survive in both coastal area and inland soils.

There were significant differences in the germination rate of ascospores at all NaCl concentrations among *R. undulata* isolates used in this study (a total of 83.3% ascospore germination rate) ($p<0.05$) (Table 2). Of isolates used in this study, ten *R. undulata* isolates (Buan, Gangneung, Gyeongju, Haenam, Incheon, Okcheon, Uljin, Suncheon, Taean, Yangyang) were germinated at up to a NaCl concentration of 100 mM (no germinations observed at 300 mM), and those isolates were all in close proximity to the coast (Table 2), suggesting that isolates collected near the coast appeared to be more resistant to the salinity compared to those further away to the coast.

It has been reported that *R. undulata* appeared to be prone to instability with regard to survival competitiveness compared with other microorganisms (Lee et al. 2005). In this regard, *R. undulata* incidence is predicted to decrease at forest fire sites since the community of soil microorganisms is restored with time (Kim 2004). Along with the fact that microorganism densities are in fact lower in sandy soils of coastal areas than in soils inland and the capability of *R. un-*

Table 2. Ascospore germinations of *R. undulata* isolates incubated in PDA with different NaCl concentrations

Collected locations	Distance from coast (km)	Host	Descriptions[a]	NaCl concentrations in PDA (mM)			
				0	50	100	300
Buan	0.3	*Pinus thunbergii*	Coast, Sand, Camping site	84.7±0.33[b]	28.4±0.38	2.1±0.14	0
Gangneung	1.4			83.2±0.29	31.1±0.18	6.2±0.14	
Incheon	0.7			85.2±0.17	49.1±0.20	4.7±0.21	
Uljin	2.2			84.3±0.12	48.3±0.31	3.1±0.11	
Taean	1.1			87.1±0.36	40.6±0.42	3.1±0.17	
Yangyang	0.6			81.3±0.13	31.5±0.29	1.5±0.23	
Andong	60.0	*Pinus densiflora*	Mountain, Loam, Burned area	78.7±0.21	29.1±0.27	-	0
Gimhae	20.9			82.1±0.15	31.5±0.17		
Gumi	95.7			83.2±0.35	29.8±0.35		
Gyeongju	21.6			79.4±0.24	41.0±0.26	2.3±0.18	
Haenam	3.8			85.9±0.22	39.4±0.23	1.9±0.14	
Hongcheon	76.4			83.1±0.27	30.1±0.37	-	
Namwon	60.0			75.3±0.36	20.1±0.48		
Okcheon	85.6			79.8±0.32	39.7±0.27	1.0±0.12	
Suncheon	8.5			84.3±0.21	40.1±0.36	1.1±0.16	
Ulsan	15.2			86.6±0.26	33.9±0.29	-	

[a]geographical condition, soil texture and descriptions of collection site; [b]Germination rate (%): Mean values±SD(Standard deviation) of 5 replicates.

dulata to germinate and grow in coastal areas could provide favored environmental conditions for the growth of *R. undulata*.

In conclusion, we demonstrated that *R. undulata* could be possible to survive in coastal areas, but showed the lower NaCl tolerance than other fungi. However, the effect of salinity on disease development is unknown. Thus, further studies should address the effect of additional environmental factors on Rhizina root rot in coastal pine forests.

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