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Research Article

Effects of Water Stress on the Endophytic Fungal Communities of *Pinus koraiensis* Needles Infected by *Cenangium ferruginosum*

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Abstract To examine the effects of water stress and *Cenangium ferruginosum* (*CF*) on the fungal endophytic community of needles of *Pinus koraiensis* (*PK*), fungal endophytes isolated from the needles of 5-year-old *PK* seedlings were compared before and after exposure to water stress conditions and artificial inoculation with *CF* ascospores. Artificial *CF* inoculation was successfully confirmed using PCR with *CF*-specific primers (CfF and CfR). For comparison of the degree of water deficit in water-stressed and control groups of *PK* seedlings infected with *CF*, the water saturation deficit and water potential were measured. Lower water potential estimates were found in the water-stressed seedlings than in the control group. The fungal endophytes isolated from the second-year needles of non-water-stressed seedlings before and after *CF* inoculation revealed that primary saprobes were approximately 30% and 71.7%, respectively, and the remaining endophytes were rot fungi or pathogens. Sixty days after *CF* inoculation, diverse fungal endophytes in the first-year needles were isolated from the water-stressed seedlings. However, some fungal endophytes isolated from the non-water-stressed seedlings were also identified. Fungal endophytes in the second-year needles of the water-stressed and non-water-stressed seedlings were approximately 8% and 71.7% of saprobes, respectively, and the remaining endophytes were rot fungi or pathogens. On the basis of the results, we conclude that water deficit and *CF* can have an effect on fungal endophytic communities in the needles of *PK* seedlings.

Keywords *Cenangium ferruginosum*, Fungal endophytic community, *Pinus koriensis*, Water stress

Cenangium dieback is a disease caused by *Cenangium ferruginosum* (*CF*) that appears when a tree is weakened by environmental stress, and it results in damage to twigs, branches, and whole trees [1]. *CF* is known to be an endophyte fungus and a primary decomposer of weakened and dying trees [2-4]. The fact that *CF* can pose a serious threat under various environmental conditions is of particular concern [5-7]. In 1989, 2007, and 2009, *Cenangium* dieback caused severe damage to major pine species (*Pinus densiflora*, *P. thunbergii*, and *P. koraiensis*) in South Korea [8]. Endophytic *CF* has been isolated from the needles of healthy *P. sylvestris*, and it has also been isolated from the shoots of *P. nigra* with no symptoms of *Cenangium* dieback [9, 10]. *CF* was not detectable from the needles of *P. koraiensis* (*PK*) [11-13]. The weak pathogenicity of *CF* suggests that it is a dehydration-induced saprobe rather than a parasite dieback [8, 9, 14]. In contrast to evidence of weak pathogenicity of *CF* observed in pine trees in other countries, *CF* on *PK* in South Korea showed strong pathogenicity. This result prompted multi-faceted studies of the morphological and cultural characteristics of *CF* [8]. In addition, the colony color, mycelial growth, and genetic characteristics varied among strains isolated from *P. densiflora*, *P. thunbergii*, and *PK* [14].

Fungal endophytes are mostly non-pathogenic and are known to have symbiotic relationships with their hosts, in which secondary metabolites are used for the biological control of other microorganisms [15, 16]. Various pathogens are also isolated when separating fungal endophytes from conifers, and many studies have been reported for using these pathogens as biological control agents [9, 16-18]. However, the correlation between most fungal endophytes and pathogens has yet to be determined.

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Among the various types of damage caused by water stress to trees, needle necrosis is a common symptom that occurs in conifer species, and it is also referred to as conifer dieback. While there have been many studies on the effects of water-stress on fungal endophyte communities isolated from the bark and sapwood of *Quercus robur* [19], few studies have reported on fungal endophyte communities in coniferous needles.

In this study of the disease development of *Cenangium* dieback, fungal endophytes were isolated from the needles of 5-year-old *PK* seedlings (after artificial *CF* inoculation) before and after exposure to water-stress conditions. Additionally, the fungal endophytes isolated from *PK* needles were analyzed before and after artificial *CF* inoculation to determine the effect of water-stress and/or *CF*.

MATERIALS AND METHODS

Identification and inoculation of *CF* and sample collection. To obtain a single spore from the apothecium, an optical microscope (Carl Zeiss Axio Imager A1; Zeiss Co., Göttingen, Germany) and a dissecting microscope (SZX-ILLK200; Olympus Co., Tokyo, Japan) were used to observe the morphological characteristics of *CF* [8]. The isolate was cultured on MSA (3% malt extract; 0.3% soytone; 1.5% agar; Difco Co., Detroit, MI, USA) culture medium at 20°C for approximately 4 wk under dark conditions prior to use in the study [14].

Ten 5-yr-old *PK* seedlings were obtained from the Forest Practice Research Center (Korea Forest Research Institute, South Korea) on April 3, 2014. The seedlings were transplanted in pots, and then rooted for 2 mon under greenhouse conditions. For comparisons of fungal endophyte communities from needles of *PK* seedlings before and after artificial *CF* inoculation, the fungal endophytes were isolated from the second-year and third-year needles of five healthy control seedlings, which did not have the first-year needles at that time.

Artificial *CF* inoculation used ascospores collected in Hongcheon, Korea. Ascospores were only harvested from the *CF* fruiting body, and they were subsequently suspended in sterile water. Each of the 10 healthy seedlings was then sprayed on needles with 10 mL of the ascospore suspension (approximately 10^4 ascospores/mL; 87.0% ascospore germination rate). *PK* seedlings inoculated with *CF* ascospores were maintained at a humidity level ≥ 90.0% for 3 days to induce spore germination and infection. During the second week of experimentation, 5 of the trees inoculated with *CF* were irrigated twice a week for normal water supply, and the other 5 trees were not given any water. After 6 wk, the fungal endophytes from the 10 inoculated trees were isolated from the first-year and second-year needles taken from the second branch from the base of the tree crown.

Needle samples for the measurements of water saturation deficit (WSD) and water potential (WP) as well as for the isolation of fungal endophytes were used immediately upon collection. The specimens used to detect *CF* were treated in liquid nitrogen for 5 min immediately after harvesting, and were stored in an ultralow temperature freezer for later use.

WSD and WP of *PK* needles. The fresh weight (FW) was measured using 20 needles. The turgid weight (TW) was measured by taking the raw weight of sterile water at 20°C and fully submerging 20 needles. The submerged needles were then allowed to absorb moisture under light conditions for 4 hr, and the moisture remaining on the surface of the leaves was then removed. The dry weight (DW) was measured after 48 hr of drying the needles in a dry oven at 90°C. Each measured value was used in the following formula to calculate the WSD: WSD (%) = (TW – FW)/(TW – DW) × 100 [20, 21]. WP (Ψw) was measured using a Dewpoint PotentiaMeter (WP4; Decagon Devices, Pullman, WA, USA). Each bundle of needles was measured 5 times.

Isolation and identification of fungal endophytes from *PK* needles. The surfaces of the needles collected for isolating fungal endophytes were sterilized (70% ethanol for 1 min, 1% NaOCl for 1 min, and rinsed in sterile water), 5 mm of each was cut at three places (the apical, middle, and basal parts of the needle), and subsequently placed on potato dextrose agar (Difco Co.) and malt agar (Difco Co.) culture media. The cut needles were then cultured for five cycles in 15°C and 25°C incubators for 15~30 days to isolate the fungal endophytes.

The isolated fungal endophytes were identified by nucleotide sequences of 18S rDNA internal transcribed spacer (ITS) regions using the BLAST tool (http://blast.ncbi.nlm.nih.gov/), thereby confirming the samples with an identity rate of ≥ 99%.

Twelve species (*Lophodermium nitens*, *Lophodermium macci*, *Lophodermium pini-excelsae*, *Biscogniauxia maritima*, *Annulohypoxylon annulatum*, *Colletotrichum gloeosporioides*, *Cladosporium sphaerospermum*, *Phomopsis eucommicola*, *Alternaria alternata*, *Botryosphaeria dothidea*, *Diaporthe eres*, and *Sydowia polyspora*) within the identified fungal endophytes were used as controls for *CF* detection tests.

Genomic DNA extraction. Genomic DNA was extracted from 1 mg of mycelium using a ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions, and freeze-dried in a chamber at −20°C for further study. Genomic DNA was extracted from the needles of *PK* seedlings using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions, with slight modifications. To extract genomic DNA from needles of *PK* seedlings, 20 needles were surface-cleaned and homogenized using liquid nitrogen. One milligram of the homogenized needle tissues was then immediately transferred

to a 2 mL PowerSoil bead tube (MoBio Laboratories) for processing.

PCR conditions and sequencing. PCR amplification was carried out using ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primer sets for identification of fungal endophytes [22]. CfF (5′-GAT CAT TAC CAG AAG TGT CC-3′) and CfR (5′-CCT AGG TGA GTT GGG GTT GC-3′) primer sets specific to *CF* were designed based on the 18S rDNA ITS region using the Lasergene PrimerSelect program (DNASTAR Co., Madison, WI, USA). The expected amplicon size using CfF and CfR primer sets was 477 bp.

PCR was performed in a total reaction volume of 50 µL containing 2 µL of genomic DNA, 1 µL (20 pM) of each primer, and 25 µL of 2 × PCR Master Mix (Promega Co., Madison, WI, USA). The cycling profile for PCR consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. PCR amplification using CfF and CfR primers was performed with an annealing temperature of 70°C for 30 sec. The amplification reaction was conducted on a Dyad Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The amplification products were analyzed by electrophoresis (Mupid-21; Cosmo Bio Ltd., Tokyo, Japan) on a 1.5% (w/v) agarose gel in 1× TAE buffer (Promega Co.) after staining with Loading Star (DyneBio Inc., Seoul, Korea), and then visualized using a UV transilluminator (GBOX; Syngene Co., Cambridge, UK).

Amplified products were sequenced using an ABI PRISM 3730XL Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis, diversity, and dominance indices. A one-way ANOVA (Tukey's test) statistical analysis was performed using the SPSS ver. 19 (IBM SPSS Inc., Armonk, NY, USA).

The Shannon index was used to calculate the diversity of the species distribution: $H = -\Sigma\ (n_i/N)\ (\log n_i/N)$ [23]. The Simpson's index was used to calculate the dominance of the species distribution: $D = \Sigma\ (n_i\ (n_i - 1)/N\ (N - 1))$ [24].

RESULTS AND DISCUSSION

Specificity of the *CF*-specific marker. In order to verify the success of artificial *CF* inoculation, *CF*-specific markers (CfF and CfR primers) were generated using nucleotide sequences from the 18S rDNA ITS region. These primers only produced a *CF*-specific amplicon, and the amplicon was not produced from the 12 strains of *PK* fungal endophytes that were used as controls. Moreover, the amplicon was not produced from *PK* needles that were not inoculated with *CF*; however, it was amplified in the non-infected *PK* needles that were mixed with *CF* mycelium (Fig. 1). The nucleotide sequence of the amplicon was a 100% match with that of the rDNA ITS sequence of *CF* (data not shown). Therefore, the CfF and CfR primers can be effectively used to amplify the *CF* marker and to detect *CF* from *PK* needles. In studies of other tree pathogens, markers specific to the respective pathogens were developed as diagnostic tools [25].

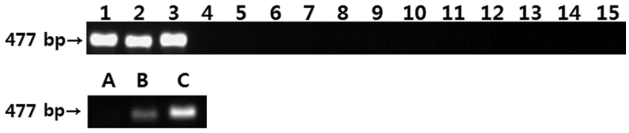

Fig. 1. The PCR products amplified from genomic DNA of fungal species mycelium (top) and needles (bottom) using *Cenangium ferruginosum* (*CF*)-specific primers CfF and CfR. *CF*-specific primers were designed using the 18S rDNA ITS region. Lane: 1~3, *CF*; 4, *Lophodermium nitens*; 5, *Lophodermium macci*; 6, *Lophodermium pini-excelsae*; 7, *Biscogniauxia maritima*; 8, *Annulohypoxylon annulatum*; 9, *Colletotrichum gloeosporioides*; 10, *Cladosporium sphaerospermum*; 11, *Phomopsis eucommicola*; 12, *Alternaria alternata*; 13, *Botryosphaeria dothidea*; 14, *Diaporthe eres*; 15, *Sydowia polyspora*; A, needle of *Pinus koraiensis* seedling non-infected with *CF*; B, lane A + C; C, *CF* mycelium.

Detection of *CF* from *PK* needles. To verify the success of artificial *CF* inoculation, PCR analyses were performed before and after inoculation. As a result, *CF*-specific amplicons were detected in all of the inoculated seedlings, whereas none were detected in the initial seedlings (Table 1).

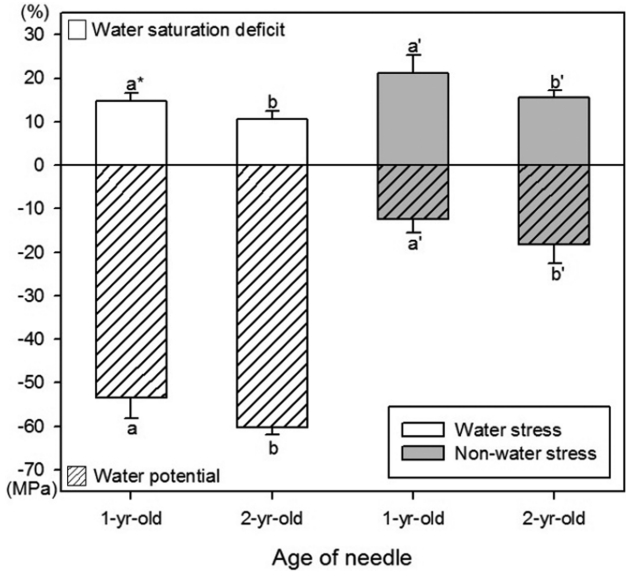

Fig. 2. Water saturation deficit and water potential of needles of *Pinus koraiensis* seedlings by water stress condition. *Significant differences using one-way ANOVA (Tukey's test, $p < 0.05$).

Therefore, artificial *CF* inoculation was successful. The size of the amplified PCR product was 477 bp, and the nucleotide sequence was a 100% match with that of *CF*. In fact, species-specific markers have been produced by various methods and utilized to detect tree pathogens [25, 26].

Comparison of WSD and WP of *PK* needles associated with water stress. When water stress treatment was administered after artificial *CF* inoculation, the seedlings having a normal supply of water (control group) appeared to be healthy based on visual observation, but the seedlings exposed to water stress (treatment group) showed overall necrotic symptoms of second-year needles (data not shown).

To compare the degree of water stress on the treatment and control groups, the WSD (%) and the WP (MPa) inside the needles were measured. As a result, in the first-year needles, the average WSD of the control and treatment groups were 21.1 ± 9.5% and 14.8 ± 4.1%, respectively. In the second-year needles, the WSD of the control and treatment groups were 15.6 ± 1.6% and 10.4 ± 2.0%, respectively ($p < 0.05$). The WP in the first-year needles was determined to be −12.4 ± 7.0 MPa in the control group and −53.5 ± 10.5 MPa in the treatment group, while it was −18.3 ± 9.5 and −54.3 ± 14.5 MPa, respectively, for the second-year needles ($p < 0.05$). The WP in the treatment group was lower in comparison to that of the control group, and for both control and treatment groups, the moisture content in the second-year leaves was lower than that of the first-year leaves (Fig. 2). Therefore, the treatment group experienced more water stress as compared to the control group.

Comparison of fungal endophyte community isolated from *PK* needles. When the fungal endophytes were isolated from the apical, middle, and basal parts of needles, the highest number was obtained when isolated from the basal parts of all seedlings, followed by the middle and apical parts (data not shown). These results have also been reported in other studies [9, 27].

In the needles of the initial *PK* seedlings before the *CF* inoculation, a total of 129 fungal endophytes were isolated, belonging to 1 kingdom, 1 phylum, 3 classes, 8 orders, 8 families, 13 genera, and 13 species, with Rhytismatales (32.6%) and Xylariales (52.2%) being the most dominant. Thirty percent were confirmed to be primary saprobes, with the remaining 70.0% being rot fungi or pathogens. When the fungal endophytes in the second-year and third-year needles were isolated and compared, the latter outnumbered the former and the fungal endophytes isolated in large numbers in the third-year needles were also represented in large numbers in the second-year needles (Table 2). The diversity index of fungal endophytes was higher in third-year needles than in second-year needles (Table 3). Thus, more fungal endophytes were isolated from the third-year needles than from the second-year needles, which implies that fungal endophyte strains increase over time. Similar results have also been reported in other studies [17, 28]. Despite the fact that the seedlings were healthy prior to *CF* inoculation, the presence of saprobes, rot fungi, and pathogens in the needles suggests that they can thrive in healthy trees as fungal endophytes, which is consistent with the results reported in other studies [9, 12, 13]. Furthermore, the abundance of rot fungi and pathogens in the needles of initial seedlings may be attributable to

Table 1. Detection of *Cenangium ferruginosum* (*CF*) from living needles of *Pinus koraiensis* seedlings

Seedlings			Age of needle (yr)	Detection	Size (bp)
Initial seedlings			2	0/5 (0)[a]	-
			3	0/5 (0)	-
CF-inoculated	After 1 wk		1	5/5 (100)	477
			2	5/5 (100)	477
	After 6 wk	Non-water stress	1	5/5 (100)	477
			2	5/5 (100)	477
		Water stress	1	5/5 (100)	477
			2	5/5 (100)	477

[a]The number of detection/5 replications (detection rate, %).

Table 2. Number and frequency (%) of fungal species isolated from living needles of *Pinus koraiensis* seedling

Fungal species	No. of isolates (%)						NCBI accession No.	Identity (%)
	Initial seedling		*CF*-inoculated seedling					
	2-yr-old[a]	3-yr-old[a]	1-yr-old[a]	2-yr-old[a]	1-yr-old[b]	2-yr-old[b]		
Lophodermium pinastri	-	1 (1.4)	-	1 (1.4)	-	1 (0.5)	FJ861986.1	99
Lophodermium macci	-	2 (2.8)	-	3 (4.1)	5 (8.3)	4 (2.1)	AF540560.1	100
Lophodermium australe	-	1 (1.7)	-	-	-	-	FJ861969.1	99
Lophodermium pini-excelsae	-	-	-	2 (2.7)	3 (5.0)	4 (2.1)	FJ861987.1	99
Lophodermium conigenum	-	-	-	2 (2.7)	1 (1.7)	-	FJ861975.1	99

Table 2. Continued

Fungal species	No. of isolates (%)						NCBI accession No.	Identity (%)
	Initial seedling		*CF*-inoculated seedling					
	2-yr-old[a]	3-yr-old[a]	1-yr-old[a]	2-yr-old[a]	1-yr-old[b]	2-yr-old[b]		
Lophodermium nitens	7 (12.1)	7 (9.9)	-	7 (9.6)	-	1 (0.5)	JF332166.1	99
Lophodermium sp.	-	-	-	1 (1.4)	-	1 (0.5)	JQ760369.1	100
Leptostroma sp.	-	1 (1.4)	-	-	-	-	KC354586.1	99
Rhytismataceae sp.	9 (15.5)	14 (19.7)	-	1 (1.4)	1 (1.7)	1 (0.5)	KC283119.1	99
Leotiomycetes sp.	-	-	-	-	2 (3.3)	2 (1.0)	JQ761252.1	99
Phomopsis eucommicola	-	-	-	-	-	3 (1.6)	AY578071.1	100
Phomopsis sp.	-	-	-	1 (1.4)	16 (26.7)	62 (32.5)	HM751825.1	99
Diaporthe phaseolorum	-	-	-	-	2 (3.3)	6 (3.1)	AF001018.2	99
Diaporthe eres	1 (1.7)	-	-	-	-	3 (1.6)	FJ478132.1	99
Diaporthe cf. *nobilis*	-	-	-	-	2 (3.3)	4 (2.1)	KC343148.1	99
Diaporthe sp.	-	-	-	-	11 (18.3)	7 (3.7)	KC145882.1	99
Diaporthales sp.	-	-	-	-	4 (6.7)	1 (0.5)	GQ996124.1	99
Annulohypoxylon annulatum	1 (1.7)	-	1 (12.5)	1 (1.4)	-	-	FJ481153.1	99
Annulohypoxylon truncatum	1 (1.7)	-	-	-	-	-	JQ303335.1	98
Hypoxylon crocopeplum	-	1 (1.4)	-	-	-	-	JN979411.1	98
Hypoxylon sp.	-	1 (1.4)	-	-	-	-	JN660823.1	92
Hansfordia sp.	1 (1.7)	-	-	-	-	-	GQ906969.1	99
Biscogniauxia maritima	3 (5.2)	1 (1.4)	1 (12.5)	2 (2.7)	4 (6.7)	-	JQ247198.1	99
Nodulisporium sp.	-	-	-	1 (1.4)	-	-	GQ906948.1	99
Pestalotiopsis sp.	-	-	-	1 (1.4)	-	-	JF439507.1	99
Arthrinium phaeospermum	-	-	-	1 (1.4)	-	-	AB220268.1	100
Xylaria primorskensis	-	2 (2.8)	-	-	-	-	FJ707473.1	99
Xylaria sp.	-	1 (1.4)	-	-	-	-	AB731127.1	99
Xylariales sp.	-	-	-	1 (1.4)	2 (3.3)	1 (0.5)	AB495010.1	99
Xylariaceae sp.	25 (43.1)	29 (40.8)	-	-	-	-	AB741589.1	96
Colletotrichum gloeosporioides	-	1 (1.4)	-	1 (1.4)	-	-	JF682848.1	100
Nigrospora oryzae	-	-	-	1 (1.4)	-	1 (0.5)	KC341981.1	99
Cladosporium sphaerospermum	-	-	1 (12.5)	6 (8.2)	2 (3.3)	1 (0.5)	EU823317.1	100
Cladosporium cladosporioides	-	-	-	24 (32.9)	2 (3.3)	2 (1.0)	JX981454.1	100
Cladosporium sp.	-	-	1 (12.5)	1 (1.4)	-	-	EU167592.1	99
Toxicocladosporium irritans	-	-	-	-	-	1 (0.5)	JN974765.1	99
Alternaria alternata	-	-	-	-	-	1 (0.5)	JF973295.1	100
Alternaria sp.	-	-	-	-	-	4 (2.1)	KF438091.1	100
Cenangium ferruginosum	-	-	2 (25)	5 (6.8)	1 (1.7)	1 (0.5)	-	-
Botryosphaeria dothidea	-	-	-	-	-	77 (40.3)	HQ660453.1	100
Peniophora sp.	-	-	-	-	1 (1.7)	-	HM535362.1	100
Sydowia polyspora	1 (1.7)	1 (1.4)	-	2 (2.7)	-	-	GQ412722.1	100
Penicillium fellutanum	-	-	-	1 (1.4)	-	-	JX091420.1	100
Penicillium sumatrense	-	-	-	2 (2.7)	-	-	HE962603.1	100
Penicillium sp.	-	-	-	3 (4.1)	-	-	HE962588.1	99
Meyerozyma guilliermondii	-	-	1 (12.5)	-	-	-	KC237294.1	100
Podospora sp.	1 (1.7)	-	-	-	-	-	FJ197969.1	97
Sordariomycetes sp.	1 (1.7)	-	-	-	-	-	GU222395.1	95
Phyllosticta papayae	-	1 (1.4)	-	-	-	-	JF414731.1	99
Preussia sp.	1 (1.7)	-	-	-	-	-	JN225886.1	99
Dothideomycetes sp.	-	1 (1.4)	-	-	-	-	JQ758763.1	99
Unidentified 1	2 (3.4)	1 (1.4)	-	-	-	-	HF674740.1	89
Unidentified 2	-	1 (1.4)	-	-	-	-	HQ823749.1	96
Unidentified 3	3 (5.2)	4 (5.6)	-	-	-	-	EF373587.1	99
Unidentified 4	-	1 (1.4)	-	-	-	-	GQ334426.1	81
Unidentified 5	-	-	1 (12.5)	2 (2.7)	1 (1.7)	2 (1.0)	-	-
No. of isolates obtained (%)	58 (100)	71 (100)	8 (100)	73 (100)	60 (100)	191 (100)	-	-

CF, *Cenangium ferruginosum*.

[a]Non-water stress.

[b]Water stress.

massive infiltrations of the organisms in trees weakened by transplantation. However, *CF* was not found in the needles of initial seedlings, and the examination of the fungal endophytes isolated from the needles of *PK* in other studies showed the same results [11-13].

In the needles of the post-inoculation seedlings in the control group, a total of 81 fungal endophytes were isolated, belonging to 1 kingdom, 1 phylum, 5 classes, 10 orders, 11 families, 14 genera, and 17 species, with Rhytismatales (21.0%) and Capnodiales (40.7%) being the most dominant. Seventy-two percent were confirmed to be primary saprobes, with the remaining 28.0% being rot fungi or pathogens. When the fungal endophytes from the first-year and second-year needles were isolated and compared, the latter outnumbered the former, with only 8 strains isolated from the former (Table 2). The diversity and dominance indices of fungal endophytes were higher in second-year needles than in first-year needles (Table 3). It has been reported that the diversity of fungal endophytes is heavily influenced by climate, and that older needles exhibit greater diversity in isolated fungal endophytes [17, 28-30]. Therefore, it is assumed that the small number of fungal endophytes isolated from the first-year needles may be due to the narrow range of climate variability under greenhouse conditions, which resulted in the infiltration of fewer fungi than when left in field. The dominant species from second-year needles was *C. cladosporioides* (32.9%), but it was not isolated from first-year needles. Instead, isolated *CF* strains accounted for 8.0%, which proves that artificial *CF* inoculation was successful (Table 2). The percentage of isolated *CF* was very low, presumably owing to the characteristics of *CF* to move to the branch after inoculating the needle, resulting in a decrease in the number of surviving mycelia in the needle [1, 31].

In the needles of *PK* seedlings water-stressed after *CF* inoculation, a total of 251 fungal endophytes were isolated, belonging to 1 kingdom, 2 phylum, 4 classes, 9 orders, 9 families, 11 genera, and 17 species, with Diaporthales (48.2%) and Botryosphaeriales (30.7%) being the most dominant. Eight percent of the fungal endophytes were confirmed to be primary saprobes, with the remaining 92.0% being rot fungi or pathogens. The dominant species from first-year needles were *Phomopsis* sp. (26.7%) and *Diaporthe* sp. (18.3%), and *Phomopsis* sp. (32.5%), and *B. dothidea* (40.3%) from second-year needles. Of the isolated strains, *CF* accounted for 0.8%. When the fungal endophytes from the first-year and second-year needles were isolated and compared, more fungal endophytes were isolated from the second-year needles, but distinct differences in the dominant fungal endophytes in the first- and second-year needles were detected. However, the dominant fungal endophytes isolated from each needle were mostly rot fungi and pathogens (Table 2). There have been reports of only a few fungi being dominant among the fungal endophytes [30, 32]. Regarding the first-year needles, the diversity index of fungal endophytes was higher than the second-year needles, but the dominance index was lower (Table 2).

The fungal endophytes isolated from the second-year needles of non-water-stressed seedlings before and after *CF* inoculation revealed that primary saprobes were 30.0% and 71.7%, respectively, and the rest were rot fungi or pathogens. In pine needles, it was confirmed that saprobes, rot fungi, and pathogens were already widely distributed before the onset of needle necrosis, and that the primary saprobes increased over time. The possibility of an increase in primary saprobe density due to *CF* inoculation cannot be eliminated, but to identify the exact cause, additional research will be required to determine the effect of *CF* on these fungal endophytes. Xylariaceae spp. were isolated in large numbers before, but not after *CF* inoculation. In contrast, *C. cladosporioides* were not isolated before *CF* inoculation, but were isolated in large numbers after *CF* inoculation (Table 2). *C. cladosporioides* is a very common, cosmopolitan, and saprobic species. It often occurs as a secondary invader in necrotic parts of many different host plants, and is a common endophytic or quiescent fungus [33-36]. The diversity index of fungal endophytes of the second-year needles after *CF* inoculation was higher than the second-year needles before *CF* inoculation, but the dominance index was lower (Table 3). In the needles of initial seedlings before *CF* inoculation, rot fungi and pathogens were high, which is assumed to be the result of weakened seedlings from transplant stress. The resulting weakness is improved over time as the tree recovers from the stress, and leads to an increase in primary saprobe density.

When the fungal endophytes were isolated from the first-year *PK* needles inoculated with *CF* in the water stress treatment and control groups, diverse fungal endophytes were isolated from the treatment group, but virtually no fungal endophytes were isolated from the control group.

Table 3. Comparison in diversity and dominance indices of fungal endophytes isolated from *Pinus koraiensis* needles

Seedlings		Age of needle (yr)	Diversity index	Dominance index
Initial seedlings		2	0.821	0.220
		3	0.920	0.212
CF-inoculated	Non-water stress	1	0.828	0.036
		2	1.128	0.127
	Water stress	1	1.044	0.116
		2	0.808	0.269

CF, *Cenangium ferruginosum*.

This can result from the needles being weakened by water stress in the treatment group, making them vulnerable to massive infiltrations of rot fungi and pathogens. The second-year needles in the treatment group only represented approximately 8.0% of primary saprobes, whereas primary saprobes accounted for over 71.7% in the control group (Table 2). The diversity index of fungal endophytes of the second-year needles in the water stress treatment groups was lower than the second-year needles in the control groups, but the dominance index was higher (Table 3). In the water stress treatment groups, the number of *Phomopsis* sp. and *Diaporthe* sp. increased, but the number of *C. cladosporioides* decreased. *B. dothidea* was isolated in large numbers in the water stress treatment groups, but was not in the control groups. The asexual states of *Diaporthe* and *Phomopsis* species have broad host ranges and are widely distributed, and occur as plant pathogens, endophytes or saprobes [37]. *B. dothidea* is a latent pathogen capable of endophytic infections, and is a primary opportunist that preferentially colonizes wounded and stressed tissue [38]. *Pinus* needle decomposition starts before the falling of needles, and the decomposition of brown and green-brown needles is related to primary saprobes such as *Lophodermium*, *Cenangium*, *Aureobasidium*, and *Cladosporium* [2-4]. Thus, we conclude that many fungal species invade during the period of weakness experienced by the first-year needles, and that the second-year needles die because of dominant rot and pathogenic fungi. When fungal endophytes are isolated from conifers, various rot fungi and pathogens are also isolated, as confirmed by various reports [9, 11-13, 17]. The second-year needles in the treatment group manifested the necrotic symptoms first, which can be aggravated by the propagation of the endophytic rot fungi and pathogens, or those that infiltrated the weakened needles. Thus, it can be assumed that the second-year needles succumbed to dieback first under water stress because of the endophytic or infiltrated rot fungi and pathogens.

Furthermore, if the correlation between the isolated fungal endophytes and *CF* can be further studied by verifying both the levels of competitiveness for secondary metabolites and resistance to dehydration impairment, then the effects of *CF* on the dieback of water-stressed seedling needles and the fungal endophyte community may be determined.

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