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RESEARCH ARTICLE

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Screening of Endophytic Fungal Isolates Against *Raffaelea quercus-mongolicae* Causing Oak Wilt Disease in Korea

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ABSTRACT

Oak wilt disease caused by *Raffaelea quercus-mongolicae* has emerged obviously in Korea. We selected antifungal isolates against *R. quercus-mongolicae* among 368 endophytic fungal isolates from different parts of oak and pine trees. The experiment was conducted in the primary and secondary screenings by dual culture test. The antifungal activity of the selected isolates was assessed in culture filtrate test based on the inhibition rates in mycelial growth, sporulation, and spore germination of oak wilt fungus. Five isolates, E089, E199, E282, E409 and E415, showed strong antifungal activity in culture filtrate test, and their antifungal activity decreased on the culture media supplemented with heated culture filtrate. Higher mycelial growth inhibitions on the unheated media were recorded in E409 (*Colletotrichum acutatum*), E089 (*Daldinia childiae*), E415 (*Alternaria alternata*) and E199 (*Daldinia childiae*) with the inhibition rates of 79.0%, 70.1%, 68.9% and 64.5%, respectively. These isolates also had the higher sporulation inhibitions on unheated media with the rates of 96.8%, 84.2%, 82.8% and 80.5%, respectively. The spore germination of the oak wilt fungus was completely inhibited by E282 (*Nectria balsamea*) on both unheated and heated media. These results showed that a higher number of potent antifungal isolates against oak wilt fungus was isolated from the petiole compared to the other parts. This study could contribute to the development of biological control approaches for the management of oak wilt disease caused by *R. quercus-mongolicae*.

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1. Introduction

Oak trees include more than 500 species of *Quercus*, which are found worldwide, and a larger number in Asia, Americas, and few in Europe [1]. Oaks play a role in producing a wide diversity of timber and non-timber products as well as ecosystem services [2]. However, oak wilt is one of several significant diseases of oak that have particularly threaten oak health worldwide [3]. The fungus *Ceratocystis fagacearum* is a major pathogen of oak wilt in the eastern United States [3], while a symbiotic fungus of ambrosia beetles is observed as a serious oak wilt pathogen in oak forests of Korea and Japan [4]. It was indicated that dead oak trees by the infestation of ambrosia beetle *Platypodidae* are associated with *Raffaelea*, an-ambrosia fungi, in both Korea and Japan [4,5]. Japanese oak wilt had been observed in Japan since 1990 and it resulted from the pathogenic fungus *Raffaelea quercivora* transported by the ambrosia beetle *Platypus quercivorus* (Murayama) [5]. In Korea, however, *R. quercus-mongolicae* causing oak wilt, which was first broken out at Gyeonggi province in 2004, is closely associated with an insect vector, *P. koryoensis* [4]. This disease then has emerged obviously across several areas of Korea [4]. The pathogenicity of *R. quercus-mongolicae* was tested *in vivo* on *Quercus acutissima* [6]. Other studies on genetic diversity and genome size of *R. quercus-mongolicae* also reported in Korea [7,8]. In order to manage this disease, several control methods such as mass trapping device to capture insect vector, silver nanoparticles, and *Streptomyces blastmyceticus* inhibiting mycelial growth of fungal pathogen were applied [9–11].

Biological control of plant pathogens has been studied in many countries including USA, Japan, and so on for a long time as essential environment-friendly methods to replace harmful control methods. Endophytic microorganism is an active biocontrol agent [12], and endophytic fungi plays an important role in controlling diseases not only in crops but also in forest trees [13]. Some entomopathogenic and endophytic fungi such as *Acremonium, Beauveria, Clonostachys, Paecilomyces, Colletotrichum,* and *Alternaria* were used to manage insects and diseases of crops [14–16]. For forest trees, several antimicrobial secondary metabolites were also synthesized by endophytic fungi such as *Collophora aceris*, *Lophodermium*

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Table 1. Antifungal isolates of endophytic fungi against *R. quercus-mongolicae* in the primary screening at 5 days after inoculation in the dark at 25 °C.

No.	Host tree species	Tissue	Isolate no.	Endophytic fungi	Inhibition zone (mm)[a]
1	*Quercus dentata*	Petiole	E066	*Pestalotiopsis lushanensis*	1.0
2		Petiole	E205	*Fusarium avenaceum*	1.0
3		Petiole	E206	*Daldinia childiae*	1.0
4		Leaf	E350	*Amyloporia sinuosa*	13.0
5		Branch	E352	*Monochaetia kansensis*	3.0
6		Stem	E356	*Antrodia sinuosa*	2.0
7		Petiole	E409	*Colletotrichum acutatum*	2.0
8		Petiole	E410	*Colletotrichum acutatum*	4.0
9	*Pinus densiflora*	Branch	E253	*Daldinia childiae*	1.0
10		Branch	E280	*Pestalotiopsis lushanensis*	2.0
11		Branch	E282	*Nectria balsamea*	1.0
12		Branch	E286	*Cladosporium cladosporioides*	6.0
13		Stem	E318	*Valsa mali*	4.0
14	*Quercus mongolica*	Petiole	E199	*Daldinia childiae*	2.0
15		Leaf	E394	*Colletotrichum acutatum*	1.0
16		Stem	E396	*Valsa mali*	1.0
17	*Quercus serrata*	Petiole	E161	*Daldinia childiae*	2.0
18		Petiole	E367	*Nectria balsamea*	1.0
19		Leaf	E415	*Alternaria alternata*	3.0
20	*Quercus acutissima*	Petiole	E089	*Daldinia childiae*	8.0
21		Petiole	E345	*Annulohypoxylon truncatum*	2.0
22	*Quercus variabilis*	Petiole	E118	*Paraconiothyrium brasiliense*	3.0
23		Petiole	E381	*Valsa mali*	3.0

[a]Distance between colonies of *R. quercus-mongolicae* and endophytic fungi.

nitens, *Cytospora* sp., *Microdiplodia* sp., and so on [17]. *Collophora aceris* isolated from stem tissues of Douglas Maple (*Acer glabrum* var. *douglassi*) was active against such pathogenic fungi as *Pythium ultimum* and *Phytophthora palmivora* [18], while *L. nitens*, a foliar endophyte of *Pinus strobus*, was antifungal to the biotrophic pathogen *Microbotryum violaceum* [19]. Another study also indicated that the new compound 13-hydroxylmacrophorin isolated from endophyte *Microdiplodia* sp. TT-12 was weakly active against *R. quercivora* causing Japanese oak wilt [20]. However, information on endophytic fungi against oak wilt pathogen (*R. quercus-mongolicae*) in Korea is limited. In our knowledge, only two research papers found antifungal activities of actinomycetes against *R. quercus-mongolicae* [21,22]. Hence, our objectives in this study were to (i) select endophytic fungi isolated from different parts of oak and pine trees against oak wilt; (ii) assess the effect of heating on antifungal activity of culture filtrates from selected isolates; (iii) indicate isolation frequency of endophytic fungi with antifungal activity from different parts of host tree. Experiments were conducted the primary and secondary screenings by dual culture assay and subsequently the assessment of antifungal activity of the culture filtrates from selected isolates based on the inhibition rates in mycelial growth, sporulation, and spore germination of oak wilt fungus.

2. Materials and methods

2.1. Pairing test (dual culture test)

For the primary screening, 615 isolates of endophytic fungi were obtained from various parts of oak and pine trees, and fungal identifications based on different gene regions such as internal transcribed spacer (ITS), large subunit ribosomal (LSU), translation elongation factor-1 alpha (TEF-1α), β-tubulin 2 (BT2), and RNA polymerase II (RPB2) were conducted in the previous study [23]. Among these, 368 isolates (including 111 isolates from leaves, 91 isolates from petioles, 112 isolates from branches, and 54 isolates from stems) were used to test antifungal activities against oak wilt pathogen (*R. quercus-mongolicae*). Testing technique using a dual culture method with modifications is as follows: Endophytic fungal isolates and oak wilt pathogen were sub-cultured on PDA medium for 7 days. Used a 5-mm diameter cork borer to excise mycelium plugs of endophytic fungi and test pathogen. Four different endophytic fungal isolates were placed on a 9-cm diameter PDA plate, equidistant, and near periphery. After 2 days, a mycelium plug of *R. quercus-mongolicae* (YY isolate) was placed on the center of PDA plate. All plates were sealed with plastic wrap and incubated in the dark at 25 °C for 5 days to inspect antifungal activities of endophytic fungal isolates. Inhibition zones were measured as distances between colonies of test fungus and endophytic fungi. Twenty three endophytic fungal isolates with inhibition zone above 1.0 mm were selected for the secondary screening (Table 1).

Twenty-three endophytic fungal isolates showed antagonistic activities against *R. quercus-mongolicae* in the primary screening were retested in the secondary screening experiment (Table 1). Endophytic fungi and oak wilt pathogen were sub-cultured on PDA medium for 10 days. A 5-mm-diameter mycelium plug of endophytic fungal isolate was placed on 9-cm diameter PDA plate at 1 cm away from the periphery. After 2 days, a 5-mm diameter mycelium plug of test

pathogen was placed at opposite side (1 cm away from the periphery). In the control, a 5-mm-diameter mycelium plug of test pathogen was placed at 1 cm away from the border of 9 cm diameter PDA plate without endophytic fungi. The experiment was designed in triplicate for each treatment. All plates were sealed with plastic wrap and incubated in the dark at 25 °C for 7 days to assess antifungal activities of endophytic fungal isolates.

Radial mycelial growth of test pathogen was measured in all treated and control plates, and then the percentage of mycelial growth inhibition (MGI) was calculated by the following formula [24]:

$$\text{MGI}\ (\%) = \frac{(C-T)}{C} \times 100 \qquad (1)$$

where C is mycelial growth of test pathogen in the control plate and T is mycelial growth of test pathogen in the treated plate.

2.2. Culture filtrate test

Eight endophytic fungal isolates with high-antifungal activity selected from pairing test were used for culture filtrates test.

Culture filtrates were prepared based on the method of previous studies [25,26]. Ten 5-mm-diameter plugs were taken from 10-day-old PDA culture plates of each endophytic isolate and were inoculated into 250 mL Erlenmeyer flasks containing 100 mL potato dextrose broth (PDB) medium. These flasks were incubated at 25 °C with constant shaking at 150 rpm for 14 days. Culture suspensions were filtered through Miracloth to separate the culture broth from mycelia. The culture broth was then re-filtered through membrane filters with a pore size of 0.45 µm. Culture filtrates of endophytic fungi were subsequently stored in the refrigerator at 4 °C until using.

To prepare assay media for the assessment of antifungal activity against oak wilt fungus, two types of media containing heated or unheated culture filtrates were used. For heated media, the same volumes of PDA medium and culture filtrate of each isolate were mixed before autoclaving at 121 °C for 15 min. For unheated media, PDA medium was autoclaved at 121 °C for 15 min

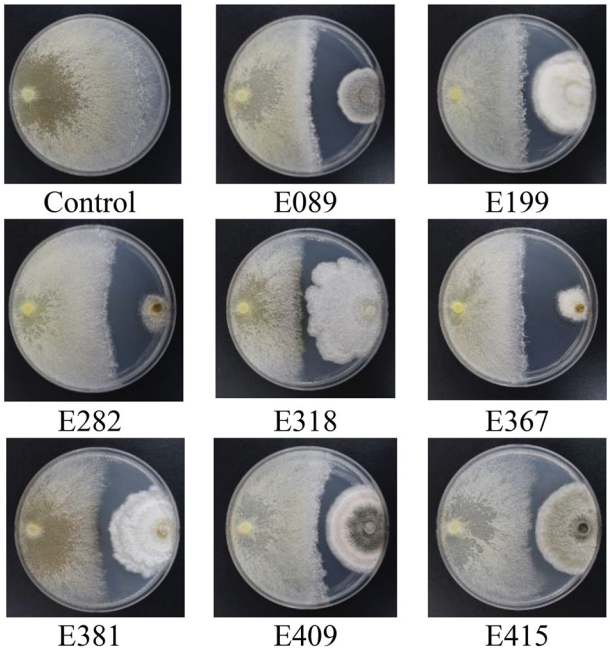

Figure 1. Dual culture test results showing mycelial growth inhibition of *R. quercus-mongolicae* by endophytic fungal isolates with strong antifungal activity at 7 days after inoculation in the dark at 25 °C.

Table 2. Mycelial growth inhibition rate (%) of endophytic fungal isolates against oak wilt fungus in dual culture test at 7 days after inoculation in the dark at 25 °C.

No.	Strain No.	Endophytic fungi	Inhibition rate (%)*	Inhibitory level†	Antifungal activity
1	E066	*Pestalotiopsis lushanensis*	0	−	None
2	E089	*Daldinia childiae*	47.9 ± 1.6^{c}	++	Strong
3	E118	*Paraconiothyrium brasiliense*	30.5 ± 2.4^{a}	+	Moderate
4	E161	*Daldinia childiae*	39.4 ± 2.1^{abc}	+	Moderate
5	E199	*Daldinia childiae*	45.3 ± 2.6^{c}	++	Strong
6	E205	*Fusarium avenaceum*	0	−	None
7	E206	*Daldinia childiae*	0	−	None
8	E253	*Daldinia childiae*	44.5 ± 2.8^{c}	+	Moderate
9	E280	*Pestalotiopsis lushanensis*	43.2 ± 4.4^{c}	+	Moderate
10	E282	*Nectria balsamea*	45.3 ± 4.2^{c}	++	Strong
11	E286	*Cladosporium cladosporioides*	42.4 ± 1.6^{bc}	+	Moderate
12	E318	*Valsa mali*	61.0 ± 4.7^{d}	++	Strong
13	E345	*Annulohypoxylon truncatum*	0	−	None
14	E350	*Amyloporia sinuosa*	33.1 ± 5.6^{ab}	+	Moderate
15	E352	*Monochaetia kansensis*	31.4 ± 0.9^{a}	+	Moderate
16	E356	*Antrodia sinuosa*	31.8 ± 2.0^{a}	+	Moderate
17	E367	*Nectria balsamea*	48.3 ± 3.0^{c}	++	Strong
18	E381	*Valsa mali*	48.7 ± 4.2^{c}	++	Strong
19	E394	*Colletotrichum acutatum*	42.0 ± 2.8^{bc}	+	Moderate
20	E396	*Valsa mali*	0	−	None
21	E409	*Colletotrichum acutatum*	48.3 ± 1.0^{c}	++	Strong
22	E410	*Colletotrichum acutatum*	44.1 ± 0.9^{c}	+	Moderate
23	E415	*Alternaria alternata*	48.7 ± 2.8^{c}	++	Strong

*Means and standard deviation with different letters are significantly different ($p < 0.05$).
†No inhibition (−); means < 45.0% (+); means ≥ 45.0% (++).

and then was mixed with culture filtrate of each isolate (without autoclave) in the same volumes. After cooling, both heated and unheated media were added 100 mg/L streptomycin sulfate before transferring to 9-cm dimeter Petri dishes. For control treatment, culture filtrates were replaced by sterilized water to mix with PDA media.

To assess antifungal activity of culture filtrates, inhibition rates in mycelial growth, sporulation, and spore germination of oak wilt fungus were measured. Agar disk with active growing mycelium of oak wilt pathogen was placed on the center of Petri dishes containing heated and unheated media, and then incubated in the dark at 25 °C for 5 days. Mycelial growth of oak wilt fungus was measured on each plate of treatments, and compared with the control to calculate rate of MGI using formula (1). All treatments were conducted in five replications.

For sporulation assessment, all plates were kept one more week at room temperature after checking mycelial growth. After that, 10 mL of sterilized water was added on the culture plates and scraped cautiously with sterilized glass rod to harvest spores. Spore suspensions were filtered through Miracloth before counting spore concentrations, and Haematocytometer was used to count the number of spores under compound light microscope. All treatments were conducted in three replications and

$$\text{SI}\ (\%) = \frac{\text{Spore No. (control)} - \text{Spore No. (treatment)}}{\text{Spore No. (control)}} \times 100$$

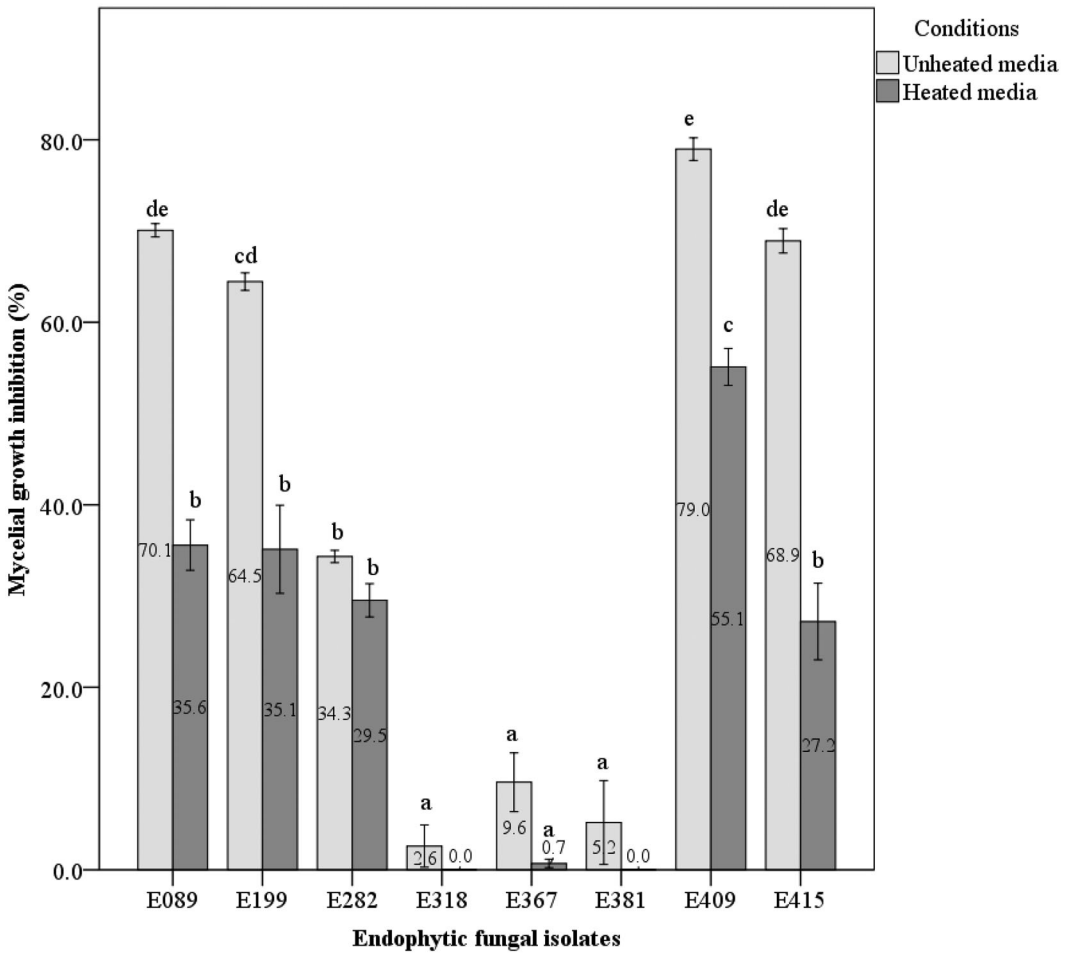

Figure 2. Mycelial growth inhibition rate (MGI %) of endophytic fungal isolates against oak wilt fungus in culture filtrate test at 5 days after inoculation in the dark at 25 °C. Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

the rate of sporulation inhibition (SI) was calculated as follows:

To test spore germination, 0.5 mL spore suspension of oak wilt fungus (10^2 cells/mL) was spread on Petri dishes containing heated and unheated media from the culture filtrate of each endophytic fungal isolate and then incubated in the dark at 25 °C. All treatments were conducted in three replications. After 3 days, the number of colonies was counted to

$$\text{SGI}\ (\%) = \frac{\text{Colony No. (control)} - \text{Colony No. (treatment)}}{\text{Colony No. (control)}} \times 100$$

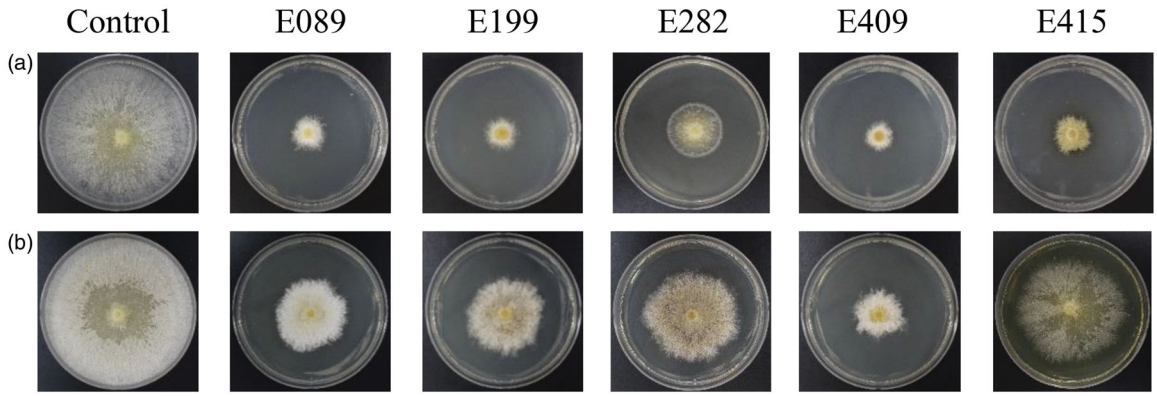

Figure 3. Mycelial growth of *R. quercus-mongolicae* on culture media containing unheated or heated culture filtrate of endophytic fungal isolates at 5 days after inoculation in the dark at 25 °C (a: unheated media; b: heated media).

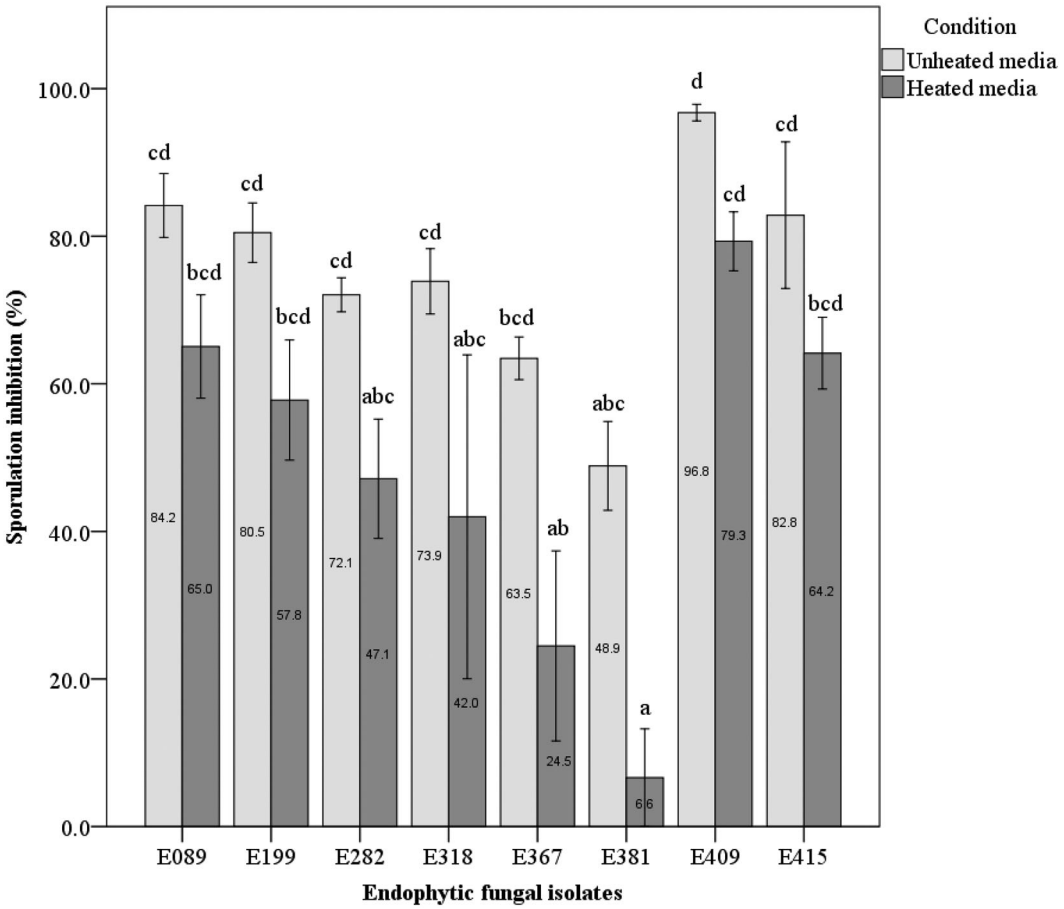

Figure 4. Sporulation inhibition rate (SI%) of endophytic fungal isolates against *R. quercus-mongolicae* in culture filtrate test at 12 days after inoculation (5 days in the dark at 25 °C and 7 days in room temperature condition). Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

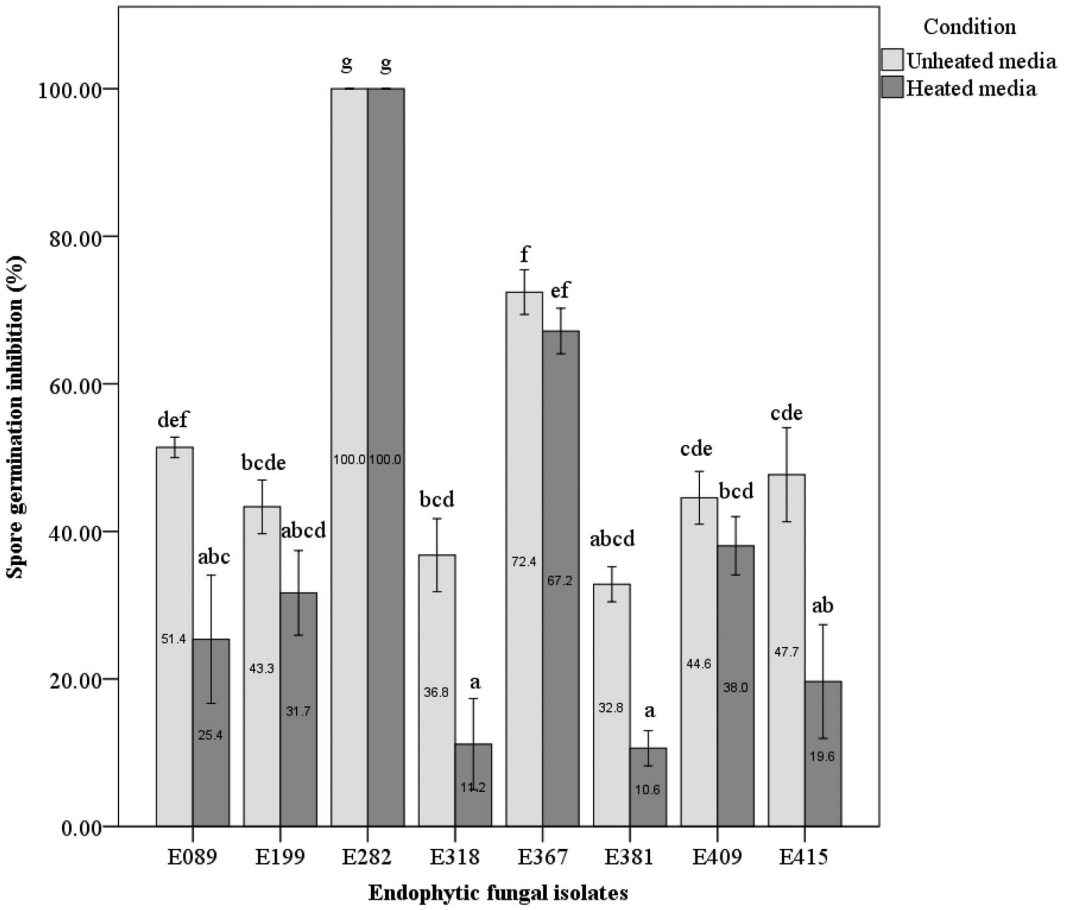

Figure 5. Spore germination inhibition rate (SGI %) of endophytic fungal isolates against *R. quercus-mongolicae* in culture filtrate test at 3 days after inoculation in the dark at 25 °C. Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

calculate the rate of spore germination inhibition (SGI) according to the formula as:

2.3. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (Tukey's HSD) test as post hoc test were used in order to test differences among endophytic fungal isolates at 5% probability level. All statistical analyses were conducted in IBM SPSS statistics version 24 for Window.

3. Results

3.1. Screening of endophytic fungal isolates with antifungal activity against R. quercus-mongolicae in dual culture test

Five endophytic fungal isolates (E066, E205, E206, E345, and F396) showed no antifungal activity in the paired test despite having antifungal activity in the primary screening (Table 2). It was inferred that the antifungal activity of these isolates in the primary screening could be formed in combination with other isolates. Mycelial growth inhibition rate of endophytic fungal isolates against oak wilt fungus ranged from 30.5% (E118) to 61.0% (E318). There was a significant difference ($p < 0.05$) in inhibition rates among endophytic fungal isolates. Among them, 10 strains (E118, E161, E253, E280, E286, E350, E352, E356, E394, and E410) had lower antifungal activities with the inhibition rates from 30.5 to 44.5%, while eight strains (E089, E199, E282, E318, E367, E381, E409, and E415) had relatively higher inhibition rates from 45.3 to 61.0% and these eight isolates were used for culture filtrate test (Table 2; Figure 1).

3.2. Mycelial growth inhibition activity of the selected isolates against R. quercus-mongolicae in culture filtrate

Mycelial growth inhibition of all selected isolates on heated media was lower than that of unheated media. This means that some of the secondary metabolites in culture filtrates were chemically changed and lost their antifungal activities after autoclaving (Figure 2).

Mycelial growth inhibition rates of eight selected isolates were significantly different ($p < 0.05$) between unheated and heated media. MGI rates of isolates on unheated media were always higher than those on heated media (Figure 2). However, the significantly

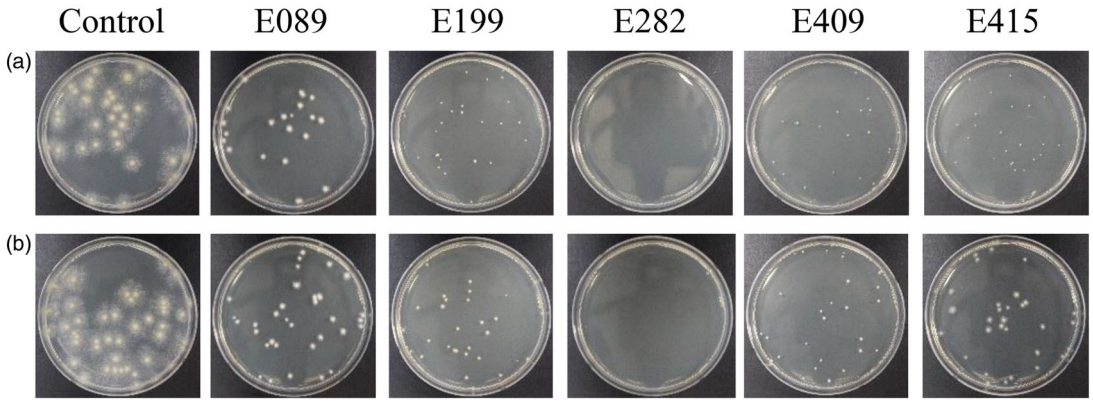

Figure 6. Spore germination of *R. quercus-mongolicae* on culture media containing unheated or heated culture filtrate of endophytic fungal isolates at 3 days after inoculation in the dark at 25 °C (a: unheated media; b: heated media).

higher MGI rates were only shown in the isolates of E089, E199, E409, and E415 ($p < 0.05$). Isolates of E282, E318, E367, and E381 had lower MGI rates than other isolates on both unheated and heated media from 10 to 40 times. The highest MGI rate was observed in E409 on unheated media with 79.0% and the lowest MGI rate was in E318 and E381 on heated media with 0%. MGI rates of E089, E199, E409, and E415 on unheated media were higher than those on heated media ($p < 0.05$) (Figures 2 and 3). On unheated media, MGI rates of E089, E415, E199 were 70.1, 68.9, and 64.5%, respectively, while MGI rates of the same isolates on heated media were 35.6, 27.2, and 35.1%, respectively (Figures 2 and 3).

3.3. Sporulation inhibition by the selected isolates

The difference in the inhibition rate of sporulation among eight selected isolates was significantly different ($p < 0.05$) on both unheated and heated media (Figure 4).

Similarly to MGI, all eight selected isolates had the inhibitory activity against sporulation on unheated media higher than on heated media. Sporulation inhibition rates ranged from 48.9 to 96.8% on unheated media and from 6.0 to 79.3% on heated media. The higher SIs were shown in E409, E089, E415, and E199 on unheated media, with the rates of 96.8, 84.2, 82.8, and 80.5%, respectively. In comparison, the lowest SI was observed in E381 with a rate of 6.6% (Figure 4). Although the inhibition rate of sporulation decreased on heated media, there was no significant difference between unheated and heated media in each isolates.

3.4. Spore germination inhibition by the selected isolates

Spore germination of *R. quercus-mongolicae* was measured by counting the number of colonies on unheated and heated media containing culture filtrates of eight selected isolates. The inhibition rate of spore germination had a significant difference among treatments on both unheated and heated media ($p < 0.05$) and the most of isolates except E282 showed higher inhibition rates on unheated media than heated media (Figure 5). The isolate E282 completely inhibited spore germination of oak wilt pathogen and reached up to 100% on both unheated and heated media. SGI rates were ranged from 36.8 to 100% on unheated media, while those on heated media were from 11.2 to 100% (Figure 5).

Although four isolates E089, E199, E409, and E415 showed lower SGI rates than E282, they could inhibit the growth of colonies after germination. The colony diameter of germinated spores for the control plate was higher than those for treatment plates on both unheated and heated media (Figure 6). The isolate E367 also had a relatively high inhibition rate, but it did show no significant difference between unheated and heated media; moreover, the colonies of germinated spores grew quickly as the control plate.

4. Discussion

4.1. Endophytic fungi against oak wilt

The antifungal activities of endophytic fungi against oak wilt were significantly different. Several fungal isolates were identified as the same species, but their antifungal activities against oak wilt were different (Table 2). This is consistent with previous findings that endophytic fungi isolated from different host tree species or tissues showed different antifungal activities against pathogens [27–29]. For instance, *Trochoderma theobromicola* DIS 376f isolated from *Cola praecuta* inhibited 19.2% mycelial growth of *P. capsici,* while *T. theobromicola* DIS 85f isolated from *Theobroma cacao* had no antifungal activity against this pathogen in hot pepper [27]. The antifungal activity against *Colletotrichum capsici* of *Trichoderma* isolates obtained from the phyllosphere

of health chili plants was significantly higher than those of *Trichoderma* isolates obtained from the rhizospheric region [28]. Three isolates of endophytic fungus *Fusarium equiseti,* Fe1, Fe2, and Fe3 obtained from roots of *Vicia villosa* and *Triticum aestivum* had various effects on reduction of root rot disease caused by *Fusarium avenaceum* and *Peyronellaea pinodella* in pea (*Pisum sativum*) [29].

Our results supported that 5 selected isolates E089 (*Daldinia childiae*), E199 (*Daldinia childiae*), E282 (*Nectria balsamea*), E409 (*Colletotrichum acutatum*), and E415 (*Alternaria alternata*) from oak and pine trees had strong antifungal activity against *R. quercus-mongolicae in vitro*. MGI and SI rates (%) of endophytic fungal isolates against oak wilt fungus decreased in the order of E409 (*Colletotrichum acutatum*), E089 (*Daldinia childiae*), E415 (*Alternaria alternata*), E199 (*Daldinia childiae*), and E282 (*Nectria balsamea*). Numerous studies have described morphological and molecular characterization of these fungal species [30–33]. However, these studies frequently focused on the pathogenicity of these species. For instance, *Colletotrichum acutatum* was reported in the most serious diseases in commercial fruit production as strawberry [34], citrus [35] and apple [36]; *Alternaria alternata* produced AAL toxin – a pathogen responsible for stem canker in tomato [37]. Some previous researches, *Colletotrichum* spp. and *Alternaria* spp. had also identified as potential endophytes in biocontrol [15,38,39]. They produced antibiotic compounds in culture, which inhibit plant pathogens [14,40]. Colletotric acid isolated from endophytic fungus *Colletotrichum gloeosporioides* showed antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea* [14]. A group of flavonoids, flavone ($C_{15}H_{10}O_2$) was extracted from the culture process of *Colletotrichum* sp. strain EG4 – an endophytic fungus isolated from *Ginkgo biloba* leaves [41], while this compound had demonstrated as an antimicrobial agent against Gram negative bacteria [42]. Monorden, a natural product from *Colletotrichum graminicola* had shown antifungal activities against foliar pathogens caused by *Alternaria alternata*, *Bipolaris zeicola*, and *Curvularia lunata* [43]. Two compounds, Alternariol monomethuyl ether and 4S-α, β-dehydrocurvularin isolated from *Alternaria* species had antifungal ability against the appressorium formation of *Magnaporthe grisea*, a rice pathogen [15]. For oak wilt pathogens, the compound 13-hydroxylmacrophorin isolated from the endophyte *Microdiplodia* sp. TT-12 was ingredient as an antimicrobial against *R. quercivora* causing oak wilt in Japan [20]. Another oak wilt pathogen in the United Sates, *Ceratocystis fagacearum*, also inhibited by endophytic bacteria namely *Bacillus pumulis*, *Pseudomonas denitrificans*, and *Erwinia herbicola* [44]. However, in our knowledge, there are currently no studies on antifungal activities of fungal endophytes against *R. quercus-mongolicae*, the oak wilt pathogen in Korea. Hence, the findings in this study could pave the way for using endophytic fungi as a potential biocontrol agent in the management of oak wilt. The fractionation of metabolites and assessment of their antifungal activities, as well as identification of the active compound need to be conducted in the further studies.

4.2. The effect of heating on antifungal activity of culture filtrate from endophytic fungi

These results observed that culture filtrate of five isolates E089, E199, E282, E409, and E415 are sensitive to heated condition, and generally lost antifungal activity after autoclaving. Only SGI of E282 was not affected by the heated condition (Figure 5). On the culture media supplemented with heated culture filtrates, decreased rates in MGI, SI, and SGI were ranged from 4.8 to 41.7%, 17.4 to 25.0%, and 6.6 to 28.1%, respectively (Figures 2, 4 and 5). The antifungal activity against *R. quercus-mongolicae* of culture filtrate from *S. blastmyceticus* also decreased in heated condition compared to unheated condition and temperature and period condition in culture also affected antifungal activity of *S. blastmyceticus* [22].

4.3. Isolation frequency of endophytic fungi with antifungal activity from different parts of host tree

The number of antifungal endophytes was significantly different in parts of host tree. A total of 18 antifungal isolates from different parts of trees was selected in the dual culture test (Tables 1 and 2). The frequencies of occurrence of isolates with antifungal activity from leaf, petiole, branch, and stem were 2.7, 8.8, 4.5, and 3.7%, respectively. The frequencies of occurrence of isolates with strong antifungal activity were also different among parts of trees. The highest frequency of occurrence was 5.5% in the petiole, followed by 1.9% in the stem, while leaf and branch had a similar frequency of 0.9% (Table 1; Figure 1). The diversity of endophytic fungi was also different among parts [23,45–48]. The most dominant species of endophytic fungi from *Pinus densiflora* was *Daldinia childiae* and the frequency of occurrence in the branch is higher than that in stem and needle tissues [23]. Dominant endophytic fungi from *Panax ginseng* depended on tissues, i.e., root, stem, petiole and leaf, of 3-year-

old ginseng plants, and *Entrophospora* sp., *Phoma radicina*, *Alternaria alternate*, and *Xylaria* sp. were dominant species, respectively [46]. The diversity of endophytic fungi from *Acalypha indica* leaves was higher than that from other tissues [47]. The number of endophytic fungal isolates from leaves of six different *Quercus* species was higher than that from petiole, stem, and branch [23]. The frequency of endophytic fungal isolates from *Tinospora cordifolia* was also the most abundant in leaves compared to those in stem and root tissues [49]. The highest frequency in leaves was explained by the greater surface area of the leaves for trapping of fungal inoculum compared to other parts [50]. However, endophytic fungi of some plants were often heterogeneous between specific tissues [51].

5. Conclusion

Eight isolates had strong antifungal activity against *R. quercus-mongolicae* in dual culture test. However, only five isolates showed strong antifungal activity in culture filtrate test and their activity was affected by heated condition. MGI and SI rates of endophytic fungal isolates against oak wilt fungus decreased in the order of E409 (*Colletotrichum acutatum*), E089 (*Daldinia childiae*), E415 (*Alternaria alternata*), E199 (*Daldinia childiae*), and E282 (*Nectria balsamea*), while SGI rates decreased in the order of E282, E367 (*Nectria balsamea*), E089, E415, and E409. The frequency of endophytic fungal isolates was different among tree parts and the number of potent antifungal isolates against oak wilt fungus was isolated mostly from the petiole. For the development of biocontrol agents against *R. quercus-mongolicae*, solvent fractionation of culture filtrates for the selected isolates E089, E199, E282, E409, and E415 and their antifungal activity test as well as identification of bioactive substances should be further studied.

Disclosure statement

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