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Antifungal Activity of Culture Filtrate from Endophytic Fungus *Nectria balsamea* E282 and Its Fractions against *Dryadomyces quercus-mongolicae*

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Abstract: A key role that fungal endophytes play in interacting with their host plant can be defined by the fact that they promote the growth of plants and enhance the tolerance of the host against plant pathogens using bioactive compounds that they produce. Several studies utilizing endophytic fungi as a source of biological control against plant pathogens were conducted, and a representative example includes *Aureobasidium protae* from common wheat (*Triticum aestivum*), which inhibited the mycelial growth of *Fusarium graminearum* and *Fusarium culmorum*, causal agents of *Fusarium* head blight disease. Our previous study indicated that three endophytic fungal isolates, E089 (*Daldinia childiae*), E282 (*Nectria balsamea*), and E409 (*Colletotrichum acutatum*), showed antifungal activities against *D. quercus-mongolicae*, an ascomycetous fungus that is reported to be associated with oak mortality in South Korea. The objectives of this study were to optimize and evaluate antifungal efficiency for these endophytic fungi against *D. quercus-mongolicae*, and this was achieved using culture filtrate retrieved from the three above-mentioned endophytes and fractions isolated from the culture filtrate. Of those, the culture filtrate from E282 showed higher mycelial growth and sporulation inhibitions on PDA medium where *D. quercus-mongolicae* was grown. In addition, three fractions, including hexane, $CHCl_3$, Et_2O, and H_2O, were tested for antifungal activities against *D. quercus-mongolicae*. The results revealed that the Et_2O fraction showed higher mycelial growth and sporulation inhibition rates. Taking these results together, the endophytic fungus, *N. balsamea*, which exhibited high antifungal efficiency, can be effectively used as a biocontrol agent for the management of oak wilt disease in the country.

Keywords: biocontrol; culture filtrate; endophytes; fraction; *Raffaelea quercus-mongolicae*

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1. Introduction

Endophytic fungi reside in the internal living tissue in most of plants, where they may balance their symbiotic relationship with host species [1]. Fungal endophytes play an important role in promoting the growth of plants and enhancing the tolerance of the host to plant pathogens [2,3]. In interaction with their host plants, endophytic fungi produce bioactive compounds to support their hosts' adaptation to abiotic and biotic stress [4,5]. In addition, endophytic fungi produce ligninolytic enzymes, which have been applied in bioethanol production [5,6]. Fungal endophytes are transmitted to their host plant in

two ways: majorly through airborne spores to the new host plant and, in some cases, seed-borne to the next generations [1,7]. The diversity of endophytic fungi has been reported from various plant species and plant organs, such as leaves, stems, roots, etc. [3,5,8]. There are an estimated 1 million species of fungal endophytes worldwide [8]; however, the number of endophytic fungi reported mostly belongs to about 800 genera, and dominant genera were identified as *Alternaria, Aspergillus*, and *Colletotrichum* [3].

Applying endophytic fungi to control pathogen displacement by antimicrobials is one of the major fields in endophytic research [9]. For instance, *Phomopsis longicolla*, an endophytic fungus isolated from *Bostrychia radicans*, produced antibacterial compounds against Gram-positive bacteria, namely *Staphylococcus aureus* and *Staphylococcus saprophyticus* [10]. Five isolates of *Aureobasidium protae* from common wheat (*Triticum aestivum*) inhibited the mycelial growth of *Fusarium graminearum* and *Fusarium culmorum*, causing *Fusarium* head blight disease from 22.5% to 38.0% and 20.5% to 30.5%, respectively, while two isolates of *Chaetomium globosum* showed higher antifungal activities against these two pathogens with inhibition rates of 48.5% to 51.0% and 39.5% to 41.0%, respectively [11]. A fungal endophyte identified as *Pezicula sporulosa* isolated from red spruce (*Picea rubens*) produced antifungal compounds such as cryptosporiopsin, 5-hydroxycryptosporiopsin, and mullein [12]. An endophytic fungus, *Phoma* sp., isolated from the cinnamon tree (*Cinnamomum mollissimum*), produced 5-hydroxyramulosin, a polyketide compound that had antifungal activity against the fungal pathogen *Aspergillus niger* [13]. In general, fungal endophytes are prolific producers of bioactive compounds [1]. However, secondary metabolites obtained from endophytic fungi might be influenced by culture conditions such as synthetic medium, pH, culture temperature, and period [14,15]. Hence, optimization of culture conditions can enhance bioactive products during the fermentation of fungal endophytes [16].

Since 2004, when oak wilt mortality was first observed in South Korea, it has been reported that *Dryadomyces quercus-mongolicae* is partly responsible for this syndrome, especially the death of *Quercus mongolica* [17–19]. Among those investigated to see whether endophytes isolated from pine and oak tree have antifungal activity against a putative pathogen associated with oak wilt disease [20,21], it was shown that three isolates, including E089 (*Daldinia childiae*), E282 (*Nectria balsamea*), and E409 (*Colletotrichum acutatum*), strongly inhibited the mycelial growth of *D. quercus-mongolicae* in vitro. In this regard, this study aimed to optimize the cultural period of endophytic fungi and see whether their culture filtrates inhibit the mycelial growth as well as sporulation and spore germination of *D. quercus-mongolicae*. Fractionation of the culture filtrate for the potential strain was then conducted, and antifungal activities of its fractions were tested as the foundation for identifying the active compound.

2. Materials and Methods

2.1. Preparation of Culture Filtrate and Experimental Design

Culture filtrate was prepared following the protocol mentioned in previous studies [21,22]. The oak wilt fungus and endophytic fungal isolates were molecularly identified based on sequence analysis of different gene regions such as internal transcribed spacer (ITS), large subunit (LSU), translation elongation factor-1 alpha (TEF-1α), β-tubulin 2 (BT2), and RNA polymerase II (RPB2) in previous studies [22,23]. Three fungal endophytes, E089 *(Daldinia childiae)*, E282 *(Nectria balsamea)*, and E409 *(Colletotrichum acutatum)*, with 100% similarities of ITS sequences from the GenBank accession numbers JX658462, HM484540, and EF464594, respectively, were inoculated into 500 mL Erlenmeyer flasks containing 200 mL Potato Dextrose Broth (PDB) (Merck, Darmstadt, Germany) medium and cultured with constant shaking at 150 rpm at 25 °C for two different periods: two weeks and three weeks. Culture suspensions were then filtered through membrane filters with a pore size of 0.45 μm. Culture filtrates obtained from culture suspensions were stored in the fridge at 4 °C for further study.

Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) medium was autoclaved and then mixed with culture filtrates or sterilized water (control) at a 1:1 ratio. After cooling, 100 mg/L streptomycin sulfate was added to the mixtures before transferring them

to Petri plates. The culture filtrate test was conducted with six treatments and a control in five replications, as follows: (1) Control: PDA were amended with sterilized water, (2) E089; 2-week culture filtrate: PDA were amended with a culture filtrate of E089 obtained after a 2-week culture period, (3) E089; 3-week culture filtrate: PDA were amended with a culture filtrate of E089 obtained after a 3-week culture period, (4) E282; 2-week culture filtrate: PDA were amended with a culture filtrate of E282 obtained after a 2-week culture period, (5) E282; 3-week culture filtrate: PDA were amended with a culture filtrate of E282 obtained after a 3-week culture period, (6) E409; 2-week culture filtrate: PDA were amended with a culture filtrate of E409 obtained after a 2-week culture period, (7) E409; 3-week culture filtrate: PDA were amended with a culture filtrate of E409 obtained after a 3-week culture period.

2.2. Assessment of Antifungal Activity of Culture Filtrates against D. quercus-mongolicae

A mycelial plug of actively growing oak wilt fungus was placed in the center of a Petri plate containing PDA amended with culture filtrates or sterilized water, and then inoculated in the dark at 25 °C for 6 days. After that, all Petri plates were placed at room temperature until day 12 for sporulation. The mycelial growth of *D. quercus-mongolicae* was measured as colony diameter for every plate of treatments and control from 3 to 12 days. The rate of mycelial growth inhibition (MGI) was calculated after incubating for 6 days when the mycelia of the oak wilt fungus had grown fully in the control plates. Formula (1) was used to compute the MGI rate, where C is the mycelial growth of the oak wilt fungus in the control plate, and T is the mycelial growth of *D. quercus-mongolicae* in the treated plate.

$$\text{Inhibition rate} = \frac{C - T}{C} \times 100\% \quad (1)$$

After measuring the mycelial growth of *D. quercus-mongolicae* on the 12th day, 10 mL of sterilized water was added to all plates, and the spores were harvested by scraping with a sterilized glass spreader. Miracloth was used to filter spore suspensions, and spore concentrations were checked using a Haemacytometer under a compound light microscope (Nikon U-III, FDX-35, Tokyo, Japan). The sporulation inhibition (SI) rate was calculated using Formula (1), where C is the number of spores in the control plate, and T is the number of spores in the treated plate.

For spore germination assessment, 500 μL of spore suspension (10^2 cells/mL) of the oak wilt fungus was evenly spread on treated and control plates. All plates were then incubated in the dark at 25 °C for 5 days to check for germinated spores. The spore germination inhibition (SGI) rate was calculated according to Formula (1), where C is the number of colonies in the control plate, and T is the number of colonies in the treated plate.

2.3. Extraction and Isolation of Fractions from Nectria balsamea E282

Based on the antifungal activities of culture filtrates, the endophytic fungus *Nectria balsamea* E282 was used for further study due to the strongest inhibitions against *D. quercus-mongolicae*. This fungus was shake-cultured in PDB at 25 °C for 3 weeks to obtain a culture filtrate. Two liters of this culture filtrate were extracted with methanol (2 L) over 2 days at room temperature. The filtrate was then concentrated at 40 °C using a rotary vacuum evaporator (N-1200A, EYELA, Tokyo, Japan) connected to a low-temperature circulator (CCA-1111, EYELA, Tokyo, Japan). The crude methanol extract was suspended in methanol–H_2O (1:1), and successively partitioned with hexane, chloroform ($CHCl_3$), diethyl ether (Et_2O), and H_2O. The resulting fractions were concentrated under decreased pressure to give residues of hexane fraction (9.1 mg), $CHCl_3$ fraction (34.2 mg), Et_2O fraction (56.4 mg), and H_2O fraction (30.4 g).

2.4. Assessment of Antifungal Activity of Fractions

Residues of the hexane fraction (9.1 mg), $CHCl_3$ fraction (34.2 mg), and Et_2O fraction (56.4 mg) were dissolved in 1 mL of acetone. The residue of the H_2O fraction (30.4 g) was

dissolved in 30.4 mL of sterilized water. Antifungal activities of fractions were assessed based on the inhibition rates in mycelial growth, sporulation, and spore germination of *D. quercus-mongolicae*.

For mycelial growth inhibition, 40 µL of each fraction was dropped onto the center of a 9 cm diameter PDA plate and gently spread on the surface using a sterilized glass rod. After acetone was completely volatilized in a laboratory laminar flow hood overnight, an active mycelial plug (5 mm) of oak wilt fungus was taken from a 5-day-old PDA culture plate and inoculated in the center of the PDA plates. For the control treatment, 40 µL of sterilized water was used instead of fractions. The experiment was conducted in triplicate for each treatment. All plates were incubated in the dark at 25 °C for 5 days to measure the mycelial growth of the oak wilt fungus on each plate of fraction treatments and compare it with the control. The calculation of mycelial growth inhibition (MGI) was performed, as described above.

To assess sporulation inhibition, all plates from the experiment of mycelial growth inhibition were kept for more than 5 days at room temperature. Spore suspensions were collected, and the calculation of sporulation inhibition (SI) was performed, as described above.

For spore germination inhibition, PDA plates containing fractions and control plates were prepared similarly to the mycelial growth inhibition assay. After that, 0.5 mL of spore suspension of *D. quercus-mongolicae* (10^2 cells/mL) was evenly spread on each plate. The experiment was conducted in three replications, and all plates were then incubated in the dark at 25 °C for 5 days. The calculations of spore germination inhibition (SGI) were performed, as described above.

2.5. Statistical Analysis

Two-way analysis of variance (ANOVA) was used to test differences between treatments and control in mycelial growth according to the cultured period. The differences in MGI, SI, and SGI rates among treatments were indicated by one-way ANOVA. Tukey's honestly significant difference (Tukey's HSD) was used as post hoc tests to evaluate statistical significance at a 5% probability level. All statistical analyses and computations were performed in IBM SPSS statistics version 24 for Windows.

3. Results

3.1. Mycelial Growth of D. quercus-mongolicae on Culture Filtrates

The mycelial growth of *D. quercus-mongolicae* had significant differences ($p < 0.05$) among treatments according to the cultured time. Mycelium growth of the fungus on 2-week culture filtrates of E089, E282, and E409 isolates was significantly higher ($p < 0.05$) than those on 3-week culture filtrates, and all of them were significantly smaller ($p < 0.05$) than those in control (Figure 1). Mycelium growth in the control spread fully across the Petri dish on day 6, with 81.0 mm. In general, mycelial growth increased during the cultured period in almost all treatments except for the culture filtrate of E282 obtained after incubating for 3 weeks (Figure 1). There were significant differences ($p < 0.05$) in each isolate between the two cultured periods. Mycelial growth of isolate E282 increased from 11.8 mm (day 3) to 81.0 mm (day 12) in the case of a 2-week culture filtrate, while that on a 3-week culture filtrate had no growth during incubation (Figure 1). For isolate E089, mycelial growth rose from 2.0 mm (day 3) to 45.4 mm (day 12) in 2-week culture filtrate and from 5.6 mm (day 3) to 49.8 mm (day 12) in 3-week culture filtrate (Figure 1). Similarly, mycelia growth of isolate E409 at day 3 was 3.4 mm and 2.0 mm in 2-week and 3-week culture filtrates, respectively, while those at day 12 were 62.0 mm and 47.8 mm (Figure 1).

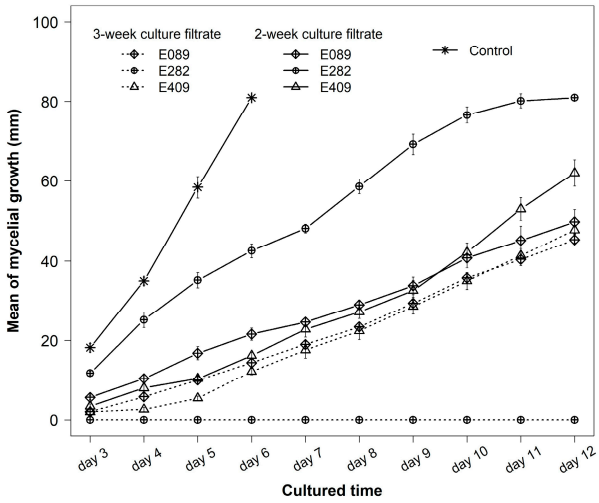

Figure 1. Mycelial growth of *D. quercus-mongolicae* on PDA medium containing culture filtrates of E089, E282, and E409 isolates during the cultured time (in the dark at 25 °C for 6 days and at room temperature for 6 days).

3.2. Inhibition Activities of Culture Filtrates against D. quercus-mongolicae

Mycelial growth inhibition rates were calculated after incubating for 6 days when the mycelial growth of *D. quercus-mongolicae* in the control plates reached maximum value. MGI rates of endophytic fungi had significant differences ($p < 0.05$) among treatments (Figure 2). MGI rates of three isolates E089, E282, and E409 in 3-week culture filtrates were significantly higher ($p < 0.05$) than those in 2-week culture filtrates (Figures 2 and 3). The highest antifungal activity against *D. quercus-mongolicae* was found in the 3-week culture filtrate of E282 with an MGI rate of 100%, while the lowest MGI rate was shown in the 2-week culture filtrate of E282 with 47.4% (Figure 2). Antifungal activities of E089 and E409 also significantly differed ($p < 0.05$) between 2-week and 3-week culture filtrates with MGI rates of 73.3% and 80.0%, 82.2% and 85.0%, respectively (Figure 2).

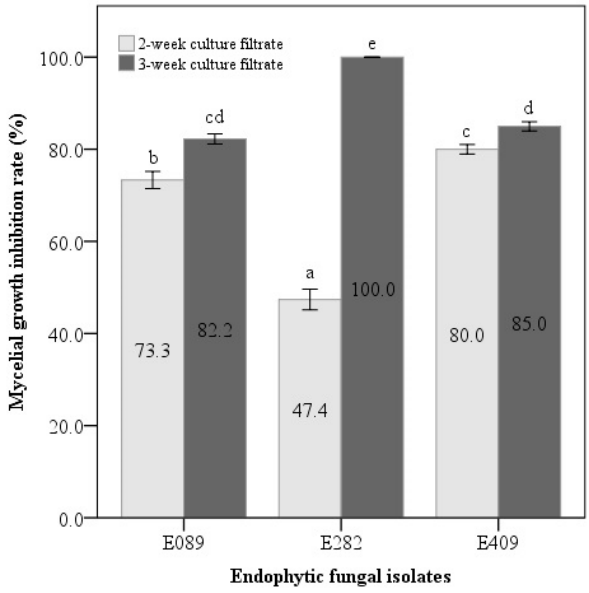

Figure 2. Mycelial growth inhibition rates of E089, E282, and E409 isolates against *D. quercus-mongolicae* after incubating in the dark at 25 °C for 6 days. Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

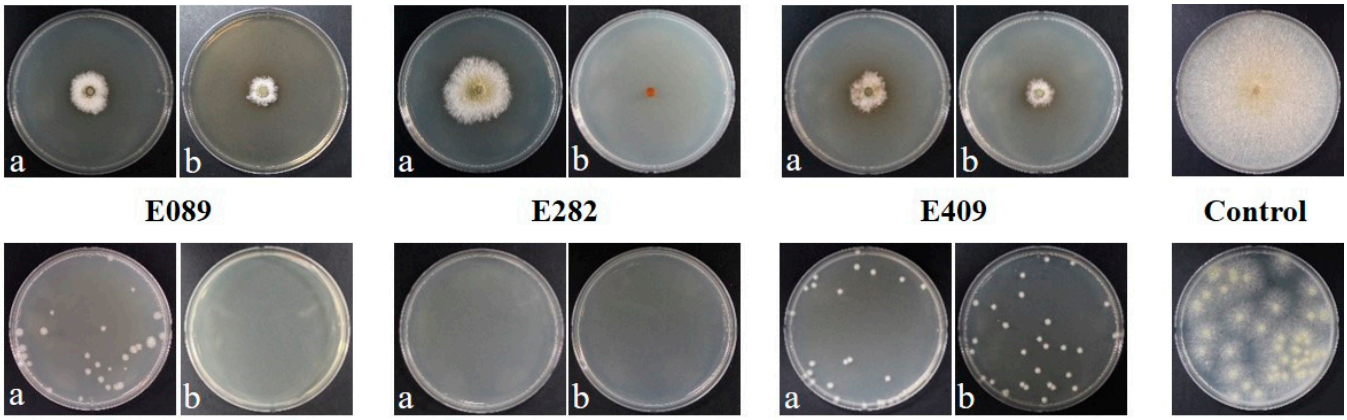

Figure 3. Mycelial growth (**above**) and spore germination (**below**) of *D. quercus-mongolicae* on PDA medium containing culture filtrates of E089, E282, and E409 isolates (**a**): 2-week culture filtrates; (**b**): 3-week culture filtrates after incubating in the dark at 25 °C for 6 days and 5 days, respectively.

Sporulation inhibition rates had significant differences ($p < 0.05$) among treatments. The SI rate of E282 in the 3-week culture filtrate was the highest with a percentage of 100% and significantly higher ($p < 0.05$) than that of 2-week culture filtrate with a rate of 80.1% (Figure 4). The SI rate of E089 also had a significant difference ($p < 0.05$) between 2-week and 3-week culture filtrates with 81.6% and 93.3%, respectively (Figure 4). Although the SI rate of E409 in the 3-week culture filtrate was higher than that in the 2-week culture filtrate, there was no significant difference ($p > 0.05$) between the two culture periods with 92.1% and 91.9%, respectively (Figure 4). The SI rate of E282 had a significant difference ($p < 0.05$) compared with E089 and E409 in the 3-week culture filtrate, while the SI rate of E409 significantly differed ($p < 0.05$) with E089 and E282 in the 2-week culture filtrate (Figure 4).

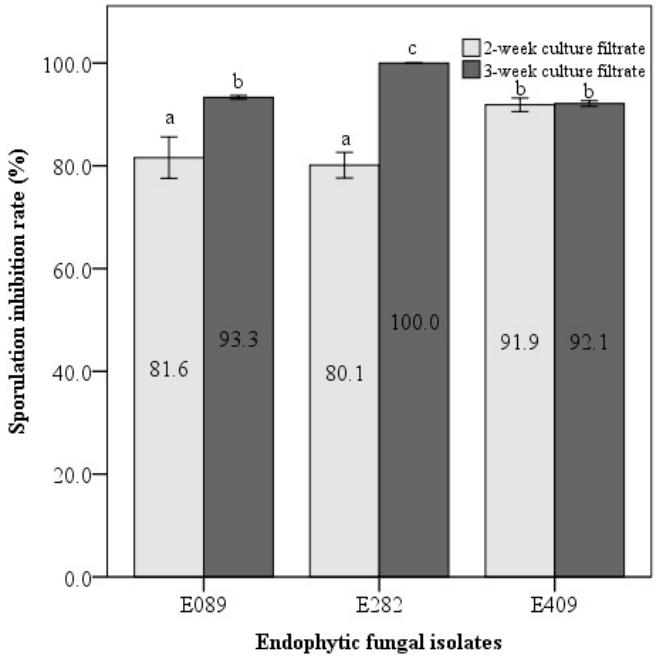

Figure 4. Sporulation inhibition rates of E089, E282, and E409 isolates with different cultured periods against *D. quercus-mongolicae* at 12 days after inoculation (in the dark at 25 °C for 6 days and at room temperature for 6 days). Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

There was a significant difference ($p < 0.05$) among treatments in the spore germination inhibition rate (Figure 5). SGI rates of E282 in both the 2-week and 3-week culture filtrates reached the highest inhibition of 100% (Figures 3 and 5). SGI rates of E089 and E409 had a significant difference ($p < 0.05$) between the 2-week and 3-week culture filtrates (Figure 5). Of these, the SGI rate of E089 was also 100% in the 3-week culture filtrate and about twice as high in the 2-week culture filtrate with a rate of 52.3% (Figures 3 and 5), while E409 showed a higher SGI rate in the 2-week culture filtrate compared to the 3-week culture filtrate with 47.6% and 33.1%, respectively (Figures 3 and 5). These results indicated that the cultured period did not affect the SGI rate of E282, but the influences might be seen in the SGI rates of E089 and E409 (Figures 3 and 5).

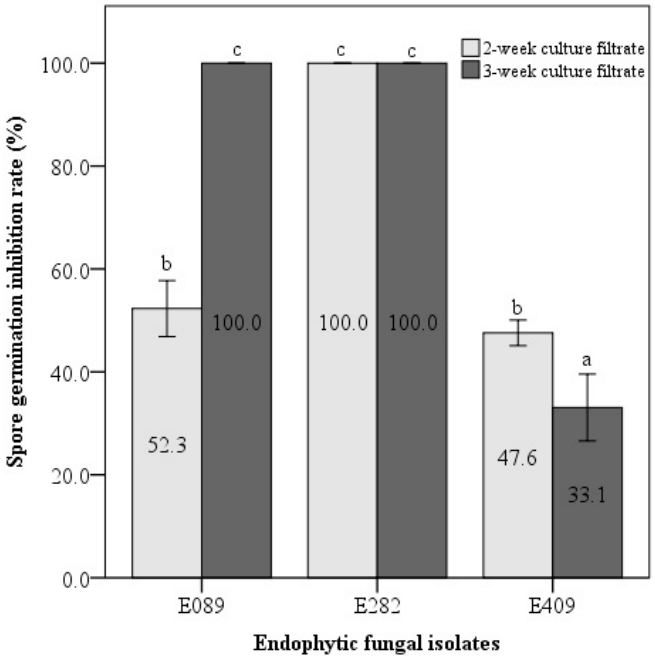

Figure 5. Spore germination inhibition rates of E089, E282, and E409 isolates with different cultured periods against *D. quercus-mongolicae* in the dark at 25 °C for 5 days after inoculation. Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

3.3. Inhibition Activities of Fractions from Nectria balsamea E282 against D. quercus-mongolicae

The three-week culture filtrate of *Nectria balsamea* E282 completely inhibited mycelial growth, sporulation, and spore germination of *D. quercus-mongolicae*. Hence, a 3-week culture filtrate of this fungus was selected for antifungal assessment of fractions. Fractions, hexane, $CHCl_3$, Et_2O, and H_2O showed a significant difference ($p < 0.05$) in mycelial growth inhibition rates against *D. quercus-mongolicae* (Figure 6). The Et_2O fraction completely inhibited mycelial growth of the fungus with an MGI rate of 100%, while the MGI rate of the hexane fraction was 80.6% (Figures 6 and 7). The lower MGI rates were presented for $CHCl_3$ and H_2O fractions with 36.0% and 12.4%, respectively (Figures 6 and 7).

Similarly to MGI rates, sporulation inhibition had a significant difference ($p < 0.05$) among fractions. SI rates of fractions decreased in the order of Et_2O, hexane, $CHCl_3$, and H_2O (Figure 8). The highest SI was observed in the Et_2O fraction with a rate of 100%, followed by the hexane fraction with an SI rate of 83.0%, while $CHCl_3$ and H_2O fractions showed lower inhibitions with SI rates of 38.1% and 3.5%, respectively (Figure 8).

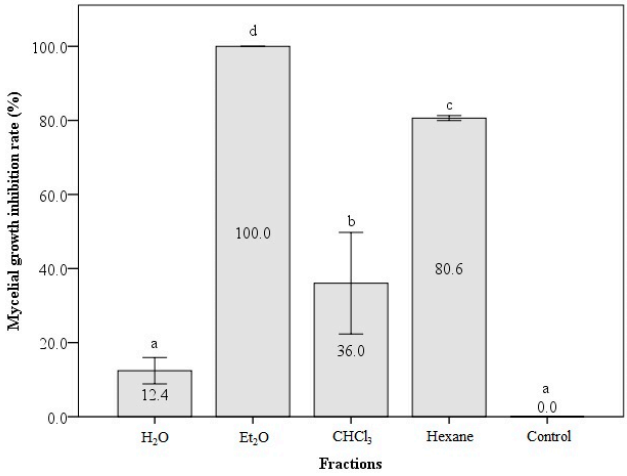

Figure 6. Mycelial growth inhibition rates of fractions against *D. quercus-mongolicae* on PDA medium containing 40 μL of fractions after incubating in the dark at 25 °C for 5 days. Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

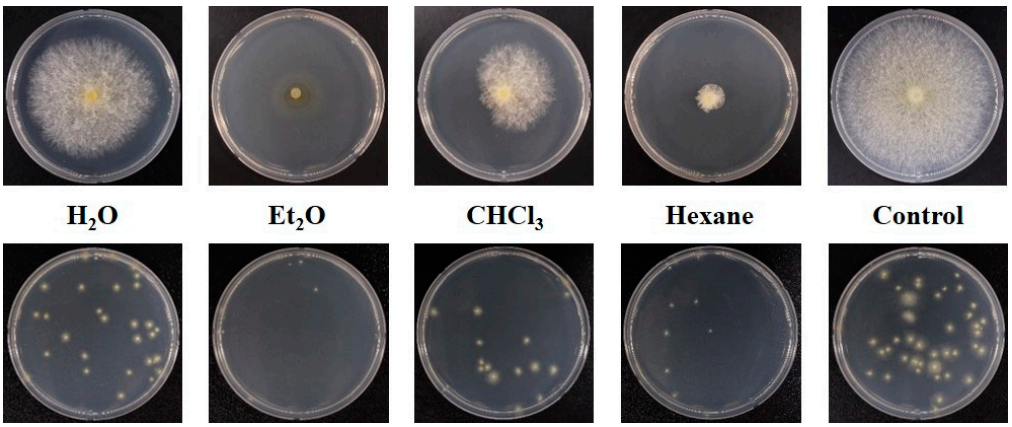

Figure 7. Mycelial growth (**above**) and spore germination (**below**) of *D. quercus-mongolicae* on PDA medium containing 40 μL of fractions after incubating in the dark at 25 °C for 5 days.

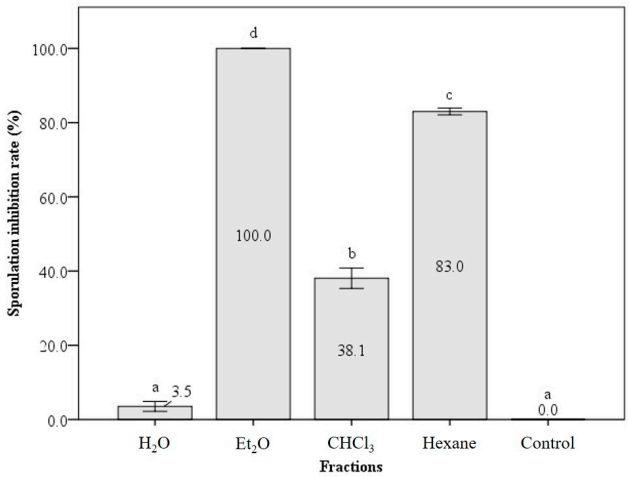

Figure 8. Sporulation inhibition rates of fractions against *D. quercus-mongolicae* at 10 days after inoculation (in the dark at 25 °C for 5 days and at room temperature for 5 days). Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

Spore germination of *D. quercus-mongolicae* was checked based on the number of colonies on the PDA medium containing fractions. There was a significant difference ($p < 0.05$) among fractions in spore germination inhibition, but no significant difference ($p > 0.05$) was indicated between $CHCl_3$ and hexane fractions (Figure 9). The Et_2O fraction had the highest SGI rate of 83.2%, while the H_2O fraction had the lowest SGI rate of 10.4%. The SGI rates of hexane and $CHCl_3$ fractions were 54.0% and 44.2%, respectively (Figures 7 and 9).

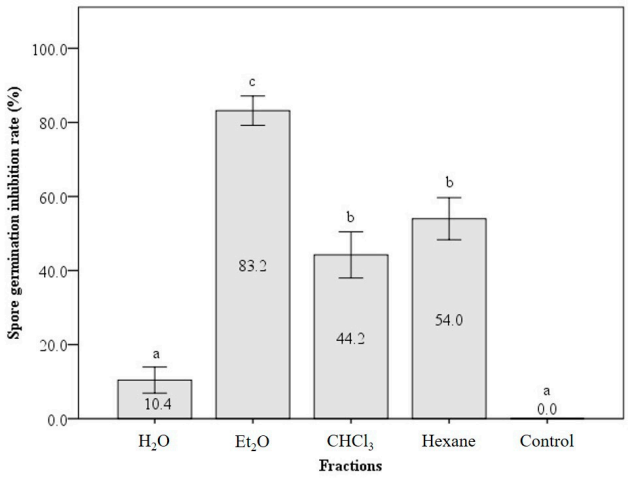

Figure 9. Spore germination inhibition rates of fractions against *D. quercus-mongolicae* on PDA medium containing 40 µL of fractions after incubating in the dark at 25 °C for 5 days. Different letters indicate a significant difference ($p < 0.05$) among treatments by Tukey's HSD test.

4. Discussion

It was confirmed in this study that the antifungal activities of endophytic fungal isolates against *D. quercus-mongolicae* were significantly influenced by cultured periods. Culture filtrates of endophytic fungi obtained after incubating for 3 weeks showed higher inhibition rates than those of 2 weeks, except for SGI rates of E282 and E409 (Figures 5 and 6). The SGI rate of E282 was not affected by culture periods when culture filtrates obtained in both culture conditions completely inhibited spore germination of the fungus with an SGI rate of 100% (Figures 5 and 6). The culture filtrate of E282 was also not influenced by the heating condition in the inhibition of spore germination of this pathogen [22]. Our results indicated that E282 (*Nectria balsamea*) had stronger antifungal activities against *D. quercus-mongolicae* than E409 (*Colletotrichum acutatum*) and E089 (*Daldinia childiae*). The present study also showed the optimal culture period of endophytic fungi, demonstrating that the 3-week culture filtrate of *Nectria balsamea* E282 completely inhibited mycelial growth, sporulation, and spore germination of *D. quercus-mongolicae* (Figures 2, 4 and 5). The antifungal activities of its fractions against *D. quercus-mongolicae* were significantly different. All fractions had the capacity to inhibit mycelial growth, sporulation, and spore germination of the oak wilt fungus. However, MGI, SI, and SGI rates of the H_2O fraction were not significantly different ($p > 0.05$) compared with the controls (Figures 6, 8 and 9). The Et_2O fraction completely inhibited mycelial growth and sporulation of *D. quercus-mongolicae* and had extreme activity in inhibiting spore germination (Figures 6, 8 and 9). Thus, this endophytic fungus is a potential biocontrol agent against *D. quercus-mongolicae*, which is reported to be associated with oak wilt disease in South Korea.

N. balsamea was recognized and specified earlier in 1884; however, it was then revised and illustrated as *Thyronectria balsamea* in 1940 [24]. *T. balsamea* caused canker disease on stems and branches of *Abies fraseri* in the United States [25]. *N. balsamea* was also found on the Hayata cone of *Pinus taiwanensis* in Taiwan [26]. To date, information associated with *N. balsamea* as an endophytic fungus is still limited. However, the family Nectriaceae has been identified as dominant endophytes in several plants, and some species belonging to the genus *Nectria* might produce bioactive compounds against plant pathogens [27–31]. For

instance, the endophytic fungus *Nectria haematococca*, isolated from branches of cacao trees, might inhibit *Crinipellis perniciosa*, the Witches' Broom pathogen on *Theobroma cacao* [27]. *N. haematococca* accounted for 18.2% of endophytic fungi isolated from the roots of *Panax ginseng*, and this fungus produced saponins (the main medicinal product of *P. ginseng*) with concentrations ranging from 0.063 to 0.115 mg/mL [29]. *Nectria pseudotrichia* 120-1NP, an endophytic fungus isolated from *Gliricidia sepium*, released nectrianolins A ($C_{22}H_{32}O_5$), B ($C_{26}H_{38}O_7$), C ($C_{15}H_{26}O$), and these secondary metabolites exhibited cytotoxic activity [32]. The fungal endophyte *N. pseudotrichia*, isolated from *Caesalpinia echinata*, also produced several leishmanicidal compounds such as xylaric acid D, piliformic acid, hydroheptelidic acid, cytochalasin D, and so on [31]. Two new polyketides, nectriacids B and C, which exhibit α-glucosidase inhibitory activity, were obtained from the culture of *Nectria* sp. HN001, an endophytic fungus isolated from *Sonneratia ovata* (a mangrove plant) in China [30].

To control the fungal pathogen, *Dryadomyces quercivora*, reported as a causal agent for oak wilt disease in Japan, a new compound, 13-hydroxylmacrophorin, was synthesized from the endophyte *Microdiplodia* sp. TT-12. However, this compound weakly inhibited the Japanese oak wilt pathogen [33]. On the other hand, chemical controls using Alamo® fungicide have been applied to prevent oak wilt disease caused by *Bretziella fagacearum* in the United States [34]. In Korea, oak wilt management by tree injection of a culture suspension of the antifungal actinomycete, *S. blastmyceticus*, showed significant preventive efficacy against *D. quercus-mongolicae*. However, this microorganism's antifungal activity was slightly lower than that of Alamo fungicide treatment [35]. Hence, our findings in this study could provide a potential endophyte source as a biological control agent for oak wilt disease in Korea. Evaluating the antifungal efficiency of the culture filtrate of *N. balsamea* E282 in planta, as well as identifying bioactive compounds, should be conducted in further studies.

5. Conclusions

Culture filtrates of endophytic fungi were significantly influenced by culture periods in the inhibitions of mycelial growth, sporulation, and spore germination against *D. quercus-mongolicae*. Culture filtrates of E089, E282, and E409 obtained three weeks after cultivation had higher MGI, SI, and SGI rates than those obtained at weeks, except for the SGI rates of E282 and E409. The three-week culture filtrate of *Nectria balsamea* E282 completely inhibited mycelial growth, sporulation, and spore germination of the oak wilt fungus. The antifungal activities of its fractions against *D. quercus-mongolicae* were significantly different, with the Et_2O fraction completely inhibiting mycelial growth and sporulation of *D. quercus-mongolicae*. It could be developed as a potential biocontrol agent against *D. quercus-mongolicae*.

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