RESEARCH ARTICLE

Antifungal Activity of Thymol against *Aspergillus awamori* and *Botrytis aclada* Isolated from Stored Onion Bulbs

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ABSTRACT

The antifungal activity of thymol against *Aspergillus awamori* F23 and *Botrytis aclada* F15 in onions was examined through direct treatment with amended media and gaseous treatment with I-plates (plastic plates containing central partitions). The protective and curative control efficacy of thymol was examined 24 h before and after the inoculation of onion bulbs with the fungal isolates. Mycelial growth, sporulation, and spore germination of the isolates were inhibited on potato dextrose agar amended with various concentrations of thymol or acetic acid (positive control). Overall, thymol produced a stronger inhibitory effect on the mycelial growth and development of the isolates than acetic acid. Following gaseous treatment in I-plates, mycelial growth, sporulation, and spore germination of the isolates were inhibited at higher concentrations of thymol or acetic acid; however, acetic acid showed a little effect on the sporulation and spore germination of the isolates. Following the treatment of onion bulbs with 1000 mg L^{-1} of thymol 24 h before and after fungal inoculation, lesion diameter was greatly reduced compared with that following treatment with 0.5% ethanol (solvent control). Onion bulbs sprayed with thymol 24 h before fungal inoculation generally showed reduced lesion diameters by isolate F23 but not in isolate F15 compared with those sprayed 24 h after fungal inoculation. Collectively, thymol effectively inhibited the growth and development of *A. awamori* and *B. aclada* on amended media and in I-plates. In addition, spraying or fumigation of thymol is more desirable for effectively controlling these postharvest fungal pathogens during long-term storage conditions.

1. Introduction

Onions (*Allium cepa* L.) are primarily cultivated in temperate and sub-tropical regions and are consumed in almost every culture and country [1]. In Korea, this crop is one of the most important and seasonal vegetables; thus, consistent supply to consumers must be ensured through long-term postharvest storage. However, onions are one of the most vulnerable vegetables to postharvest fungal pathogens, such as *Aspergillus* spp., *Botrytis* spp., *Fusarium* spp., and *Penicillium* spp., under storage conditions [2–7]. These pathogens causing postharvest diseases often lead to the deterioration of vegetable quality and nutrient composition, eventually resulting in postharvest losses and shortening of storage duration.

In onions, conventional postharvest disease control relies on the use of chemical fungicides before or after harvest. However, consumers have been paying increasing attention toward safer food for consumption and prefer less harmful control measures to the environment and human health. To cope with this consumer trend, alternative control measures, such as the use of antifungal microorganisms or natural products, have been extensively explored [8–11]. However, controlling postharvest diseases at the desirable level is challenging due to the lack of multiple options for disease control, although postharvest biocontrol has been explored for a long time [12]. In this context, plant extracts and antagonistic microbes may be promising alternatives without further regulation of use as postharvest products [13–15]. Therefore, studies on the biocontrol of the storage diseases of onions, including the practical use of plant-derived materials, may offer not only economic (in terms of crop loss) but also human health (in terms of safety) benefits [16].

In particular, essential oils have garnered much attention due to their environmentally sound characteristics [17,18]. Plant-derived essential oils often contain diverse chemical components such as terpenes, aldehydes, ketones, and phenolic compounds [19–22]. Previously, Maqbool et al. [22] reported that cinnamon oil exhibited antifungal activity against *Colletotrichum gloeosporioides* in banana. Moreover, thyme oil exhibited control efficacy against *C. gloeosporioides* in avocado and *Penicillium digitatum* in citrus lemon [23,24]. Similarly, Arrebola et al. [25] reported that lemongrass oil from *Cymbopogon citratus* inhibited the mycelial growth of *Botrytis cinerea* and *Rhizopus stolonifera* in peach fruit. In addition, Vitoratos et al. [26] tested various plant essential oils against *B. cinerea, Penicillium italicum*, and *P. digitatum*, and observed that lemongrass essential oil from *C. citratus* and tea tree oil from Melaleuca plants showed antifungal activity against *B. cinerea*. Among these plant-derived compounds, thymol [a colorless crystalline monoterpene phenol (2-isopropyl-5-methylphenol) isolated from the essential oil of thyme (*Thymus vulgaris* L. thymoliferum)] inhibited the growth of many plant fungal pathogens, such as *Alternaria alternata, B. cinerea*, and *C. gloeosporioides* [27–30]. Moreover, thymol vapor as fumigation treatment inhibited postharvest brown and gray mold rot in apricot [31]. Occasionally, thymol exhibits synergistic antifungal activity with salicylic acid (an inducer of systemic resistance of the host) against *Fusarium solani*, a postharvest fungal pathogen of many fruits and vegetables [32]. Another recent study showed that thymol protected tobacco under salt stress (abiotic stress) through increased sodium ion efflux and elevated nitric oxide and glutathione levels [33]. Further, thymol has been regarded safe by the Food and Drug Administration and Environmental Protection Agency of the United States [34].

To this end, in the present study, antifungal activity of thymol against *Aspergillus awamori*, which causes black mold rot, and *Botrytis aclada*, which causes gray mold rot of onions, was investigated through direct treatments with amended media and gaseous treatment with I-plates (plastic plates containing central partitions). Moreover, the protective and curative efficacy of thymol against the fungal isolates on onion bulbs was examined.

2. Materials and methods

2.1. Fungal isolates

The *A. awamori* isolate F23 and *B. aclada* isolate F15 derived from diseased onion bulbs, which were maintained on potato dextrose agar (PDA) at 4 °C for long-term storage in our laboratory, were used in this study. The *A. awamori* F23 (KACC 47249) and *B. aclada* F15 (KACC 47250) have been deposited to the Korean Agricultural Culture Collection (KACC) (Wanju, Korea). Isolate F23 was initially deposited to KACC as *Aspergillus niger*; subsequently, it was re-identified as *A. awamori* as observed in our other work [7]. These isolates of *A. awamori* and *B. aclada* were cultured on PDA for 5 days at 28 °C and 20 °C, respectively. Spores of the cultures were harvested using sterile distilled water with 0.03% Tween 20, and spore concentration was adjusted to 1×10^6 spores mL^{-1} using a hemocytometer for use as inocula.

2.2. Ethanol concentration for thymol-amended media

Various concentrations of absolute ethanol as the solvent for thymol were tested to determine the maximum concentration that does not affect fungal growth. The final ethanol concentration in PDA was adjusted to 0%, 0.01%, 0.1%, 0.2%, 0.5%, and 1% of the total medium volume. Then, $2 \mu L\ L^{-1}$ of the spore suspension (1×10^6 spores mL^{-1}) of *A. awamori* F23 and *B. aclada* F15 was drop-inoculated on PDA and further incubated at 28 °C and 20 °C, respectively. Mycelial growth (mm) of isolates F23 and F15 was determined 6 and 7 days after incubation, respectively. The experiment was conducted two times with three replicates each.

2.3. Fugal growth and development on thymol-amended medium

Various concentrations of thymol [0 (unamended control), 1, 10, 50, 100, 200, and 250 mg L^{-1}] or glacial acetic acid (GAA; positive control) [0 (unamended control), 1, 10, 100, 200, 500, and 1000 $\mu L\ L^{-1}$] were added to the PDA medium. Thymol dissolved in absolute ethanol was added to the medium (final ethanol concentration = 0.1% of the total medium volume). Spore suspensions ($2 \mu L$ of 1×10^6 spores mL^{-1} of 0.03% Tween 20) of *A. awamori* F23 and *B. aclada* F15 were drop-inoculated at the center of the culture plate containing PDA amended with various concentrations of thymol or acetic acid and incubated at 28 °C and 20 °C, respectively. Mycelial growth (mm) of isolates F23 and F15 was determined 6 and 7 days after incubation, respectively. Relative mycelial growth was determined as mycelial diameter on the thymol- or acetic acid-amended medium divided by mycelial diameter on the unamended control medium. Quadratic regression analysis of relative mycelial growth on log$_{10}$-transformed thymol or acetic acid concentrations was applied to fit the dose-response

curves. From the dose-response curves, the EC_{50} values and 95% fiducial limit for the fungal isolates were determined. The experiment was conducted two times with three replicates each.

Next, the spores of *A. awamori* and *B. aclada* isolates on thymol or acetic acid-amended PDA were harvested using 10 mL of 0.03% Tween 20 per plate. Mycelial debris was removed after passing through two layers of sterile cheesecloth. Harvested spores were counted using a hemocytometer after adjusting to proper spore concentration. To evaluate the germination rate of the harvested spores of isolates F23 and F15, spore suspension (2 μL of 1×10^6 spores mL^{-1} of 0.03% Tween 20) was dropped on a sterile glass slide. The glass slide with two drops (sub-replication) of spore suspensions deposited at a distance was placed on a wet filter paper in a petri plate to avoid drying of the spore drops. Germination (%) of the fungal spores was determined 18 h after incubation at 28 °C for *A. awamori* F23 and 20 °C for *B. aclada* F15. The experiment was conducted twice with three replicates (glass slide) each.

2.4. Fungal growth and development in I-plates containing volatile thymol

Various concentrations of thymol (5 mL) [0 (unamended control), 10, 100, 200, 500, and 1000 mg L^{-1}] (final ethanol concentration = 0.1% of the total medium volume) or GAA (positive control) [0 (unamended control), 10, 100, 200, 500, and 1000 μL L^{-1}] were applied to one side of the I-plates (plastic plates containing central partitions) (Fisher Scientific, Pittsburgh, PA, USA) without medium. On the other side (PDA) of the plates, spore suspensions (2 μL of 1×10^6 spores mL^{-1} of 0.03% Tween 20) of *A. awamori* F23 and *B. aclada* F15 were drop-inoculated. The plates were incubated for 6 days at 28 °C for *A. awamori* F23 and for 7 days at 20 °C for *B. aclada* F15. Mycelial growth, sporulation, and germination rate of the fungal isolates were evaluated as described above. Determination of relative mycelial growth, quadratic regression analysis, and determination of the EC_{50} values and 95% fiducial limit were also conducted as described above. The experiment was conducted two times with three replicates each.

2.5. Disease evaluation on thymol-treated onion bulbs

To test the control efficacy of thymol against *A. awamori* F23 and *B. aclada* F15, the fungal isolates were inoculated on half-cut onion bulbs originating from Muan, Jeonnam Province, Korea, which were purchased from the Gyeongdong Market, Seoul,

Korea [5]. Briefly, the half-cut onion bulbs were treated with 0.5% ethanol (solvent control), GAA (2000 μL L^{-1}) (positive control), and thymol (1000 mg L^{-1}) at concentrations determined based on EC_{50} values from previous tests. In addition, fluazinam (400 μL L^{-1}, commercial concentration) was used as the positive fungicide control. The test chemicals were sprayed on the inner layers of the half-cut onion bulbs 24 h before (i.e., protective efficacy) and after (i.e., curative efficacy) inoculation with the fungal isolates. For inoculation, the isolates were grown on PDA as described above, and mycelial plugs (5 mm in diameter) were placed on the inner layers of the bulbs [three points (sub-replicates) per bulb]. Agar plugs without mycelia were used as uninoculated controls. Next, the inoculated bulbs were placed on mesh screens in plastic containers ($16.0 \times 23.0 \times 12.5$ cm^3) with three layers of wet paper towels. The containers were tightly closed and incubated at 28 °C for *A. awamori* F23 and 20 °C for *B. aclada* F15. At 5 days after incubation, lesion diameters (mm) on the inner layers of the inoculated onion bulbs were determined. The experiment was conducted two times with three replicates (bulbs) each.

2.6. Statistical analysis

All experiments were arranged in a completely randomized design, and the data were analyzed using Statistical Analysis Systems software (SAS Institute, Cary, NC, USA). Quadratic regression analysis of the relative mycelial diameters of *A. awamori* F23 and *B. aclada* F15 was performed using polynomial regression in SigmaPlot® 2001 for Windows (SPSS Inc., Chicago, USA) to obtain the best fit equation for thymol and acetyl acid. The EC_{50} values and 95% fiducial limit of the isolates to thymol and acetyl acid was determined using probit analysis. The log_{10}-transformation of sporulation data and arcsine-transformation of spore germination data were applied for statistical analysis. For the data of lesion diameters on onion bulbs subjected to thymol and other treatments, contrast analysis was used to compare the means before and after inoculation with the fungal isolates. General linear model procedures were used for the analysis of variance and the means were separated using the least significant difference test at $p < 0.05$.

3. Results

3.1. Ethanol concentration for thymol-amended media

In experiments 1 and 2, mycelial growth of *A. awamori* F23 and *B. aclada* F15 on PDA amended with

various concentrations (0.01%, 0.1%, 0.2%, 0.5%, and 1% in the total medium volume) of ethanol slightly differed compared with that on ethanol-unamended control medium (0%; Supplementary Table S1). However, mycelial growth of *A. awamori* differed between PDA amended with 0.01 and 1% and ethanol-unamended control medium in experiment 2. Similarly, *B. aclada* also showed slight differences in mycelial growth between ethanol-amended PDA and ethanol-unamended control medium (Supplementary Table S1). Therefore, $<0.5\%$ ethanol of the total medium volume was considered appropriate as the solvent concentration for thymol and applied in further *in vitro* and *in vivo* tests on the antifungal effect of thymol.

3.2. In vitro *antifungal effect of thymol on PDA*

To test the *in vitro* antifungal effect of thymol, mycelial growth, sporulation, and spore germination of *A. awamori* F23 and *B. aclada* F15 were examined on the thymol- or GAA (positive control)-amended PDA (Figures 1–3). Mycelial growth of these isolates was inhibited on media amended with various concentrations of thymol or acetic acid, as shown in dose-response curves in Figure 2. On thymol-amended media, the EC_{50} values against *A. awamori* F23 were 2.2 and 2.3 ($\log_{10}$ value) mg L^{-1}

in experiments 1 and 2, respectively. Meanwhile, on acetic acid-amended media, the EC_{50} values against the isolate were 6.0 and 8.8 ($\log_{10}$ value) μL L^{-1} in experiments 1 and 2, respectively. Notably, *B. aclada* F15 was effectively inhibited by both thymol and acetic acid, with the EC_{50} values of 1.4 and 1.2 ($\log_{10}$ value) mg L^{-1} for thymol and of 2.7 and 2.6 ($\log_{10}$ value) μL L^{-1} for acetic acid in experiments 1 and 2, respectively (Figure 2).

Furthermore, in *A. awamori* F23, thymol significantly ($p < 0.05$) inhibited sporulation at the concentrations of 200 and 250 mg L^{-1} (Figure 3A) and spore germination at the concentrations of 100 to 250 mg L^{-1} (Figure 3B) in experiments 1 and 2. Acetic acid as a positive control slightly affected sporulation of isolate F23 but reduced spore germination to some extent at the concentrations of 10 to 1000 μL L^{-1} in experiment 1 and of 1000 μL L^{-1} in experiment 2. Similarly, the sporulation and spore germination of *B. aclada* F15 were inhibited at 10 and 50 mg L^{-1} of thymol in both experiments (Figure 3). In addition, acetic acid affected sporulation of isolate F15 at 1000 μL L^{-1} in experiment 1 and at 500 and 1000 μL L^{-1} in experiment 2. Furthermore, acetic acid affected the germination of isolate F15 only at 500 μL L^{-1} in repeated experiments (Figure 3). Overall, thymol regardless of test concentrations more significantly ($p < 0.05$)
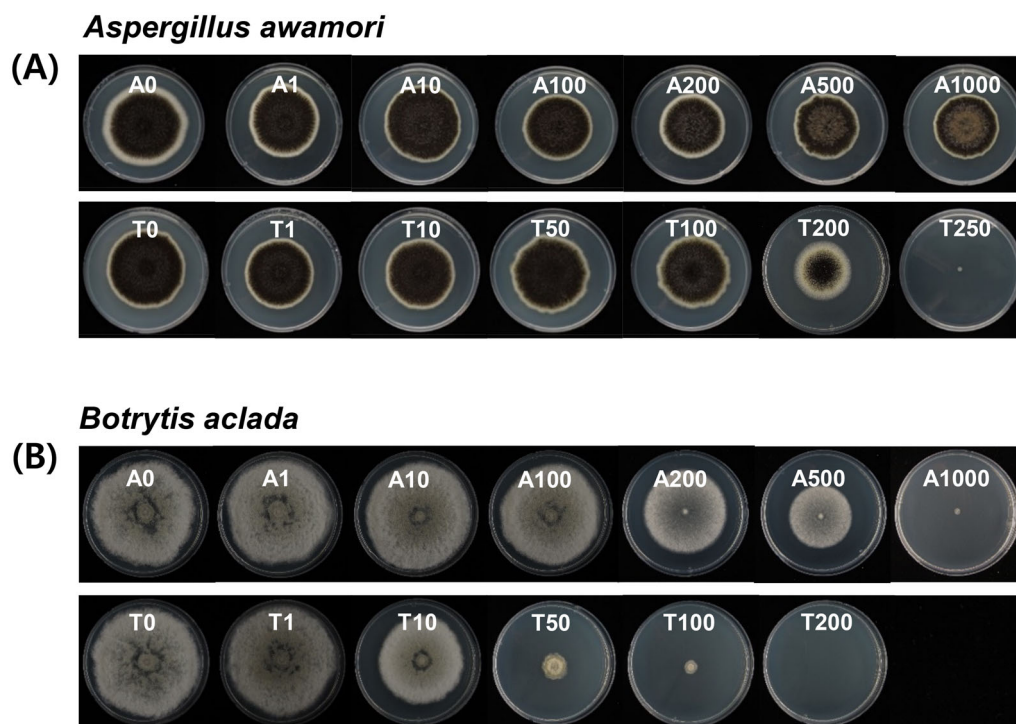

Figure 1. Mycelial growth of (A) *Aspergillus awamori* F23 and (B) *Botrytis aclada* F15 on PDA amended with various concentrations of glacial acetic acid (GAA, positive control; 0, 1, 10, 100, 200, 500, and 1000 μL L^{-1}) or thymol (0, 1, 10, 50, 100, 200, and 250 mg L^{-1}). Spore suspensions (2 μL of 1×10^6 spores mL^{-1}) of isolates F23 and F15 were drop-inoculated on potato dextrose agar (PDA) and mycelial growth (mm) was measured 6 and 7 days after incubation at 28 °C for *A. awamori* F23 and at 20 °C for *B. aclada* F15, respectively. A: GAA; T: thymol.
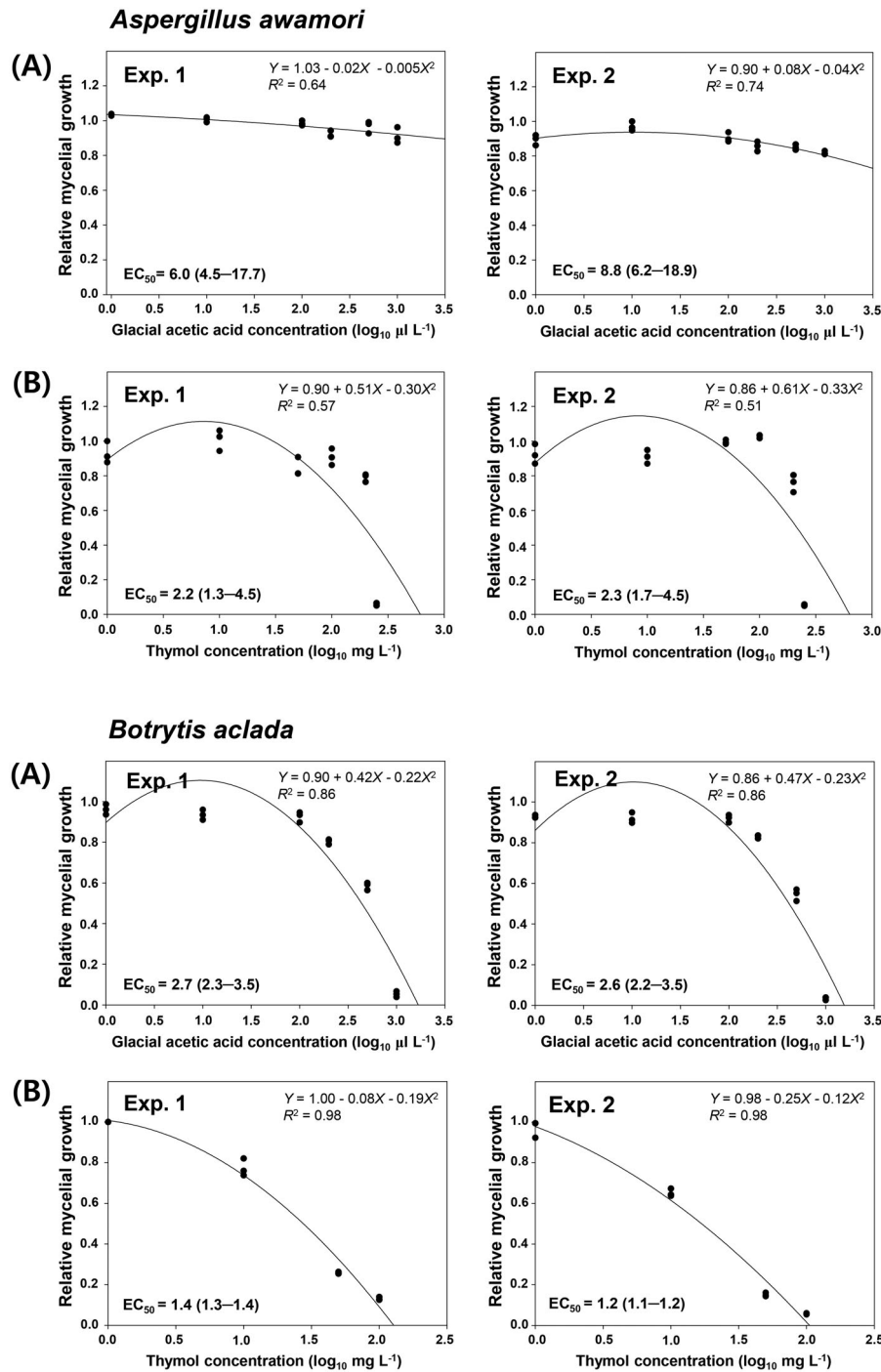
Aspergillus awamori

Botrytis aclada

Figure 2. EC_{50} values and 95% fiducial limits for *Aspergillus awamori* F23 and *Botrytis aclada* F15 based on relative mycelial growth on PDA amended with various concentrations of (A) GAA (positive control; 0, 1, 10, 100, 200, 500, and 1000 μL L^{-1}) or (B) thymol (0, 1, 10, 50, 100, 200, and 250 mg L^{-1}). Spore suspensions (2 μL of 1×10^6 spores mL^{-1}) of the isolates were drop-inoculated on PDA and then mycelial growth (mm) was measured 6 and 7 days after inoculation at 28 °C for *A. awamori* F23 and at 20 °C for *B. aclada* F15, respectively. The experiment was conducted with three replicates each.

inhibited the sporulation and spore germination of *A. awamori* F23 and *B. aclada* F15 than acetic acid (Figure 3).

3.3. In vitro *antifungal effect of volatile thymol in I-plates*

Various concentrations of thymol or GAA in the gaseous form were applied in I-plates, and dose-response curves between the relative mycelial

growth of *A. awamori* F23 and *B. aclada* F15 and volatile thymol or acetic acid were described (Figures 4 and 5). The EC_{50} value of volatile (gaseous form) thymol against *A. awamori* F23 was 3.0 ($\log_{10}$ value) mg L^{-1} in experiments 1 and 2. Meanwhile, the EC_{50} values of volatile acetic acid against isolate F23 were 7.0 and 6.3 ($\log_{10}$ value) μL L^{-1} in experiments 1 and 2, respectively (Figure 5). Likewise, the EC_{50} values of volatile thymol against *B. aclada* F15 were 2.2 and 2.4 ($\log_{10}$ value)
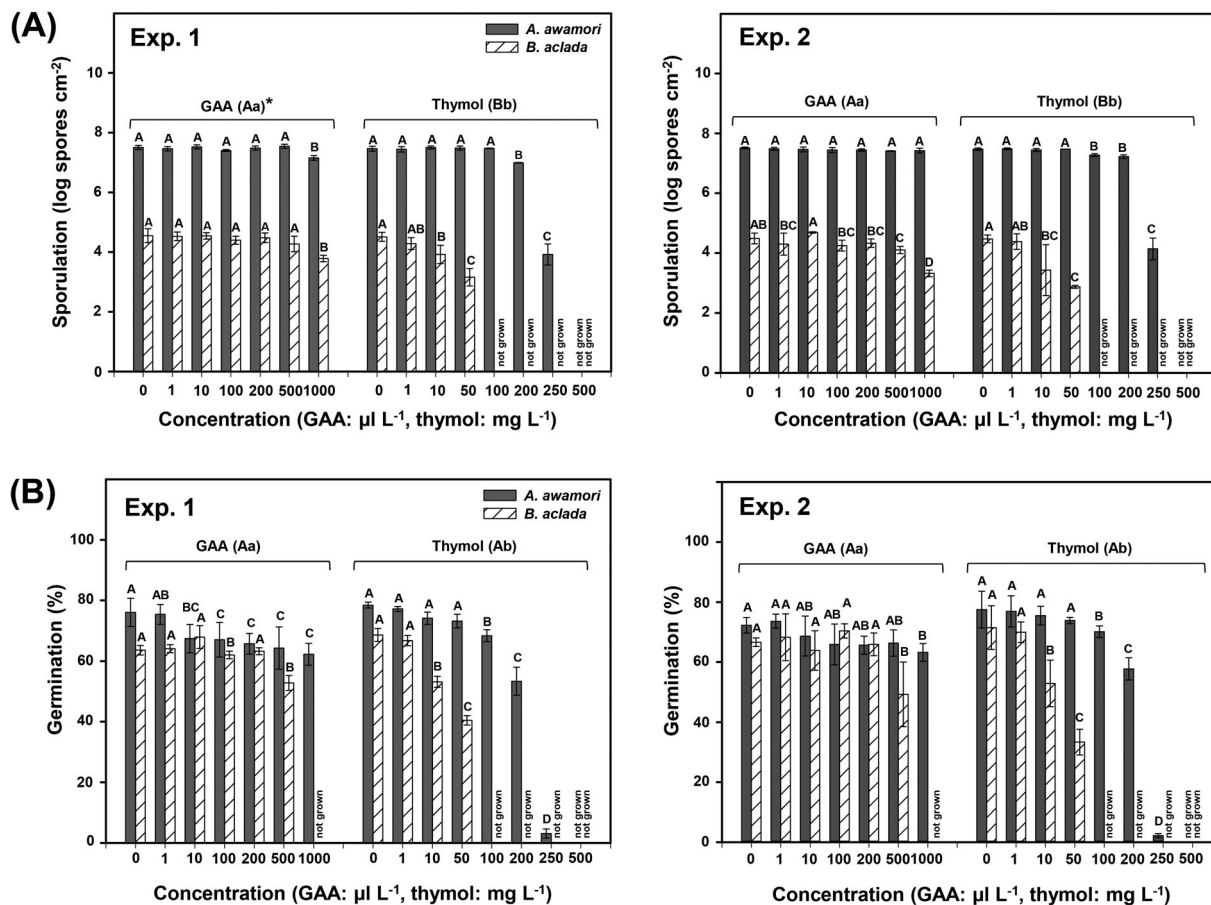
Figure 3. (A) Sporulation ($\log_{10}$ spores cm^{-2}) and (B) spore germination (%) of *Aspergillus awamori* F23 and *Botrytis aclada* F15 on PDA amended with various concentrations of GAA (positive control) or thymol. Spore suspensions (2 μL of 1×10^6 spores mL^{-1}) of the isolates were drop-inoculated on PDA, and sporulation was determined 6 and 7 days after inoculation on PDA amended with GAA or thymol at 28 °C for *A. awamori* F23 and at 20 °C for *B. aclada* F15, respectively. Spore germination was determined 18 h after incubation at the same temperatures. Bars represent mean ± standard deviation of three replicates. Values followed by the same letters are not significantly different ($p < 0.05$) according to the least significant difference test following arcsine transformation. *Uppercase and lowercase letters indicate significant differences between GAA and thymol for *A. awamori* F23 and *B. aclada* F15, respectively.

mg L^{-1} in experiments 1 and 2, respectively; however, the EC_{50} values of volatile acetic acid against isolate F15 were 5.5 and 5.3 ($\log_{10}$ value) μL L^{-1} in experiments 1 and 2, respectively (Figure 5).

In *A. awamori* F23, volatile thymol significantly ($p < 0.05$) inhibited sporulation at 200 to 1000 mg L^{-1} (Figure 6A) and spore germination at 500 and 1000 mg L^{-1} in at least one of two experiments (Figure 6B). However, volatile GAA slightly affected the sporulation of isolate F23 in repeated experiments, except at 1000 μL L^{-1} in experiment 2. Likewise, the spore germination of isolate F23 was somewhat inhibited at the tested concentrations in both experiments (Figure 6). Similarly, the sporulation of *B. aclada* F15 was significantly ($p < 0.05$) reduced at 200 and 1000 mg L^{-1} of volatile thymol compared with untreated controls in experiments 1 and 2 (Figure 6). However, the germination of isolate F15 was greatly reduced at 10 to 1000 mg L^{-1} in experiment 1 and at 1000 mg L^{-1} in experiment 2, compared with the untreated controls. In

contrast, volatile (gaseous form) acetic acid did not significantly ($p > 0.05$) affect the sporulation and spore germination of isolate F15 at all tested concentrations in repeated experiments (Figure 6).

3.4. In vivo *disease inhibitory effect of thymol on onion bulbs*

To evaluate the disease inhibitory effect of thymol on onion, half-cut bulbs were treated with 1000 mg L^{-1} thymol 24 h before (i.e., protective efficacy) and after (i.e., curative efficacy of thymol) inoculation with *A. awamori* F23 and *B. aclada* F15 (Table 1). The bulbs sprayed with tymol 24 h before (pre-treatment) fungal inoculation produced greatly reduced lesion diameters by both isolates, compared with those sprayed with 0.5% ethanol (solvent control) in experiments 1 and 2. Further, GAA (2000 μL L^{-1}) (positive control) and fluazinam (400 μL L^{-1}) (fungicide control) significantly ($p < 0.05$) reduced lesion diameters in both
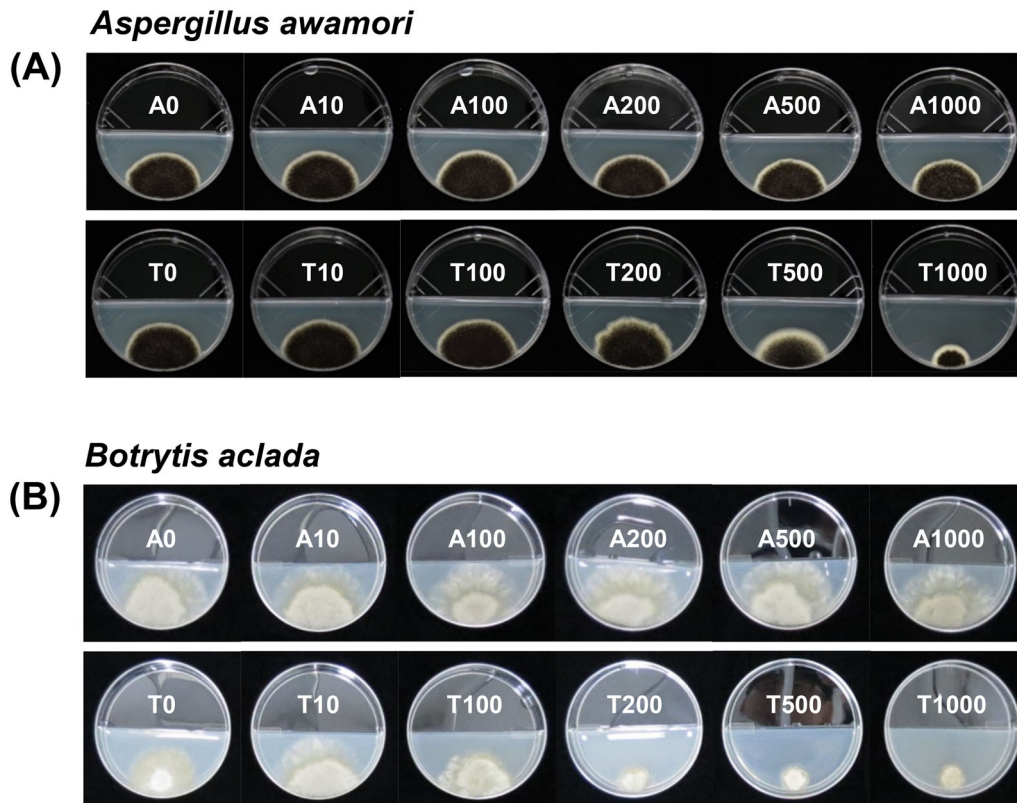
Aspergillus awamori

(A)

Botrytis aclada

(B)

Figure 4. Effect of gaseous treatment at various concentrations of GAA (positive control; 0, 10, 100, 200, 500, and 1000 μL L^{-1}) or thymol (0, 10, 100, 200, 500, and 1000 mg L^{-1}) on the mycelial growth of (A) *Aspergillus awamori* F23 and (B) *Botrytis aclada* F15 on PDA in I-plates. Spore suspensions (2 μL of 1×10^6 spores mL^{-1}) of the isolates were drop-inoculated on PDA and mycelial growth (mm) was measured 6 and 7 days after incubation at 28 °C for *A. awamori* F23 and at 20 °C for *B. aclada* F15, respectively. A: GAA; T: thymol.

experiments. Thymol showed comparable disease control efficacy against *A. awamori* F23 and *B. aclada* F15 to fluazinam, except against isolate F15 in experiment 1 (Table 1).

Meanwhile, bulbs sprayed with thymol 24 h after (post-treatment of thymol) fungal inoculation showed significantly ($p < 0.05$) reduced lesion diameters caused by *B. aclada* F15 but not by *A. awamori* F23 compared with those sprayed with ethanol in experiments 1 and 2 (Table 1). As observed above, post-treatments with acetic acid (positive control) and fluazinam (fungicide control) significantly ($p < 0.05$) reduced lesion diameters caused by isolate F15 in both experiments and by isolate F23 in experiment 1 (Table 1).

Furthermore, bulbs sprayed with thymol 24 h before fungal inoculation (i.e., protective efficacy), produced significantly ($p < 0.05$) reduced lesion diameters caused by *A. awamori* F23 compared with those sprayed with thymol 24 h after inoculation (i.e., curative efficacy) in experiments 1 and 2 (Table 1). However, unlike that in *A. awamori* F23, in *B. aclada* F15, thymol treatment showed no significant ($p > 0.05$) effect on lesion diameter between 24 h before and after fungal inoculation in repeated experiments. Acetic acid significantly ($p < 0.05$) reduced lesion diameters by isolate F23 but not by

isolate F15 when applied 24 h before inoculation compared with those when applied 24 h after inoculation in repeated experiments. Fluazinam treatment before fungal inoculation showed better disease inhibition against isolates F23 and F15 than treatment after inoculation in experiment 2. Regardless of the pre- or post-treatment, acetic acid and fluazinam produced overall potent inhibitory effects against diseases caused by *B. aclada* F15, as observed with thymol treatment (Table 1).

4. Discussion

In our previous study [7], we reported that *Aspergillus* spp. (63.9%, occurrence frequency), *Fusarium* spp. (6.4%), *Penicillium* spp. (15.5%), *Rhizopus* spp. (5.2%), and others (9.0%) were the predominant fungal species of diseased onion bulbs originating from Muan, Korea, which were purchased at the Garak Market, Seoul, Korea. In that study, *A. awamori*, *Fusarium oxysporum*, *Penicillium brasilianum*, and *Rhyzoctonia oryzae* were the predominant fungi in onion bulbs. In another study [2], *B. aclada* (44%) was the most commonly isolated fungal pathogen from onions at cold storage houses in Muan, Korea. Since these species are important postharvest fungal pathogens of onions in
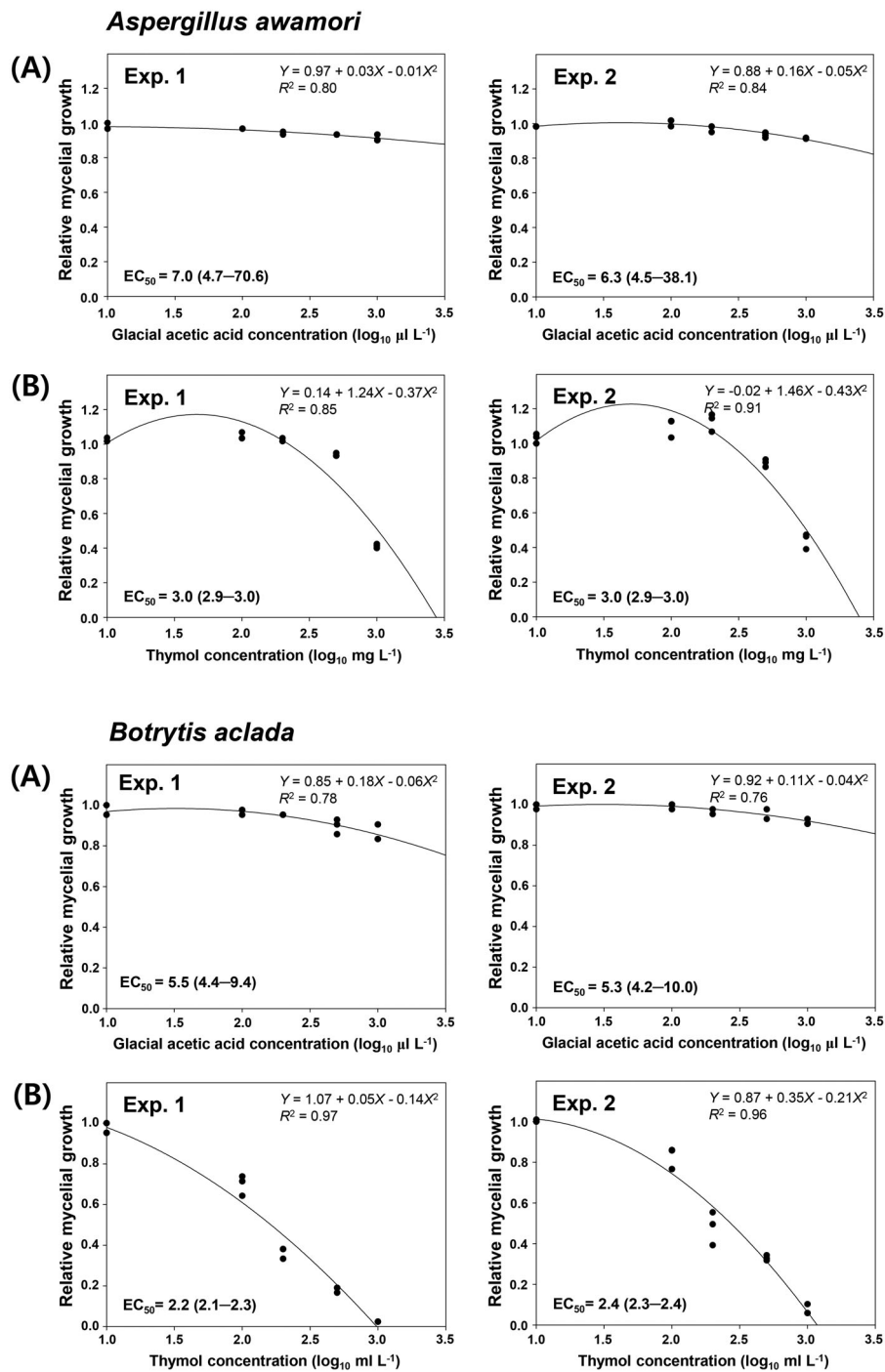
Aspergillus awamori

Figure 5. EC_{50} values and 95% fiducial limits for *Aspergillus awamori* F23 and *Botrytis aclada* F15 based on relative mycelial growth on PDA after gaseous treatment at various concentrations of (A) GAA (positive control; 0, 10, 100, 200, 500, and 1000 μL L^{-1}) or (B) thymol (0, 10, 100, 200, 500, and 1000 mg L^{-1}) in I-plates. Spore suspensions (2 μL of 1×10^6 spores mL^{-1}) of the isolates were drop-inoculated on PDA and mycelial growth (mm) was measured 6 and 7 days after incubation at 28 °C for *A. awamori* F23 and at 20 °C for *B. aclada* F15, respectively.

Korea, they should be controlled through appropriate measures under long-term storage conditions. Therefore, we attempted to establish an environmentally sound and effective control method and tested the antifungal activity of thymol against *A. awamori*, the causal agent of black mold rot, and *B. aclada*, the causal agent of gray mold rot, using *in vitro* amended media and I-plates and *in vivo* artificial inoculation trials. We demonstrated that

thymol effectively inhibited the growth and development of *A. awamori* F23 and *B. aclada* F15 on amended medium and in I-plates. In addition, spraying thymol on onions efficiently controlled these postharvest fungal pathogens under cold storage conditions.

Essential oils from various host plants have been tested against several plant pathogens because plant-derived essential oils exhibit environmentally
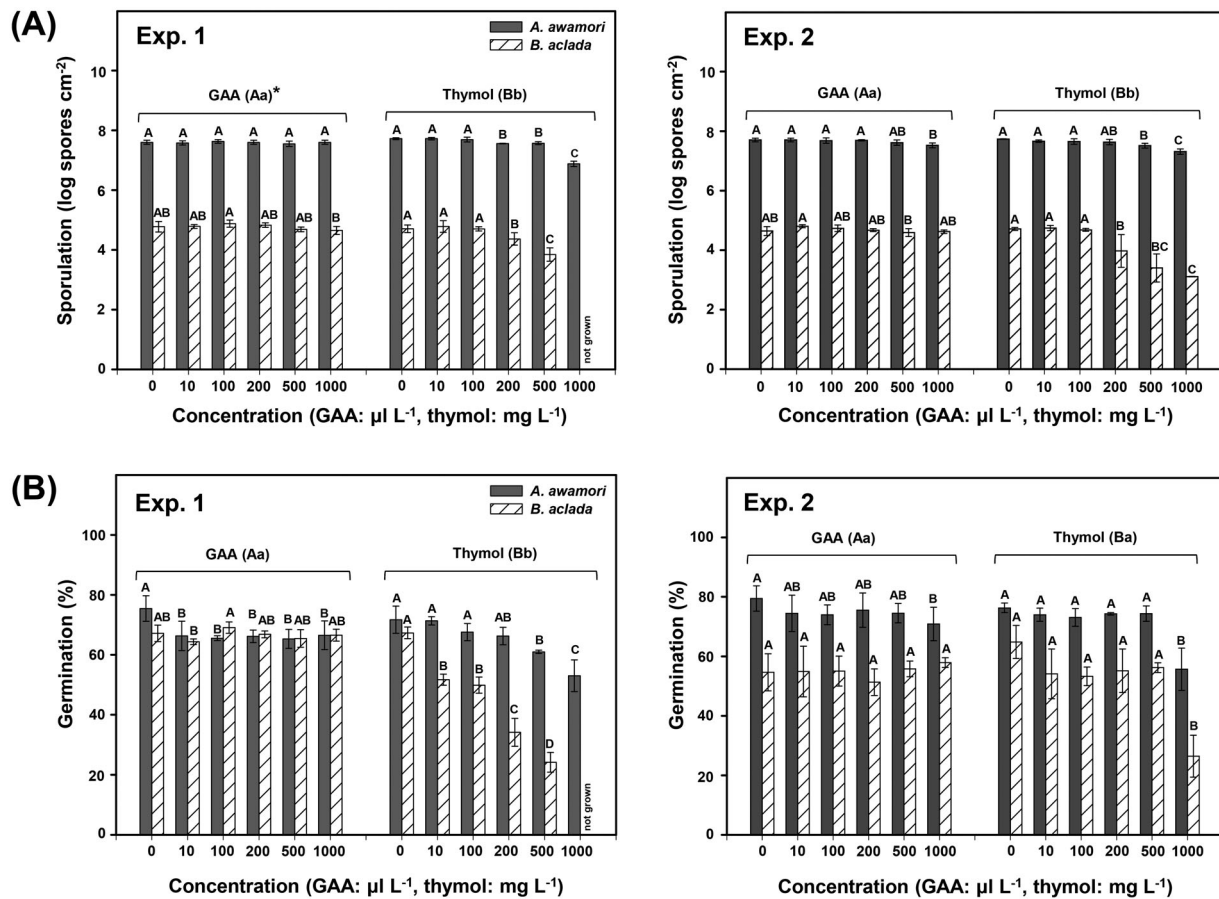
Figure 6. (A) Sporulation ($\log_{10}$ spores cm^{-2}) and (B) spore germination (%) of *Aspergillus awamori* F23 and *Botrytis aclada* F15 on PDA after gaseous treatment at various concentrations of (A) GAA (positive control; 0, 10, 100, 200, 500, and 1000 μL L^{-1}) or (B) thymol (0, 10, 100, 200, 500, and 1000 mg L^{-1}) in I-plates. Spore suspensions (2 μL of 1×10^6 spores mL^{-1}) of the isolates were drop-inoculated on PDA, and sporulation was determined at 6 and 7 days after inoculation on PDA amended with GAA or thymol at 28 °C for *A. awamori* F23 and at 20 °C for *B. aclada* F15, respectively. Spore germination of the isolates was determined 18 h after incubation at the same temperature. Bars represent mean ± standard deviation of three replicates. Values followed by the same letters are not significantly different ($p < 0.05$) according to the least significant difference test following arcsine transformation. *Uppercase and lowercase letters indicate significant differences between GAA and thymol for *A. awamori* F23 and *B. aclada* F15, respectively.

Table 1. Lesion diameters on the inner layers of onion bulbs sprayed with thymol, glacial acetic acid (GAA, positive control), ethanol (solvent control), and fluazinam (fungicide control) 24 h before and after inoculation with *Aspergillus awamori* F23 and *Botrytis aclada* F15.

	Lesion diameter (mm)			
	Experiment 1		Experiment 2	
Fungus/treatment[a]	Sprayed before inoculation	Sprayed after inoculation	Sprayed before inoculation	Sprayed after inoculation
A. awamori F23				
Ethanol	18.17 ± 1.41 A[b]	21.72 ± 1.55 A	15.33 ± 0.84 A	15.42 ± 0.53 B
GAA	7.77 ± 1.78 B*[c]	15.93 ± 0.28 B	11.89 ± 0.19 B*	19.59 ± 1.29 A
Thymol	8.00 ± 0.50 B*	19.95 ± 1.55 A	8.89 ± 0.82 C*	15.11 ± 0.70 B
Fluazinam	8.17 ± 0.33 B	12.17 ± 3.21 C	8.94 ± 0.39 C*	13.11 ± 1.68 B
B. aclada F15				
Ethanol	8.89 ± 1.40 A*	11.28 ± 0.25 A	8.61 ± 0.42 A	9.11 ± 0.63 A
GAA	6.21 ± 0.51 B	7.05 ± 0.78 B	7.67 ± 0.44 B	7.50 ± 0.29 B
Thymol	5.55 ± 0.08 B	7.68 ± 1.36 B	5.72 ± 0.39 C	6.17 ± 0.16 C
Fluazinam	2.50 ± 0.33 C	4.67 ± 1.76 C	5.39 ± 0.25 C*	6.44 ± 0.34 C

[a]Mycelia agar plugs (5 mm in diameter) of the isolates were inoculated on the inner surfaces of onion bulbs 24 h before and after spraying with thymol (1000 mg L^{-1}), GAA (2000 μL L^{-1}), 0.5% ethanol, and fluazinam (400 μL L^{-1}) at 28 °C. The onion bulbs inoculated with *A. awamori* and *B. aclada* were incubated at 28 °C and 20 °C, respectively, and lesion diameters (mm) were measured 5 days after inoculation. The onion bulbs inoculated with agar plugs without mycelia as uninoculated controls did not produce symptoms.
[b]Value is represented as mean ± standard deviation of three replicates with four sub-replicates each. Values followed by the same uppercase letters are not significantly different ($p < 0.05$) before and after fungal inoculation according to the least significant difference test.
[c]Asterisks indicate significant differences ($p < 0.05$) in values before and after inoculation according to the contrast analysis.

friendly control characteristics [17,18,20,35]. In a previous study, Rasooli et al. [36] reported that essential oil extracts from *Thymus eriocalyx* and *Thymus xporlock* exhibited antifungal effects against *A. niger*. In addition, the mycelia of *A. niger* treated with essential oils exhibited irreversibly damaged

cell walls and membranes and organelles in transmission electron microscopy. Similarly, Mossini et al. [37] conducted *in vitro* trials to examine the effects of *Azadirachta indica* (neem) extracts against *Penicillium verrucosum* and *Penicillium brevicompactum* and observed significant reductions in mycelial growth and sporulation but no inhibition of ochratoxin A production by *P. verrucosum* and *P. brevicompactum*. Recently, De Melo et al. [35] reported that essential oils of *Lippia gracilis* and *Lippia sidoides* exhibited significant, antifungal activity against *Lasiodiplodia theobromae*, the causal fungus of diplodia pod rot of cocoa.

Based on many studies on the antifungal effects of plant-derived essential oils, we tested various concentrations of thymol for antifungal activity against *A. awamori* F23 and *B. aclada* F15 in the present study. High concentrations of thymol as amendment in culture media or in gaseous form in I-plates significantly inhibited the mycelial growth, sporulation, and spore germination of both isolates. Overall, thymol produced a stronger inhibitory effect on the mycelial growth and development of the isolates than acetic acid (positive control). This antifungal activity of thymol may be attributed to its phenolic components that can disrupt fungal cell wall or membrane integrity as well as interfere with ergosterol biosynthesis [38,39]. In a previous study, Ahmad et al. [38] reported fungistatic and fungicidal effects of thymol against 111 isolates of *Candida* and demonstrated that the mode of action of this compound is closely related to the mechanism described above. In another recent study, Ranjbar et al. [40] assessed the antifungal effects of thymol against *A. niger* and *Penicillium commune* causing postharvest rot on pomegranate fruits. Scanning electron microscopy revealed cell deformation of *A. niger*, resulting from cell membrane destruction and cell wall strength loss. Interestingly, fungal growth was inhibited due to reduced cellulase and pectinase activities following thymol treatment [40]. Furthermore, Chen et al. [41] reported, for the first time, the anti-oomycete activity of a sulfonate derivative of thymol against *Phytophthora capsici*, the causal agent of Phytophthora blight of pepper. Therefore, thymol may serve as an environmentally sound alternative control measure against postharvest storage fungal pathogens on onions.

In the present study, we used acetic acid as the positive control against *A. awamori* F23 and *B. aclada* F15 because it has been reported to effectively control various storage fungi [8,42,43]. In a previous study, Liu et al. [42] reported that acetic acid vapors reduced the colony diameter and germination of *Monilinia fructicola*. However, we observed weak effect of acetic acid on the mycelial growth, sporulation, or spore germination of *A. awamori* F23 and *B. aclada* F15 when applied as the gaseous form in I-plates. Chu et al. [8] have reported similar results in sweet cherries. They fumigated *M. fructicola* and *Penicillium expansum*-infected sweet cherries with acetic acid and noted that acetic acid effectively reduced blue mold rot but not brown rot. Thus, acetic acid treatment in the gaseous form may be not greatly effective against certain fungal pathogens.

In the case of postharvest diseases, primary inoculum is typically already present, as derived through exposure to contaminated soil, transported vehicle, storage house atmosphere or wounds during transportation and packaging [44–46]. El-Mougy et al. [47] treated garlic cloves with essential oils before storage, which produce desirable protective effects against black mold rot. Raju and Naik [48] sprayed onion bulbs with various systemic and non-systemic fungicides before storage and observed protective effects, as evidenced by reduced mycelial growth and disease incidence of *A. niger*. In the present study, spraying onion bulbs with $1000\,mg\,L^{-1}$ thymol 24 h before and after fungal inoculation greatly reduced lesion diameters compared with spraying 0.5% ethanol (solvent control). Onion bulbs sprayed with thymol 24 h before fungal inoculation (i.e., protective efficacy) generally showed reduced lesion diameters by both *A. awamori* F23 and *B. aclada* F15. However, post-treatment of the pathogen-inoculated onion bulbs with thymol only provided a curative effect against *B. aclada* F15, but not against *A. awamori* F23. Here, the reason why thymol failed to show a curative effect against *A. awamori* F23 might be the rapid penetration of the fungal isolate into onion tissues after inoculation.

Taken together, thymol, a plant-derived essential oil, can effectively inhibit the mycelial growth and development of *A. awamori*, the causal agent of black mold rot, and of *B. aclada*, the causal agent of gray mold rot, on *in vitro* amended media and I-plates as well as in *in vivo* artificially inoculated onion bulbs. In particular, spraying or fumigating thymol on onion bulbs can efficiently control these postharvest fungal pathogens. Additional studies on the gaseous treatment of thymol in onions to control various other fungal pathogens under long-term storage conditions are warranted.

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