

Broad-Spectrum Activity of Volatile Organic Compounds from Three Yeast-like Fungi of the *Galactomyces* Genus Against Diverse Plant Pathogens

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ABSTRACT

The application of antagonistic fungi for plant protection has attracted considerable interest because they may potentially replace the use of chemical pesticides. Antipathogenic activities confirmed in volatile organic compounds (VOCs) from microorganisms have potential to serve as biocontrol agents against pre- and post-harvest diseases. In the present study, we investigated *Galactomyces* fungi isolated from rotten leaves and the rhizosphere of cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*). VOCs produced by *Galactomyces* fungi negatively affected the growth of phytopathogenic fungi and the survival of nematodes. Mycelial growths of all nine examined phytopathogenic fungi were inhibited on agar plate, although the inhibition was more intense in *Athelia rolfsii* JYC2163 and *Cladosporium cladosporioides* JYC2144 and relatively moderate in *Fusarium* sp. JYC2145. VOCs also efficiently suppressed the spore germination and mycelial growth of *A. rolfsii* JYC2163 on tomatoes. The soil nematode *Caenorhabditis elegans* exhibited higher mortality in 24 h in the presence of VOCs. These results suggest the broad-spectrum activity of *Galactomyces* fungi against various plant pathogens and the potential to use VOCs from *Galactomyces* as biocontrol agents.

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1. Introduction

Globally, we are observing trends moving toward improving the quality and safety of fresh fruits and vegetables by using biocontrol agents and natural materials. Extensive studies have been performed to explore and develop strategies based on microbial antagonists to biologically control pathogens [1,2]. The mechanisms of these microbial antagonists underlying the biocontrol activity, including competition for nutrients and space, enzyme secretion, toxin production, mycoparasitism, volatile organic compound (VOC) release, and resistance induction in plants, are likely to be involved in the antagonistic function [3]. VOCs are low-molecular-weight organic compounds that easily reach the target by vaporizing and diffusing in air. Recently, an increasing number of studies have focused on determining the function of VOCs [4] and their efficiency in reducing the viability and proliferation of devastating plant pathogens [5–7].


The *Galactomyces* genus of fungi (Saccharomycetales: Dipodascaceae) has long been considered a potential biological control agent

owing to its capacity to inhibit the growth of other phytopathogenic fungi by using nonvolatile antimicrobial compounds [8–10]. The inhibition of mycelial growth acts not only on living planted crops but also on harvested fruits, which makes them potentially valuable in controlling both pre- and post-harvest diseases. In our previous study, we observed that *G. candidum* JYC1146 could inhibit the growth of coexisted fungi by producing VOCs. Such capability was not displayed by the other five genera of the fungi examined in the same study [11]. Moreover, because all the three phytopathogenic fungi (*Botrytis cinerea*, *Fusarium incarnatum*, and *Colletotrichum gloeosporioides*) showed significant inhibition in growth, VOCs from *G. candidum* JYC1146 exhibited a potentially broad spectrum of activities against sympatric phytopathogens [11].

Broad-spectrum antipathogenic activity has been noted in some bacterial strains but has rarely been observed in VOCs from fungi. In the case of the bacteria, the broad-spectrum antipathogenic activity can be achieved by releasing lytic enzymes, causing permeability changes in the membrane, producing

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hydrogen cyanide, and secreting siderophores [12]. These mechanisms act on structures shared by several cellular organisms. Thus, despite these studies mainly focusing on phytopathogenic fungi, antipathogenic activities might also occur in other phytopathogens. Although numerous studies have examined the effects of VOCs on pathogenic fungi, their effects on the free-living stages of nematodes have rarely been studied [13,14]. Nematodes cause crop losses by both directly attacking host plants and acting as the vectors of other pathogens [15]. Various methods have been developed against the damage from nematodes including genetic host resistance, cultural practices, chemical applications, government regulatory measures, and biological control [16,17]. However, as the only metazoan phytopathogen with the largest body mass, nematodes are usually harmful for the agricultural environment [18].

The antipathogenic activity of VOCs from yeast-like fungi, *Galactomyces*, was confirmed in our previous study to be potentially broader than that from other *Galactomyces* species. In the present study, we further examined the effect of VOCs from three *Galactomyces* strains, *G. candidum* JYC1146, *G. candidum* JYC546, and *G. geotrichum* JYC549, on nine phylogenetically distant phytopathogenic fungi (belonging to the three classes: Agaricomycetes, Dothideomycetes, and Sordariomycetes) grown on agarose plates. To more directly simulate adoption on postharvest diseases, the effects of VOCs were examined *in situ* with the phytopathogenic fungi growing on the plate and harvested fruits of marketed cherry tomato. The mortality of the soil nematode *Caenorhabditis elegans* was also examined to estimate the protection of VOCs against the infection of plant nematodes.

2. Materials and methods

2.1. Fungal inoculum

Three *Galactomyces* strains were isolated from rotten leaves (*G. candidum* JYC1146) and the rhizosphere of cherry tomato (*L. esculentum* var. *cerasiforme*) (*G. candidum* JYC546 and *G. geotrichum* JYC549) for examining the antagonistic effects of VOCs on fungi.

Nine phytopathogenic fungi used for examining the response to VOCs included four fungi, which caused tomato wilt disease (*F. proliferatum* JYC2153 and JYC2155 and *F. incarnatum* JYC2156) and southern blight disease (*Athelia rolfsii* JYC2163); *Col. gloeosporioides* JYC2139, which causes ripe rot in grapes, bitter rot in apples, and several diseases in fruits and vegetables; and four fungal strains, namely *F. incarnatum* JYC2140, *Cladosporium*

uredinicola JYC2141, *Cl. cladosporioides* JYC2144, and *Fusarium* sp. JYC2145 isolated from rotten strawberries collected from the northern and central regions of Taiwan. Both *Galactomyces* and phytopathogenic fungi were grown on yeast extract peptone dextrose (YPD) agar plates containing 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar.

2.2. Effects of VOCs on the mycelial growth inhibition of the nine phytopathogenic fungi

The *Galactomyces* strains used for testing the mycelial growth inhibition were first cultured for 24 h to an OD_{660nm} of 0.8 (approximately 1.26×10^7 cells/mL); 20 μ L of the *Galactomyces* culture was spotted on the center of a 9-cm plate. The plate was placed “mouth to mouth” with another plate by using a piece of pathogenic mycelium (approximately 5×5 mm²) in the center. The two plates were wrapped together with parafilm [11]. Each of the nine examined phytopathogenic fungi was tested using all the three *Galactomyces* strains and one control (paired with the plate without the *Galactomyces* culture) with six replications.

Areas of the mycelium occupying the plate surface were photographed and measured using Image J after being incubated at 28 °C for 6 days [19]. The mycelial growth inhibition (MGI) was calculated using $([R_c - R_{exp}]/R_c) \times 100\%$, where R_c is the mean area of the control fungi and R_{exp} is that of the examined phytopathogenic fungi treated with the candidate *Galactomyces* strains [20].

2.3. Effects of VOCs on *Athelia rolfsii* JYC2163

Among the nine phytopathogenic fungi, *A. rolfsii* JYC2163 intensively responded to VOCs from the *Galactomyces* fungi and was the only fungus generating spores under laboratory conditions. We selected it for further investigation of spore germination and mycelial growth *in situ* on tomatoes under treatments of VOCs.

For the investigation of the effects of VOCs on spore germination, conidial suspensions were prepared by pouring 3 mL of Tween 80 solution (Sigma-Aldrich, St. Louis, MO) on 9-cm potato dextrose agar (PDA) plates colonized by *A. rolfsii* JYC2163. The conidia were gently scratched with a sterile scalpel to pull out spores into Tween 80 solution. The concentrations of the suspensions were measured and adjusted at approximately 5×10^5 conidia/mL by counting the conidia by using a hemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany). The conidial suspension (100 μ L) was spread on a YPD agar plate (approximately 5×10^4 conidia per plate) and the cell culture

grown in YPD broth of each *Galactomyces* strain (approximately 1.26×10^7 cells/mL) was inoculated on another YPD plate. The two plates were then placed “mouth to mouth” and wrapped with parafilm. All the three treatments and one control whose conidial plate was paired with a blank YPD plate were repeated six times. After incubation at 25 °C for 24 h, the spores were washed from the plate by using 100 μ L of sterile water, and the number of germinated spores in 1 μ L of the conidia suspension was counted using a hemocytometer.

The effect of the *Galactomyces* VOCs in the post-harvest control of *A. rolfsii* JYC2163 on tomatoes was examined according to the modified method suggested by Huang et al. [21]. Cherry tomato fruits with no apparent diseases or visible wounds were bought from a local market. Tomatoes of a similar size were surface sterilized in 70% ethanol for 5 s, washed twice with sterile distilled water, and blotted on paper towels to remove water. Three wounds, approximately 2-mm deep, were made on the stalk, bottom, and side of the tomato on its skin by using sterile pipette tips. The fungus was transported by injecting 20 μ L of *A. rolfsii* suspension with 1.7×10^7 conidia into each wound. Each of the five infected tomatoes was placed in a plastic box along with four YPD plate. In each of the treatments, the plates were cultured by one of the three *Galactomyces* strains (for examining the effect of VOCs) and the blank YPD as control. Each of the four treatments was repeated six times, and finally, 30 tomatoes were examined in total. The boxes were covered; high relative humidity was maintained using a moistened tissue paper to favor the post-harvest onset of the disease. After incubation for 8 days at 25 °C, each tomato was photographed, and the symptom and health areas were calculated using Image J. The areas of five tomatoes sharing the same condition in one box were summarized, and the disease severity was estimated by the ratio of symptom area [symptom area/total area].

2.4. Lethal effect of VOCs on free-living nematodes

The lethal effect of VOCs on the fourth larval stage (L4) of the *C. elegans* strain N2 was examined using the aforementioned “mouth to mouth” method. Approximately 100 nematodes were each examined in the nematode growth medium (NGM; 3 g of NaCl, 2.5 g of peptone, 17 g of agar, 5 mg of cholesterol, 1 mL of 1 M CaCl₂, 1 mL of 1 M MgSO₄, 25 mL of 1 M KH₂PO₄, and 1 L of H₂O) covering with a YPD plate cultured by each of the *Galactomyces* strains, *Saccharomyces cerevisiae* (for examining the common VOCs of the yeast such as

ethanol), or a blank plate as control. All the treatments were repeated six times. For maintaining the growth of *C. elegans*, the plates were inoculated with using *E. coli* OP50 cells as the food resource. All the nematodes were treated for 24 h in dark at 25 °C, and survival of 30 randomly selected worms was recorded by their movements or response to touch by using a needle.

2.5. Statistical analysis

Four parameters (MGI values of the nine phytopathogenic fungi, number of germinated spores of *A. rolfsii* JYC2163, disease severity of tomatoes infected by *A. rolfsii* JYC2163, and mortality rate of nematodes) were adopted to represent the effects of VOCs and analyzed for statistical significance. Different MGI values obtained using the same VOC treatment were compared using the beta regression model for inherently proportional data with the phytopathogenic fungi acting as the fixed effect. To fit the model, three data points with the minus value closed to 0 were considered as 10^{-8} . Significant differences between the treatments were compared using the Tukey’s all-pairwise comparisons. A similar model was also adopted for comparing disease severity with the treatment of fungal VOCs as the fixed effect. Numbers of germinated spores of *A. rolfsii* JYC2163 were first modeled using the generalized linear model (GLM) with Poisson’s error, and post-hoc multiple comparisons were performed using Tukey’s all-pairwise comparisons. The mortality rates of nematodes were modeled using the GLM with binomial error and Tukey’s all-pairwise comparisons as the post-hoc analysis. All the analyses were performed using R [22] with packages lmtree, betareg, emmeans, multcompView, and multcomp.

3. Results

3.1. Effects of VOCs on the mycelial growth inhibition of the nine phytopathogenic fungi

The growth of all the nine phytopathogenic fungi was inhibited by the presence of VOCs from the *Galactomyces* fungi. However, the inhibition levels varied among the treatments (Table S1 and Figures 1 and 2). Compared with the other examined fungi, *Fusarium* sp. JYC2145 was less inhibited in response to VOCs from each of the three *Galactomyces* strains, whereas *A. rolfsii* JYC2163 and *C. cladosporioides* JYC2144 showed higher inhibition levels (Figure 2).

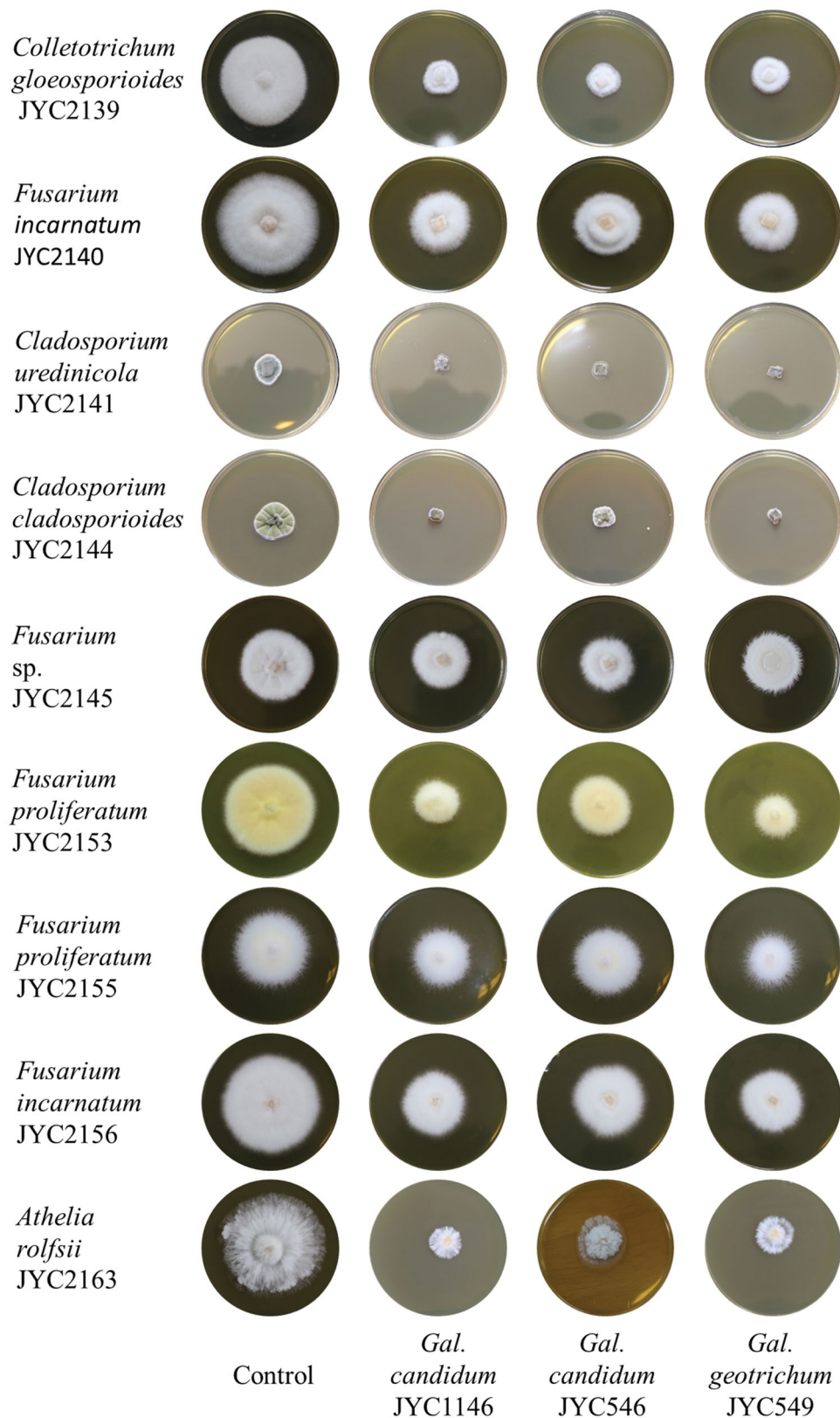


Figure 1. Mycelial growth of the phytopathogenic fungi under the effects of VOCs secreted from the three *Galactomyces* yeast strains.

3.2. Effects of VOCs on the spore germination of *Athelia rolfsii* JYC2163

Spores treated with VOCs showed significantly lower germination (likelihood ratio test: $df=3$, $X^2 = 113.23$, $p < 0.001$). The number of germinated

spores observed in $1 \mu\text{L}$ of the conidia suspension was 44.83 ± 21.11 for the control and 18.33 ± 7.37 , 19.00 ± 14.53 , and 17.33 ± 6.56 for treatments with *G. candidum* JYC1146, *G. candidum* JYC546, and *G. geotrichum* JYC549, respectively (Figure 3). Compared with the control treatment,

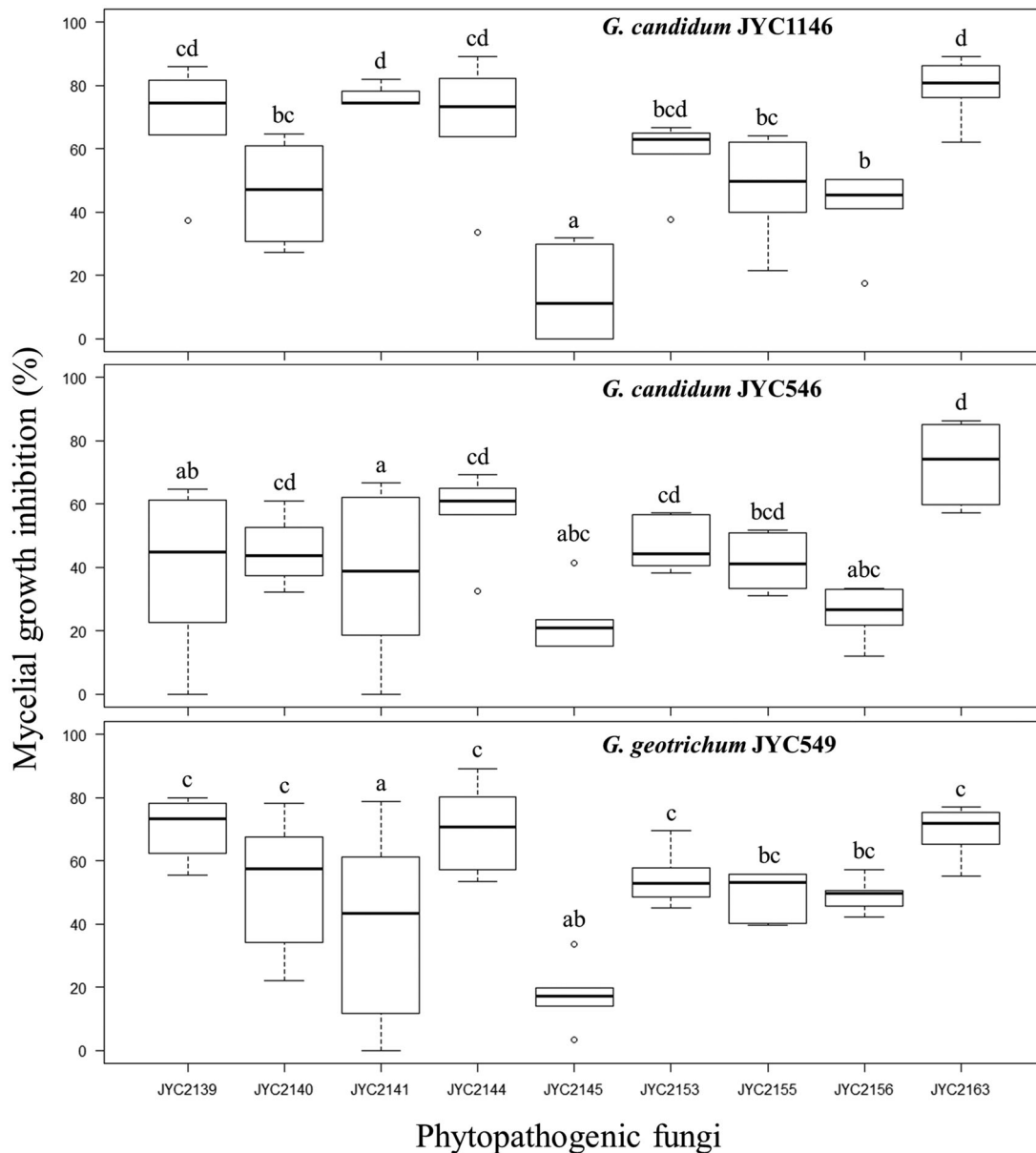


Figure 2. Mycelial growth inhibition (MGI) value of the phytopathogenic fungi under the effects of VOCs secreted from the three *Galactomyces* strains. Letters indicate significant pairwise differences (Tukey's all-pair comparisons using the beta regression model, $p < 0.05$).

approximately 38%–42% of spores germinated under VOC treatments, and no significant difference was observed among the different *Galactomyces* strains.

3.3. Effects of VOCs on the mycelial growth of *Athelia rolfsii* JYC2163 on tomatoes

The infected tomatoes treated with VOCs from all the three *Galactomyces* strains demonstrated significantly lower disease severity compared with the control (likelihood ratio test: $df=3$, $X^2 = 22.28$, $p < 0.001$). Without VOC treatment, 30.08% \pm 8.52% of the fruit surface was occupied by the mycelium, whereas for treated fruits, 8.02% \pm 3.33%, 14.30% \pm 5.84%, and 12.96% \pm 7.54% of the

surface were occupied by the mycelium when treated with VOCs from *G. candidum* JYC1146, *G. candidum* JYC546, and *G. geotrichum* JYC549, respectively. Although *G. candidum* JYC1146 displayed the most efficient reduction in disease severity, no statistical significance was observed among the three *Galactomyces* strains (Figure 4).

3.4. Lethal effects of VOCs on free-living nematodes

VOCs from all the three *Galactomyces* strains significantly increased the mortality rate of *C. elegans* in the L4 stage (likelihood ratio test: $df=4$, $X^2 = 158.32$, $p < 0.001$). The background mortality at 24 h

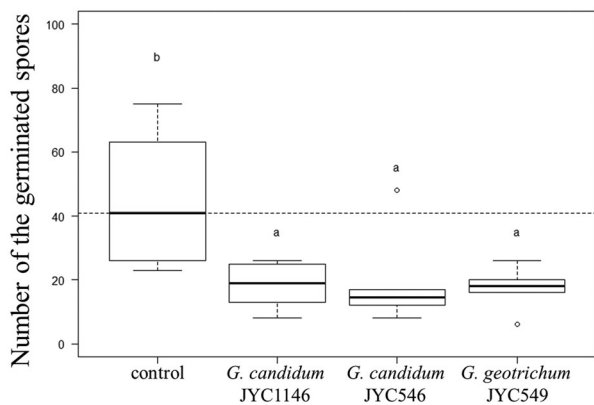


Figure 3. Germination of spores from *Athelia rolfsii* JYC2163 after the treatment of VOCs secreted by antagonistic fungi for 24 h. The dashed line indicates the median number of germinated spores without the effect of the antagonistic yeast. Letters indicate significant pairwise differences (Tukey's all-pair comparisons using the generalized linear regression model with Poisson's error, $p < 0.05$).

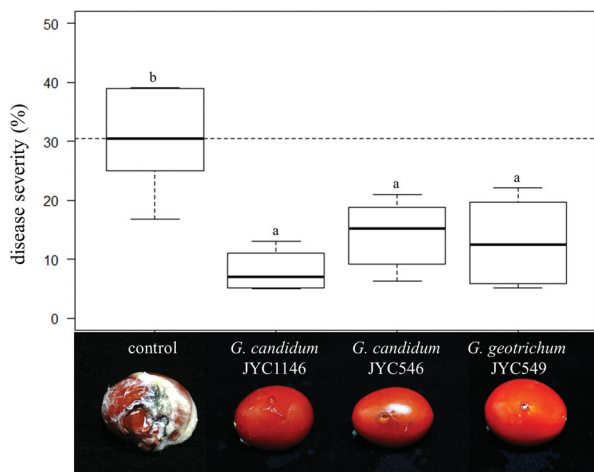


Figure 4. Efficacy of VOCs produced by the antagonistic fungi in postharvest control of *Athelia rolfsii* JYC2163 on the marketed cherry tomato. The dashed line indicates the median number of disease severity [symptom area/total area] without the effect of the antagonistic yeast. Letters indicate significant pairwise differences (Tukey's all-pair comparisons using the beta regression model, $p < 0.05$).

was $7.78\% \pm 5.44\%$ without VOC treatment. Treatment with *S. cerevisiae* showed a slightly increased mortality ($17.22\% \pm 6.47\%$), which was marginally significant under the statistical analysis. The lethal effects exerted by the three *Galactomyces* strains were significantly higher than those exerted by treatments with both the control and *S. cerevisiae*. Although the intensities were significantly different among the three *Galactomyces* strains ($52.22\% \pm 12.59\%$ for *G. candidum* JYC1146, $55.56\% \pm 14.86\%$ for *G. candidum* JYC546, and $40.56\% \pm 8.28\%$ for *G. geotrichum* JYC549), their effects might be rather similar, because extreme high mortality was observed in one sample treated with *G. candidum* JYC546 (Figure 5).

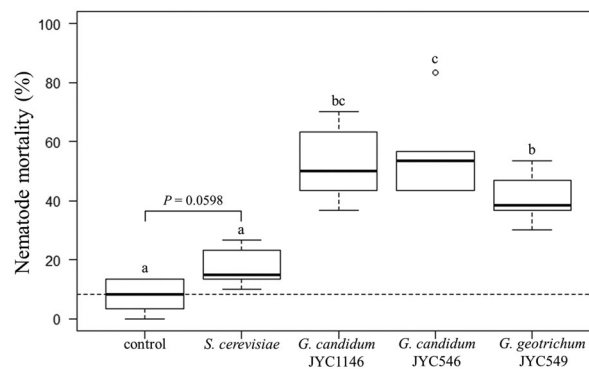


Figure 5. Lethal effects of the VOCs produced by the fungi on the fourth larval stage of *C. elegans* strain N2. The dashed line indicates the median mortality rate without the effect of the yeast. Letters indicate significant pairwise differences (Tukey's all-pair comparisons using the generalized linear regression model with binomial error, $p < 0.05$). The p value between the control and treatment of *S. cerevisiae* suggests a marginal significance.

4. Discussion

Fungi commonly exhibit antagonistic activities to manipulate the surrounding microbiota for their own interests. Some of them have also been commercially applied in controlling pre- or post-harvest infections of plants [23]. *Galactomyces* fungi have been found in various habitats, and some of them are widely applied in the food industry and enzyme production [24]. They are also well known for flavor production [25]. Although a few studies have revealed the effect of *Galactomyces* fungi on plant health, in our previous study, we observed their potential in controlling pre- or post-harvest diseases [11]. In the present study, we further confirmed their broad-spectrum antagonistic activities against phytopathogenic fungi and free-living nematodes by their production of airborne VOCs.

4.1. *Fusarium* sp. JYC2145 was less inhibited by VOCs

The broad spectra of antagonistic activities have been reported in some bacterial and yeast species [12,26]. Herein, all the nine phytopathogenic fungi examined in the present study displayed growth inhibition in the presence of VOCs from the *Galactomyces* fungi; however, the levels of inhibition varied. *Fusarium* sp. JYC2145 showed a relatively moderate response to the presence of the *Galactomyces* fungi. The other four *Fusarium* strains examined in this study displayed moderate to high growth inhibition; therefore, such resistance might not be entirely because of the phylogeny. Except for *Fusarium* sp. JYC2145, which has never been noted to attack living plants, the other four *Fusarium* strains are common plant pathogens. To break the defense of living plants, several fungal strains display

local adaptation to the current host cultivars and trade off those less encountered [27,28]. Although a similar tradeoff between the epidemic (onto or into the host) and survival (often outside the host) phases remains poorly documented [28], the different inhibition levels among the *Fusarium* strains in the present study suggest a tradeoff between the capability of competition against aggressive fungi and exploitation of living plants.

4.2. *Cladosporium cladosporioides* JYC2144 and *Athelia rolfsii* JYC 2163 were highly inhibited

The growth of *Cla. cladosporioides* JYC2144 and *A. rolfsii* JYC 2163 was highly inhibited by VOCs from the *Galactomyces* strains. *Cla. cladosporioides* has been reported to occasionally cause blossom blight in strawberries and leaf spots in tomatoes [29–31]. Despite causing plant diseases, the facultative infection and relatively low damage to the host plant make *Cla. cladosporioides* a less important phytopathogenic fungus. By contrast, it is also a potential biocontrol agent because of its antifungal activity against coexisting phytopathogenic fungi [32]. Hence, growth inhibition caused by the *Galactomyces* fungi might be a negative factor in the adoption of multiple biocontrol agents. Biocontrol agents potentially offset each other's potency. In the case of controlling infection in the invasive tropical shrub *Mimosa pigra* in Australia, the efficiency of the fungal plant pathogen *Phloeospora mimosae-pigra* was significantly reduced in the presence of another biocontrol agent, the stem-boring moth *Neurostrota gunniella* [33]. Because the interaction network of microorganisms in the soil is more complex than that in the culture medium, the *in vivo* effect of the *Galactomyces* fungi on living plants may be worthwhile to investigate in the future.

The growth of *A. rolfsii* JYC 2163 was also highly inhibited by the *Galactomyces* fungi. *A. rolfsii* (syns. *Sclerotium rolfsii*) is a soil-borne facultative plant pathogen, which causes southern blight disease in several host plants. Members of *Athelia* cause economic crop (fruits and vegetables) losses in tropical, subtropical, and warm temperate countries and are difficult to control because of the variety in host range, fastidious growth, and ability to produce persistent sclerotia [34,35]. Fungal metabolites have been considered potential biocontrol agents to avoid negative effects on the environment and human health by chemical agents [34,36]. The reaction of VOCs from the *Galactomyces* fungi is similar to that of 6-pentyl- α -pyrone (6PAP), which is produced by numerous *Trichoderma* species. 6PAP has been previously noted to suppress the disease caused by *A. rolfsii* when applied onto the surface of the soil

[36]. Although the mechanism underlying the anti-fungal activity has never been examined in *A. rolfsii* disease, 6PAP has been observed to inhibit mycelial growth and conidia germination of *F. moniliforme* [37]. Although the efficiency of VOCs from the *Galactomyces* fungi has not been examined in living plants, the reduced damage to tomatoes and the marginal efficiency in several developmental stages of disease from *A. rolfsii* JYC 2163 suggested their potential in controlling postharvest infections.

4.3. Lethal effects on the free-living nematodes

Some studies have suggested that animal or plant residues incorporated into the soil release VOCs, causing biofumigation, and some chemicals from plants have been observed to be directly toxic to the infectious stage of nematodes [13,38]. Such effects explain the efficiency of soil amendments, which are frequently adopted as environmentally friendly strategies to replace the application of chemical nematocides [18]. However, little is known regarding chemicals released from soil microorganisms. Fungi which negatively affect the fitness of soil nematodes can be characterized into two main groups, nematophagous fungi, which exploit nematodes for nutritious substances, and, endophytic fungi, which colonize plant roots and reduce the incidence of plant disease by enhancing plant resistance or releasing enzymes or toxins against nematodes [39,40]. The *Galactomyces* fungi examined in the present study are more likely to be endophytic fungi, which release volatile toxins. Although a similar effect was observed in *S. cerevisiae* in our study, nematodes exposed to VOCs from the *Galactomyces* fungi demonstrated a more intense effect. Some chemicals released from the fungi were detected to be toxic to nematodes. Acetic acid, which inhibits the movement of infesting juvenile nematodes, was detected during the fungal growth of *Paecilomyces lilacinus* and *T. longibrachiatum* [41]. Trans-2-decenedioic acid detected in *P. ostreatus* caused irreversible damage to the free-living nematode *Panagrellus redivivus* [42]. Despite their potent effects on nematodes, these chemicals released from nematophagous or saprophagous fungi might also be disadvantageous to plants and less likely to be released from endophytic fungi. In addition to the *Galactomyces* fungi, *F. oxysporum* and *Neotyphodium* spp. are known to release toxic compounds against nematodes and enhance plant growth. However, mechanisms underlying nematode resistance in most of the plant–fungus associations are poorly understood [40,43]. Thus, precise analytical methods, such as solid-phase microextraction–gas chromatography–mass spectrometry, should be applied in future

studies to confirm whether volatile compounds produced by these *Galactomyces* fungi.

5. Conclusion

Traditional methods of soil amendments (compost, oats, and straw) limit the incidence of plant disease by manipulating soil communities. However, the efficiency exhibited by opportunistically increasing the population of antagonistic or competitive soil microorganisms is uncertain [34]. In the present study, VOCs from *G. candidum* JYC1146, *G. candidum* JYC546, and *G. geotrichum* JYC549 displayed significant antibiotic activity, which demonstrates potential in controlling plant diseases caused by phytopathogenic fungi and nematodes. The adoption of these yeast-like fungi isolated from the natural environment in agricultural systems is more environmentally friendly and less harmful for human health. The effect of *Galactomyces*, as a potential biocontrol agent, on living plants and stability of VOCs released in the environment is worth examining in future investigations.

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Author contributions

STC and JYC originally formulated the idea and developed the methodology, STC collected samples and carried out experiments, MCC performed statistical analyses and built statistical models, STC, MCC and JYC wrote the manuscript. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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